Dear Participants of the 5th World Congress on Alternatives and Animal Use in the Life Sciences,

The abstract book contains 614 abstracts of the plenary lectures of 272 oral presentations and 342 posters to be presented at the 5th World Congress on Alternatives in August 2005 in Berlin, Germany. The programme and the abstract book allow you to make your best selection of presentations among seven themes, which will be discussed in 29 sessions, 15 workshops and in the poster sessions.

Usually, the first author of an abstract is slotted to give the oral presentation or to be present during the poster session to discuss your comments and questions. Otherwise, the presenting author is marked with an asterisk (*).

We hope that you will find the abstracts particularly helpful to set priorities when two or more sessions are scheduled at the same time. In case you do not manage to attend a session or to meet an author, the contact addresses are given for each contributor.

It is obvious from the list of sponsors, the programme and from the number of abstracts submitted that the most important topic of the 5th World Congress will be in vitro testing of cosmetic ingredients, in particular acute local toxicity testing. More abstracts have been submitted to sessions and workshops of Theme 5 “Safety testing, validation and risk assessment” than to any of the other themes. Moreover, more than 60 abstracts have been submitted to session 5.4 “Development and validation of alternatives for dermal toxicity testing”. We have, therefore, decided to hold an additional session on in vitro dermal toxicity testing. According to the number of abstracts submitted, session 5.6 on “In vitro approaches for determining acute systemic toxicity” is the second most popular topic, probably since ECVAM and ICCVAM are collaborating on a project to predict acute oral toxicity from cytotoxicity data. Finally, session 7.2 “Innovative approaches for alternative methods development” is number three in the hierarchy of the most attractive sessions.

The high scientific quality of the abstracts demonstrates that the development of in vitro methods is currently at the forefront of research in the life sciences and no longer a by-product of cell and molecular biology. The EU Commission as well as the 25 Member States should, therefore, provide more funding to meet the challenges of EU Directive 86/609 for the protection of experimental animals and in particular those of the 7th Amendment of the EU Cosmetics Directive and the new EU Chemicals Directive (REACH).

The staff of the ALTEX editorial office has enjoyed providing you with the abstract volume. We hope that you and all your colleagues will enjoy the 5th World Congress in Berlin, in particular by meeting colleagues from all over the world, who are actively engaged in promoting the 3Rs Principle of Bill Russell and Rex Burch.

With best wishes
from the ALTEX Team in Zurich (CH)
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Plenary Lectures

Lecture

**NIH funding of the 3Rs (reduce, refine, replace)**

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The National Institutes of Health (NIH) is charged with pursuing fundamental scientific knowledge about the nature and behaviour of living systems and with applying that knowledge to extend healthy life and reduce the burdens of illness and disability. The use of animal and other models of disease has been an essential component of NIH’s efforts and successes in fulfilling its mission. The NIH has played an active role in contributing to a better understanding and utilisation of animal models of disease, including lower phylogenetic species, and in supporting science that has led to refinements in techniques and practices that have reduced pain and distress in the laboratory animal. As NIH looks to the future, the NIH Roadmap and other scientific initiatives are creating an exciting world of opportunities that will allow NIH to continue its commitment to the 3Rs.
Systems Biology and the 3Rs

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Pancreatic beta-cells, the only physiological source of insulin production, die by apoptosis in early type 1 diabetes mellitus (T1DM). Apoptosis is an active, gene directed process, and recent observations by our group suggest that beta-cell fate following exposure to immune mediators is a complex and highly regulated process, depending on the duration and severity of perturbation of key interacting gene networks. This departs from the traditional view of phenomena, based on the study of signaling pathways by intuitive inferences based on the study of individual pathway components. Identification of complex and interacting gene/protein patterns poses a formidable challenge, but the sequencing of the human genome, and of the genome of several other species, makes it possible to address it by the use of new high throughput technologies, such as microarray analysis and proteomics. To fully use these data we will need a global multivariate strategy, as proposed by the systems biology approach. This approach seeks to devise models based on the comprehensive, qualitative and quantitative analysis of all constitutive parts of a cell or tissue with the ultimate aim of explaining biological phenomena through the interaction of all its cellular and molecular components.

Against this background, we are utilising microarray analysis, detailed promoter studies and in silico analysis to clarify the pattern and regulation of gene expression in primary rat beta-cells and in human islets exposed for different time points to the pro-apoptotic cytokines IL-1β + IFN-γ. The data obtained are deposited at the “Beta Cell Gene Expression Bank”, which is already accessible at http://t1dbase.org/cgi-bin/enter_bcgb.cgi. The ultimate goal of this open access resource is to identify and annotate all genes expressed in rat, mouse and human beta-cells.

By allowing us to obtain massive and integrated information on limited amounts of tissue, and by increasing the predictive power of different biological and in silico models, this novel approach may lead to a decrease in the number of animal experiments. This potential impact of the systems biology approach on the “3Rs” will be discussed at the lecture.

ECVAM’s progress in implementing the 3Rs in Europe

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Starting with the animal welfare Directive from 1986 and continuing until most recent chemicals and cosmetics legislation, Europe has laid the ground for the implementation of alternative methods. In order to meet these political expectations, a couple of technical and strategic developments became necessary:
- An analysis of current in vivo test performance to set benchmarks for alternatives
- An analysis of the frequency (prevalence) of toxic health effects in different areas of test application
- An inventory and database of the alternative methods available
- A coached development of lacking tests also making use of novel technologies
- An acceleration and international harmonisation of the validation process and regulatory implementation
- A development of quality assurance systems for in vitro methods such as Good Laboratory Practice and Good Cell Culture Practice
- A transition from single tests as stand-alone replacements to the composition of test strategies and their validation.

The European Centre for the Validation of Alternative Methods (ECVAM) has played a proactive role in all these processes co-ordinating many stakeholder activities. A review of the state of these developments shall be given, in order to show how a new type of evidence-based toxicology is emerging which is based on validated and quality controlled test strategies.
Lecture

**Education in alternatives to animal experimentation. Who shall be educated?**

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The contribution of animal experimentation to human welfare can not be ignored. Although the value of animal experimentation is recognised, one’s sentiment to love animals must be remembered even in scientific communities. The well-educated liberalists have established a new idea for laboratory animal welfare, 3Rs that is a key of alternative studies.

Because the basement of 3Rs is sentiment and ethical mind of human beings, the early education is essential. The primary school pupils, high school students shall be educated. The university students in particular medical, dental, veterinary and biology schools shall be educated. These students are the future science or biology teachers for children and students. The post-graduate students in biomedical sciences shall be educated before they are planning animal experimentation for their thesis. The tutors, lecturers and professors to look after biomedical postgraduate students shall be educated because they are teachers for future researchers and scholars and also they are researchers who are making experimental protocols. All of members in education at not only academic institutions but also research institutions shall be educated.

Finally but most importantly, the citizens shall be educated to understand the alternatives to animal experimentation as tax payers who are patrons of any scientific activities including animal experimentation.

Lecture

**Willi Halle’s registry of cytotoxicity**

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In toxicology, we are forced to test hazard potential in animals as alternates to men. Inter-species sensitivity differences are then bridged by applying safety factors to NOAEL’s observed in animals. However, in general, data from acute systemic toxicity tests are not used to derive NOAEL’s for risk assessment procedures, so that valuable information derived from these studies is often reduced to the crude information needed for classification and labelling: A rough estimate of the LD50, or an estimate of a toxicity class. Cell biologists have therefore since long investigated whether the loss of cellular functions in vitro can be used to predict lethal doses in vivo, in particular the pioneers of the principle of basal cytotoxicity, Björn Eckwall and Willi Halle.

In the late 60ies of the last century, Willi Halle started in former East Germany to collect published IC50 values from cytotoxicity studies provided these had met his defined acceptance criteria. Once LD50 values of these chemicals became available, the data were entered into a data base, the Registry of Cytotoxicity (RC). However, Willi Halle did not receive the necessary support for his pioneering work before the reunification of Germany, and only after the Berlin Wall fell, his work received the attention and support it deserved. With support of the German Ministry of Education and Research (BMBF) and continuous support by ZEBET, the BfR is currently in the lucky position to hold the RC as an electronic database – with 537 chemicals the largest collection of in vitro IC50 and related LD50 values. Willi Halle’s model for prediction of acute oral LD50 shows particular strength in the prediction of the absence of oral toxicity, which holds for the majority of industrial chemicals.
Laboratory training models are essential for developing and refining surgical skills, especially for microsurgery. The lack of an accurate vascular model has necessitated the use of living models when bleeding, and vascular liquid filling is required. To avoid the use of live anaesthetised animals in surgical training, particularly in training on procedures that must simulate the living human in terms of ability of bleeding, and liquid filling of vessels to practice vascular and microsurgical procedures. We have developed a new method using human cadavers for surgical training by connecting the vessels of the cadaveric specimen to coloured liquid reservoirs and using a pump to provide pulsating pressure transmitted to the vessels. This method provides a condition that simulates live surgery in terms of bleeding, pulsation, and fluid filling of the vascular tree, being an excellent alternative model. It can be applied to the whole cadaver or to a particular cadaveric parts (head, arm, leg…) or to an isolated organ (heart, liver, kidney…) and can be applied in the same manner to ethically sourced animal cadaveric specimens for veterinary surgical training instead of using live healthy animals. We used this model in courses were rats, rabbits, and other small animals been used for practising vascular dissection and anastomosis saving hundreds of live healthy animals in few courses.

Utilising this technique will eliminate, and forever the use of live anaesthetised healthy animals for surgical training. Video and PowerPoint presentation.
Lecture

Teaching humane science: A European perspective

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In an increasing number of European countries some specific system of training exists for persons (wanting to become) involved in animal experimentation. Aiming at furthering the harmonisation and standardisation of such education programmes the Council of Europe adopted in its Convention ETS 123 for the protection of vertebrate animals used for experimental and other scientific purposes (1986) the recommendations of the Federation of European Laboratory Animal Science Associations. FELASA developed educating and training programmes for laboratory animal caretakers (Cat. A), research technicians (Cat. B), scientists (Cat. C) and laboratory animal science specialists (Cat. D). In addition FELASA started in 2004 an accreditation system to guarantee the quality of laboratory animal science education and training programmes. It is generally expected that the European Union will also include mandatory training of personnel involved in animal experimentation in its Directive 86/609/EC which is currently being revised.

The principles of the 3Rs can be recognised throughout the curriculum of the recommended training programmes. Apart from training skills each programme focuses on developing an attitude towards the humane treatment of the animals used for scientific purposes. Without neglecting the importance to seek possibilities to replace the use of animals, the emphasis is laid on adequately designing animal experiments. Students are thought that proper a priori statistics as well as standardisation of procedures can reduce the numbers of animals needed without jeopardising the quality of the results. Also the principle that methods that induce less discomfort are beneficial to both animals and science are elementary in the training programmes.

Lecture

Online learning to teach humane science

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Training programs for animal researchers focus on reducing the use of animals in research and on refining animal handling and treatment when animal use is necessary. As an adjunct to face-to-face training, online learning provides depth in knowledge of concepts, prepares a learner for personal training, and reinforces lessons learned.

In the USA, online training of scientists on the ethics of animal research is addressed in many institutions by the courses Working with the IACUC and Writing a Protocol for Research in Animals, administered through Research Training Org (www.researchtraining.org) and the AALAS Learning Library (www.aalaslearninglibrary.org). These courses encompass all US regulatory and ethical requirements. Since their release in 2001, over 40,000 US researchers have completed these training courses, and access continues to grow monthly at the rate of 1,500 individuals.

AALAS has undertaken an initiative to expand the course curriculum to fully support the 3Rs via promoting the competence of all members of the animal research team. The AALAS Online Learning Committee has developed curricula for five categories of research personnel: researchers, technicians, managers, veterinarians, and institutional animal care and use committees. Initial courses for researchers feature ethical decision-making, mouse bioengineering, breeding, methodologies, pain and distress, and anaesthesia. Additional courses are in progress for managers and veterinarians. To better integrate with institutional training programs of different sizes and types, the AALAS Learning Library architecture provides access on the basis of individuals and groups and allows a customisation of course materials for tailoring to an institution’s specific needs.
Although veterinary schools increasingly have mainstreamed alternatives in their curricula and many resources are available for secondary education, alternatives are not widely adopted for teaching high school biology in the United States, a growing paradox. Viewing the practice of dissection as recalcitrance of teachers is an oversimplified perspective. Three barriers mitigate against adoption of alternatives in classrooms. First, a curricular gap exists; dissection is not considered in course outlines. Though common in high school biology, dissection receives little attention in research and curricular standards. Second, instrumental and technical support for science laboratories has been reduced. County districts formerly provided resources integrated with laboratories in lesson plans, supported by subject matter specialists. Now teachers must acquire their teaching materials to enhance their courses. Small budgets are sufficient only for a few clerical supplies. Information is available on abundant, though costly, resources (website: http://www.vetmed.ucdavis.edu/Animal_Alternatives/altsearch.htm). Planning ahead is required for resources available on loan. Third, to teachers, supplying motivating and informative materials for students is of prime importance. Teachers dream of motivating students to learn, and seek to inspire them. High quality laboratory exercises are difficult to muster. Consideration of whether to use animal specimens and other resources in high school classrooms is not supported within the texts of curricular standards and science frameworks, nor are such resources and relevant expertise offered by school districts. Thus, the teachers’ highest goal of inspiring their students in biology becomes ever more unattainable.

Lecture
Three barriers obstructing mainstreaming alternatives in K-12 education

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Teaching humane science: Should live animals be used when educating future biomedical scientists?

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National laws and regulations require that individuals who work with laboratory animals must have appropriate skills and qualifications for performing experimental procedures. FELASA has produced proposals concerning educational and training requirements for technicians and scientists working with laboratory animals. Maximal implementation of refinement and reduction through ensuring that all staff is competent with respect to the species of animals they are going to work with will usually require animals to be used in hands-on practicals in well structured courses. Handling and restraining animals require that conscious animals are used, whereas injection techniques, assessment of the effect of anaesthesia and euthanasia can be trained on fully anaesthetised animals in non-recovery practical sessions. The use of live animals on mandatory courses for scientists allows the teaching of the most humane attitudes to animals as well as proper ways to handle, restrain and anaesthetise animals. On-the-job training is unlikely to be of the same quality and result in uniform good results as compared with high quality courses. On training courses it is possible to emphasise the importance of prioritising animal welfare above the scientific results, and to introduce the students gradually to humane techniques by using AV-materials and dummies. Experience demonstrates that the course-item that young scientists rank as the most important is the practical sessions during which they are taught how to handle the animals, how to gentle and condition them and how to restrain them without stressing the animals and how to perform common simple procedures on anaesthetised animals.
Reduction and refinement alternatives in veterinary instruction in the US

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Veterinary medical educators have traditionally used live animal models for instruction in handling, diagnostic and surgical techniques. Animals used in laboratories can experience from very little to a substantial degree of stress and pain. Having recently received enhanced scrutiny from regulatory agencies and criticism from animal protection groups, veterinary medical instructors in the U.S. should be increasingly looking for alternative approaches to animal laboratories. Current alternative methods typically involve cadavers, models and computer simulations. Continuing resistance to adoption of alternative methods results from lack of evidence of the existence and effectiveness of such methods. A systematic approach for reduction and refinement of animal use in training of basic and clinical veterinary skills begins with assessment of the necessary procedural knowledge required. The continuum of training should involve video presentation, model or cadaver demonstration and practice, simulations, and laboratory animal use only when unavoidable. Competency at each step should be assessed and required for progression to the next phase. Veterinary trainees and instructors must also be educated as to the value of using a more humane approach. Outcome assessments should be developed to monitor efficacy of training exercises, improvements in clinical skills, and student and instructor confidence. Additional efforts and resources should be directed to development of more advanced models and simulations in veterinary medicine. Our presentation will outline specific indications, barriers, and benchmarks for alternatives in this field. Implementation of alternative methods for teaching has potential to greatly reduce pain and distress in animals used for veterinary medical instruction.

Lecture
Animal use in higher education in Bosnia and Herzegovina and Serbia and Montenegro

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The work represents the results of independent questionaries on opinions and experiences on animal use in higher education of the students studying at Faculty of Sciences in Banjaluka (Bosnia and Herzegovina), Faculty of Veterinary Medicine in Belgrade (Serbia and Montenegro) and Faculty of Sciences in Novi Sad (Serbia and Montenegro). The questionnaire explores their personal experience and opinions about animal use at the higher educational institutions providing reliable results on the number of used animals and procedures performed in animal use in higher education at the region.

The work presents the results of one year long research of the author working on behalf of the group within the Student Organisation of Faculty of Science in Banjaluka which has been dealing on assessment of the animal use in higher education and promotion of alternatives and the 3R concept. The presentation of the results shows information on the current situation on animal use in higher education in Bosnia and Herzegovina and Serbia and Montenegro, the source of experimental animals, most often experimental procedures within particular subjects, survey on students’ attitude toward dissection and vivisection, the presence of alternatives and current methods used for increasing welfare of the used animal in higher education.

Also, within the conclusion the author proposes possible forward steps in improving the presence of alternatives and the 3R concept in higher education at the region.
Studies show that the use of live animals in medical education reduces the ability to retain information, thereby reducing actual learning, due to the stress that the student experiences when causing suffering and death to his “patient”. Today there are hundreds of substitute models that generate effective learning. The objective of this study is to understand the perception of students about the use of animals in education and its importance in medical learning. A questionnaire was responded to by 61 medical students. Results showed that 54.1% believe that animal use is fundamental to learning the medical profession, 59% don’t feel comfortable in replicating this learning in their professional practice, 72.1% aren’t aware of substitute methods, 55.7% believe that it is ethical to use live animals, 70.5% acknowledge unpleasant feelings during the process, 60.7% don’t recommend the practice as a good learning method and 55.7% would prefer the use of substitute models if they were capable to produce good learning. The contradiction in the responses regarding the necessity of animals for good learning must be due to a lack of knowledge about efficient substitute methods for medical teaching. In light of the emphasis on humanisation and positive doctor-patient relationship in professional education, we should stimulate the publication and use of these methods, reflecting on the true importance of this type of education. It is necessary to emphasise the option of conscientious objection and compliance with Federal Law 9605/98 (makes illegal the use of live animals when substitutes exist).

**Poster**

**The use of animals in medical education: A paradigm shift**

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Animals are not moral agents and so cannot be accused of holding virtues and vices, cunning and slyness, and so on, even though this is classic terminology for horses and foxes respectively. Humans on the other hand have choices of what they do, and as moral agents are held accountable for their actions. In animal research the “right actions” will often determine the welfare of the animals in their care and in their use. Having the right attitude towards animals therefore is one essential contribution to refinement and good laboratory practices. In this context come several activities that are important for the well-being of animals such as providing of good conditions for their housing and husbandry, daily and regular checks of animal health and welfare, good statistical design and research strategies, provision of pain relief etc. So in what ways do we incorporate this notion into our training and ethical teachings? Too often such teaching deals only with the 3Rs, but making people with responsibilities for the care and use of animals aware of their duties is more fundamental than any debate over animal rights.
THEME 1, SESSION 1.1

Poster

The protective effect of selenium against cadmium cytotoxicity in WEHI 164 cells

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Selenium is an essential trace element that occurs in active site of antioxidative enzymes directly involved in redox reactions. Numerous literature data indicate also that selenium plays an important role in protection against toxic effects of cadmium which is one of the most toxic substances in the environment.

The aim of our study was to evaluate the effect of 48-hour preincubation of WEHI 164 mouse fibrosarcoma cells in the presence of sodium selenite (0.3 µM) and medium containing 2% foetal bovine serum (FBS) (with decreased selenium concentration) on their viability after exposure for 24 h to cadmium chloride (7 µM). In order to intensify the effect of cadmium, hydrogen peroxide (H₂O₂) at the concentration of 0.1 mM was added to culture medium. Cytotoxicity of the chemicals was determined using MTT reduction assay.

We observed that the viability of WEHI 164 cells pre-treated with selenite and exposed to cadmium was increased in comparison to the cells exposed to cadmium alone. We also showed that the viability of the cells growing in medium containing 2% of FBS was significantly decreased in comparison to control cells cultured in the presence of 10% FBS. In our study H₂O₂ had no significant effect on cadmium cytotoxicity. These results suggest that the concentration of selenium in culture medium can have a significant influence on WEHI 164 cells viability and decrease cadmium cytotoxicity.

Poster

Preclinical cell culture models for early onset colon cancer: a novel approach for cancer prevention

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Introduction: Germline mutation in the tumor suppressor and DNA mismatch repair genes represent primary predisposing genetic defects for early onset familial/hereditary colon cancer. Similar mutations in mice produce intestinal adenomas. Reliable cell culture models with quantifiable carcinogenic risk offer a mechanism-based approach for rapid screening of efficacious preventive agents. Methods: The cell cultures maintained in serum containing DME/F12 medium were monitored for growth kinetics, cell cycle progression and anchorage-independent colony formation (AICF) that represent quantitative endpoint biomarkers. Results: Subculturable colon epithelial cell lines developed from APC [+/−] and APC [+/−]/Mlh1 [+/−] mice exhibited aberrant hyperproliferation and high incidence of AICF. Treatment with low dose combinations of mechanistically distinct Coxibs, polyamine synthesis inhibitor and Thymidylate synthase inhibitor produced cytostatic growth arrest, altered cell cycle progression and inhibited AICF representing in vitro surrogate endpoints for carcinogenesis in vivo. Conclusion: These data validate a novel cell culture approach for rapid screening and for rational prioritising of efficacious combinations of chemopreventive agents for subsequent preclinical and clinical trials on colon cancer prevention.

Support: The Irving Weinstein Foundation and NCI MA0#NO1-CN-75029-63
In light of growing awareness of the need to improve animal welfare and to refine, replace and reduce the number of animals needed for demonstration purposes, a video was produced to replace the gonadectomy, hormonal treatment and necropsy in rats, traditionally made by veterinary students during reproduction physiology classes. The first module of this video, set in a laboratory environment, demonstrates restraint and intraperitoneal injection, inhalation anaesthesia, surgical preparation of patient, main surgical procedures, postoperative recovery and hormonal treatment. In the second module physiological response to hormonal treatment is shown and discussed by way of animated diagrams. Covered topics include reproductive anatomy, hormonal function, hypothalamus-pituitary-gonad axis regulation and surgical technique. The 20 minutes DVD was filmed and edited by interns at University Multimedia Studio, which resulted in low cost but high professional quality. Using such an alternative resource we are now able to save many rats from pain, suffering and death. Moreover, the always short class time can be better employed for fruitful exchange of views between teachers and students about the main theme of the class: physiology.
Experiments with animals continue to be a part of curricula at many medical schools. We believe that all undergraduate medical students should be both theoretically and practically informed about the existence of alternatives to the use of animals in research and in education. Therefore we have prepared a course based on the 3Rs concept. This course takes place in the first and the second study year. During the course students learned and practically mastered, among others, the following topics:
- The 3Rs concept – scientific background, ethical and legislative considerations.
- Mammalian cells cultivated in vitro as an alternative to experiments on animals.
- Non invasive students self-experimentation.
- Invasive (volunteered) self-experimentation.
- Screen-based alternatives (interactive computer programmes).

We prepared a written anonymous questionnaire to evaluate student’s opinions about the course and their attitudes towards the alternatives. Results of the survey showed that our students were generally satisfied with the course and it seems that both experiments with cells in vitro and human experimentation could be a suitable alternatives in medical education.

Due to the fact that we organised the similar survey in 1995 and 2000 years, it was possible to analyse changes in the students’ attitudes during the last 10 years. One general tendency is obvious, students are currently less strict in their opposition against animal experimentation, and substantial part of our respondents even required animal experimentation. Reason behind these changes will be discussed.

This work was supported by Czech Republic Ministry of Education Research Project MSM 0021620820.

In 1985, all 126 medical schools in the US offered a live animal laboratory as a requirement for teaching physiology, pharmacology, and/or surgery. Currently, 80% of medical schools have eliminated these labs from their curricula. The remaining 20%, although not requiring participation, still offer live animal labs to civilian medical students.

Until recently, the University of Virginia School of Medicine (UVa) in Charlottesville continued to teach emergency techniques to medical students using a canine laboratory. Surgical procedures were performed on approximately 100 beagles (euthanised at the end of the lab). Medical students, physicians, veterinary technicians, and members of the community initiated a group effort, working with faculty and administration, to eliminate the use of live animals and implement superior training methods. In November 2004, a new life-saving techniques course was implemented using a human patient simulator and other stand-alone stations, allowing students to practice techniques such as chest tube insertion, cricothyroidotomy, and venous cut-down for intravenous fluids.

Elimination of the canine lab marked a turning point for medical education at UVa, and follows a general trend since 1994 of a declining use of animals in medical education as determined in the 2001 survey by Drs. Hansen and Boss. Advantages of using human-based training methods include anatomical accuracy, repeating procedures for proficiency, and long-term cost benefits. Simulated human tissues and body fluids provide a realistic experience. Successful strategies for continuing this trend of replacing animal laboratories for training medical students, based on this case study, will be discussed in detail.

This work was supported by Czech Republic Ministry of Education Research Project MSM 0021620820.
Lecture

RECAL: Creating computer-based alternatives using a sustainable learning objects approach

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The mainstay alternatives to using animals in higher education are multimedia computer-assisted learning (CAL) programs simulating pharmacology practical classes. They are intrinsically tied to the authoring application used to create them, are not editable and, with ever changing operating systems, rapidly become obsolete – the only options then being to recreate at further expense or to abandon.

The RECAL project, funded by the Lord Dowding Fund, is developing methods and tools to break this cycle of redundancy and reinvention. The approach is to disaggregate existing CAL programs to separate the learning objects (media elements, sequencing and runtime instructions) from the runtime environment. This allows the learning objects to be changed independently of each other and thereby facilitates reuse and sharing.

Development has so far focused on a Macromedia Flash runtime tool that can read a standards-based XML parameter file, call down the appropriate resources from the repository, in which the resources are catalogued and stored, and provide the interface for the user. Over time, new run-time shells can be built for new platforms or applications.

Rendering the pedagogical design is achieved using IMS Simple Sequencing, which can describe a single learner’s navigation path(s) through a group of learning activities. A RECAL editor will allow teachers to create or adapt both the content and sequencing.

The RECAL project, by adopting principles of standards, objects and reusability, has both improved the long-term viability of CAL alternatives and facilitated their adaptation by teachers to meet local needs and processes.

Poster

The current Japanese students’ activities for alternatives in veterinary education

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This paper presents the activities of “Network of Japanese Students for the Ethical Treatment of Animals in Education”, which include Alternatives Tour in Japan in 2003, the result of questionnaires about animal use in Japanese veterinary education, on-going activities, the trend of ethical education in national veterinary universities, and our perspective and mission for the future. In Japanese veterinary education, quite a few animal experiments and related practices, which are not in line with the concept of 3Rs, are still conducted, but in recent years some changes have been observed.

Some teachers have developed alternatives to the use of animals in education and some practices that harm and kill animals have been replaced with alternatives.

Besides that, there’s increasing number of students who cannot accept or sometimes express objections to traditional harmful use of animals from their ethical standpoints.

Such students began to organise groups for animal welfare within and beyond universities. The aforementioned network was established by two veterinarians in 2002. Now our network has developed nation wide. In the Alternatives Tour, voluntary members of our network held presentations of alternatives at all sixteen Japanese veterinary universities. During this tour, we distributed a questionnaire intended for students and teachers about animal use in veterinary education. We’re working for specific issues, as well as enlightening teachers and students on the concept of alternatives.

Our present goal is to establish the client donation program in Japanese universities.
Surgeons have to be well trained to achieve sufficient skills before operating on humans which is often done in animal laboratories. In order to reduce or even replace the use of animals we developed a new technique for production of anatomical models which can be used for skilled training of surgical techniques. The models are made by casting anatomical specimens from human cadavers (e.g. heart, lung) in a flexible silicon resin mold which is then duplicated using differentially hardened polyurethane. The phantoms are characterised by nature-like qualities of tissues and organs and they show all important anatomical details.

If necessary the models are adjusted to mechanical or electronic devices in order to simulate the function of the organs.

By this procedure a model for coronary artery surgery on beating heart was made which allows to avoid training courses on pigs or sheep. Another phantom consists of a retrostius of the human body with an aneurysmatic aorta which is successfully used for training of aorto-iliaco bypass surgery. Finally a phantom for training of microvascular surgery was developed consisting of artificial blood vessels sized between 1 and 5 mm which in future may replace the use of rodents for training.

Due to its nature-like characteristics the models can be used not only for skilled training of surgeons but also for testing newly developed surgical instruments and devices which is usually done in animal laboratories.

Euthanasia of animals for use in veterinary education in North America has declined as new resources and methods have been developed at veterinary schools and mainstreamed into the curricula. This process has included substantial curricular changes in four areas, coinciding with students no longer performing terminal surgeries. First, the creation and preparation of models and plastinated organs, prosections of tissues and organs, and software programs for teaching anatomy have supplanted the former practice of dissection conducted by students. Some teaching materials are available for purchase (website: http://calf.vetmed.ucdavis.edu/). Second, ongoing experiences are now provided to afford clinical exposure throughout the four years of veterinary medical education. In this arrangement, students’ clinical skills with clients assume a higher priority in their training. Third, students practice with mechanical devices, surgical tools, and physiological instrumentation as preparation for the manipulative aspects of animal handling and surgery. Fourth, students practice as surgeon and anaesthetist in spaying and castrating dogs, benefiting from close surgical supervision from faculty. The animal’s post-operative recovery is monitored by students. These changes have replaced students’ previous optional choices for alternatives. This presentation highlights innovative curricula developed at North American veterinary schools by teaching faculty, including a new webpage with information on veterinary curricular contributions. Veterinary students have welcomed the mainstreamed curricular experiences in animal handling, anatomy, physiology, and surgery.
Lecture

The InterNICHE policy on the use of animals and alternatives in education

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The InterNICHE “Policy on the Use of Animals and Alternatives in Education” is a comprehensive document in 10 sections that addresses all aspects of work with animals and alternatives in life science education. The Policy presents guidelines to ensure effective and fully ethical acquisition of knowledge and skills. It includes a definition of alternatives in education and of harm, and presents individual policies on dissection, the sourcing of animal cadavers and tissue, work with live animals for clinical skills and surgery training, and ethical field studies. It also addresses the use of animals for the production of alternatives themselves. While the ideal “replacement alternative” is defined as “non-animal” within the 3Rs philosophy of Russell and Burch (1959), the Policy highlights a shortcoming of the 3Rs approach for education. Not only is there a requirement for some students to work with animals, animal tissue and clinical procedures in their education, there is widespread evidence of the ability to fully meet all teaching objectives in ways that are neutral or beneficial to individual animals and that do not involve animal experimentation or killing. As well as non-animal learning tools like multimedia computer simulation, digital video, training models and mannekins, replacement alternatives also include the use of ethically-sourced animal cadavers for dissection and skills training, and apprenticeship into clinical practice with animal patients. A definition of “ethically-sourced”, and of ethical educational opportunities within clinical work, are included in the Policy which demonstrates the possibilities for full replacement of harmful animal use in education.

Poster

A package of simulation softwares as an alternative of animal use with enhancing teaching quality for pharmacology practical classes

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Introduction: Large number of animals have been replaced by the use of simulation softwares in life science education with certain limitations that the dissection, tissue mounting and animal experimentation skills cannot be gained with the use of simulation softwares. To overcome such limitations in a part and further reduction of animal use, a package of simulation softwares has been developed for undergraduate pharmacology practical classes.

Objectives: The simulation softwares have been developed with two main objectives. First is, to provide alternative of animal use for pharmacology practical classes. Second is, to provide simulation softwares that not only reduce animal use but also enhance the teaching quality and attract the experts of education for their use in teaching.

Methods: The softwares have been developed by a pharmacy teacher with the help of computer-programmers.

Results: Majority of pharmacology experiments has been covered in the simulation with provision of student evaluation mode with password protection. The package covers experiments of perfused and isolated frog’s heart preparation, frog’s rectus abdominus muscle preparation, rat and guinea pig ileum preparation as per pharmacy syllabus.

Discussion: The software will be useful for computer aided learning and practice examination and to replace number of animals. Various standard steps of animal experiments have been incorporated to thoroughly ground the users (students) with the procedure of the animal experiment. These features will be helpful not only for teaching but also for training the students for real tissue experiments and hopefully attract the teachers for their use in teaching.
The InterNICHE Alternatives Loan System is an evolving library of alternatives for application in life science education and training. Based in Slovenia, the alternatives are available for free loan worldwide. The Alternatives Loan System was established during 2001-2002 as a resource primarily for borrowers to familiarise themselves with the diversity and quality of existing alternatives, and to trial individual products. Over 100 CD-ROMs, videos, simulators and training mannequins are included for their pedagogical value and potential to replace common dissections and animal experiments within all life science disciplines. Borrowers include teachers, students, animal ethics committees, government ministries, organisations and campaigners in over 30 countries. The Alternatives Loan System has serviced over 100 loans, comprising over 1000 usages of individual alternatives. The loans have successfully given access to alternatives where none existed before, provided a resource for demonstrations at conferences, outreach tours and training, and supported the work of campaigners. As a tool for facilitating implementation, the value of the Loan System is indicated by a number of positive results: significant teacher use and the high number and wide geographical range of loans, subsequent purchase and implementation of products, direct replacement of harmful animal use, and providing an international resource for campaigners. Small-scale “micro-Loan Systems” have now been established in Brazil, Russia, India and Japan.

Poster
The InterNICHE alternative loan system: facilitating implementation through access to alternatives
Monika Percic¹ and Nick Jukes²
¹InterNICHE Alternatives Loan System, Ljutomer, Slovenia; ²InterNICHE, Leicester, UK

To expand upon the achievable outcomes of established approaches, we developed a clinical skills curriculum grounded in universal skills, self-direction and self-evaluation that emphasised beneficial, and eliminated harmful, animal involvement. The novel aspect of this design was the focus on universal skills as foundational to professional physical performance as basic sciences are viewed as foundational to clinical cognition. The goal was preparing pre-clinical veterinary students in the psychomotor, behavioural, perceptive and cognitive skills necessary for safely and successfully performing procedures in the apprenticeship format of the clinical years. Course content and delivery progressed from basic skills training on simple/abstract learning tools to integrated skills training on procedural simulation learning tools to performance in closely supervised apprentice training with patient learning tools. The course was temporally progressive in complexity, challenge, and responsibility while allowing unlimited learning tool repetition. Training in problem solving, knowledge integration and life-long learning skills were threaded throughout. Evaluation was by faculty observation, pre- and post-training testing, and student logbook review. While the course structure provides a promising framework for limiting animal use and harm in veterinary clinical skills education, progress in professional performance was affected by student acceptance of the pedagogy. In fact, improvement in metacognition, self-reliance, and physical performance closely paralleled individual student acceptance of the curricular approach, and was limited in highly teacher-dependent learners. Factors that promoted acceptance were learning tool design, student introspection, and instructor feedback. To overcome these problems curricular redesign will address student compliance and validation studies will be conducted.

Lecture
Observations on a novel universal skills-based approach to veterinary clinical skills education
Lara Rasmussen, J. B. Kitchen and B. M. Aldridge
Western University of Health Sciences, College of Veterinary Medicine, Pomona, USA
Poster

Virtual dog for demonstrating the cardiovascular effects of drugs

Ramasamy Raveendran
JIPMER, Pondicherry, India

Introduction: Dogs are used for undergraduate medical, dental, pharmacy and veterinary practical classes to demonstrate the effects of drugs on the blood pressure (BP) and heart rate (HR). This experiment can be replaced by simulating it on a computer using Computer Assisted Learning (CAL) software. Since CAL rather than the live experiment is sufficient to meet the objectives of a practical class, a CAL software to simulate the effects of drugs on dog BP and HR was developed.

Methods: The software (developed in VisualBasic for Windows) displays a simulated chart recorder on which the animated tracings of BP and HR are recorded continuously. The user can choose any drug (and the dose) from the list provided, administer it to the virtual dog and measure its effects on BP and HR. When the software is run under the "tutorial mode", it allows the user to interactively test all the drugs in the list and observe their effects. In the "examination mode", an unknown drug is given and the student is asked to find out its nature by comparing its effects with those of known drugs.

Discussion: The software can serve as a replacement for the dog experiment. Its design, user-friendliness and realistic simulation of drug effects will make it an acceptable alternative for the live experiment. Since the software is available free of cost, it will be useful for students and teachers who cannot afford similar, commercially available packages. The software will be demonstrated to the delegates.

Poster

Role of computer-based technology as an animal alternative - digital transformation in animal science and zoology curricular

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Animal science and zoology teachers have to be prepared to expose students to the virtual laboratory, an innovative digital technology that transforms conventional animal dissections into multimedia learning. The students can perform dissections on screen as full virtual reality simulations with a very high degree of interactivity. In the present digital age, keyboard, mouse and interactive multimedia software packages on CD-ROM can no doubt not only supplement black board learning but also play a significant role in the conservation of animals in animal sciences. Further, computer-aided CD-ROM alternatives may aid the creativity of teachers and give them new perspectives. The practical advantages include financial savings, the wise use of resources and reduced environmental impact. The programmes allow quick access and can easily be used during a lecture or in a practical course. Creative utilisation of this technology can provide a highly effective learning/teaching aid. The process of developing something new or reforming something old, using creative methods to improve the curriculum design is an important process. The zoology practical curriculum has been changed by the Bharathidasan University, Tiruchirapalli, Tamilnadu, South India at graduate (Bachelor of Science) and post-graduate (Master of Science) level. At post-graduate level, dissections involving the killing of frogs, calotes, rat and sharks have been substituted with the available CD-ROMS. In India, the attitude of teachers towards animals dissections is changed/changing. The responsibility lies with the planners of the curriculum and the teachers. Education is a challenge.
Lecture

**Learning research skills in the life sciences without using animals**

*Garry Scroop*

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Practical teaching for students in the life sciences is commonly focused on traditional, recipe-driven experiments, mostly in animals, and completed in a 3 hour session. They are intended to complement factual material presented in lectures but given that the protocols are designed for all students to obtain the same result, their ability to motivate students is poor and recycling of practical results from previous years is common practice. The Department of Physiology in Adelaide has abandoned this traditional approach in its second year courses in Medicine and Science and replaced it with student-driven research projects where the central theme is to provide practical experience in the scientific method of problem-solving. Five to seven students, working as self-sufficient research teams, conceive, design and execute individual research projects, lasting an entire 12 week semester, using themselves and colleagues as the experimental subjects. They are supported by an academic staff member acting as project supervisor and they work in a small laboratory module equipped with the basic research infrastructure appropriate for data collection and analysis of the physiological system under investigation. Student performance is assessed progressively with each assessment designed to reinforce the research experience. Although developed in the context of physiology, the focus is more on “process” than “content”, and as such the concept can be applied in any discipline at any stage of education. This new teaching methodology not only provides the students with an important life-long learning experience but is also an unambiguous opportunity to remove animals from teaching.

Poster

**Empowerment and training in the use replacement alternatives in education – the success stories in Italy, India and Romania**

*Massimo Tettamanti1, S. Ramkrishna*2, *Alina Bodnariu*4, *N. Sathyanarayana*2, *Shiraneep Pereira*1 and *Prema Veeraraghavan*1

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Introduction: Most often, life science education comprises of mandatory didactic experiments that involve live animals. A students desire not to participate in vivisection and choice “not to kill” i.e. “conscientious objection” is both a human rights issue and an animal rights issue.

Methods: Empowerment, through education and training in the use of alternatives, creation of alternatives libraries and promulgation of laws for “conscientious objection” or a ban in the use of animals in education were used to propagate 100% replacement of animals in teaching.

Results: Since 2000, a series of training/talks in the use of alternatives in veterinary, pharmacology, medicine and biology education have been conducted in Italy, India and Romania, where over 1000 of teachers/students have participated to learn the use of alternatives like CD’s, models, mannekins etc. This has resulted in a drastic reduction in the use of animals in several universities, 100 percent replacement in yet others and the creation of virtual labs.

Discussion: The realisation that the use of live animals in education is inferior or harmful from a pedagogic, psychological and ecological point of view and knowledge of the negative impact of vivisection on the psyche of a student has resulted in this change. The paper discusses the success stories in replacing the use of animals in education, in Italy, India and Romania and the efforts of teachers, students and animal welfare personnel that made this possible.
Introduction: Portuguese Secondary Schools, as the majority of educational institutions around the world, use animals in lab works. Although no accurate numbers can be found, visits to several schools, and informal conversations with teachers point out that a significant number of mice, rabbits, pigeons, and organs of different farm animals are used. In this way, teachers try to comply with programs which completely ignore the existence of alternative methods to the use of animals in education. The programs are intended to adolescent students (ages 16 to 18) who will at higher educational levels, accept the use of animals for experimental purposes without any constraint.

Methods: Secondary School curricula study enabled us to organise the first official Humane Education Course in Portugal. The 25 hours Course has been accepted by the Ministry of Education, and covers areas important for the implementation of a humane school system, such as animal sentience, animal rights and welfare, anthrozoology, and alternative methods to the use of animals in education. Different loan systems for alternatives were used.

Results and Discussion: The twenty teachers had a fruitful “hands on” experience, and became the ambassadors of a long due change in mentalities, and attitudes. Their relationship with computers, and multimedia changed. These are now currently used as tools for a humane teaching. These teachers are demonstrating the powerful and encouraging network effect.
The use of animals in veterinary education is becoming a subject of a moral debate and is often opposed on educational and practical grounds. However, the experienced discomfort of the animals in relation to the purpose of their use should play a major role in this debate. For example, the grade of discomfort will be different for animals used for practising handling skills or for surgical training.

Many alternatives have been developed and are already in use in veterinary education. However, would it be feasible and desirable to replace all experimental animal use in veterinary education?

The debate on the use of animals in veterinary education should include the question who benefits:
1. the animal patients.
2. the animal owners.
3. the veterinary students.

When the latter appears to be the case, students should at least have a mandatory training in ethical aspects of the use of experimental animals and in the application of the 3Rs.
The veterinary profession has its origins in agriculture, assisting farmers to maximise the production and profitability of their animals. In developed countries increasing social affluence has allowed expenditure on companion animals to the point where the majority of contemporary veterinarians work almost entirely with these species. Social attitudes towards animal welfare have similarly developed and are reflected in the evolving attitudes of veterinarians, assisted by the marked feminisation in the last decade of a previously male-dominated profession. Nevertheless, our surveys of the world’s leading national veterinary associations reveal that official veterinary positions lag behind those of the general public on a range of important animal welfare issues, including the close confinement of veal calves in small crates, of laying hens in “battery cages”, and of pregnant sows in gestation crates. Formal veterinary education is the factor most responsible for these shortcomings. Although humane alternatives are being introduced, harmful animal use in surgical and pre-clinical training remains commonplace in veterinary courses worldwide, and studies have demonstrated that veterinary students are likely to view animals as being less sentient towards the end of their veterinary education, suggesting a process of desensitisation. Animal welfare, bioethics, critical reasoning and related topics comprise a very small part of most veterinary curricula. The “Concepts in Animal Welfare Syllabus” launched in 2003 by the World Society for the Protection of Animals and the School of Clinical Veterinary Science at the University of Bristol was created to address these shortcomings. It provides training in critical reasoning skills and education about a range of animal welfare issues, including farm and companion animal welfare, wildlife, animals used in experiments, and alternatives, which replace, reduce and refine animal use in research and education.

Poster

Invitrotrain: Training courses on alternative test methods for the hazard identification of chemicals

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The Invitrotrain project covers the development, validation and – most importantly – the demonstration of in vitro methods for testing of chemicals and prediction of toxicity. In the framework of five training courses established for scientists in the field of toxicology, pharmacology, and chemistry, multiple alternative (non-animal) methods will be educated at the bench and general aspects of the replacement of animal testing by in vitro procedures for the identification of hazardous properties of chemicals will be addressed by lectures. Special focus is placed on the performance of scientifically validated in vitro methods which are accepted in regulatory toxicology and the statistical evaluation of the test results. The aims of the training courses are to provide the attendees with sufficient experience, so that they may apply the techniques to their own needs and to disseminate the use of in vitro alternative methods.

The training courses will address multiple alternative methods:
- skin corrosivity and phototoxicity
- penetration models
- acute eye toxicity
- reproductive toxicology
- ecotoxicology

The courses will take place biannually (February and July) at the Freie Universität Berlin, Institute of Pharmacy.

The project is sponsored by the European Commission (EFRE 20002006 2/15
Previously the significant reductions that can be obtained by thinking carefully about what a researcher is trying to achieve, and then devising experimental designs to achieve that objective efficiently, have been demonstrated.

This talk will outline some educational strategies that have been used to try and disseminate this message and convince scientists that specifying clear objectives can reduce animal use. It will also discuss how to assess their success.

Educational material has been developed that enables a group of scientists of varying backgrounds to analyse the essential aspects of a research problem. This has been used mainly in group work to improve objective setting through debate, and to enhance the participants’ ability to plan a programme of work.

This leads on to consideration of different types of experimental design with a facilitator knowledgeable in this field, and discussion of the most efficient in each circumstance.

Another approach suitable for larger groupings is to give opportunity for the groups to study flawed designs and to provide comments that a publication referee might make on the designs, then in a plenary session give guidance on how the designs could be improved to give better use of animals.

Some skills in reduction by good design may be imparted by these sessions but the main aim (apparently achieved) is to foster a willingness to explore the possibilities for more efficient animal use.

In discussion of reduction through good research strategy ECVAM Workshop 29 (ATLA 26, 283, 1998) looked at an approach which began with specifying the experimental questions, then making testable hypotheses from these, and distinguished confirmatory experiments which tested hypotheses from exploratory ones which could produce data on which a hypothesis might be constructed. This approach could offer a way of evaluating published work for efficiency in animal use and for referees and editors to comment on scope there may have been for reduction in the experiments presented for publication. However the usual layout of a biomedical paper does not lend itself to assessment along these lines. In a random selection of papers from quality journals, the experimental question was unclear in over half the experiments reported making it difficult for the reader to criticise the efficiency of the design used, and there were many flaws in analysis or presentation that had escaped editor and referee scrutiny.

This talk will consider how to persuade editors and referees that reduction is worth pursuing and that they could help promote reduced animal use by insisting on presentation in a way that allowed a proper evaluation of the efficiency of the experimental approach used.
Lecture

Mainstreaming user-friendly curricula on alternatives for research scientists

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Alternatives have the potential to arouse interest and involvement when they are presented within the audience’s context of research interests. Targeted presentations designed and presented for specific courses, workshops, or symposia complement other information and profile the practical value of bibliographic searching techniques for accessing information on alternatives. Courses at UC Davis where we routinely offer presentations include: for veterinary students, Mouse Behaviour and Biology (website: http://www.vetmed.ucdavis.edu/Animal_Alternatives/phr408-Mice.html); for graduate students, The Mouse as an Experimental Model for Human and Animal Diseases; for undergraduates, Introductory Companion Animal Biology; and, for junior medical and veterinary faculty, Mentored Clinical Research Training Program. We offer workshops each year for veterinary laboratory animal residents from the California National Primate Research Center and the Center for Laboratory Animal Science. Workshops also are delivered on a tutorial, hands-on basis for visiting veterinarians and librarians, including USDA Animal and Plant Health Inspection Service Preceptor Veterinary Fellows each year. These small groups are instructed on-site. For symposia on emerging techniques, we developed presentations on new methods of imaging (website: http://www.vetmed.ucdavis.edu/Animal_Alternatives/imaging.html) and cell culture and explants (website: http://www.vetmed.ucdavis.edu/Animal_Alternatives/cell.htm). Each presentation is targeted to the particular users and their current topics of attention. Special web pages are prepared and configured that users can access. This method of instruction offers support to users in efficient searching within their context of the course material or work setting, such that the alternatives curricula supplement their needs.

Poster

Focus on alternatives: Recent initiatives in the UK, working together to replace animal experiments

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Focus on Alternatives brings together representatives from British non-profit organisations which fund the development, or promote the acceptance, of methods that replace the use of laboratory animals in research, education and testing. Organisations currently represented on FoA include Dr Hadwen Trust, FRAME (Fund for the Replacement of Animals in Medical Experiments), The Humane Research Trust, Lord Dowding Fund, RSPCA, St Andrew Animal Fund and UK Human Tissue Bank (UKHTB).

The strategy taken by FoA is to work by lobbying, facilitating access to information, educating animal users, and by organising workshops and meetings on specific topics of concern.

Current initiatives include:
1. Human Volunteers for Research Testing
2. Serum-Free Media for Cell Culture
3. Donation of Human Tissue for Research
4. A Workshop on Septic Shock Research

The aim of these initiatives and their current status will be reported.
Poster

**Rat uterus in vivo as an alternative to adult bovine female for the study of pathogenesis of immunological infertility**

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Introduction: Pathogenesis of immunological infertility in bovines can most appropriately be studied in a bovine female. High procurement and management costs are limiting factors. Thus, suitability of rat to act as a “biological incubator” representing a “simulated repeat breeder cow” harbouring high titres of antisperm antibodies was considered.

Methods: Bovine sperm xeno-immunised rats (n=6) were challenged by intrauterine infusion of bovine sperm. Sperm motility, viability, acrosomal integrity in the uterine flushings and tissue reaction were studied in these rats against non-immunised controls (n=6) at 15 and 30 min of incubation.

Results and Discussion: In the xeno-immunised rats, sperm motility, viable sperm percentage and sperms with intact acrosomes reduced dramatically. Vigorous phagocytic activity and spermophagy was also evident compared to non-immunised controls. Macroscopically, xeno-immunised uterine horns showed congestion, turgidity and increased oedema compared to no appreciable changes in the non-immunised rats. Histopathologically, Arthus reaction was observed in the immunised rats evidenced by congestion and degenerative changes in blood vessels with thickening of vascular wall and fibrinoid necrosis whereas, non-immunised rats, showed only occasional erythrocyte clumping and vascular oedema. Thus, a model of the pathogenesis of immunological infertility with respect to events like immobilisation of the sperm, inhibition of sperm migration through the female genital tract, and possibly, inactivation of acrosomal enzymes presumed essential for fertilisation, and inhibition of sperm attachment to and penetration of ova, could be explained.

Poster

**Norwegian veterinary training based on animal alternatives**

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¹NOAH for Animal Rights, Oslo, Norway; ²Norwegian School of Veterinary Science, Laboratory Animal Unit, Oslo, Norway

This presentation describes the steps taken by a Norwegian veterinary student to complete her veterinary education using alternatives to laboratory animals. This included the use of computer simulations, student self-experiments in physiology, dissections on superfluous material from the pathology department and naturally dead animals, and surgical training through beneficial procedures in veterinary clinics.

The presentation will also discuss the various ethical issues involved and the range of attitudes that students, teachers and veterinary schools in general must tackle when planning clinical teaching and training that may involve animals or animal material. The merits of providing courses without animal material for conscientious objectors, or alternatively phasing out animal use for an entire student class regardless of individual views, will be discussed.

Possible alternatives to laboratory animal use, and beneficial or neutral work with animals, will be described, building upon the authors experiences with databases such as NORINA (http://oslovet.veths.no) and organisations such as InterNICHE (http://www.interniche.org).

The Veterinary School in Oslo has a standing committee on the use of animals in teaching. The work of this committee will also be described.
With the rapid increase of transgenic mice, there has come an increase in their exchange between laboratories. In one sense this welfare concern is no different from the welfare of any mouse no matter whether or how it has been modified, e.g. by genetic engineering, or natural or artificial mutation processes. This movement of animals has meant that receiving laboratories are very likely to be inexperienced in looking after a particular “strain”, its performance and its phenotype. We started to develop mouse “passports” in 1998 so that receiving laboratories could be provided with some form of benchmarking in order to help them make better welfare assessments of the animals. These passports built on our existing “score sheets” we normally use for animals undergoing experiments that are based on clinical signs that an animal may show. However, these sheets go further than that inasmuch as they provide photographs and videos (when useful) of the phenotype and also any abnormal post-mortem changes. They record factors such as a description of the strain its origin, and benchmarking of its reproductive performance. They record any defect, its incidence, prevalence, age of occurrence, any treatment or alleviation or strategies to avoid or ameliorate. The details are for scientific staff but more importantly for the animal care staff who need to know what to expect in terms of clinical signs, performance, when to expect it, the number of animals likely to be affected, what to do about it, how to prevent it, and so on.

Poster

Mouse passports to refinement

David Morton¹ and Karen Helliwell²
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In the spirit of the Three Rs we developed a web based self paced course that will provide the tools to practice the most humane science. The course demonstrates that humane science is the best science, and raises the question – “if one is not practicing humane science, is their research compromised?” This course, available at http://caat.jhsph.edu, comprising 12 lectures, each about 30 minutes long will change the way the most fundamental aspects of animal-based research are practiced. The lectures are supported by discussions of the content with faculty. Some examples of what course participants learn include: Using the wrong experimental design wastes resources, time and animal lives; humane endpoints allow achievement of experimental endpoints while minimising or eliminating pain, distress and discomfort to animals; non invasive technologies minimise animal pain and distress and allow collection of high quality data from fewer animals; and enrichment addresses the question when is enrichment not appropriate? The course is designed to enhance ones research contributions and help identify replacement alternatives when possible, or allow implementation of reduction and/or refinement alternatives when replacement is not possible. Thus, the course encourages the practice of the most humane science. We envision that the course will help internalise the Three Rs and the ethic of preventing harm as guiding principles in the conduct of animal-based studies. Further, it will demonstrate that taking appropriate care of research animals is both a benefit to the broader public attitudes towards animal research and to individual scientific aims.

Poster

Enhancing humane science: Improving animal welfare

James R. Owiny and Alan Goldberg
Johns Hopkins University, Comparative Medicine, Baltimore, USA
Poster

New strategies for experimental zoology

Santi G. Pal
Dept. of Zoology, University of Calcutta, India

For years many designs of experimental zoology were utilised to record and to repeat observational data, using many different forms of small-sized invertebrate organisms. Often the latter were processed or treated in a destructive manner.

There is evidence to suggest that many different kinds of experiments have resorted to using invertebrate organisms which carry the eggs, eggmass or young offspring in their abdomens. For instance, isopod land crustaceans carry their young in their marsupium. These life stages require careful manipulation during the execution of a detailed and rigorous destructive experiment, particularly when soil pollution is involved soil pollution. There are similar other examples, in which insects or molluscs undergoing vitellogenesis or in a state of mating and copulation are treated with chemicals, hormones or other interferences. Such operations should be rationalised and unnecessary handicaps on the insects or molluscs should be avoided as far as practicable. Smaller invertebrates are exceedingly important models for studies in genetics, cell biology, developmental biology, neurobiology, ethology and other new emerging areas. The former would likely provide enormous data to such segments of science if experimentators seriously adhered to the principles of welfare and wellbeing of such important and key organisms.

Conceptually, each biological species represents an icon of evolutionary adaptation against an environmental pattern. This includes insults from nature as well as from human interference. Now, finally, more prudence should be exercised while planning zoological experiments for the collection of data for a variety of disciplines. The state of the art needs pruning and dissection.

Lecture

Animals and alternatives in biomedical education in Baltics

Osvaldas Ruksenas
Vilnius University, Biochemistry-Biophysics, Vilnius, Lithuania

Scientific environment in all three Baltic countries – Estonia, Latvia and Lithuania – during post-soviet period is comparable therefore review of situation with use of alternatives in biomedical education is based on data from Lithuania.

Use of animals for various biomedical purposes has decreased substantially during the last decade in Lithuania – from 21,000 animals in 1996 to 8,000 in 2004 in total and from 7,000 to 1,400 for teaching particularly. There are several reasons for this decrease: I) Introduction of legislation regulating use and care on laboratory animals; II) demand from students for the use of alternatives in teaching; III) increasing number of available alternative methods and equipment; IV) increasing economy enabling purchase of alternative teaching materials. However, there are some factors limiting more rapid increase in the use of alternatives in education – most of them are relatively expensive, in many institutions of higher education there is lack of computers and modern audio/video equipment necessary for implementation of alternatives, relatively large number of students still have difficulties with foreign languages and this restricts their access to materials in these languages. One of the most attractive and acceptable alternatives to the use of animals is self-experimentation.
Scientific papers are published in a wide range of journals that do not necessarily focus primarily on the Three Rs. The purpose of publishing is not only to report scientific results, but also to enable others to evaluate both the scientific and ethical validity of the work conducted. Advances within the Three Rs made during the course of research should be easily detectable for those searching the scientific literature. This imposes clear responsibilities on authors and journals alike, since they are often operating under pressures of time and space. These pressures must not, however, prejudice the dissemination of new knowledge within the Three Rs which other research groups expect to find when using the scientific literature to plan their own experiments. This presentation describes ways to promote the spread of advances within the Three Rs, highlights the pitfalls to avoid when publishing, gives links to guidelines that may be of help when writing scientific papers, and illustrates using specific examples how animal experiments have in fact been reported in recent years. A comparison between papers reporting experiments on traditional mammalian species and those using fish species will also be presented.

Educational experiences with pets and wildlife can facilitate the development of positive attitudes toward animals, as well as help children learn about themselves and their place in the world. Unfortunately, the use of live animals in schools is unregulated and lacks standardised guidelines; in non-formal education programs such as 4-H, oversight policies for live animal projects are highly variable and difficult to enforce. Furthermore, logistical, ethical, and economic restrictions limit opportunities for many youth to interact with live animals. Therefore, educational interventions that utilise alternatives to live animals present an important resource for educators in schools and community-based programs. This paper presents innovative, research-based interventions that utilise alternative approaches to teaching elementary school-aged children about animals. One curriculum, Animal Ambassadors, uses no live animals in its instruction; hands-on materials, including rubber foot molds, plaster tooth casts, and imitation animal coats are organised into learning kits that accompany printed materials. Animal Ambassadors supports state and national science standards and is applicable for schools and community-based programs. Data will be presented that demonstrate the positive effect of the Animal Ambassadors curriculum on children’s knowledge of, and attitudes toward animals, as well as on science process skills. Other interventions to be discussed include Animal Science curricula for common agricultural species (sheep, swine and rabbits) that have been designed to be effective with or without the accompanying use of live animals. These curricula also support state and national science standards and were designed principally for use by 4-H Youth Development Programs.
Lecture

**Education system in Japanese research laboratories for animal experimentation in a global pharmaceutical company**

*Makoto Suzuki*

Comparative Medicine and Biology Support, Pfizer Nagoya Laboratories, WWSS, Chita-gun, Aichi, Japan

Research laboratories of pharmaceutical companies have their original education systems for the researchers involved in animal experimentation as a global base. Depending on relevant laws and/or regulations, there are some inconsistencies or variations in each system among countries. We, Pfizer have several laboratories in the USA, UK, France, Belgium and Japan, which would lead to enable continuous exposure to the cutting edge sciences in the world. On the other hand, this causes some complexities in terms of unifying a scientific/technical education system for animal experimentations under the different situation in each country. In the case of Japan, there is the law/regulation concerning animal welfare and protection, which focused the care and use of laboratory animals from the scientific and humanitarian standpoints. Since it is not clearly presented about the practical implementation in the Japanese regulation, it is difficult to clearly make an interpretation and to practically indicate what kind of education system should be set up in each animal experimentation facility. Therefore, many laboratories in Japan including our Pfizer Nagoya laboratories have been investigating the appropriate system based on the self-imposed restraint manner complying with the governmental guidance/notification. The creation of an “Institutional Animal Care and Use Committee”, which mainly takes charge of education, would be the first approach in each facility, and it would come to the surface as an ideal model in time. This presentation refers to our present education system and future plan of Japanese laboratories as a global company, reviewing the difference of Japan’s laws/regulations.

Poster

**An international course on alternatives to animal use**

*Marc Teunis¹, Henny De Vos Burchart-Lodewijks¹, Jan Van Laake¹ and Coenraad Hendriksen²*

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With the intensifying demand for suitable replacements for animal experiments in the laboratory, the need for qualified technical and scientific personnel is steadily growing. Furthermore, it will become increasingly important to have specialised technicians to develop better and simpler models than animals for addressing fundamental scientific questions. In order to address this need we are developing a course on alternatives to animal use in life sciences research. EU and USA students engaged in bachelor-level education in life sciences can participate in this course.

The up to date program of this 6 month course includes many aspects of reduction, replacement and refinement. During the course, students will get acquainted with theoretical and practical aspects of physiology of the laboratory animal and with various Three Rs research areas such as: Risk assessment in toxicology and vaccine testing; humane endpoints; telemetry; databases, bioinformatics and computer modeling; animal welfare and housing; law issues; validation; genomics and proteomics; surgery and the use of models and simulators; cell culture models, and alternatives in education.

One important feature of the course will be the project Mission Alternative. Throughout the course participants will work together with institutions or industry to solve an existing problem or answer a scientific question, regarding alternatives to animal use. Currently, we are attracting lecturers from abroad to enhance the international character of the course. To facilitate participation for international students this course will be developed in close collaboration with the international office of our university and foreign contacts.
Lecture

Educating scientists on alternatives: A continuous process

Jan van der Valk

Already from the start of their study in one of the biomedical sciences should students become aware of the fact that animal experimentation is no more a matter of such and that in several instances Three Rs models can and should be used. During laboratory classes, whenever possible, animal free teaching models should be considered and used. Also, when it is regarded essential to have the students work with experimental animals, they should be aware of the consequences and the concerns of the society.

By Dutch law, every scientist designing animal experiments should be qualified. The Laboratory Animal Science course, where replacement, reduction and refinement (Three Rs) are the main themes, offers this qualification.

During the scientist’s career there should be continuous pressure to consider the Three Rs when animal experiments are planned. The most important one is the animal ethics committee requesting that Three Rs models have been considered before an animal experiments proposal is approved. This requirement and subsequent verification of an expert on alternatives ensures that scientists are (made) aware of possible Three Rs models in their field of interest.

Several journals now require a statement that the Three Rs have been considered and applied before a manuscript discussing animal experiments is accepted. Furthermore, several scientific organisations focus on the development, acceptance and information exchange of Three Rs models.

Education on Three Rs models should not be a one-time event, but a continuous process that makes scientists also aware of new developments that can be applied to replace, reduce and refine animal experimentation.

Lecture

The use of animals in research, testing and teaching in New Zealand – a legal perspective

Neil Wells
Unitec New Zealand, School of Natural Sciences, Auckland, New Zealand

Part 6 of the Animal Welfare Act 1999 (New Zealand) provides that a person may only use an animal for the purposes of research, testing or teaching if the person is a code holder or is authorised by a code holder through an animal ethics committee.

Legal provisions such as the Animal Welfare Act are not intended to be, nor should they be used as, an insurance policy. The concept of submitting animal use protocols through an animal ethics committee “just in case” is an abuse of legal process and involves both the applicant and the animal ethics committee in misuse of time and expense.

Part 6 of the Animal Welfare Act 1999 embodies the legal provisions of the 1984 amendment to the Animals Protection Act 1984 and practices that developed until 1998. Where Part 6 of the Act does not apply to a particular animal use Part 1 will apply. This paper focuses on determining which part of the Act applies to specific animal use in research, testing and teaching and provides a guide to determining when low level (in terms of suffering) use of animals in research, testing or teaching must or may not require animal ethics committee approval.
Poster

A survey of U.S. veterinary schools for alternatives to the use of live animals in teaching

Robert A. Willems and Diane Biederman
United States Department of Agriculture, Animal Care, Raleigh, North Carolina, USA

The purpose of this study is to survey the veterinary schools in the United States to determine how those schools are currently using live animals for teaching their students. Veterinary schools in the U.S. are registered as research facilities with the United States Department of Agriculture (USDA) as required by the federal Animal Welfare Act (AWA). Federal rules promulgated under the AWA require the schools to consider alternatives to painful procedures on animals used in teaching, employing the principle of the “Three Rs” of Russell and Burch (replacement, reduction and refinement). The surveys for this study were conducted by USDA veterinarians responsible for inspecting those schools for compliance with the AWA in their use of animals. The surveys cover the 5 year period from 2000 to 2004. Using data from the surveys, this study will attempt to determine how U.S. veterinary schools have replaced the use of live animals with alternatives in the various courses in their veterinary curricula. The study will show the types of veterinary courses in which live animals are being used for teaching, the teaching procedures in which these animals are used, the number and species of animals being used, and what alternatives to the use of live animals the veterinary schools in the U.S. have employed over the past 5 years. The study will also attempt to identify current trends in the use of live animals for teaching veterinary students.
Workshop 1.4
Multi-media exhibits of alternatives in education

Lecture
Humane endpoints in biomedical research: An interactive CD ROM

Iris Boumans and Coenraad Hendriksen
Netherlands Centre Alternatives to Animal Use (NCA), Animal, Science and Society, Utrecht, The Netherlands

The CD ROM “Humane endpoints in biomedical research” is an interactive program for educational and training purposes, aimed at increasing the awareness and competence regarding humane endpoints amongst those working with laboratory rats and mice. This CD ROM is intended to be incorporated into laboratory animal science training programmes and can be applied as an alternative in favour of the refinement of animal experimentation by any one.

Both normal and abnormal behaviour and signs of pain and distress are shown, as well as general clinical signs and clinical signs that are typical for a number of specific biomedical research areas (those in which animals may be subjected to severe suffering). An overview is given of parameters that can be used when applying humane endpoints. The information on “Humane endpoints in biomedical research” is accompanied by more than one hundred additional images and video clips. The information on pathology may facilitate the correct assessment of suffering, and taking measurements for follow-up experiments. Relevant laws, guidelines and reports are included too. Finally, the acquired knowledge can be assessed via a number of interactive tests.

The CD ROM will be available in Dutch and English. At the 5th World Congress on Alternatives and Animal Use in the Life Sciences, an opportunity will be given to view and explore this CD ROM.
A number of studies have demonstrated that interactive, multimedia computer-based learning programs are effective in meeting many of the learning objectives of classes which use animals in university teaching. Here we describe a number of programs, developed over several years, which simulate animal preparations frequently used to teach pharmacology and physiology to undergraduate students (www.sheffbp.co.uk). They have been developed by experts in the field, usually to support their own teaching, and may be used in a variety of ways e.g. to support conventional practical class teaching, or for self-directed learning by students. The programs generate simulated tissue responses e.g. muscle contractions or nerve action potentials, either from actual experimental results or from predictive models. Responses are presented on the monitor screen, in a form comparable to that in the real experiment i.e. storage oscilloscope or scrolling chart recorder. Students are expected to simulate many of the tasks associated with practical class teaching such as determining experimental parameters, collecting data in much the same way as they would in the laboratory, data reporting and communication. They work, usually in small groups, at their own pace and most take readily to this form of teaching. In many cases the programs use text and high-quality graphics to describe the preparation, the apparatus, methods and the underlying physiology and/or pharmacology. Some contain self-assessment questions or student-centred tasks to test accuracy of data collection, data interpretation, knowledge of underlying principles etc. Examples from a number of programs are used to illustrate these features.

Lecture

Interactive, computer-based alternatives to using animals in university teaching

David Dewhurst
University of Edinburgh, Learning Technology, Edinburgh, UK

InterNICHE has been working internationally to promote and implement alternatives in higher education for 17 years, facilitating the replacement of harmful animal use and building a broad network with contacts in over 50 countries. From the InterNICHE experience, successful international work requires qualities and practices from organisations that include: A bold and positive vision, a specific focus and an awareness of the links between issues; a commitment to pro-actively catalyse sustainable change and create win-win solutions; the design of organisational structures conducive to participatory democracy, alliance building and the organic growth of the network; the practice of solidarity and support for local initiatives rather than empire building; and the provision of resources and training for action and capacity building. The presentation will draw on examples of InterNICHE projects such as the production and multi-language translations of printed, video, and website resources; the Alternatives Loan System for trial of software, mannekins and simulators anywhere in the world; the international Humane Education Award for local development and implementation of alternatives, including freeware; support for student conscientious objectors; and conferences, outreach visits, and training in alternatives for teachers. The challenges met within such work will also be explored, and suggestions of how to overcome them will be given.

Lecture

Internationalising alternatives in higher education

Nick Jukes
InterNICHE, Leicester, UK
The Norwegian Reference Centre for Laboratory Animal Science and Alternatives has maintained a website since 1996, featuring the NORINA database (http://oslovet.veths.no). NORINA contains information on nearly 4,000 audiovisual aids and other materials that can be used as alternatives or supplements to animal use in teaching and training. The website also includes TextBase, a database with information on 1,100 textbooks of relevance to the 3Rs. The website has been totally rebuilt in 2005. The databases are now linked to the other textual information on the website, with many new features. The site includes information on guidelines for animal research, the care and use of fish, current legislation, course material (including three compendia), links to databases within the 3Rs and a Virtual Tour of the Centre. The website has now an additional shorter Internet address: www.norinadatabase.org.

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The European Resource Centre for Alternatives in higher education (EURCA)

Marjolein van Boxel¹, David Dewhurst² and Jan van der Valk¹

¹Netherlands Centre Alternatives to Animal Use, Animals, Science and Society, Fac. of Veterinary Sciences, Utrecht University, Utrecht, the Netherlands; ²University of Edinburgh, Faculty Group of Medicine and Veterinary Medicine, Edinburgh, UK

Practical pharmacology, physiology, laboratory animal science, anatomy and dissection classes intend to teach students practical skills, as well as factual knowledge and procedures regarding data handling, experimental design and communication. In many curricula animals are used, even though animal-based classes are resource and time intensive, require technical support, equipment and notably animals. Use of animals, certainly for education purposes, is questioned nowadays. There is a widespread availability of non-animal alternative models, such as computer-based simulations, static and interactive video, post-mortem material and in vitro methods. These non-animal models are often less expensive, certainly in the long run. In the context of Russell and Burch’s 3R principle, application of alternative models contributes to the reduction of animal use. Moreover, several studies have demonstrated that the effective knowledge gain is equivalent to that of animal classes. Additional advantages of many alternatives include their suitability for tutor-independent training, possibilities for self-assessment and the inherent combination of theoretical and practical components. The choice for the adequate model to use depends on individual tutors to clearly define learning objectives. The European Resource Centre on Alternatives in higher education has an on-line database aimed at helping teachers making a well-balanced decision. The EURCA database (http://www.eurca.org) offers extensive information on high-quality peer-reviewed models. Furthermore, EURCA demonstrates models from its database and offers advice to teachers at national and international meetings.
Guidelines for the housing and husbandry of animals used in research and teaching provide research establishments, Institutional Animal Ethics Committees (AECs), practitioners, government regulators and the public with benchmarks against which housing and husbandry practices can be compared. Guidelines that are regularly reviewed to include current knowledge provide evidence for and documentation of good contemporary practice. In New South Wales (NSW), the Animal Research Review Panel (ARRP), a statutory body appointed under the NSW Animal Research Act, is developing species-specific guidelines for the housing and care of laboratory animals and, to date, has published guidelines for dogs (1999), rabbits (2003) and rats (2005). Utilising resources of the NSW Department of Primary Industries and recognised external authorities on particular species, the ARRP commissions an exhaustive search of published literature relating to the behaviour, husbandry and care of the species of interest. Information is collated on enclosure design, care and management, social needs, environmental enrichment, nutrition, and environmental variables such as lighting, temperature, humidity, ventilation and sound. Recommendations are listed for each topic. Draft guidelines are circulated for three months to all accredited animal research establishments for comment, consultation and emendation and advice is sought from members of AEC’s, animal house managers, animal technicians and researchers. Further, expert comment is sought from international authorities. After consultation, the document is amended, and posted on the website – Animal Ethics Info-link – www.animalethics.org.au. The acceptance of aspects of housing and care which have been recommended to promote species-specific needs will be reviewed.
Comprehensive phenotyping means that a statistically valid sample of animals is subjected to a battery of clinical, anatomical and neurological tests to characterise the phenotype of the strain. Some types of comprehensive phenotyping are sensitive enough to distinguish between different inbred strains of mice. Comprehensive phenotyping is important to detect the subtle effects transgenesis can have on the phenotype of genetically modified (GM) animals. This in turn provides information that helps to both optimise the assessment of GM animal wellness and to establish appropriate endpoints for the GM strain. Neonatal experiences (such as maternal behaviour and the cage environment) can strongly influence the resultant behaviour of offspring. This is of particular concern for GM mice that will be used for phenotyping. Since appropriate environmental enrichment promotes the expression of normal behaviour, reduces variability between animals, and promotes breeding success, this refinement could be particularly important to ensure accurate, statistically valid phenotyping using the least number of animals. We are interested in exploring the literature on the impact the neonatal environment has on GM animal behaviour since environmental enrichment in mouse breeding colonies represents a significant opportunity for refinement for large numbers of animals.

**Poster**

Implications of the neonatal environment on comprehensive phenotyping of genetically modified mice

*Julie Comber and Gilly Griffin*

Canadian Council on Animal Care, Guidelines Development, Ottawa, Canada

Comprehensive phenotyping means that a statistically valid sample of animals is subjected to a battery of clinical, anatomical and neurological tests to characterise the phenotype of the strain. Some types of comprehensive phenotyping are sensitive enough to distinguish between different inbred strains of mice. Comprehensive phenotyping is important to detect the subtle effects transgenesis can have on the phenotype of genetically modified (GM) animals. This in turn provides information that helps to both optimise the assessment of GM animal wellness and to establish appropriate endpoints for the GM strain. Neonatal experiences (such as maternal behaviour and the cage environment) can strongly influence the resultant behaviour of offspring. This is of particular concern for GM mice that will be used for phenotyping. Since appropriate environmental enrichment promotes the expression of normal behaviour, reduces variability between animals, and promotes breeding success, this refinement could be particularly important to ensure accurate, statistically valid phenotyping using the least number of animals. We are interested in exploring the literature on the impact the neonatal environment has on GM animal behaviour since environmental enrichment in mouse breeding colonies represents a significant opportunity for refinement for large numbers of animals.

**Lecture**

Mandated environmental enrichment in rodents: Possible positive and negative consequences for research

*William T. Greenough*

Beckman Institute, University of Illinois at Urbana-Champaign, Urbana, USA

Developed as a research paradigm to study the effects of experience, and particularly of learning, upon the brain, enrichment for laboratory rodents has come to be viewed in some quarters as a potential method for enhancing animal well-being in the laboratory. Remarkably little research has attempted to measure “well-being” in a quantitative manner, although appropriate measures (e.g. of chronic stress levels) are certainly possible. The term “enrichment” presupposes the positive nature of such manipulations, such that I propose the use of the term “housing supplementation” as a more neutral description of the addition of inanimate and possibly animate elements to the housing environment. Changes that add complexity and perhaps cognitive and motor challenges to animals’ environments can have dramatic effects on brain structure, gene expression and physiology as well as behaviour, including recovery from illness and injury. Here I summarise research regarding the effects of supplemented housing upon brain, peripheral physiology, and behavioural measures. In general, addition of supplemental elements beyond food, water and bedding to the housing environment is associated with changes in synapse structure and number, closely coupled macroglial changes, such as increased axonal myelination and astrocytic ensheathment of synapses, and capillary volume in various cerebral cortical and extracortical brain areas. Behaviourally, animals in supplemented environments perform better in complex, appetitively-rewarded tasks. Measurements of indices of chronic stress such as adrenal weight show little difference among groups reared in individual, social, or social-supplemented housing, although individual differences in response to stress are evident in all three housing conditions.
Lecture
Importance and effects of enrichment on physiology and behaviour in mice

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Institute for Animal Welfare and Behaviour, Veterinary School Hannover, Hannover, Germany

The phenotype of an individual is formed by its genotype and environment. The heritability scales the genotype influence on the phenotype, thus as lower the heritability as stronger is the influence of the environment. For quality and welfare reasons (reduction) laboratory animal science focused the last decades on reducing the variability of experimental results by standardisation. Genotype (inbred strains) and environment (hygiene, diet, climate, housing) were standardised, thus the number of animal used for experimental purposes could be reduced in most countries within Europe. As welfare concerns are increasing it seems to be necessary to evaluate the necessity of changing the common standard housing of laboratory animals according their needs. Such changes are summarised by the concept of Environmental Enrichment. This term itself is confusing, as the common standard housing of laboratory animals already contains environmental enrichment items such as bedding, nesting material, social groups. So the question is, which other environmental changes are suitable and necessary. Many studies showed that changes of the environment (enrichment) will change the phenotype including the behaviour of laboratory animals. Some studies also show that a change of the environment can change the mean and often increases the intra- and inter-individual variability of phenotypic variables, thus finally more animals may be necessary to obtain valid results. Therefore changes of the environment need to be balanced against an increasing number of necessary animals. For this balance it is prerequisite to know the importance of enrichment for the animal, which can be estimated only in consumer demand and not in simple choice experiments.

There is no doubt, that housing which results in stereotype behaviour needs to be changed, like the stereotype digging behaviour of gerbils shows a clear deficiency of standard housing. This stereotype behaviour can be reduced by adding a burrowing system.

For other changes of the environment it is necessary to know the objective beneficial effect for the animal as well as the effect on the experimental result (mean and variance). Only when these two effects are known, the benefit for the animal can be balanced against the influence of the experimental result.

Poster
Using telemetry to study physiological and behavioural parameters in “companion-housed” adult male mice

Hans Peter Kaesermann, Andreas Rettich, Kurt Buerki and Margarete Arras
University of Zurich, Institute of Laboratory Animal Science, Zurich, Switzerland

Housing laboratory mice in stable, compatible groups allows them normal social behaviours. Adult males, however, tend to fight and are therefore frequently housed individually. Social isolation induces distress affecting physiology and behaviour. As compromise, two males are kept in one cage, but separated within by a grid divider, which allows indirect social contact by vision and smell but prevents fighting. The aim of the study was to investigate the influence of such housing on some physiological and behavioural parameters related to the welfare of an animal.

Before starting experiments, in 16 adult male NMRI-mice a telemetry system was implanted, measuring heart rate (HR), body core temperature (BT) and locomotor activity (ACT). These mice showed no significant differences in body weight, food and water intake as well in HR, BT and ACT being alone or with a female as companion behind the divider.

In contrast, when an adult male served as companion, these mice showed an increase in water intake and a decrease in body weight, whereas food intake was unchanged. Additionally ACT and BT were significantly increased. HR showed the most prominent effect: A constant and significant increase over 10 days which did even not return to baseline the next 8 days, although the male companion was removed.

In conclusion, dividing a mouse Type3 cage, allowing limited social contacts between animals, may beneficially influence the well-being of an individually housed male mouse. But it seems important which gender is selected as companion, otherwise contrary effects on welfare may occur.
Lecture

Refinement alternative for animal housing-enrichment

Timo Nevalainen
National Laboratory Animal Center, University of Kuopio, Kuopio, Finland

Revised Appendix A of the Council of Europe Convention calls for environmental enrichment and group housing for all gregarious species unless there are scientific or veterinary reasons not to do so. Enrichment is considered as refinement as it should promote animal wellbeing. Interference with an experimental outcome could be a scientific reason, and fighting between incompatible animals a veterinary reason for not implementing enrichment. Any refinement to improve animal welfare requires scientific validation to ensure it is truly beneficial to animals (efficacy) and does not detract from the scientific integrity of the study (safety). The outcome may simply be a change in the mean, and this may not matter as it should affect all groups, but changes in variance will lead to more animals being used, itself an ethical issue. While refinement aims can mostly be connected to research data, reduction alternative suffers from lack of research to base regulations on. It is obvious that changes in variance may be strain-, facility- and enrichment-specific, which makes overall guidelines difficult. Indeed, instead of trying to assess impact of enrichment on every determination, it could be more productive to look at effects on variance of welfare indicators with the understanding that low variance there is likely to show as low variance in other determinations. And at the same time aim at most uniform welfare of the animals in the study. COST Action B24 is a new scientists’ network focusing on both efficacy and safety of animal housing, including environmental enrichment.

Poster

Harmonisation of the care and use of fish in research

Adrian Smith, Renate Johansen and Gunvor Knudsen
Norwegian School of Veterinary Science, Laboratory Animal Science, Oslo, Norway

This presentation gives a report from an international consensus meeting held in Oslo in May 2005. The meeting is part of work conducted at the Norwegian School of Veterinary Science in collaboration with the authorities to establish a National Platform for Alternatives along the lines of the model developed by ECOPA (http://ecopa.vub.ac.be). Fish account for 95% of all research animals used in Norway. The current revisions of the Council of Europe’s Convention ETS 123 and the EU Directive 86/609, and in particular the new guidelines on the care and use of fish, have created an acute need for the exchange of current knowledge on fish welfare. There are many areas requiring more research before a consensus on best practice can be reached. Researchers spent three days discussing key topics such as welfare and ethics, pain assessment, anaesthesia and analgesia, health monitoring, handling and procedures, reporting fish experiments and guidelines for implementing the Three Rs in fish research. Ways of extrapolating from experience with the care and use of terrestrial animals to fish research were also explored in detail. A wide range of fish species, living in very different environments, were discussed during the meeting. A website containing all the reference material from the meeting has been established and will be further developed in the future: http://oslovet.veths.no/fish.
The effects of single versus pair housing were investigated on behaviour and physiology of 24 male Sprague-Dawley rats. Animals were housed on a reversed light cycle in minimally enriched environments either alone (n=8) or as a pair (n=16) over a 10 week period. Body weight (BW) and food consumption were measured weekly, and liver, paired adrenal gland and brain weights were collected at necropsy. Locomotor activity over a 30 minute test period was measured following administration of saline at three timepoints throughout the study or a stimulatory dose of heroin (0.3 mg/kg) at the end of 10 weeks. Faeces (light and dark cycle samples) were collected at 3 timepoints throughout the 10 week period (baseline, mid-study and 10 weeks) for faecal corticosterone evaluation 24 hours following exposure of each rat to a novel environment for 30 minutes. Faecal corticosterone levels were determined by ELISA. At necropsy, minor bite wounds were noted on 3 of the pair-housed animals. There were no differences in BW, food consumption, or liver:BW ratios between single or pair housed animals. Paired adrenal gland:BW ratios were significantly increased and brain:BW ratios were decreased in singly housed rats. Singly housed rats appeared to be more sensitive to the stimulatory effects of heroin, however, no differences were noted at any time in faecal corticosterone levels between single vs. pair-housed animals. Our results suggest that housing paradigm does not affect rat response to a novel environment; however, housing paradigm may affect rat responsiveness to stimulants and may alter specific physiologic processes, leading to organ weight variations.
Pain of low and middle grade is difficult to detect in laboratory mice because mice are prey animals that intend to protect themselves from their predators by hiding signs of weakness, injury and pain. Therefore, telemetry was used to exclude the influence of the investigators presence with the aim, to identify indicators of low to middle-grade post-operative pain. Male mice bearing telemetric transmitters were subjected to vasectomy, either without pain therapy or with the application of two different analgesic regimens. Postoperatively, all the animals exhibited no overt signs of pain. The telemetrically measured locomotor activity behaviour remained stable, which confirms the intended stage of low/middle-grade pain and the absence of intolerable pain. Body core temperature showed negligible increase, suggesting, that post-surgical inflammation was of no influence.

The only group of animals showing significant changes of the hearts actions (heart rate, heart rate variability), that point to pain and sympathetic activation were the animals with no analgesic treatment. For these animals also the food intake was significantly diminished and body weight was slightly reduced.

Both analgesic regimens were able to prevent any changing of heart actions and also the post-operative food consumption and body weight were unchanged when animals received pain therapy.

The results show, that i.) the method was able to identify signs of low to middle grade pain in mice by telemetry, which could not be clearly detected otherwise in this species ii.) analgesic regimens acted successfully in relief of low to middle-grade post-operative pain in mice.
Most objections to animal experimentation concern the experiments themselves. This paper addresses two aspects of laboratory animal welfare separate from the experiments: 1) housing conditions, and 2) routine procedures. Ninety published studies were reviewed to assess the effects of standard laboratory housing conditions on the behaviour of rodents, particularly mice and rats. Preference studies show that mice and rats value opportunities to take cover, build nests, explore, forage, and gain social contact, behavioural needs that are often thwarted by institutional laboratory housing systems. We also reviewed eighty additional studies to assess the potential stress associated with three routine laboratory procedures: Handling, blood collection, and gavage (force-feeding). Pronounced and significant changes in stress indicators (e.g. concentrations of corticosterone, heart rate, blood pressure) occurred for all three procedures, indicating fear, stress, and/or distress. These literature reviews depict a life where chronic lack of stimulation is exacerbated by regular stressful episodes. Resulting physiological (e.g. stunted brain development) and behavioural symptoms (e.g. stereotypies) compromise both scientific and ethical integrity. Providing animals with naturalistic living environments where they can engage in strongly motivated behaviours, while not obviating humane concerns, is feasible and ethically desirable.

Poster

Major animal stressors overlooked in the laboratory environment

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This study was conducted in mice to examine changes in body weight and heart rate (HR, using telemetry) in response to restraint stress. Animals were weighed (controls) or weighed and then restrained for 1 hour each day for 14 days. Following humane killing, the vas deferens and tail artery were mounted in organ baths for concentration-response curves to noradrenaline (NA) and electric field stimulation (EFS). There was a significant difference between the two groups in body weight gain; restrained animals showed no significant weight gain; control animals gained ~3 g over 14 days. HR increased significantly during restraint on all days and remained elevated during the entire restraint period. A degree of habituation was observed, with these effects on HR being somewhat reduced by the end of the experimental period. In tissues from stressed animals, there was an increase in the maximum response of vas deferens to NA and concentration-response curves in tail arteries were significantly shifted to the left, indicating increased sensitivity of the tissues to NA. The EC50 in tissues from restrained animals was 2x10^-8M and in tissues from control animals was 1x10^-7M. Contractions in response to EFS (1 Hz and 10 Hz) in tail artery segments from stressed animals were also significantly increased. Thus, despite some apparent habituation of the heart rate response to restraint, underlying changes in sympathetically innervated tissues persisted.
Lecture

Workshop of international experts held on the definition, recognition, assessment and alleviation of animal distress in the laboratory

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Distress in animal research can be difficult to address and is often overlooked, despite legislative and regulatory mandates in the U.S. to minimise animal distress and pain. Consequently, on February 11-14, 2004, The Humane Society of the United States, as part of its Pain and Distress Campaign, held a workshop of international experts to discuss the state of knowledge on animal distress and whether it is possible to define and measure distress in operational terms for application to animal research. Although the U.S. Department of Agriculture has proposed to define distress, current US regulations do not define the term. The seventeen participants represented the fields of animal welfare, applied ethology, veterinary medicine, physiology, ethics, and animal protection. Workshop discussion included topics such as what an operational definition of distress should encompass; concepts of distress and suffering; causes, prevention, and measurement of distress; and incorporation of distress into regulation. The lack of definition of distress hinders progress toward a comprehensive consideration of laboratory animal distress in the U.S., which has consequences for both animal welfare and quality of science. The participants agreed that creating a meaningful and practical definition is a challenge, but this can be addressed by crafting a general description of what might constitute animal distress, supported by a set of specific examples. An executive summary produced from the workshop includes the description of distress agreed upon by the participants, as well as additional information and conclusions drawn from the workshop discussions.

Poster

Responses in mice to restraint of cage-mates

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Performing stressful procedures in view of cage mates may cause stress in observer animals. However, it is not known if stressful procedures in close proximity to, but not in view of cage mates is stressful for the (observer) cage mates. Radiotelemetry and post mortem in vitro studies of the vas deferens were used to determine the effects of stress on observers. Heart rate (HR), body temperature (T) and locomotor activity were recorded for 1 hour following weighing of a cage mate, or 1 hour during restraint of a cage mate and the hour following return of the restrained mouse to the cage. This procedure was repeated for 15 days. HR, T and activity were increased in observers of both restraint and weighing of cage mates. Analysis of the AUC showed that HR and T in observers were significantly higher during restraint of a cage mate than after weighing of a cage mate. When mice were returned to the cage after weighing or restraint, HR and T were significantly higher in the cage mates of restrained animals. Results from previous studies have shown that chronic stress causes the vas deferens to become hypersensitive to noradrenaline. In this study, vas deferens from observers of restraint had a significantly increased responsiveness to noradrenaline. These results indicate that stressful procedures should be conducted in isolation from other mice. Furthermore, they show that monitoring stress using a single parameter may not give an accurate indication of the stress experienced by experimental animals.
Halothane and ketamine are two commonly used anaesthetics. There have been numerous studies to determine the effect these anaesthetics have on hormone levels. For instance, ketamine increases prolactin and cortisol levels in rhesus monkeys, but has not been found to affect testosterone in cynomolgus monkeys. In rats, no effect was observed on thyroxine, triiodothyronine, oxytocin, or LH pulses. Halothane was also observed not to have any effect on testosterone, LH, and FSH in the rat, but did increase ACTH-like and corticosterone-like immunoreactivities up to 24 hours after exposure. A previous study in this laboratory found that testosterone levels in rat plasma were increased up to 24 hours after ketamine anaesthesia. This study investigates the effect of ketamine and halothane on male rat plasma testosterone and LHRH and will discuss the validity of using anaesthesia in experimental conditions when hormones are to be measured.
Lecture

Evidence based surgical analgesia in the laboratory mouse

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The mouse can replace larger laboratory animal species for the study of diseases and treatments. Although rodent models are widely used to study physiological mechanisms and treatment of pain, most clinical dosing of analgesics for laboratory mice is based on “best guess” extrapolations. There is lack of evidence to indicate the extent to which mice suffer from postoperative pain and for adequate treatment. If mice do suffer significant morbidity from pain, the physiologic effects of this may have an important impact on the research model being studied, as well as welfare implications. The authors have developed a model to test the hypothesis that pain or other morbid conditions related to surgery might impair mobility and food or water intake, and that effective analgesic doses, timed appropriately, might act to attenuate behavioural abnormalities in C57Bl/6 female mice. We will present a scientific evaluation of mice in a surgical pain setting, evidence that analgesics can improve weight loss and behavioural abnormalities following surgery, and contextual information from the rodent basic science literature that suggests strategies for surgical pain alleviation in mice.

Poster

Anaesthetic and analgesic management for mitral valve surgery in sheep

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Mitral valve grafts were implanted in six sheep (50-66 kg BW) during cardiopulmonary bypass via a left-sided thoracotomy.

After IM premedication (midazolam at 0.6 mg/kg, ketamine at 4 mg/kg), anaesthesia was induced IV (midazolam at 0.1 mg/kg, propofol at 4 mg/kg) and first maintained IV for surgical preparation (propofol at 10 mg/kg/hr). Oxygen was insufflated (5 L/min) via a cannula in the endotracheal tube. In the operating room, anaesthesia was maintained with isoflurane (end-tidal 1.2-1.4%) in oxygen, plus fentanyl (5 µg/kg bolus, 2-10 µg/kg/hr) and lidocaine (2 mg/kg bolus, 1.8 mg/kg/hr) IV. Intercostal nerve blocks (3rd to 8th intercostals spaces) were performed with lidocaine/bupivacaine (1/1 mg/kg) before the first incision. All sheep were heparinised (100 UI/kg) but not at all times antagonised (protamine). Ephedrine, dobutamine and norepinephrine were administered to effect to maintain arterial blood pressure. Respiratory support was provided and adjusted according to arterial blood gas values. Intrapleural bupivacaine was administered during closure of the thorax. Postoperatively, regular and frequent evaluations, using composite pain scores were performed and analgesic therapy adjusted accordingly (Intercostal nerve blocks, carprofen at 4 mg/kg SID, dexamethasone at 1 mg/kg BID, fentanyl infusion at 2-5 µg/kg/hr or buprenorphine at 15 µg/kg QID).

In view of their peri-operative cardiovascular stability, their calm recoveries and their low pain scores, it would appear that all sheep received adequate analgesia. One sheep showed considerable respiratory depression and prolonged recovery. This resolved after the administration of naloxone.

The anaesthetic and analgesic protocol used were suitable for cardiac surgery in these sheep. The use of pain scores allowed for satisfactory analgesic therapy in these animals.
Lecture

Controlled release of analgesics for rodents

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Analgesics are administered to laboratory animals to relieve pain due to surgical procedures. Opioids are the analgesic of choice for moderate to severe pain. Morphine, the prototypic opioid analgesic, is widely used in animals and provides excellent analgesia. Sustained analgesia requires repeated parenteral administration, an inherently stressful procedure. The objective of this study is to develop a controlled release gel formulation of morphine that can maintain analgesia in mice for 3 to 5 days following a single administration.

In vitro tests were conducted on 12 formulations of morphine in biodegradable polymer gel and five formulations were selected for in vivo pharmacodynamic testing. Eight experimental groups of 6 mice were subjected to tail flick analgesia testing. The groups were: morphine gel formulations A2r, A3, A3r, A5 and A7; blank gel control; negative control (no injection); and positive control (morphine suspension). The baseline tail flick latency was determined for each mouse, gel was injected subcutaneously, and the analgesic effect measured. Analgesic effect was similar in formulations A2r, A3, A3r and A5, lasting for 12 to 24 hours. The analgesic effect for formulation A7 lasted for 120 hours and was significantly higher (p<0.01) than the untreated control group. Analgesia from morphine suspension lasted 28 hours. There was no analgesic effect from the blank gel. Biodegradable gels with morphine can provide analgesia to mice for 5 days following a single subcutaneous injection. This methodology can enhance the treatment of rodents subjected to surgery or other painful procedures by eliminating repeat dosing and providing sustained delivery of drug.

Poster

The combined use of epidural analgesia and fentanyl patch for relief of post-operative pain

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The ethical use of animals includes the minimisation of discomfort, distress, and pain. Pain cannot always be evaluated easily in animals and one must assume that animals experience pain in a manner similar to humans, and therefore pain management appropriate to the species, the procedure, and the circumstances must be provided. One procedure which requires careful post-operative pain management is thoracic surgery (sternotomy and thoracotomy). There are many options available for management of post-operative pain after thoracic surgery including intermittent and/or continuous delivery of analgesia via intravenous, oral or intramuscular routes. However, these protocols require continued intermittent handling of the patient at a time of greatest patient sensitivity. Analgesia for 48 hours can be provided with minimal handling of animals post-surgically by the combined use of fentanyl patches and epidural anaesthesia.

This paper discusses a refinement of analgesia through the use of fentanyl patches combined with epidural analgesia (morphine, fentanyl or buprenorphine), for control of post-operative pain in dogs and pigs after thoracic surgery.
Lecture

Are 20 kHz ultrasonic vocalisations a reliable indicator of post-operative pain in rats?

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¹University of British Columbia, Animal Welfare Program, Faculty of Land and Food Systems, Vancouver, Canada; ²University of Newcastle upon Tyne, Comparative Biology Centre, Newcastle upon Tyne, UK

Our objective was to determine whether ultrasonic vocalisations (USVs) can be used as an objective measure of post-operative pain in rats. 40 male CD-IGS rats underwent either anaesthesia or anaesthesia plus surgical implantation of a cardiovascular telemetry device into the abdomen for an unrelated study. Within each treatment group, animals were given Parecoxib (i.v.) at 1 mg/kg, 5 mg/kg or 20 mg/kg pre-operatively, to produce a graded response. Pre-operatively and at hours 1, 2, and 4 post-operatively we recorded the number of 20 kHz USVs produced by isolated animals during a 10 min period. At hour 1 we also recorded the frequency of five behaviours (twitch, writhe, stagger/fall, back arch, belly press) that have previously been effective for pain assessment following abdominal surgeries, and produced a composite score. No animals produced USVs during the pre-operative recording. Post-operatively, 27% of control animals and 22% of surgical animals produced USVs during one of the three recording periods, and these USVs were not associated with the composite pain score. Overall, the composite pain scores were higher for the surgical group than the control group (Mann-Whitney test, U22, 18 = 320, p<0.001). However, the different levels of analgesic did not produce a dose-dependent response, possibly due to analgesic efficacy. Differences in USVs pre- and post-operatively suggests that 20 kHz USVs may be indicative of distress, but the lack of association with other pain scores demonstrates that they are not indicative of post-operative pain under these testing conditions.

Poster

Investigation for objective indicator of anaesthetic depth in laboratory animals: Visual evoked potentials for ketamine anaesthesia in mice

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In many research laboratories, various anaesthetics are used for anaesthetising mice. Whereas the animals can be reliably maintained for several hours to be anaesthetised with additional doses of these anaesthetics, the anaesthetic level is roughly monitored by somatic reflex, respiratory rate and heart rate. In the present study, we performed intermittent recording of visual evoked potentials as a reliable indicator for the depth of anaesthesia to make sure the level obtained by conventional ketamine-based anaesthetics, which are commonly used for laboratory mice.

Adult mice were anaesthetised with ketamine (80 mg/kg) and xylazine (20 mg/kg) and a monopolar stainless wire electrode was put on visual cortex. When the withdrawal reflex appeared, additional anaesthesia of ketamine only or a mixture of ketamine and xylazine were added. After this addition of anaesthetics, averaged VEP in response to stimuli of flashing LED light and the withdrawal reflex were monitored every ten minutes until the withdrawal reflex appeared again. In each mouse, the amplitude of VEPs was normalised relative to the VEP that was recorded just after the appearance of the withdrawal reflex.

The withdrawal reflex of the mice with ketamine and xylazine appeared later than that with ketamine alone. The relative amplitude of the mice with ketamine and xylazine was significantly lower than that of the mice with ketamine alone.

This result showed that the lower amplitude of VEP could reflect deeper anaesthetic stage and this method could be used as a reliable indicator for the depth of anaesthesia to reduce animal suffering.
Wherever animals are used in research, minimising pain and distress is as important and objective as achieving the experimental results. This is important for good welfare and for good science. In recent years, much attention has been focused on recognising and controlling the adverse effects of scientific procedures on animals, and also on the need to improve the environment in which laboratory animals spend their lives.

Significant and immediate improvements to animal husbandry and scientific procedures can be made in a number of ways. The Joint Working Group on Refinement was convened by the BVA(AWF) / FRAME / RSPCA and UFAW to ensure up-to-date information on good practice is available. The JWGR has a broad range of membership with representatives from science and industry, veterinary and animal welfare. The group has produced a series of reports setting out good practice for the following:

- Removal of blood from laboratory mammals and birds
- Refinements in rabbit husbandry
- Refining rodent husbandry: the mouse
- Refining procedures for the administration of substances
- Laboratory birds: refinements in husbandry and procedures
- Refinement and reduction in production of genetically modified mice
- Refinements in telemetry procedures
- Husbandry refinements for rats, mice, dogs and non-human primates used in telemetry procedures
- Refining dog husbandry and care

The poster to be presented at the 5th World Congress (on behalf of the organisations that form the JWGR) will provide further details including references and highlighting the availability for many of these reports to be downloaded for free from the “Laboratory Animals” website: www.lal.org.uk.

Refinement in animal experiments means to diminish pain, suffering and harm. The use of analgesics in experiments is applied refinement and an essential part of good veterinary care for laboratory animals.

Profound knowledge is needed to rule out the most effective pain management protocol by use of general anaesthesia, regional anaesthesia and systemic analgesics. This has to take in account the side effects of used drugs to physiological parameters, behaviour and pathophysiological patterns. Recommendations for pain treatment in common experimental procedures help researchers to find out the most suitable medication for their specific experimental design in the respective animal species. Analgesic treatment should be the standard in all not insignificant painful experimental procedures. It should be pointed out that less stressful and painful protocols lead to better and more valid results.

Indeed the use of analgesics is not recommended in all experiments because effects and side-effects of the drug may interfere with the expected results.
Poster
Rehabilitation of laboratory New Zealand rabbits

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Introduction: Modern legislations on animal experimentation almost always include the credo of the Three Rs, which most often takes care of the “well being” of the experimental animal but does not always protect the “life” of the animal. The concept of the 4th R – rehabilitation of laboratory animals is a befitting continuum of Three R credo. In this paper we present a case study on the rehabilitation of 181 rabbits used in experiments.

Methods: Direct observation of these rabbits, and veterinary intervention were used to assess the condition of the rabbits.

Results: It has been observed that in rehabilitating these animals often succumb to spinal chord damage even if they have not been experimented on, due to the small dimensions of the cages that they are restrained, in laboratories, allowing them no freedom of movement.

Discussion: Rehabilitation of rabbits has to be done remembering that rabbits are delicate and sensitive animals and that cannot be directly rehabilitated in a natural environment with many incitements as they tend to be over active and this could be fatal. The paper discusses the finer nuances in their successful rehabilitation, physical environment, stabling options, degree and scaling of freedom of movement, social interaction and essential veterinary care. Ethological and behavioural studies on rehabilitated animals besides being a source of direct information to help refine and better understand the problems encountered in rehabilitation will open new views in learning and understanding laboratory animal care and use.

Poster
Back to basics: Human influence in animal experiments

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Over time our knowledge and perception with regards to laboratory animal science has changed considerably. Advances particularly in molecular sciences have lead to the development of sensitive animal models, but there are basic issues that still need to be addressed. The influence of environmental variables on test outcome in particular is one area where knowledge is lacking. Although many physical variables have been investigated, one variable that has received little attention is the human factor in animal experiments. Yet, humans play a vital part in all animal research, and differ both between labs and within.

This paper reviews a large amount of information on how direct and indirect contact with humans alters an animal’s physiology and behaviour. Crucially, it will give examples of how this impacts on test results of commonly used tests. For instance, studies carried out at the Central Science Laboratory have shown that experimenter identity was a highly significant variable in a standard anxiety test, despite the fact that all experimenters followed identical procedures.

The paper will finish by discussing ways of reducing this influence. Being aware of human factors in animal research is one step towards more consistent test results.
Introduction: From our point of view monitoring of anaesthetic depth during major surgical interventions in large laboratory animals is essential, especially when NMB’s are being used. EEG is principally suitable for monitoring anaesthetic depth but much experience in interpreting results or specially designed computer-based systems are necessary. The system used in our study was validated with measurement of oxygen consumption, another parameter for intra-operative stress.

Methods: 6 German Landrace pigs undergoing thoracic surgery were monitored. Anaesthetic agents (Propofol/Fentanyl/Pancuronium) were administered total-intravenously. Pigs were ventilated mechanically. EEG monitoring was performed using BIS Monitor 2000 XP (AspectMS, Leiden, Netherlands). Target parameters were Bispectral Index (BIS – a number between 0, representing isoelectric EEG, and 100, representing wide-awakeness) and suppression ratio (SR – ranges from 0-100, represents the percentage of isoelectric EEG in the past 63 seconds). Further parameters were: Oxygen consumption, blood pressure, ECG.

Results: The electrode fitted with the proportions of the pig’s forehead. Stable measurements were possible over a period of many hours. According to measurement of oxygen consumption reliable anaesthetic depth could be expected while BIS values were lower than 60 and SR was higher than 10%.

Discussion: Thus, the situation seems to be comparable to human patients for whom the system is well evaluated. Intra-operative stress can nearly be excluded while BIS is lower than 60 and SR higher is than 10%.

Poster

Monitoring anaesthetic depth during surgery in pigs

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Introduction: From our point of view monitoring of anaesthetic depth during major surgical interventions in large laboratory animals is essential, especially when NMB’s are being used. EEG is principally suitable for monitoring anaesthetic depth but much experience in interpreting results or specially designed computer-based systems are necessary. The system used in our study was validated with measurement of oxygen consumption, another parameter for intra-operative stress.

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Discussion: Thus, the situation seems to be comparable to human patients for whom the system is well evaluated. Intra-operative stress can nearly be excluded while BIS is lower than 60 and SR higher is than 10%.
Introduction: Postoperative patency and flow control are essential for success and validation of vascular graft implantation research projects. Angiography represents for many the gold standard in the follow-up after vascular surgery, but brings inherent risks: Longer anaesthesia, supplementary animal stress, possible occlusion, ischaemia, pain or limping. We studied the implementation of LVDU as an alternative to angiography in three animal species.

Methods: We appraised vascular flow and patency by LVDU, after graft implantation in different research projects in 3 rats, 5 rabbits and 25 swine at different time points: immediate, 1 and 4 weeks post-surgery. LVDU was validated by angiography immediately after surgery in 17 of the pigs and at necropsy (4 weeks post-operatively) in all the animals. During the immediate post-surgical control, bi-dimensional ultrasound with Doppler was performed using a linear Vingmed®, 10 MHz probe before the animal awakening. Sedation was no longer than 20 minutes for further examinations. Procedures and techniques were adapted for each species.

Results: Angiography confirmed ultrasonographic findings in 94% of the 17-pig cases and in 100% of the other. LVDU provides excellent image quality, allowing for reliable evaluation and follow-up of the graft and flow characteristics. Stress, inconveniences, evaluation time and supplementary risks were reduced. As a consequence of angiography, 4 pigs had ischaemic complications, 2 having to be euthanised before the end of the protocol.

Discussion: LVDU is an accessible, non-invasive technique, providing rapid, safe, repeatable and reliable results. This excellent alternative avoids angiography-related risks, therefore contributing to the animal welfare. It optimises the standards and allows an easy evaluation of surgical results.

Lecture

Linear Vascular Doppler Ultrasound (LVDU): A reliable and promising non invasive tool for assessment, follow up and quality control after vascular surgery

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Lecture

Magnetic Resonance Imaging of animal brain in vivo

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The tremendous success of Magnetic Resonance Imaging (MRI) in medicine is based on its ability to visualise soft tissues at high spatial resolution and with excellent sensitivity to pathophysiological alterations. This particularly applies to studies of the central nervous system. In contrast to techniques using ionising radiation, MRI offers completely non-invasive examinations and therefore not only facilitates repeated follow-up examinations but also allows for monitoring disease progression and therapy efficacy.

The past two decades have witnessed a continuous growth of MRI studies of the central nervous system of laboratory animals. In fact, advances in imaging neuroscience of knockout and transgenic animals as well as of models of human brain disorders are expected to help bridging the gap between molecular biology and clinical applications. Moreover, the structural information obtainable by MRI has been complemented by an increasing number of techniques that attempt to characterise the functional state of the tissue rather than its mere morphologic appearance. Prominent examples include magnetic resonance angiography of the intracranial vasculature, functional assessments using a wide range of contrast media, diffusion-weighted imaging for an early demarcation of ischaemic lesions as well as for an in vivo assessment of the axonal connectivity by identifying white matter fibre tracts, and localised magnetic resonance spectroscopy which offers a neurochemical characterisation of the cellular metabolism and composition.

The purpose of this lecture is to give a brief overview of the potential of magnetic resonance studies of animal brain ranging from insects to monkeys.

Poster

The effect of training for long term restraining of rats evaluated by telemetry

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The aim of this telemetric study was to evaluate the possible stress and distress by long term restraining of conscious rats by the use of two different restraining methods, “Bollmann cage” and “Scandidact rocket”, and to evaluate the effect of training for the restraining method.

Telemetry was used to measure blood pressure and heart rate in female, Sprague Dawley rats, as these parameters are known to increase due to stress and distress.

On the first day of experimentation, blood pressure and heart rate were measured for 3 consecutive hours in the rats normal housing environment without any disturbances.

On the following 10 days of experimentation, the rats were once daily restrained for 3 consecutive hours, without any prior training, by one of the two restraining methods without changing the method of restraining during the experimentation period.

Blood pressure and heart rate data was measured as mean values of two minute intervals every 15 minutes from the start of restraining.

All data was compared to data extracted at the same time points on the first day of experimentation as an indicator of the impact of training by the repeated restraining.

The results will be presented and the importance and length of specific training for routine laboratory procedures will be discussed.

We furthermore conclude that non-invasive, telemetric obtainable recordings of blood pressure and heart rate in combination with other parameters like e.g. relevant hematological measurements and behaviour observations are very useful and reliable in the assessment of stress and distress.
Non-invasive imaging utilises specific traceable molecules to monitor spatial and temporal biological events within live animals. Since the same animal can be used throughout a study, the need to sacrifice individuals from each group at specified time points is avoided. Hence, imaging methods allow the number of animals used in a study to be reduced. As a single animal is used, individual variation is avoided, improving the reliability of experimental data. Furthermore, imaging over extended periods of time can potentially reveal mechanistic details of disease- and toxicity-related events.

Many imaging techniques obviate the need for visible clinical signs and, thus, substantially refine experimental procedures. Indeed, developments in this field may eventually allow animal studies to be replaced with clinical trials in human volunteers.

The advantages of non-invasive imaging are most poignantly highlighted by two recent approaches. The first involves the use of bioluminescent reporter systems that involve transforming mammalian cells, tumours, bacteria and viruses with genes encoding enzymes that catalyse production of bioluminescent metabolites. The genes are placed under the regulation of DNA elements that only initiate gene expression in response to a specific biological molecule or event. Thus it is possible to monitor disease progression and its response to therapeutic intervention in real-time. A second technique utilises quantum dots (nanocrystals of semi-conducting materials) which when excited by a pulse of light emit electromagnetic signals of pre-determined wavelengths. Quantum dots conjugated to biological molecules can be used as tissue- or disease-specific markers or to monitor molecular interactions.

Radio-telemetry provides an alternative means of obtaining physiological measurements from awake and freely moving small laboratory animals, without introducing stress artefacts. Until now research has mainly focussed on the responses of laboratory subjects to experimental changes but not to general laboratory handling and procedures. Traditionally, a variety of techniques have been employed to obtain physiological measurements in the laboratory. When monitoring conscious animals, it is possible to use invasive methods such as sensors, and/or electrodes. Non-invasive methods such as surface electrodes for monitoring an electrocardiogram or a tail cuff for monitoring blood pressure can also be used. Although wireless radio-telemetry technology for monitoring laboratory animals has existed for years, it has only been in the last ten years that affordable, reliable, and easy-to-use commercial products have been readily available for monitoring a variety of signals. Telemetry technologies and improved laboratory tests have allowed us to better assess the well being and physiological status of each of our research subjects. The advantages of implantable wireless telemetry transmitters include: providing a humane method for monitoring conscious, freely moving laboratory animals; eliminating stress related to the use of restraint, which can alleviate a potential source of experimental artefacts and inter-animal variability; reducing animal use by 70% in single studies, and by more than 90% in multiple studies and allowing 24 hour data collection, for days, weeks, or months, without special animal care. The application possibilities and benefits of long-term measurements of physiological parameters in mice will be presented on the basis of recent results.
Lecture

Impact of the method of restraint on plasma corticosterone, heart rate and body temperature in laboratory mice

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Radio-telemetry is a frequently used tool to measure physiological parameters in freely moving animals. In this study, radio-telemetry was used to assess the value of heart rate (HR) and body temperature (BT) as quantitative measures of acute stress in mice. Female C57BL/6 mice (n=9 per group) were subjected to different methods of restraint: lifting by the tail for ~5 seconds (LT), ~10 seconds of restraint by hand (RH) and 5 minutes in a plexiglas restrainer (PR).

It was found that restraint caused a tachycardia, irrespective of which method. However, during the following 90 minutes where HR recovered to baseline, HR was found to be significantly higher in the PR group compared to the LT group. RH caused an intermediate recovery to baseline of HR. These results showed a correlation with the plasma corticosterone data found in a preceding experiment, showing a gradual increase over the three methods, with LT having the lowest concentrations and PR the highest. Hyperthermia was also found after each of the three procedures, however, as BT changes are rather slow, this parameter does not seem to be a useful parameter to quantify an acute stress response.

Poster

Faecal corticosteroid concentration vs. total faecal corticosteroids. Which measure reflects better the total amount of circulating hormone?

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Alternative methods to the utilisation of blood and its derivatives in laboratory animals are particularly interesting, especially in the hamster due to its small size and difficulties in obtaining serial blood samples. Steroid hormone metabolites quantification in faeces, widely used in studies of free-ranging or intractable animals, shows up as a non-invasive, non-stressor, economical, animal saving technique. It allows longitudinal studies as it enables frequent sampling of the same individual that can be used as its own control. However, we remain naïve about factors that may influence the accuracy of these techniques. The aim of this study was to evaluate the relevance of cortisol faecal metabolite concentration to assess physiological stress response. Ten adult female Syrian hamsters were ovariec-tomised and all faeces voided by each of them collected daily during five days before and five days after surgery. Cortisol faecal metabolites were extracted and quantified by radioimmunoassay. We determined per gram faecal corticosteroid concentrations, total 24-h faecal output and total 24-h faecal corticosteroid production. Surgery affected considerably faecal output and using “per gram” vs. “total” corticosteroids yields different conclusions: while concentrations increased significantly immediately after the ovariectomy and decreased on the subsequent days, “total” excreted corticosteroids varied in a symmetrical pattern. Then, the relative, pergram measure of hormones may not reflect the total amount of circulating hormones because these measures are comparable only if the volume of material in which the hormone is contained is the same between the two groups.
Lecture

Non-invasive telemetry in comparison with invasive telemetry and its use in toxicology studies

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Conscious dog telemetry is routinely used for in vivo cardiovascular safety pharmacology studies, generally involving invasive recovery surgery for implantation of transmitters for collection of electrocardiogram (ECG) and arterial blood pressure data. Whilst the data quality is high, and dogs can be reused over a number of years, advances in non-invasive telemetry systems may allow similar data to be obtained from a less invasive procedure. These types of systems are also conducive for inclusion in toxicology studies. Here the ECG data quality surpasses that historically recorded by conventional means at "snap-shot” time-points from restrained dogs, which can lead to stress and heart rates above the normal resting values. Collecting higher quality, long-term data in toxicology studies often eradicates the requirement for separate safety pharmacology studies, thus conferring a 3Rs improvement, since an overall reduction in animals would be achieved, as well as a refined technique providing extra valued data from the same animals. Add to this the resource efficiency and the reduced compound requirements by only running one study, may save valuable time in the drug development process. The presentation will compare the advantages/disadvantages of invasive versus non-invasive telemetry, and will present data from example studies where non-invasive telemetry has added valuable data.

Lecture

Non-invasive monitoring of stress hormones in mice: A technique opening new perspectives in biomedical and animal welfare research

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In mice, the monitoring of endocrine functions is seriously constrained by the adverse effects of blood sampling. Therefore, the aim of our investigation was to establish a non-invasive technique to monitor corticosterone concentrations. In a first step, radiometabolism studies were performed revealing that Corticosterone Metabolites (CM) were predominantly excreted via the faeces and that peak excretion occurred after a lag time of about 10 h. HPLC immunograms showed that corticosterone was extensively metabolised and suggested a newly developed 5α-pregnane-3β,11β,21-triol-20-one Enzyme Immunoassay (EIA) to be suited for measuring faecal CM in mice. In a second step, the biological relevance of this EIA was investigated. Experiments comprised monitoring faecal CM after different treatments including administration of ACTH and dexamethasone, respectively. The results clearly demonstrated that pharmacological stimulation and suppression of adrenocortical activity was reflected accurately by means of CM measurements in the faeces. Furthermore, the technique proved sensitive enough to detect effects of routine laboratory procedures like blood sampling or injections. Even the naturally occurring diurnal variation of glucocorticoids could be monitored reliably. Thus, our study provided substantial information about the metabolism and excretion of corticosterone in laboratory mice. Furthermore, the developed EIA proved a powerful tool to monitor adrenocortical activity by measuring faecal CM. This non-invasive technique avoids blood sampling related stress effects and can reduce the total number of animals used for research. Since it also allows frequent sampling of individual animals over time, it contributes to implement the “3R concept” and opens new perspectives in biomedical and animal welfare research.
Lecture

**Positron Emission Tomography (PET) enables in vivo quantitative functional organ specific pharmacokinetics using limited numbers of animals**

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Biologically interesting compounds can often be labelled with radioactive isotopes of carbon (C-11, $t_{1/2}=20$ min; nitrogen (N-13, $t_{1/2}=10$ min), oxygen (O-15, $t_{1/2}=2$ min.) and fluorine (F-18, $t_{1/2}=110$ min).

These isotopes emit positrons, which in turn annihilate to two $511$ keV γ-radiations, which makes it possible to observe compounds labelled with these isotopes with special Positron Emission Tomography (PET) cameras. Since a few years small animal PET cameras are being introduced in addition to the cameras for clinical human use, which were also useful for bigger animals (dogs, pigs).

Positron Emission Tomography (PET) is a quantitative functional procedure. A PET system yields the absolute tracer concentration during the acquisition time. This time dependent concentration can be converted into a quantitative description of function (like receptor binding, oxygen consumption etc. depending on the compound used) by pharmacokinetic modelling.

This makes it possible to obtain time dependent quantitative information on physiological and biochemical processes in selected organs, using a minimal amount of animals: One animal yields several time activity curves under chosen conditions.
Many animals are bred specifically for use in laboratories; the genetic variation between individuals is minimised, and housing and husbandry is standardised. The rationale is to increase the validity of scientific findings, and allow a reduction in number of animals used. Non-human primates used in laboratories present a very different case. For each primate species commonly used (Macaca mulatta, Macaca cynomolgus and Callithrix jacchus), there is great variation in rearing practices, housing, enrichment and training both among, and often within, facilities. In some cases, the consequences of the variations have been quantified (e.g. upper- and lower-tier housing) and the impact upon the scientific findings is known (e.g. in restrained versus unrestrained sampling), but in many cases the impact is not known (size of gang groups; rotational hand-rearing in Callithrix). While a degree of standardisation should be the aim in certain situations to ensure an acceptable level of welfare, reduced variation in the scientific output, and therefore fewer study animals, standardisation should not necessarily be the aim for keeping primates in laboratories. For example, individuals may respond to a specific enrichment device, housing system or training protocol quite differently. In this paper, I use examples to illustrate that housing, enrichment, and training should be tailored to individual needs of primates to ensure their welfare is maximised, and the science is not compromised.
Whenever and wherever, nonhuman primate research will stimulate heated debate. For many researchers in this field, that is just the reason to stay low profile. For some this will be a lifelong decision. In the Netherlands we started an unique experiment to design and execute a dialogue trajectory that brought the primate researchers, governmental administrators and animal welfare advocates together. This process, that took more than two years, consisted of 3 highly structured, closed (dialogue) meetings to build bridges of mutual understanding. Central in this approach was the explicit willingness of the participants to meet each other on neutral grounds and to commit oneself to an “objective” deliberation of the complexity of pro and cons of primate research. This resulted in a map that visualises the interrelationships of all the problems experienced by the stakeholders to reduce, refine or replace primate research. This map represents the common ground shared by the participating stakeholders. This milestone was subsequently used to formulate several policy scenarios to facilitate Three Rs initiatives in primate research. The Dutch Society for Laboratory Animal Science, installed a special workgroup (with members of the Animal Welfare Organisation) to keep close contact with the implementation of the policy scenarios. In the paper the methodology and the pitfalls will be described to inspire others to follow a road that might turn the spiral of secrecy and violence into a constructive situation, beneficial for the animals.

Poster

**Campaign for a legal ban on primate experiments**

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People for Animal Rights Germany / Doctors Against Animal Experiments Germany, Braunschweig, Germany

Introduction: Primates are sentient beings with high cognitive abilities. It is increasingly recognised by both the public and scientists that their use in experiments is ethically not justifiable. Methods: People for Animal Rights Germany have started a campaign in October 2004 which aims for an amendment of the German Animal Welfare Law to outlaw primate experiments. The campaign includes informing the public, petitions and also work on a juridical and political level. Results: In some EU countries (Austria, Sweden, Netherlands) experiments at least on great apes have been made illegal. In Germany no experiments on great apes have been conducted since 1991. Our campaign wants to see all primate experiments included in a future ban. Discussion: Our campaign is also seen in the light of the currently ongoing amendment of the Directive 86/609. A ban on primate experiments should not be limited to one country, although it would be useful for the work on European level, if one country took the lead. The difficulties we are facing arise from the powerful pro-vivisection lobby who often predict the end of medical research, if experiments on primates would be outlawed. That this is a) not true and b) even if so, primate experiments would ethically not justifiable, is the message of the campaign.
Lecture

Environmental enrichment objects for the improvement of locomotion of caged rhesus macaques (*Macaca mulatta*)

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This study focussed on two aims: First, to examine whether the locomotion behaviour of caged rhesus macaques at the Paul-Ehrlich-Institute was reduced compared to rhesus macaques in their natural environment. Second, to investigate whether locomotion of caged rhesus macaques could be improved by using two locomotive objects: Tread-mill and rotary barrel.

For this purpose two groups of caged laboratory monkeys (5/6 individuals, mixed sex and age) were used. Data were collected using a scan sampling method.

A time schedule figured out that the reduction of locomotion in caged rhesus monkeys was considerably less than expected compared to free living individuals. Using either a metal treadmill or a rotating wooden barrel, both objects were able to increase the locomotion of the examined groups. Although all animals used both, the wheel and the barrel, the adult animals did so to a considerably lesser extent than the young monkeys. None of the objects were used preferentially. A continuous supply of the objects, as oppose to a discontinuous supply, did not influence the frequency of use. A frequently available object remained of steady interest. The increase in locomotion due to the objects resulted in both, a reduction in social interaction and in aggressive behaviour.

Lecture

Positive reinforcement training: A lesson in refining animal studies

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Many of the routine husbandry practices and repetitive experimental procedures to which laboratory animals are subjected during the course of a single study may cause considerable distress and suffering even in the absence of any lasting visible physical trauma. This is because many animals may not like to be handled, restrained, removed from home cages or kept in isolation. Equally, an animal may become distressed if it learns to associate any one of the above activities with painful or invasive procedures such as surgical implants or the fitting of monitoring devices that may follow.

Positive Reinforcement Training (PRT) is a simple and effective way to greatly refine laboratory practices. This involves teaching animals to co-operate with researchers by using a system of rewards. In turn, this permits procedures to be conducted under calmer conditions thus allowing, for instance, blood samples to be taken quickly and safely, with less risk of injury to the animal or to the technician.

Working with, rather than against, animals in research labs is not only safer but is necessary to buffer or eliminate stress reactions that reflect impaired well-being and that could invalidate “scientific” research data, and increase animal usage. Indeed, it is now recognised that stress can dramatically affect responses to drugs, and alter disease progression and susceptibilities to many infections and illnesses.

This poster aims to illustrate how PRT can benefit both animal welfare and science by referring to its advantages and disadvantages as seen in small animal and primate research.
Lecture

Training laboratory-housed non-human primates: A survey of current practice in the UK

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Laboratory-housed non-human primates may experience a range of potentially stressful interactions with humans, including physical restraint, venepuncture, injection, catching and cage-change. Training the animals to co-operate using positive reinforcement is one means by which staff can significantly reduce or eliminate the adverse impact of such procedures and, therefore, is a refinement technique. Use of training can enhance not only animal welfare but also quality of science, because suffering in animals can result in physiological changes that are, at least, likely to increase variability in experimental data and, at worst, may invalidate research findings. We surveyed use of training in over half of UK establishments using and breeding primates, utilising a mixed-mode questionnaire. The survey demonstrated that there is widespread awareness of training as a refinement technique, and appreciation of its diverse benefits, but that training is not used as widely or as fully as it might be. This is due to real constraints (e.g. staff, time and a lack of confidence in ability to train), but also perceived constraints which can be overcome through information sharing and education (e.g. supposed lack of information on how to train, and overestimation of the time investment needed). There is also variation between establishments in the purposes of training and the techniques used, with a reliance on negative reinforcement in some. We conclude that there is considerable scope for refinement of common scientific, veterinary and husbandry procedures through use of positive reinforcement training, and refer to some resources designed to help establishments take action.

Poster

To improve the well-being of captive primates: The new primate centre of the C.N.R. in Rome

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The Primate Centre of the C.N.R. of Rome, since 1984 hosts a colony of about 30 tufted capuchin monkeys (Cebus apella), living in social groups. In our centre the monkeys are utilised exclusively for ethological studies. After 20 years we have built a new facility that was opened in November 2004 and consists of 820 square metres divided into four large exhibits designed for tree-living primates and a small service area. The shape and dimensions of the exhibits varies to reflect the needs and social groupings of the species on display. The most important characteristics of the new structure are: More space per animal, fresh air, grass and ground instead of concrete substrate. The outdoors of the exhibit is enriched by landscaping with trees, bushes, logs, stones, ropes. All these permit the monkeys to show more natural behaviours.

The new structure was planned based on advanced theories in the field which are extremely functional for the well-being of the animals and the needs of the research.

The new centre carries out an important scientific didactic and divulgation activity in primatology. In fact, we have produced books and CD-ROMs for students. Now we have set up a permanent multimedia information point (Totem) and didactic panels for the visitors of the Centre. This is an attempt to transfer to a huge audience numerous pieces of information about biology and behaviour of nonhuman primates and to clarify the significance of the biological and psychological similarities differences between human and nonhuman primates.
In November of 2003, Johns Hopkins University made a commitment to protect science through animal welfare refinements at the institution. An integral aspect of the plan was to eliminate all specific pathogens known to confound research data from our rodent colonies. January 2005 was set as a target deadline to eliminate all conventional or diseased rodent colonies from our Baltimore Medical Campus. Our goal was to convert all rodent colonies to specific pathogen free status with minimal scientific interference and expense. We utilised a number of strategies including revamping our sentinel program and importation quarantine services, decontaminating laboratory space and equipment, developing re-derivation and cross-fostering services, renovating existing facilities, abandoning outdated facilities, utilising ventilated and non-ventilated micro isolator housing, minimising the number of animals housed outside of central facilities, mandating the use of aseptic procedures, and expanding the training of animal care staff, principle investigators and their staff. Although we did not meet the anticipated deadline for the complete conversion in animal health, we have currently eliminated approximately 70% of our conventional rodent populations. The creation of our cross-fostering program and the use of high risk return housing have greatly contributed to our success. We have faced a number of challenges such as; equipment shortages, unique investigator equipment needs, limited procedure space, disease outbreaks, developing traffic patterns, and investigator reluctance. The sheer magnitude of this project was a major factor in extending our deadline. We are now aggressively pursuing our goal and plan to achieve 100% success by January 2006.
Ischaemia/Reperfusion (I/R) is the cessation of blood supply to an organ for a temporary period followed by restoration of flow and is the cause of complications following surgery, thrombosis or organ transplantation. I/R injury is caused by the reactive oxygen species (ROS) generated by the vascular endothelium. Besides injury, ROS generated by the vasculature are also involved in endothelial cell signalling. Studies of endothelial responses have till date involved an extensive and indiscriminate use of animals. Here, we propose to drastically reduce the use of animals by an artificial capillary system (Fibercell-CellMax) that mimics endothelial cells in the vasculature. Cells isolated from a few mice and rats, expanded into larger cultures were subjected to \textit{in vitro} I/R conditions. Earlier \textit{in situ/in vivo} studies from our lab using rat and mouse lungs have shown that the pulmonary endothelium was the predominant source of ROS generation with ischaemia. We also found that the cessation of flow triggers the closure of an endothelial K+ channel (KATP) resulting in ROS generation and all the other events that cause I/R injury.

This project investigated the endothelial ROS generation with a variety of KATP channel openers to evaluate their potential therapeutic applications. The ROS levels as monitored by H2DCF-DA fluorescence showed a sharp increase with ischaemia and was significantly reduced by the presence of K+ channel openers such as cromakalim and lemakalim. These results indicate that the KATP channel was an important component of the ROS generation in the endothelium with ischaemia.

### Lecture

**An \textit{in vitro} flow adaptation chamber replaces animals in an Ischaemia/Reperfusion model to study oxidant generation**

\textit{Shampa Chatterjee}

University of Pennsylvania, Institute for Environmental Medicine, Philadelphia, USA

The interaction of animal care personnel with the animals in their charge can be influenced by a number of factors. It has been demonstrated, particularly in the farm animal welfare literature, that the interaction of animal care personnel with animals can have a strong effect on the animals’ behaviour, productivity and wellness. Among species commonly used in biomedical research, it has been reported that mice are the least preferred species in terms of human-animal interactions in animal care facilities. In reviewing the literature and observing animal care personnel interacting with mice, it appears that the following factors may influence the manner in which mice are perceived:

- Their small size, their particular behavioural characteristics, and husbandry constraints (such as housing in ventilated racks in barrier facilities). In addition, we are interested in whether animal care personnel perceive genetically modified mice differently than non-genetically modified mice, and whether this in turn has an effect on their interaction with the animals. The ability to carefully observe an animal’s behaviour is key in assessing the animal’s wellness, and in establishing appropriate study endpoints in order to minimise pain and distress. The difficulties in assessing mouse behaviour, in particular the behaviour of genetically modified mice, will be discussed.
Lecture

Reduction of captivity stress on single housed pigeons through an enriched environment program

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Repetitive stereotypical behaviours have developed in pigeons that have been chronically housed in our vivarium for a number of years. We were not sure if these stereotypies were due to boredom and frustration or distress experienced from the barren, single-housed environment in which the birds live. Under current conditions, there is no opportunity for the pigeons to engage in species-specific behaviour such as contact with conspecifics, foraging or locomotion. We tested the bird’s faecal corticosterone levels under three distinct conditions.

Eighteen white carneaux pigeons were chosen and stereotypical behaviours were recorded and monitored for a period of four weeks. Next, birds were put into a crowded condition, two in a single home cage. In the last condition, 3 sets of 6 birds were group housed in a 5”x8” flight cage. The cage included various enrichment items. Faecal samples were collected in all three situations and are in the process of being assayed. The expected results are that faecal corticosterone levels will be high in the control group, higher in the crowded condition and lower in the flight cage.

Expectations are based on the evidence that corticosterone is a clear indicator of stress in birds (Harvey et al., 1980; Harvey et al., 1984) which unfailingly increases after a range of stressful events such as crowding, handling and captivity (Siegel, 1980).

Introduction and use of the saphenous vein blood-sampling technique in the mouse

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Introduction: The conventional method within safety assessment for obtaining multiple time-point blood samples in mouse studies has involved euthanising an animal for each sample. By adopting the saphenous vein blood-sampling technique makes it possible to obtain between one and three additional samples from each mouse, considerably reducing the number of animals required to achieve the objective of these studies.

Method: Thermostatically controlled warming cabinets are used to increase the body temperature of the mice prior to sampling, the mouse then being placed into a restraining device and the leg to be sampled is held so that a slight pressure is applied to the saphenous vein. The vessel is then punctured and the blood collected into an appropriate sample tube.

Results: Since the introduction of this technique within safety assessment, it has been used routinely for pharmacokinetic and toxicology studies, with an approximate 50% reduction in the number of animals required.

Discussion: This technique does not appear to cause the animals undue stress, although slight bruising and swelling around the sampling site is occasionally recorded.

200 µl of blood is the maximum practical volume obtainable at each time-point, making it a prerequisite that the required experimental analysis can be accomplished with this relatively small volume.

Nevertheless, saphenous vein blood-sampling has proved itself to be an extremely positive welfare initiative, capable of producing scientifically valid results, whilst substantially reducing the overall numbers of mice.
The implantation of new biomedical devices into living animals without previous biocompatibility evaluation is possible based on current legislation. The HET-CAM test offers a 3R compliant partially immunodeficient model allowing the simulation of transplantation experiments prior to animal testing. To compare these data with data acquired by animal experimentation, a meniscus regeneration experiment was repeated using the HET-CAM assay.

The meniscus implant was cut into 5x5 mm pieces and applied onto the CAM. Samples were incubated for 3 days followed by blood sampling, digital documentation and histological evaluation. The meniscus devices were tested in a sheep model by the Dept. of Orthopaedic Surgery and data were supplied for comparison.

Analysis of the sheep knee joint showed good integration and vascularisation of the implant. HET-CAM analysis revealed a firm attachment of the samples and a high vascularisation. A synovial hypertrophy observed in the sheep was visible as hypertrophy of the CAM reticular connective tissue. Both models showed an inflammatory response and a foreign body tissue reaction. Analysis of synovial smears and blood withdrawn from the CAM indicated a lymphocytosis.

Fertilised, 10 days incubated chicken eggs can show a tissue response similar to an animal model. Biocompatibility and -incompatibility can be tested prior to in vivo implantation. The routine application of the HET-CAM test would allow the exclusion of unsuitable prototypes and facilitate the selection of a biomaterial type, thus reducing the side effects caused by improper materials and allowing a reduction in animal numbers.

Biodegradable scaffolds are crucial for transplantation of tissue engineered cells. Animal models for scaffold evaluation usually cover the mid-term and late tissue reactions, but the early phase tissue response occurring during the first days after implantation is not documented. The HET-CAM test was used to compare the early phase tissue reaction in synthetic and natural scaffold materials.

All samples were tested on the CAM for three days followed by digital documentation and histological analyses.

All samples were completely integrated into the connective tissue of the CAM and showed different stages of degradation: The Hyalograft C® scaffold was completely disintegrated into single fibres while the Chondrocell® scaffold and the hyaluronic acid/polycaprolactone prototype had maintained their original shape. Both synthetic materials led to an inflammatory tissue response and thrombus formation in single blood vessels was observed. Fibrous tissue present in the surrounding of the biomaterial showed a beginning encapsulation of the implant. The normal CAM structure was altered and atypical cells were observed. The collagen implant only provoked a mild tissue response indicated by the presence of single lymphocytes within the scaffold.

While only mild reactions were observed after implantation of the collagen scaffold, transplantation of synthetic biomaterials led to alterations of CAM structure and the presence of atypical cells. Although the long-term impact of these observations remains to be evaluated, CAM testing is a valuable tool for short term analysis and intermediate in vitro/in vivo biomaterial characterisation.
Spontaneous granulosa cell tumour (GCT) of gerbil is a frequent pathology in this species and follows the natural tumour development along the animal life. We analysed a total of 241 GCT from 167 females. The largest incidence of GCT is in gerbils 2 to 3 years old, being most frequent in virgins. The ovarian macroscopy is normal – with incipient microscopic GCT – or shows developed tumour (cystic, solid or mixed). Based on the microscopy GCT are classified in incipient, cystic, solid and mixed. All tumours consist of nests and cords of cubical tumour cells; there are luteinic cells, Call-Exner bodies, pseudofollicles, incipient cysts, necrosis, and mitosis, except for the last one in the incipient tumours. Invasion compromises more frequently the ovarian hilus, periovary fat, and fimbriae. The largest number of metastasis is in the omentum. All the tumours with necrosis are malignant, as they also show metastasis. Malignant GCT represents 88% of the present sample. Natural death happened in just four virgin females with malign GCT, 2 to 3 years old. The information acquired and documented in this study is being used in lectures of Basic Pathology – Neoplasm – given to graduate students in our University. This tumour can be used as animal model of the disease giving opportunity of studies in the development of this ovarian tumour, including genetic, hormonal and immune modulation, as well as action of antineoplastic drugs.

Rheumatoid Arthritis (RA) is an inflammatory auto-immune disease that affects approximately 1% of the population and results in disability and joint deformity. As no cure exists, new therapeutic mechanisms are explored with animal models such as Adjuvant Arthritis. In this model rats are injected intradermally in the base of the tail with Freund’s Complete Adjuvant, resulting in an inflammatory response with necrosis at the injection site and a systemic immune reaction resulting in swollen joints causing severe pain and discomfort comparable to RA.

Attempts to reduce pain and discomfort in the animals are limited so far to extra bedding material, long-spouted water bottles and maximally two animals per cage.

In order to reduce both animal discomfort and the number of animals we are investigating the possibilities to refine data acquisition and analysis by applying the Cat-Walk system and infrared thermography. Additionally, a new generation of analgesics is being screened for their effectivity to reduce pain without affecting the immunological parameters of the arthritis model. Preliminary data suggest induction of early arthritis coincides with irregularity of the paw pattern and prolonged crossing time on the Cat-Walk system, indicating possibilities for refinement in data acquisition. Further results will be discussed.
The original concept first put forward by Morton and Griffiths in 1985 was that the observation of clinical signs and their degree of deviation from normality could indicate the level of animal suffering. However, it is easy to be led astray if only affected animals are observed, and they are only compared with other controls in the experiment. What is vitally important is that both control and experimental animals are compared with normal naïve animals. Some examples of such misleading comparisons will be given, and the use of simple systems of comparison offered in their place.


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**Poster**

**The importance of control groups and normal in the assessment of animal wellbeing**

*David Morton*

Birmingham, UK

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Technological advancements enabling investigators to genetically engineer mice and rats quickly and efficiently have created a deluge of models available for research today. The rise in magnitude and complexity of genetically modified animals has resulted in an increased number of models with reproductive and adverse phenotypic challenges requiring special care to appropriately house and maintain these valuable models. This presentation will describe a systematic approach to providing clinical care to a large and diverse population of transgenic mice and rats. Topics to be covered include clinical observation training, managing adverse phenotypes including clinical assessment and disposition, record keeping and data reporting, and determination and refinement of endpoints.

Kathleen Murray

Charles River Laboratories, Transgenic Services, Wilmington, MA, USA

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**Lecture**

**Working with genetically engineered rodents: A comprehensive program of veterinary care**

*Kathleen Murray*

Charles River Laboratories, Transgenic Services, Wilmington, MA, USA
Lecture

Promoting laboratory animal welfare and refinement in neuroscience and behavioural research through an Institute for Laboratory Animal Research (ILAR) report

Jennifer Obernier and Joanne Zurlo
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Over the last twenty years in the United States, the main vehicle for promotion of the 3Rs has been inclusion of language reflective of the 3Rs in federal regulation, including the Animal Welfare Act, the US Government Principles for the Utilisation and Care of Vertebrate Animals used in Testing, Research, and Training, and Public Health Service Policy on Humane Care and Use of Laboratory Animals. While an important step in the general acceptance of the 3Rs within the research community, these regulations do not provide practical guidance to scientists on implementing the 3Rs. Such guidance is particularly needed for the neuroscience research discipline, which has seen a doubling in membership to its professional society over the last decade. In order to address this, ILAR published a report “Guidelines for the care and use of mammals in neuroscience and behavioural research.” This report provides practical information on applying the 3Rs to common research methodologies, such as food and fluid regulation. Neuroscience specific methodologies, such as implanting neural probes, are also discussed. In addition, this report introduces the scientist to advances in refinement strategies, such as using behaviour to monitor animal pain. Since its publication in 2003, more than 24,000 copies of the report have been distributed and it is publicly available on the National Institutes of Health (NIH) website.

Poster

Lack of references to refinement measures in published animal-based research of genetic diseases

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Animal models are valuable research tools in studying debilitating human diseases; but for ethical reasons it is particularly important to focus on refinement in this kind of research. Since the possibility to publish is an important driving force in research, scientific journals strongly influence which approaches are chosen by researchers. To study to which extent refinement is reported, we analysed publications from 2003-04 using a recognised severe model, R6/2 Huntington’s disease mice. These mice show a rapid disease progress, with motor deficits progressively worsening until premature death at about 14-18 weeks. Of a total of 27 papers, 24 reported experiments in which mice reached the age where clinical symptoms appear. We distinguished between A) acute or ex-vivo, B) live-animal research with a fixed cut-off point and C) survival experiments. The papers were reviewed for references to: Welfare assessment, humane endpoints, housing adaptations and compliance with official recommendations. Despite that the majority of the papers reported compliance with official recommendations (Tot: 19/24, A: 7/9, B: 4/6, C: 8/9), less than a third reported that humane endpoints (Tot 6/24, A: 0/9, B: 1/6, C: 5/9) and/or housing adaptations (Tot: 7/24, A: 0/9, B: 3/6, C: 4/9) were applied. Even for survival studies, where animals develop severe symptoms and ultimately become unable to eat and drink, half of the papers failed to report such refinements. Journal publishing policy could play an important role in self-regulating research; however on the basis of the reported observations we conclude that this potential is presently poorly utilised.
Poster

Replacing the use of live stimulus animals by scent cards in the social recognition test for mice

I. Anna S. Olsson

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In the conventional test of social recognition used in behavioural phenotyping of mice, mice to be tested are confronted with juvenile stimulus mice; however van den Bos et al. (ATLA 30, 299-304, 2002) have demonstrated that for rats, live stimulus animals can be replaced by scent stimuli. We present a pilot study of a similar approach in mice. Adult male mice (N=12; equal numbers of two different genotypes) were housed in same-genotype pairs, and cards were scent-marked by leaving them in the mouse cages for 2 weeks. The behaviour of individually tested mice was studied in test cages with clean or scent-marked cards. In the first test, mice were exposed to one clean card (C) and one card with an unfamiliar scent mark (S1). After a 5 minute interval, the test was repeated with the same scent card as in the first test, together with a different, unfamiliar scent card (S2). The time spent in contact with the cards was measured, with the hypothesis that mice showing social recognition in the second test would spend more time in contact with the new than with the recently encountered scent card. Indeed, mice spent more time (Wilcoxon-signed-ranks test; Z=-2.43; p=0.015) with S2 (70.22±5.90 s) than with S1 (43.01±5.92 s), confirming the hypothesis. The results indicate that social recognition in male mice can be demonstrated using scent cards rather than live animals, thereby reducing the number of animals needed for testing.

Lecture

A behavioural study on beagle dogs rehabilitated from a laboratory in India

Shiranee Pereira1, Priyadarshini Govind*2, Massimo Tettamanti2 and Preetha Babu2

1 CIBA, Chennai, India; 2 International Centre for Alternatives in Research and Education (I-CARE), Chennai, India

Introduction: 283 beagle dogs in the age group of 1-10 years, that were bred and used in a laboratory, in India, were rehabilitated and homed. Families who adopted the dogs observed severe behavioural/psychological trauma and/or physiological ailments. Through this study it is proposed to record observable signs of physical/psychological/psychological trauma in rehabilitated beagle dogs.

Methods: A random sample of 36 dogs was studied by way of 1) direct observation, 2) a comprehensive questionnaire and 3) interaction with the owner of the dog.

Over 40 physiological/psychological/physical parameters were recorded by way of a score sheet or descriptively and were ascertained for 0, 90, 180 and 360 days post rehabilitation. Some of the parameters that were studied included apathy, aggression, fear, compulsive disorders, response to human presence and touch, phobias, stereotypes, ability to bond with human beings, physical deformities, physiological complaints etc.

Results: Apathy, extreme fear, tendency to hide, anxiety with an obvious differential response to men and women were observed in almost all the dogs. Stereotypies, pica behaviour, coprophagia and compulsive disorders were common. Almost all showed stress related skin disorders. A few beagles were aggressive.

Discussion: The paper discusses and qualifies the physiological and psychological status of these rehabilitated beagles, their biological implications, interplay of hormones in canine stress disorders, interferences in experimental responses, inferences of canine studies and implications in extrapolating the same to human health and well being.
Poster

The genetic absence epilepsy in Imp:DAK rats – a new model

Joanna Piasecka-Zelga and Jan Stetkiewicz
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Spontaneous spike and wave discharges (SWD) on the cortical EEG occur in 40-60% of our Imp:DAK outbred stock of Wistar rats. The pattern of these high-voltage spindles in EEG records is similar to that occurring in petit-mal epilepsy seizures in humans.

Following the Hanover principles of mating and selection based on the EEG, in F4 generation we obtained two lines: 1) SWD[+] in which seizures occur in 100% of rats; and 2) SWD[-] without seizures in three-hour EEG recording period. We used 60 rats (30 male and 30 female) divided according to occurrence of seizures (30 of SWD[+] line and 30 of SWD[-]). To find differences between these two lines, we applied the following behavioural tests: open field test, radial maze test, conditional passive and active avoidance tests and hot plate test. We performed also morphometry of some regions of the brain, and assessed the activity of acetylcholinesterase (AChE) in the brain and blood.

The SWD[+] rats showed impaired short-term spatial memory and long-term memory, and significantly lower sensitivity to pain. In the SWD[+] rats, morphometry revealed lower density of neurocytes and glia cells in II-IV layer of the brain cortex. The activity of AChE in midbrain, cerebellum, and blood of the SWD[+] rats was lower.

We conclude that these two lines may be used in pharmacological or neurotoxicological studies according to the aim and endpoints of the study.

Lecture

Advancing the 3Rs in pet nutrition research

Len Sauers, Dan Carey, Mark Tetrick, Kathy Boebel, Matthew Greenwood, Gail Kuhlman, Sheri Kubaszewski and Sarah Adams
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Pet food companies that manufacture premium products often conduct extensive research in the area of pet nutrition so as to 1) evaluate the nutritional adequacy of new products, 2) assess nutrient bioavailability of raw materials, and 3) evaluate the efficacy of new nutritional technologies. Since aspects of this research involve the use of animals, it is important that strict animal welfare guidelines are established, along with a research program aimed at developing alternative methodologies. At the Iams Company, several alternative methods have been developed to advance the Three Rs in all areas of pet nutrition research. Protocols have been developed which allow feeding studies, which have historically been done under laboratory-controlled conditions, to now be conducted in the homes of pet owners. Reapplication of in vitro methods, which were developed for the health care sector, has reduced the number of evaluations that must be conducted in animals. Advances in analytical methodology have reduced the need to use animals for evaluating protein quality. Development of new state-of-the-art automated systems for the collection of faeces and urine can significantly reduce the need to cage animals. Over the past two decades, use of animal alternatives by many industries has not only advanced animal welfare, but has led to methods that provide better data, which are faster and cheaper to generate. Not only is investment in alternatives by the pet nutrition industry a positive step in improving animal welfare, it will also lead to faster, more cost-efficient and better results.
Posters

**The prediction of respiratory sensitising potential of chemicals in a modified local lymph node assay**

*Helga Tuschl, Astrid Hrdina and Beate Fekete*
ARC Seibersdorf research GmbH, Toxicology, Seibersdorf, Austria

The local lymph node assay is now widely used to identify the sensitising potential of chemical contact allergens: Mice are dermally exposed to test substances and lymphocyte proliferation induced in the draining lymph node assessed by incorporation of radiolabelled thymidine. Improvements of this assay, like flow cytometric determination of B lymphocytic phenotype or T-cell activation/memory phenotype have been described. In addition, differential cytokine profiling, based on expression analyses of cytokine mRNAs or ELISAs of cytokines produced by lymph node cells *ex vivo* have been applied. It is now generally agreed that respiratory sensitisers can be differentiated from contact allergens by the induction of distinct cytokine profiles. In the present investigation we applied a modified local lymph node assay and measured cytokine production of *ex vivo* stimulated lymph node cells by a cytometric bead array and flow cytometry. The respiratory sensitisers trimellitic anhydride, phthalic acid anhydride and toluene 2,4-diisocyanate could be very well distinguished from the contact sensitisers Oxazolon, 2,4-dinitrochlorobenzene or α-hexyl cinnamaldehyde and the irritant sodium dodecyl sulphate. Interleukin-4 is a differential marker well suited to define respiratory sensitisation after topical application of chemicals in the modified local lymph node assay. Regarding the low numbers of animals used and the possibility to differentiate between contact and respiratory sensitisers, this test fulfils the criteria of reduction and refinement according to the principles of the 3Rs.

**The rabbit as an animal model in vaccine safety research; effects of experimental procedures**

*Cynthia M. Verwer*, *Geert van Amerongen*, *Ruud van den Bos* and *Coenraad F. M. Hendriksen*

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Vaccination with whole cell pertussis vaccine is and will continue to be part of the vaccination programmes in many parts of the world. In order to improve the whole cell pertussis vaccine, research is performed to gain insight in the side-effects and the components that causes these side-effects. In previous studies the results (particularly body temperature) were difficult to interpret due to the large variation in experimental outcome. A research-protocol has been set up in which stress-reducing measures, as housing condition and handling from birth on, have been taken up in order to guarantee the welfare of the animals as well as to reduce variation in experimental outcome. Social behaviour of the animals was continuously monitored with special interest in hierarchy. Behavioural tests were performed to determine tameness, anxiety and coping strategies. Testosterone and corticosterone were measured to support the findings in the behavioural tests. Body temperature was continuously monitored by means of telemetry to measure the inoculation response. Leucocyte counts and immunoglobulins were measured to determine the effectiveness of the vaccine. Handling has a statistically significant effect on the overall behaviour of the rabbits. Experimental procedures had no effect on body temperature. Difference between vaccine concentrations could be distinguished, although the inoculation response between the handled and non-handled groups differed in quantity and quality. Based on the results in body temperature it can be concluded that the rabbit can be an appropriate animal model to investigate the safety of the whole cell pertussis vaccine.
Biotechnical and other experimental procedures are believed to have an effect on the outcome of an animal experiment. In this study we focussed on the effects of individual housing of Wistar outbred rats in a toxicology experiment. A parental generation was dosed, via the Benchmark dose approach, with a brominated flame retardant (TBBPA). The offspring was kept on the same doses as their mothers till the end of the study. Per gender the offspring were randomly single or social (N=5 per cage) housed. The experimental protocol followed the OECD415 guidelines, enhanced for endocrine and immunological endpoints. Part of the male animals was used in an immunisation study to test the immune response to Sheep Red Blood Cells (SRBC). The other animals were used in neurobehavioural studies. At the end of the study part of the animals went for necropsy. During necropsy a large number of organs and tissues were dissected for further (bio)chemical or histological analysis. The following endpoints were addressed: sperm quantity and quality, cellular composition, immunological subpopulations and NK activity, thyroid hormones, clinical plasma components, TBBPA kinetics, in vitro biotransformation of TBBPA, activity of P450 factors, activity of steroidogenesis and histopathology of multiple organs. Although there were significant differences, a major problem in interpreting is the influence of age, bodyweight and gender, which markedly alter many of these variables. The consideration to house rats socially or individually should be based on the purpose of an animal experiment and the sensitivity of differences in parameters that serve this purpose.
Infectious disease studies can cause varying degrees of pain or distress from exposure to infectious agents or toxins and subsequent morbidity and mortality. Although the consideration of alternative methods prior to performing animal studies is mandated by United States regulatory authorities, many scientists continue to view this requirement as intrusive and not beneficial to their work. Another view might be that the development, evaluation, and implementation of alternative methods are a critical part of the refinements made in any scientific endeavour where methods are modified to produce the highest quality results. Refinements in infectious disease studies consist primarily of assessment strategies and selection of humane endpoints. This begins with an understanding of the research’s impact on the animal. Although animal models are used as biological systems, many scientists seem unable to consider the impact of research methods on the animal and their effects on the results. Why is it difficult for some scientists to recognise and understand the full effects of an experimental manipulation on an animal? Why are alternative methods not a priority for scientists? What can the regulatory agencies do to further encourage and enforce the use of alternative methods? How does an IACUC resolve these issues? Identifying the barriers to the development, evaluation and implementation of alternative methods may assist in greater application of alternatives.

Lecture

Impediments to humane endpoints in infectious disease research

Karl Andrutis¹ and Alicia Karas²

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Infectious disease studies can cause varying degrees of pain or distress from exposure to infectious agents or toxins and subsequent morbidity and mortality. Although the consideration of alternative methods prior to performing animal studies is mandated by United States regulatory authorities, many scientists continue to view this requirement as intrusive and not beneficial to their work. Another view might be that the development, evaluation, and implementation of alternative methods are a critical part of the refinements made in any scientific endeavour where methods are modified to produce the highest quality results. Refinements in infectious disease studies consist primarily of assessment strategies and selection of humane endpoints. This begins with an understanding of the research’s impact on the animal. Although animal models are used as biological systems, many scientists seem unable to consider the impact of research methods on the animal and their effects on the results. Why is it difficult for some scientists to recognise and understand the full effects of an experimental manipulation on an animal? Why are alternative methods not a priority for scientists? What can the regulatory agencies do to further encourage and enforce the use of alternative methods? How does an IACUC resolve these issues? Identifying the barriers to the development, evaluation and implementation of alternative methods may assist in greater application of alternatives.
Animal well-being in an experimental study is often related to the endpoint being used. Therefore, one of the major targets in refinement strategies is replacement of potential painful or stressful endpoints by earlier, humane endpoints. This strategy has been adopted by the scientific community as well as by regulatory bodies, such as European Pharmacopoeia, and by organisations such as WHO and OECD. Humane endpoints can be defined as the point at which an experimental animal’s pain and/or distress can be terminated, minimised, or reduced by actions such as killing the animal humanely, terminating a painful procedure, or providing treatment to relieve pain and/or distress (CCAC, 1998).

This workshop will present the outcome and will discuss the conclusions and recommendations from the 2nd International Conference on the Use of Humane Endpoints in Animal Experiments for Biomedical Research that was held as a satellite meeting to the 5th World Congress, August 20-21. The objective of the conference was to review progress made since the first International Conference that was held in Zeist, November 1998. Important issues that were addressed are the recognition and assessment of adverse effects in animals and the determination, validation, implementation and acceptance of humane endpoints. Furthermore, new techniques, new approaches and new strategies using non-invasive methods were presented. Other key-issues were the training of observers and the use of recently developed remote sensing devices, such as telemetry and biophotonic imaging. The conference was initiated and organised by the Working Group on Humane Endpoints (HELP).

Urinary biomarkers as humane endpoints were reviewed seven years ago (Poon and Chu, Proceedings of the International Conference on Humane Endpoints in Animal Experiments for Biomedical Research, Nov. 1998, Zeist. Hendriksen and Morton, eds., pp 85-88). Since then, many new urinary biomarkers associated with a broad range of toxicity have been reported. 4-Hydroxynonenal, a lipophilic aldehyde, is identified as a biomarker of lipid peroxidation induced by chemicals such as TCDD, while 8-isoprostaglandin F2α is a specific marker of free-radical catalysed peroxidation of arachidonic acid. Elevated urinary metallothionein-1 is correlated with exposure to heavy metals. Creatinuria has been repeatedly observed to associate with testicular injury. Increased Clara cell protein (CC16) is associated with lung damage following ozone exposure. Kidney injury molecule-1 (KIM-1) is a novel urinary biomarker of proximal tubular damage. Sucrose excretion has been used to measure the intestinal permeability change following drug induced gastrointestinal damage. Microalbuminuria has been shown in our laboratory to be a sensitive indicator of kidney tubular dysfunction in rodents. NMR and MS based proteomic and metabolomic studies have identified new proteins and metabolites in urine that are associated with organ toxicity. Thus, parvalbumin alpha is recognised as a biomarker of skeletal muscle toxicity, the metabolite N-methylnicotinamide is a potential biomarker of peroxisome proliferation. Many metabolite profiles have been documented that characterise toxic expressions of specific chemicals and pharmaceuticals. Validating and incorporating these new target and mechanism specific endpoints in toxicological studies will enhance the power of detecting toxicity, and reduce the number of animals used.
Attempts by the regulated industries and animal advocates to ascertain the amount of monies spent to shepherd alternatives through to acceptance have been mitigated by the lack of accountability by and the sheer number of federal agencies in many of the industrialised countries providing some funding for these activities. In addition, while Europe has provided a centralised focus for its resources and prioritisation, most countries do not have readily accessible accounting of annual spending. In order to prioritise replacement methods and also identify immediate needs for “picking the low hanging fruit” in the field of alternatives, a document must be collated on an annual basis to provide genuine transparency of federal funding. Those members of the regulated industries that fund research, development and validation of alternative methods should also more readily present data on annual dollars allocated for this work.
A plethora of regulations require that many chemicals and chemical products are tested for efficacy and/or safety. When permitted to operate effectively and without bias, the ECVAM/ICVAM/OECD validation process can be used to independently establish that new animal and non-animal test procedures are sufficiently relevant and reliable for their stated purposes and should be considered for regulatory use. However, it is clear that many currently-accepted animal tests and candidate non-animal tests do not, and could not, meet the agreed criteria for necessity, test development, prevalidation, validation and acceptance. Do we therefore need an invalidation process to parallel the validation process, so that such methods could be independently reviewed and declared irrelevant and/or unreliable for their claimed purposes? Examples will be given of animal tests and candidate non-animal tests which ought to be evaluated in this way, and which could ultimately be declared to be invalid.

**Poster**

**Do we need an invalidation process for animal and non-animal tests?**

*Michael Balls and Robert Combes*

FRAME, Nottingham, UK

In the Netherlands, laboratory animal use declined since 1978, but has slowed down since mid nineties. There are numerous factors influencing the use of animals in research. On the one hand there is a policy addressed towards the replacement, reduction and refinement of laboratory animal use, such as through financing 3R projects and regulation of animal experimentation. On the other hand recent developments, such as the EU programme REACH, may lead to an increase of animal use. In the annual report “Animal use barometer”, which is initiated by the animal welfare organisation “Sophia Vereeniging” and carried out by the Netherlands Centre Alternatives to animal use (NCA), trends in the animal use and the affecting societal factors are evaluated, both on the national level as on the international level. Insight in these processes is aimed to result in the development of a more specific and effective policy towards a decrease in laboratory animal use. The developments in 3R research and implementation and advances that lead to either an increase or decrease of laboratory animal use are monitored specifically. Insight will be provided of trends and developments, and specific recommendations will be given.

**Poster**

**The Animal use barometer – an annual report**

*Iris Boumans and Coenraad Hendriksen*

Netherlands Centre Alternatives to Animal Use, Animal, Science and Society, Utrecht, The Netherlands
Lecture

NIH policies and strategies

Norka Ruiz Bravo
National Institutes of Health, Bethesda, MD, USA

The National Institutes of Health is the Nation’s medical research agency supporting research to improve the quality of human health in the United States and around the world. NIH develops research policies and scientific priorities using a collaborative process that involves its numerous stakeholders, including policies related to the use of animals in research. We actively seek scientific and public input and strive to develop approaches that serve the interests of the NIH, the research community, and the public. Examples will illustrate the process.

Lecture

The current OECD health effects test guidelines for REACH are in urgent need of revision

Robert Combes
FRAME, Russell and Burch House, Nottingham, UK

The European Commission (EC) has stated that all testing to satisfy the new REACH legislation for chemicals risk assessment must be undertaken according to the OECD health effects test guidelines (TGs). Each guideline has been analysed with respect to its design and its scientific and animal welfare implications, the extent to which it makes use of modern techniques, and its suitability to be used in the REACH system for the testing of large numbers of chemicals. The results of this analysis were published recently (ATLA 32, 163-208, 2004). It was found that some of the TGs required by the EC in its annex to the REACH proposals are unnecessary and that many others need to be updated to make use of modern methods and new knowledge, and to use current approaches for applying refinement and reduction strategies, to improve the scientific and animal welfare aspects of the procedures used. This report raises the serious question of why the OECD secretariat, and the various national co-ordinators and government experts who represent the 30 Member Countries of the OECD, have allowed the TGs to become so outdated and unsuitable. This presentation discusses the above issues, and makes recommendations for improving this highly unsatisfactory situation in the light of ongoing initiatives by the OECD to improve the process of test guideline development. It is recommended that this focuses on updating TGs, as well as producing new ones, particularly those based on advanced, non-animal approaches to testing.
Before a drug enters human clinical trials, pharmacokinetic/dynamic parameters ("ADME") and toxicity must be assessed, and few non-animal methods are currently accepted. Animals are also used for quality control in drug production. Because of the global nature of the drug market, the International Conference on Harmonization (ICH) was established in 1990 to align regulatory requirements across key regions. ICH consists of regulators and industry groups from Europe, Japan, and the U.S., as well as several observers. Amongst its other activities, ICH publishes consensus guidelines for preclinical testing which have contributed to a decrease in duplicative animal testing across regions. However, the system is fallible: In 2001, Japan requested additional preclinical testing of Oxycontin in beagles, even though the drug had been on the U.S. and European markets for 30 years. In addition, ICH guidelines have largely not incorporated validated 3Rs methods. The coalition of animal protection groups known as ICAPO (International Council on Animal Protection at the OECD) has formed a sister organisation, ICAPI, to address animal testing issues at the ICH. ICAPI has made a formal request for observership status at ICH meetings in order to liaise more efficiently with other global pharmaceutical stakeholders. Though ICH activity has tended to focus on the retrospective alignment of methods long accepted in all member regions, ICAPI is well positioned to help expand this focus to harmonising acceptance of emerging alternatives by bringing 3Rs methods to the table in a timely manner, thus facilitating their incorporation into ICH guidelines and their global adoption.

**Poster**

**International harmonisation of testing for pharmaceuticals: animal protection at the ICH**

*Sadhana Dhruvakumar*

People for the Ethical Treatment of Animals (PETA), Research and Investigations, Norfolk, VA, USA

In Canada, the responsibility for overseeing the care and use of animals in science lies with the Canadian Council on Animal Care (CCAC). Each of the three programs of the CCAC (Assessment, Guidelines and Education, Training and Communication) operates through a peer-based system. The development of CCAC guidelines affords the opportunity for an interface between scientists using animals as tools to obtain scientific data; animal welfare scientists trying to understand the “needs” of animals used in science; animal care personnel; animal welfare and community representatives. The use of a peer-review approach ensures that CCAC guidelines are based on sound scientific evidence and expert opinion that takes into consideration current societal values and the interests of the animals. Examples from recently published CCAC guidelines (the care and use of wildlife; the care and use of fish; and laboratory animal facilities) will be used to demonstrate the process in action. In particular, the development of guidelines in areas where there is a lack of scientific evidence will be discussed, including short-term and long-term strategies for addressing the need for research to support guidelines’ development.

**Lecture**

**Developing guidelines – the Canadian experience**

*Gilly Griffin and Clément Gauthier*

Canadian Council on Animal Care, Ottawa, Canada

In Canada, the responsibility for overseeing the care and use of animals in science lies with the Canadian Council on Animal Care (CCAC). Each of the three programs of the CCAC (Assessment, Guidelines and Education, Training and Communication) operates through a peer-based system. The development of CCAC guidelines affords the opportunity for an interface between scientists using animals as tools to obtain scientific data; animal welfare scientists trying to understand the “needs” of animals used in science; animal care personnel; animal welfare and community representatives. The use of a peer-review approach ensures that CCAC guidelines are based on sound scientific evidence and expert opinion that takes into consideration current societal values and the interests of the animals. Examples from recently published CCAC guidelines (the care and use of wildlife; the care and use of fish; and laboratory animal facilities) will be used to demonstrate the process in action. In particular, the development of guidelines in areas where there is a lack of scientific evidence will be discussed, including short-term and long-term strategies for addressing the need for research to support guidelines’ development.
Poster

Biosimulation and its contribution to the 3Rs in animal and human research

Hanne Gürtler
Living United Consult, Alleroed, Denmark

Animal experimentation and testing in human subjects are today a necessary part of the development of new drugs. The use of experimental animals and human subjects is, however, a source of concern for the European public in general.

Biosimulation holds great hopes for the future in reduction, refinement and replacement of animal and human experimentation in drug development. Biosimulation, however, is a complex and difficult research field to understand and hence to appreciate by the public.

BioSim, a new EU sponsored Network of Excellence on Biosimulation – a New Tool in Drug Development has as a vehicle for its dialogue with the public established a workpackage on Bioethics and Dialogue with Public.

The objectives of the workpackage are to establish how and to which extend biosimulation can contribute to the resolution of bioethical issues in drug development by reducing, refining and replacing animal and humane experimentation, establish how biosimulation can contribute to the implementation of the 3Rs (reduction, refinement and replacement) in animal and human research, establish if and how the 3Rs principle in animal research can be expanded to experiments on humans, increase awareness among European research groups involved in biosimulation of the 3Rs principle in animal research and improve the knowledge and understanding of biosimulation and its potential for resolving key bioethical issues in drug development in the European public.

The objectives and the workplan of the BioSim workpackage on Ethics and Dialogue with the Public will be presented.

Poster

Living United – a new international initiative on animal welfare and animal-human relations in action

Hanne Gürtler
Living United Consult, Alleroed, Denmark

Animal welfare is a topic which interest many people. The way we use and exploit animals today and the conditions we provide for them raise many concerns in the public in many countries.

The views on animals and their use are today much polarised in the society as a whole and among different stakeholders and have over the years led to many confrontations between protectors and users of animals. The present speed of the technological development and the globalisation will not reduce these tensions. There is therefore a need for generating a common understanding on animal welfare issues and for developing practical and applicable solutions which balance the needs and welfare of animals and humans.

The implementation of new welfare initiatives is a common challenge for all users of animals. Therefore there should be an increased dialogue, exchange of experiences and collaboration between the different users on the implementation of new welfare initiatives.

The purpose of the Living United Initiative is to bridge science, business and society and hereby contribute to an improved dialogue between science and society on issues related to animal welfare and animal-human relations, an increased dialogue, exchange of experiences and collaboration among users and between users and protectors and a decreased polarisation in society through broad stakeholder involvement, constructive dialogue, joint efforts and innovative communication.

The objectives of the Living United Initiative and the Living United Stakeholder Forum for communication of balanced solutions will be presented.
Posters

**Population control of dogs and cats: a proposal for interacting education, research and society**

*Stelio Luna, Alfredo Lima, Paulo Steagall and Bruno Minto*

FMVZ-UNESP, Veterinary Surgery and Anaesthesiology, Botucatu-SP, Brazil

The population of dogs and cats in Brazil is growing without control, as the Brazilian people have not assimilated the concept of responsible ownership. The increased population of dogs and cats lead to several public health problems. The capture and euthanasia is expensive, difficult and ethically compromising. The ovariosalpingohysterectomy and orchiectomy are the best methods to minimise overpopulation, as well as the conscience of the owner regarding the responsible ownership, registration and identification of the animals. This study aimed to perform surgical sterilisation of dogs and cats, to reduce their population, integrating the program with education and research, by using the same animals. The animals were selected from owners of low income. The surgeries were performed by undergraduate and graduate students and residents under supervision, in practical classes and research projects. In 2004, 1028 dogs and cats were castrated, being about 70% female and 30% male dogs and cats. This project has reduced the population of dogs and cats, has provided an alternative method for teaching anaesthesiology and surgical technique and allowed the elaboration of 15 clinical research projects as an alternative to the use of animals specifically for teaching and research purposes. The student acceptance of the method was excellent, increasing their responsibility and dedication when compared to the use of the animals from the experimental kennel. The use of animals for research was avoided, integrating a public health campaign for the population, teaching and research under ethical principles.

Lectures

**The development and implementation of public policies governing the use of animals in the life sciences – influences and outcomes**

*Margaret Rose*

University of New South Wales, Prince of Wales Clinical School, Sydney, Australia

The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes is the key policy document governing such use of animals in Australia and has been the basis for similar documents in New Zealand, Singapore, and Hong Kong. First published in 1969 as an initiative of the scientific community, the seventh edition was published in 2004. Today, revision of this code is the responsibility of a diverse group representing research institutions, funding bodies, regulators and animal welfare organisations; extensive public consultation is an essential part of this process. The principles of the 3Rs, the primacy of practitioner responsibility and the oversight by Animal Ethics Committees are fundamental elements of this code which, in recent years, has been incorporated into state legislation. Since the inception of this code there have been significant changes in community involvement in and expectations of how and why animals are used for scientific purposes. The ways in which this code, associated legislative changes and other public policies governing such use of animals have evolved in Australia against a background of increasing expectations of transparency, accountability and consultation are discussed and the involvement and influence of various stakeholders in setting policies and achieving outcomes is considered. As a scientific activity where the practitioner is expected to take primary responsibility for the welfare of the animals, the use of scientific evidence to inform such policies and the involvement of scientists in their formulation also is examined.
Lecture

Challenges and opportunities of animal welfare organisations in influencing and making public policy

Ursula G. Sauer
Deutscher Tierschutzbund, Akademie für Tierschutz, Neubiberg, Germany

For ethical and scientific reasons, the German Animal Welfare Federation strives for an end to all animal experiments. Therefore political activities lately have concentrated, amongst other issues such as cosmetics testing or the use of non-human primates in research, on the new EU Chemicals Policy, REACH. Its aim to submit not only new but also existing chemicals to an extensive evaluation scheme encompasses the danger that this might lead to a substantial increase in animal testing. To avoid this, the challenge to fundamentally revise an entire area of legislation should be taken as an opportunity to bring about a change in paradigm in safety testing and evaluation. The German Animal Welfare Federation has subjected the concept of the REACH system to an in depth scientific evaluation and has put forward detailed proposals that ensure that the new Chemicals Regulation will serve to improve human health and environmental protection without the use of live animals. These proposals are continuously being brought forward to national and European politicians and members of relevant authorities, as well as to the public and other stakeholders representing industry and environmental or consumer protection organisations. Institutions that indirectly influence the new EU Chemicals Policy are also addressed, such as the OECD – responsible for international test guideline acceptance, or national and European funding institutions who can play an essential role in promoting the further development of appropriate non-animal test methods.

Poster

The ethics of research involving animals

Harald Schmidt
Nuffield Council on Bioethics, London, UK

The issue of animal research has aroused intense debate, particularly in the UK. It is unhelpful to view the discussion as being only between those who are in favour of research and those who are against it, since between these two poles of the spectrum there are a range of further positions. In May 2005, the Nuffield Council on Bioethics, an independent UK body examining ethical issues raised by new developments in biology and medicine, published a report on “The ethics of research involving animals”. This talk will focus on the conclusions made in the report, and present recommendations for future policy and practice. The principal ethical question considered is: is it permissible for one species to cause pain, suffering and death to another to achieve aims that benefit primarily the former species? The report discusses the ethical arguments to foster an understanding of the debate, identify areas of agreement and understand what lies behind the remaining disagreement. The report concludes that further moral argument alone cannot provide a universal answer as to whether or not research on animals is justified, but practical advances in scientific method can reduce areas of conflict. For this reason, the importance of the 3Rs, and especially of the need to find replacement alternatives, cannot be overstated. Considering this, the report makes recommendations on improving access to information, the role of funding bodies, ethical review processes and implementation of the 3Rs.
Lecture
The role of national associations in promoting animal welfare

Ann Turner
American Association for Laboratory Animal Science, Memphis, USA

Voluntary associations play a significant role in the United States and globally in deliberating issues and establishing societal policies. Effective associations have advanced knowledge, skills, and attitudes in the arts and sciences because they promote research; disseminate information; provide education, training, and a forum for deliberation; and influence public policy in the governmental and private sectors. This presentation will enhance the understanding of how associations function and reach consensus on important society topics. A taxonomy of associations will be presented and the major functions of each type of association will be explored.

The role of the American Association for Laboratory Animal Science (AALAS) in advancing animal welfare will illustrate the impact of national associations on animal welfare and further clarify how associations function. AALAS is the largest and oldest voluntary association in the United States devoted to the advancement of responsible laboratory animal care and use to benefit people and animals. The association encourages research; publishes research findings, opinion articles, and educational materials; develops position statements on animal science topics; provides education and training in print and electronic formats; promotes professional standards through certification; and sponsors an annual conference devoted to scientific and practice excellence. These endeavours promote the philosophy of the Three Rs (refinement, replacement, and reduction). Specific examples of how AALAS has promoted animal welfare throughout its 55-year history will be used to illustrate the dedication to implement the Three Rs philosophy.

Poster
The importance of understanding societal expectations and awareness on animal testing and alternatives

Sonja Van Tichelen
Eurogroup for Animal Welfare, Brussels, Belgium

The debate on animal welfare and in particular animal testing contains many different views, variations of tolerability, knowledge and emotions but it is widely recognised that the use of animals in research is a huge and growing social and political concern.

The opinion of a society and its citizens are and should be a key driver for policy makers. Legislation is a reflection of the values and morals of a given society in a given time. For policy makers it is essential to understand what citizens know and expect in order to produce laws which are accepted and respected. For politicians their popularity can depend on their stance for a particular cause.

Understanding societal expectations is of interest to all other stakeholders. Consumer research informs producers about what their clients know and feel about animal testing. Differences between regions, age and social profile give international corporations the opportunity to fine-tune marketing messages and to adapt their animal testing policy.

For the animal welfare movement, the views of its members and society are an integral part of its advocacy work. It assists in deciding priorities, manage expectations and deliver information needs.

An overview of recent opinion polls and consumer research will attempt to give a insight in what citizens and consumer in this day and age think about the use of animals for scientific purposes and what the consequences are for policy makers, industry, scientists and other relevant stakeholders.
Lecture

**International efforts to identify data gaps for the development of science-based guidelines for laboratory animal care**

*Joanne Zurlo*¹ and *Hilton Klein*²

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The goal of guidelines and regulations for laboratory animals is to provide for optimal animal welfare while facilitating the best science. While many guidelines are based on published data and scientific principles, others are based more on expert opinion and can vary widely among countries. In 2003, ILAR hosted an internationally-sponsored workshop on the Development of Science-based Guidelines for Laboratory Animal Care. The purpose of the workshop was to bring together experts from around the world to assess the available scientific knowledge that impacts current and pending guidelines for laboratory animal care. Platform presentations focused on issues ranging from mechanisms of regulation development across different countries to data-based studies on the effects of environmental enrichment on research outcomes. In discussion sessions, participants were tasked with evaluating the current scientific literature on animal housing and environmental enrichment, identifying gaps in the current knowledge in order to encourage future research endeavours, and assessing the effects of current and proposed regulations on facilities, research, and animal welfare. During the point/counterpoint session to discuss the pros and cons of harmonisation of standards, most of the panel members expressed positive attitudes about working toward some form of harmonisation. There was a consensus among participants to continue the dialogue and to pursue an international effort to both identify data gaps and address the challenge of filling the gaps through additional research. Several activities have emerged as a follow-up to the workshop, and these will be discussed.
Establishing the 3Rs principle around the world: A plea for international standards

Andrzej Elzanowski
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Even the best national 3Rs implementation standards have little impact on other countries. In contrast, the international standards have substantial effects through empowering animal advocates in public and legislative debates. While the rhetoric of national pride acts against adopting even best foreign standards, it usually works (with a partial exception for the superpowers) for compliance with internationally sanctioned rules.

A good example of an international standard in action is the Guidelines for Euthanasia of Experimental Animals (Close et al., 1996, 1997) adopted as the European standard by the EC. Such international standards are badly needed in other areas, such as the use of animals in education; application of humane endpoints beyond those in safety evaluation, which are covered by the OECD’s (2000) document; individual marking (tagging) of laboratory and wild animals; and, first and foremost, ranking of experimental procedures in terms harms to animals. The importance of an internationally accepted harm scale is hard to overestimate as it would permit differentiate the acceptability of generic procedures depending on their harm grade (e.g. to accept only lowest grade procedures in education).

With many countries worldwide having little if any regulations of animal research and testing, any reasonable standard adopted by an international committee is better than none, both in terms of its immediate effects when used by animal protectionists, and as a draft for future improvements. Hence a plea to use international gatherings, such as the World Congress on Alternatives, to at least initiate work on the many missing 3Rs implementation standards.
Lecture

**NIEHS: Program in alternative test method research, development and validation**

*Jerrold J. Heindel, William S. Stokes and Christopher Portier*

NIEHS, RTP, NC, USA

The NIEHS has developed several programs to promote basic research and to translate these discoveries into alternative test methods by promoting their development, validation and acceptance by regulatory agencies. The NIEHS Division of Extramural Research and Training (DERT) supports investigator-initiated basic research that can lead to the development of alternative test methods as well as the actual development of alternative toxicity tests via its grants program. It also supports the development and prevalidation of alternative toxicity tests via its small business innovation research (SBIR) grants and contracts and its small business technology transfer (STTR) program that specifically stimulates coordination between university researchers and small businesses.

The NTP works to develop more efficient mechanism-based testing strategies such as genetically engineered models and the implementation of microchip based gene expression technologies for use in improving *in vivo* assays and development of *in vitro* assays. It also supports workshops to promote the development of alternative toxicity tests.

The NTP Interagency Center for the Evaluation of Alternative Test Methods (NICEATM), in coordination with the Interagency Coordinating Committee for the Validation of Alternative Test Methods (ICCVAM), coordinates and directs the independent validation studies necessary to evaluate the scientific validity of alternative test methods for their proposed regulatory application. ICCVAM then forwards the NICEATM validation recommendations to the appropriate federal agencies for consideration of acceptance and incorporation in test guidelines, regulations and policies.

DERT, the NTP and NICEATM have resources available to both fund and promote their particular aspects of the NIEHS alternative test development and validation program.

Lecture

**The Netherlands Centre for Alternatives to Animal Use (NCA)**

*Coenraad Hendriksen*

National Centre for Alternatives, AL Bilthoven, The Netherlands

The Netherlands Centre for Alternatives to Animal Use (NCA), established in 1994, is the central point in the Netherlands for coordinating research and disseminating information on alternatives to animal experiments. The centre is part of the Department for Animals, Science and Society of the Faculty of Veterinary Medicine within the Utrecht University.

The NCA collaborates closely with the Dutch Alternatives to Animal Experiments Platform (Dutch Platform) and the Programme Committee Alternatives to Animal Experimentation of the Netherlands Organisation for Health Research and Development (ZonMW). The aim of the NCA is defined as: “To stimulate the development, validation and application of alternatives to animal experiments in the Netherlands.”

The focus of interest is divers. First, NCA supports ZonMW in a number of activities, such as the publication of a newsletter and the management of an online data base of 3Rs studies performed in the Netherlands and by having a website (www.nca.nl.org). NCA also participates in training courses on Laboratory Animal Science. Additionally, NCA performs own research projects in the area of the development and validation of 3Rs approaches in the quality control of immunobiologicals, the use of human tissue, the implementation of humane endpoints in animal experimentation and the application of new technologies such as genomics. Finally, NCA co-ordinates the activities of the European Resource Centre for Alternatives to animal use in higher education (www.eurca.org), in collaboration with the University of Edinburgh.
The FRAME Reduction Committee was established in 1998 and has recently been reorganised into the FRAME Reduction Steering Committee and a number of working parties. Members of the Committee are representatives from industry and academia, with expertise in statistics, experimental design, animal welfare and alternatives research. The Committee’s main objective is to reduce the number of animals used in research, education and testing without compromising the quality of research or hindering scientific progress.

The Committee facilitates the implementation of reduction initiatives by providing educational material, training aids and guidelines for journal editors. In addition, it aims to improve awareness of the potential ethical and scientific rewards that can be gained from implementing initiatives to reduce the number of animals used in biomedical research, via a number of different worldwide forums.

The Committee’s working parties work towards three specific goals, which together form the Reduction Initiative, namely implementing reduction, broadcasting the message, and assessing how successful reduction is.

Some of the Committee’s recent initiatives are described and information about key resources given. The FRSC is the only group that has the sole commitment of reducing the number of animals used for experimental purposes. It provides invaluable and important information to researchers, the Government and other animal welfare organisations to expedite reduction initiatives where no alternatives are yet available.

Poster

The FRAME Reduction Steering Committee

Michelle Hudson
Fund for the Replacement of Animals in Medical Experiments, Nottingham, UK

In this paper I want to vindicate that the 3R is the major ethical justifiable concept to overcome the confrontation of two often used ethical positions. Anthropocentric libertarianism with the claim of nearly no animal research control/regulation and on the opposite side the animal rights/liberation movement with the claim of the immediate abolish of every animal exploitation. In a pluralistic society no such extreme positions can become the only way of legal enforcement of morality. In modern theories explaining the relation of law and morality it is often used the comparison of legal positivism and natural law. Ostensible legal positivists like Kelsen and Hart defend the separation of law and moral whereas natural law theorist like Fuller (The Morality of Law), Devlin (The Enforcement of Morals) and Finnis (Natural Law and Natural Rights) defend a strong relation. But as I want to show that the concept of natural law/rights is not very helpful for bio/ethical considerations. Because the main problem is still unsolved, which position of natural law/rights is supported by morality: The libertarian or the animal rights? Then I want to use for defending 3R the analysis of J. Waldron from his famous anthology (The Theory of Rights) where he as editor resumes, that if metaethical cognitivism is untenable, then rationally resolvable disputes in bio/ethics become possible only between those who share certain fundamental values or principles in common. The 3R will be the justified starting point for the search of such values and principles.
Introduction: The concept of the 4th R rehabilitation of laboratory animals can best be described as a continuum of Three Rs credo, in keeping with the spirit and philosophy of the founders of this doctrine, Russel and Burch.

Methods: Borne from the philosophy and belief in Ahimsa, the concept of the 4th R is today defined and elaborated in the guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) Govt. of India and is a new law proposal with the Govt. of Italy. In countries like Italy and Switzerland the concept of 4th R is grown out of several years of arduous effort of both scientific and animal welfare personnel.

Results: The CPCSEA guidelines state that personnel using experimental animals have a moral responsibility for the animals after their use and investigators are responsible for the aftercare and/or rehabilitation of animals post-experimentation. An ongoing project for the promulgation of a new legislation in Italy for laboratory animal care and use, includes the concept of the 4th R.

Discussion: The Government of India is now posed to implement and work out rehabilitation measures, working with funding agencies, scientific institutes and animal welfare organisations. The paper discusses the evolution and status of the concept of the 4th R in India, Italy and Switzerland, its legal status, the ethics and philosophy of the concept and practical implications in propounding the credo of 4Rs in the care and use of laboratory animal care and use.

Poster
The success of the concept of the 4th R: A new era in laboratory animal care
Shiranee Pereira¹, Massimo Tettamanti², Cristina Del Tutto³, Giulio Schmidt³ and Prema Veeraraghavan²
¹ CIBA, ICAR, Chennai, India; ² International Centre for Alternatives In Research and Education (I-CARE), Chennai, India; ³ Camera Dei Deputati, Govt. of Italy, Rome, Italy

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Lecture
The status of the concept of the 3Rs in India
Shiranee Pereira
CIBA, ICAR, Chennai, India

In India the CPCSEA is a statutory body of the Government of India and draws its powers from Prevention of Cruelty to Animals Act 1960. The Act states that the “duty of the committee is to take all such measures as may be necessary to ensure that animals are not subject to unnecessary pain and suffering before, during and after the experiment” and “experiments on animals be avoided wherever it is possible to do so if other devices and the like may equally suffice”. This can be traced as the first legal indication of the concept of alternatives in India. The PCA Act 1960, was conceptualised by Rukmini Devi Arundale, theophist and philosopher, a contemporary to founders of the classical concept of the 3Rs – Russel and Burch. The Breeding of and Experiments on Animals (Control and Supervision) Rules 1998 further reiterates the concept of alternatives. The CPCSEA, proactively works to propagate the concept of the 3Rs and an independent expert committee has been constituted to suggest and implement alternatives in research, education and drug testing. There have been profound changes made in education, in the use of equines in the production of immunobiologicals, manufacture of tissue culture vaccines etc. The CPCSEA in 2004, further recognised the concept of the 4th R rehabilitation of laboratory animals as a national policy which states that personnel using experimental animals have a moral responsibility towards these animals after their use.
Lecture

Increasing use of alternative methods in Latin America and the Caribbean

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Centre for Toxicology and Biomedicine (TOXIMED), Laboratory for Alternative Methods, Santiago de Cuba, Cuba

A project to promote awareness and acceptance of the Three Rs principle of Russell and Burch in Latin America and the Caribbean was initiated in 1998 under the leadership of the Centre for Toxicology and Biomedicine (TOXIMED) in Santiago de Cuba. Information about alternatives has thereby been provided to about two hundred and fifty academic and research institutions from many countries of that region. As a main part of the initiative, short courses, lecture series and practical trainings have been delivered to hundreds of attendees from those sectors. In seven years many scientists and educators have been persuaded of the advantages of using alternatives and they have started to include them in their laboratories and classrooms. Nevertheless, this is so in a reduced group of countries yet. Papers referring to reduction, refinement and/or replacement are being increasingly published in scientific Latin American journals. Furthermore, there is almost no scientific event on laboratory animals or related fields that does not include at least a session about alternatives. The goal of this paper is to present the achievements with the incorporation of the Three Rs principle in the daily work of some Latin American and Caribbean institutions.

Lecture

The 3Rs in Brazil

Ekaterina Rivera
Federal University of Goiás, Brazil

Brazil is a young and continental country and, for this latter reason, you can find different economical, social, cultural and educational statuses throughout its extension. The same principle applies to the scientific field, where you have areas of excellence in some regions and, in others, little knowledge of many subjects.

In relation to the 3Rs we had been working trying to reach the whole country. This strategic plan consists in:
1. Using educational programs in universities and research institutes where animals are used;
2. Holding discussions in Brazilian regulatory agencies (more specifically, Environmental and Health), showing the need of international harmonisation in required tests with animals;
3. Implementing Ethical Committees on Animal Use and their importance on guiding researchers and teachers on the application of the 3Rs.

It has been continuous and hard work, but it was worth doing. The results will be presented and they are so encouraging that keep us stimulated to continue this work and we can see forward on a promising future.
**Poster**

**Diffusion of the 3Rs principle in Latin America**

*María P. Vinardell*

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The knowledge of the 3Rs principle in different countries of the Latin America area is still very limited, depending on the area we consider, and makes necessary its diffusion. In order to introduce this concept, we have organised in the last seven years several courses of alternative methods in different countries such as Cuba, Chile, Argentina and Brazil and in faculties of Pharmacy and Medicine. Our common language, facilities the diffusion, understanding, and discussion of the real situation of their institutions. The people attendant these courses were in all the cases professionals in the research and education area and in all the cases were very receptive and, were prepared to diffuse these ideas to their colleagues.

After an introduction on the history of the 3Rs we focused the course in the most relevant alternative methods of Toxicology *in vitro* with theoretical and practical classes.

Other important part of the course, especially for teachers was the introduction of the different alternatives in education and the manner to look for information of new alternatives and their advantages in front of the traditional practices with animals. Finally, we presented the more relevant webs to find information on the 3Rs principle all around the world.

This kind of courses constitutes an excellent manner to approach people to the 3Rs principle and facilities their diffusion, especially when the attendants are educators who will give information to students and futures professionals.

**Poster**

**Consensus platforms as a tool to reach harmonisation within an expanding Europe**

*Robert Vincent*

Depart. Biology and Society, Faculty of Earth and Life Sciences, Free University of Amsterdam, Amsterdam, The Netherlands

In biomedical research test animals are frequently used in lab experiments. Since 1980 the use of these animals decreased and got partly replaced by alternative methods. Subsequently the EU stimulated this trend by introducing art 23 of Directive 86/609/EEC where the Commission and Member States have to make efforts to develop and validate alternative methods in order to use fewer experimental animals and apply less painful procedures. In order to join forces in this process “ecopa” (European consensus-platform for alternatives) was founded. Ecopa is composed of several national platforms. A consensus platform is officially formed when the four major stakeholders – governmental institutions, animal welfare, academia and industry – are united. Within this context a project was started with the purpose to create consensus on the 3R strategy and a meeting was organised between all stakeholders of the national platforms. The aim of this meeting was to achieve consensus concerning the interpretation and ethical implication of: 1) pain and suffering in relation to research aims and 2) the (un)-equality of certain species (e.g. the tendency to designate a special status to primates), in relation to the concept of replacement. These topics were discussed last June in Ljubljana (Slovenia) by means of a tailor-made consensus methodology. This approach disclosed a spectrum of professional and cultural values that are relevant in accomplishing harmonisation within an expanding Europe. The results of the meeting will be presented and discussed.
One of the past bottle-necks in the funding schemes was the lack of global European co-ordination of the identification and validation of alternative methods for regulatory purposes. The European Commission and its relevant DGs have recognised this and many initiatives were taken.

ECVAM closely collaborates with national, international and industrial research funding bodies to make the missing alternatives available. On European Commission level, ECVAM offers to play a proactive role in this process by encouraging development activities and being involved in several FP6 projects, which deliverables will hopefully feed into ECVAM’s validation studies.

ECVAM funds directly and manages feasibility and pre(validation) studies which could reduce, refine and replace the use of animals for cosmetics, chemicals and other products testing, thus meeting the legislative requirements.

ECVAM funds and organises on a regular basis workshops and taskforce meetings, which help to identify promising alternative methods and testing strategies and feedback to research programmes or validation activities.

Although the commitment and willingness of the European Commission to contribute to co-ordination and funding of 3Rs efforts through its own research and validation funding, significant results can only be obtained if there is a joint effort between scientists, the Member States, industry and NGOs.
Lecture

FRAME – A scientific charity working for better science and animal welfare

Robert D. Combes
FRAME, Nottingham, UK

FRAME (Fund for the Replacement of Animals in Medical Research) was founded as a scientific charity in 1969 in London with the objective of promoting the development and validation of alternatives to animal experimentation. Since the early 1980s, FRAME funds a research programme focused on laboratory research at the University of Nottingham Medical School and at its offices nearby. This research has encompassed all the Three Rs (replacement, refinement and reduction) of Russell and Burch. Substantial contributions have been made by FRAME scientists in many areas, including skin and eye toxicity using human cell models, embroyotoxicity, genetically modified animals, endocrine disruption, genotoxicity, carcinogenicity, toxicogenomics, the use of non-human primates, testing legislation, the non-invasive use of volunteers and animal welfare. FRAME also has two scientific committees, on reduction and toxicity respectively, and a comprehensive web site which provides a useful resource on information on alternatives and how to search for it in the literature. FRAME works closely with industry, regulatory agencies and governments at national and international levels and is active in campaigning for legislative reform worldwide. FRAME receives funding from a variety of sources, including the general public, charitable trusts, industry and research grants to enable it to pursue its ultimate goal of replacing all animal experiments with advanced, scientifically justified and robust non-animal methods.

Lecture

Research in in vitro toxicology funded under the 6th Framework Programme. Review and opportunities for the forthcoming 4th call

Maria Dusinska and Beatrice Lucaroni
European Commission, Research Directorate General, Brussels, Belgium

The development of novel, alternative in vitro tests has been a priority for the various European Community research programmes since the 80s. The current Framework Programme (FP6) funds research on non animal testing methods mostly under thematic priority 1.

In the first three years of the programme, 55.5 million € have been invested in developing robust and effective in vitro methods that will withstand the requirements of international validation. The funding instruments used are: Integrated Projects (IP), Specific Targeted Research Projects (STREP) and Specific Support Actions (SSA).

Research topics currently supported by the EC encompass the application of in vitro cell and sensor technologies to replace in vivo animal studies, in vitro test strategies predicting human acute, chronic and reproductive toxicity. Hepatotoxicity, cellular (this includes research on human embryonic stem cells) and organ toxicity and specific types of toxicity such as allergies are also included.

Funding is also devoted to pharmacotoxicity and kinetic studies applied to product screening and the development of pharmaceutically relevant lead compounds. SSAs range from activities to raise awareness on the use of alternative methods in New Member States and Candidate Countries, to the promotion of new biosensor-based technologies for the Three Rs, and the analysis of the mechanisms of nuclear hormone receptors to potentially bridge the gaps between in vitro and in silico.

This presentation will provide a review of currently available funding under FP6 relevant to Three Rs research, with detailed information on forthcoming possibilities offered by the 4th call (deadline November 2005).
Lecture

**Funding opportunities through the Johns Hopkins Center for Alternatives to Animal Testing (CAAT)**

*Alan Goldberg*

The Johns Hopkins Center for Alternatives to Animal Testing, Baltimore, USA

CAAT established its Research Grants Program shortly after the Center’s creation in 1981. The program originally was intended to provide critical seed money for scientists to develop new *in vitro* methods for risk assessment. As the need for more research in the refinement area became apparent, CAAT expanded its grants program, starting in 2001, to include refinement projects as well.

For the 2006-2007 funding period, CAAT solicited projects in the following areas:

- **Refinement** ($25,000 maximum): grants focused specifically on the issues of alleviating pain and/or distress in laboratory protocols.

- **Developmental Toxicology and Developmental Neurotoxicology** ($50,000 maximum): projects using *in vitro* methods, embryonic stem cells, or species such as *c. elegans* or zebrafish to address these areas.

- **Immunotoxicology** ($50,000 maximum): grants focused on basic mechanisms as they relate to toxicity.

We follow a stringent, peer-reviewed process for selecting grant recipients. To date, CAAT has funded nearly 300 grants for a total of about $5.5 million.

In 2004, CAAT further expanded the refinement program, adding the Animal Welfare Enhancement Awards. These awards ($6,000 each) are aimed at the people who work directly with the animals, such as lab technicians, animal technicians, and veterinarians. The focus of the awards is to improve housing, handling and/or experimental situations for laboratory animals.

For more information about CAAT grants, please see [http://caat.jhsph.edu/programs/grants/grants.htm](http://caat.jhsph.edu/programs/grants/grants.htm).

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**“Three Rs” R&D in the European Union: Funding tools and opportunities of Framework Programme 7**

*Beatrice Lucaroni and Maria Dusinska*

European Commission, Research Directorate General, Brussels, Belgium

The Seventh Framework Programme, FP7, refers to the next research programme in a series of multi-annual Framework Programmes that have been the European Union’s main instrument for funding research and technological development since 1984.

The Commission’s proposals for FP7, published on April 6, 2005, will cover the period 2007-2013.

The total budget for FP7, as proposed, is of 73 billion Euro, with a yearly average of around 10 billion Euro.

The adoption of the research framework programme will be subject to a co-decision procedure, in which the Commission, Council and the Parliament play an equally important role. The new proposal is based on a year-long process of consultations with interested parties. The debate on FP7 continues throughout 2005 and 2006 and includes negotiations among the European institutions, Member States and stakeholders.

Subtitled “Building the European research area of knowledge for growth”, FP7 is designed to respond to the competitiveness and employment needs of the EU through four specific programmes:

1. **Co-operation**: mainly collaborative research in 9 areas, and joint technology initiatives
2. **Ideas**: basic research, through the European Research Council
3. **People**: training, fellowships
4. **Capacities**: infrastructures, helping SMEs, regions of knowledge, science and society

Details of these programmes will be set out later in the year.

The presentation will provide an update of FP7 preparations, focussing on scientific areas and funding tools relevant to research in the Three Rs, particularly in the sections devoted to Human Health, Environment as well as Science and Society.
Lecture

The beneficial partnership between the European (ecopa) and the National Consensus Platforms (NCPs) for Alternatives to Animal Experimentation is growing

Peter Maier
3R Research Foundation, Münsingen, Switzerland

Ecopa represents the pan-European umbrella organisation of national consensus platforms (NCP) consisting of those four parties (animal welfare organisations, industries, governmental institutions and academia) interested in fostering research, development and implementation of alternatives to animal experiments. NCPs and ecopa adhere to the 3R concept of refinement, reduction and replacement. They exhibit a type of non-governmental organisations combining the parties involved in an area of interest being diverse in their views and nature, but agreeing to find a consensus.

Members of ecopa are the NCPs in Europe (voting members) but also associate members (individuals, societies, associations, institutions). Ecopa was founded 2002 in Brussels by NCPs from now 15 European states and legally recognised on April 21, 2004 as an international not-for-profit organisation.

Examples will be presented how ecopa strives (see: http://ecopa.vub.ac.be) i) to raise public, government and scientific awareness in Europe for a better acceptance of alternatives in animal experimentation, ii) to facilitate the exchange of scientific information, expertise and experience between National Consensus Platforms, EU, government, industry, animal welfare and science institutions, iii) to organise conferences and seminars, publish documents, collect and circulate information and iv) to support scientific and educative initiatives. The final goal is the further development and implementation of 3R methods in Europe and worldwide. This is driven by the ongoing EU specific-support-action CONAM (Consensus networking on Alternative Methods within Europe). Examples of interactions between the 3R Research Foundation (the NCP of Switzerland) and ecopa demonstrate the beneficial outcome of this networking.

Lecture

Research directions in toxicology at the US National Toxicology Program

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In its more than 25 years of existence, the NTP has been a leader in toxicology testing and research within the United States and contributed significantly to the scientific knowledge used in making public health decisions. Dramatic advances have occurred in computer science and molecular biology during the last decade of the 20th century and beginning of the 21st century. In August 2003, the NTP set forth a vision for the 21st century: to support the evolution of toxicology from a predominantly observational science at the level of disease-specific models to a predominantly predictive science focused upon a broad inclusion of target-specific, mechanism-based, biological observations. The NTP Roadmap, developed with input from leading researchers in academic, industry, government, and advocacy groups, addresses the goals of the NTP vision for the 21st century and provides a framework for setting NTP research priorities to achieve the most efficient and effective research portfolio possible. The NTP Roadmap identifies the challenges and opportunities confronting the program and discusses the directions for the NTP in three main areas: (1) refining traditional toxicological assays, (2) developing rapid, mechanism-based, predictive screens for environmentally induced diseases, and (3) improving the overall utility of toxicology for public health decisions. The NTP Roadmap is posted on its web site (http://ntp.niehs.nih.gov) or available in hardcopy from the NTP Liaison and Scientific Review Office (phone: +1-919-541-0530).
Lecture

An overview of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs)

Vicky Robinson
National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), London, UK

This presentation provides an overview of the NC3Rs, including background and examples of current activities.

In May 2004, Lord Sainsbury, Parliamentary Under-Secretary of State for Science and Innovation, announced the establishment of the NC3Rs. The NC3Rs became operational after the first meeting of its board in September 2004.

The NC3Rs provides a UK focus for the promotion, development and implementation of the 3Rs in animal research. The Centre brings together stakeholders in the 3Rs in government, academia, industry and animal welfare organisations to facilitate the exchange of information and ideas, and the translation of research findings into practice that will benefit both animals and research.

The NC3Rs funds high-quality 3Rs research, organises workshops and symposia to disseminate and advance the 3Rs and is developing a range of 3Rs information resources and guidelines. Further information on the NC3Rs can be found on the new, comprehensive 3Rs web resource at www.nc3rs.org.uk.

ZEBET’s funding program for the development of alternatives to testing in animals

Horst Spielmann, Manfred Liebsch, Andrea Seiler and Susanne Boy
ZEBET, National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments, BfR, Federal Institute for Risk Assessment, Berlin, Germany

Since 1990 ZEBET at the BfR has funded research in Germany according to the 3Rs principle in order to reduce testing in animals for scientific and regulatory purposes. An annual budget of 300,000 € allowed ZEBET to support on an average of 10 concurrent projects for 2-3 years. To date 87 projects have been funded; most of them at university laboratories. The program is advertised at the national level and around 20% of the applications have received funding. Several successful projects have initiated international validation studies in the field of regulatory toxicology, e.g. phototoxicity, skin and eye irritation, ecotoxicology, pyrogenicity. ZEBET has also funded biostatistical support for validation studies. Other projects helped to replace the production of monoclonal antibodies in the ascites mouse by culturing ascites cells in advanced bioreactors, the production of polyclonal antibodies in chicken eggs rather than in rabbits, the use of embryonic stem cells in embryotoxicity testing, establishing transgenic cell lines for drug metabolism in humans and quality control and standardisation of commercial human skin models.

Funding by the German government via ZEBET and the BfR has proven remarkably successful, since some of the new methods developed in the program have been accepted for regulatory testing at the international level. Moreover, a considerable number of projects achieved international recognition and were awarded for contributing to refining, reducing or replacing testing in experimental animals.
The marketing and use of chemicals for various purposes require hazard and risk assessment with respect to human health and the environment. Depending on the respective legal context specific information including data on (eco)toxicological properties is required in order to perform hazard and risk assessment. For mutual acceptance of data reasons internationally standardised and accepted testing methods are required. The majority of testing methods in toxicology – and to a certain extent in ecotoxicology – involve testing with vertebrate animals. For animal welfare reasons the OECD has committed to the 3R principle already 1982, simultaneously encouraging to discover, develop and validate alternative testing systems. In recent years a number of in vitro test methods have been developed, validated and published as OECD test guidelines, many of them with the engagement of the German ZEBET. Especially with the new European Chemicals Policy REACH, which aims at improved environmental and human health safety without additional animal testing wherever possible, the development of alternative testing methods and “intelligent testing strategies” including also QSARs is of utmost importance.

Lecture

Status and perspectives of alternative testing methods for chemical safety assessment in the regulatory context

Petra Greiner
Abteilung IV1, Umweltbundesamt, Dessau, Germany
Poster

A critical analysis of the 2002 EU statistics on the use of laboratory animals for scientific purposes

Christina Grindon and Nirmala Bhogal
FRAME, Nottingham, UK

The European Commission (EC) recently published its fourth report on the number of animals used in the EU. Although, a set of standardised tables are now used, the new reporting format still fails to provide an adequate level of information regarding the use of laboratory animals. The reports are only published every three years. As such there may be a tendency to ensure that fewer animals are used, or reported as being used, in those years. Further underestimates of animal use within the 15 Member States arise from the exclusion of animal breeding for, for instance, the generation of transgenic strains rather than for direct experimentation. Neither do the statistics indicate whether the animals used were normal, genetically modified or contain harmful genetic defects, which is especially relevant given that mouse transgenics research has persistently increased over the past decade. The UK statistics include animals used in breeding programs and in transgenic experimentation such that the UK domestic statistics report almost 1 million more animals than appeared in the UK submission to the EU statistics. Further discrepancies will be discussed, but it is clear that changes are required to improve the way in which statistics on laboratory animals in the EU are reported. This will allow trends to be discovered more readily and areas for reduction, refinement and replacement initiatives to be identified.

Lecture

Three years of animal welfare in the German Constitution – the balance from an animal welfare perspective

Roman Kolar
Animal Welfare Academy, Akademie für Tierschutz, Neubiberg, Germany

The inclusion of animal welfare into the German Constitution in 2002 gained world-wide attention. With great expectations animal welfare organisations had been lobbying and campaigning to reach this goal for more than a decade, and they had good reasons to do so: in several concrete cases the regulations of the German Animal Welfare Act had been overruled by basic rights laid down in the Constitution, such as the freedom of science, the freedom of education and the freedom of professional choice. According to a decision of the German Federal Constitutional Court, licensing authorities were not allowed to reject applications for animal experiments on scientific or ethical grounds.

Three years after the change in the Constitution has taken place not much seems to have changed concerning the practice of animal experimentation, and the regulation thereof, in Germany. Therefore, it is time for a review of the situation. This presentation looks at case studies from different areas of research involving animals to analyse which consequences the change of Constitution actually has brought about. Licensing procedures as well as specific cases of animal husbandry and care are examined. Also, findings of a survey among licensing authorities and members of ethics committees undertaken in 2005 are used to assess the practical implications of the change in the German legislation.

This review is to yield a list of concrete measures that would need to be taken by the government and the authorities to pay regard to the animal welfare requirements resulting from the amended Constitution.
Lecture

Botulinum testing – time to kill the LD$_{50}$

Andre Menache
Scientific Consultant to Animal Aid, Tonbridge, UK

The classic LD$_{50}$ test, developed in 1927, has, since the end of the 1970s, been widely criticised for both scientific and animal welfare reasons. In 2002 the original LD$_{50}$ test (OECD 401) was deleted from the OECD guidelines, and replaced by modified versions of the LD$_{50}$, requiring fewer animals. These are: the fixed dose procedure (TG 420), acute toxic class method (TG 423) and the up-and-down procedure (TG 425).

One of the few remaining instances where the classic LD$_{50}$ is still used today is the mouse LD$_{50}$ test, in the potency and safety testing of botulinum toxin, used in both cosmetic and therapeutic preparations.

The European Pharmacopoeia has set the regulatory framework for non-animal testing of botulinum toxin type A for injection (No. 2113; 5th edition EP). A non-animal immunoassay – the SNAP-25 endopeptidase assay – has shown excellent results with respect to the estimation of the potency of type A toxin in therapeutic preparations (ATLA 31, 381-391, 2003). Similarly, two rapid, non-animal assays have also been developed for botulinum toxin type B.

The only remaining obstacle to regulatory approval of these non-animal methods would appear to be the validation process. There is a moral imperative to give priority to the validation process in view of the fact that this test requires over 80,000 mice in the UK alone every year.

Lecture

Strategies to reduce animal testing in US EPA’s HPV program

Chad Sandusky$^1$, Megha Even$^1$, Kristie Stoick$^1$ and Jessica Sandler$^2$

$^1$PCRM, Toxicology and Research, Washington, DC, USA; $^2$PETA, Federal Liaison, Norfolk, VA, USA

The High Production Volume (HPV) program was launched in the US by the EPA in 1998. In an effort to reduce the number of animals killed in this large testing initiative, members of the animal welfare community met with government officials and negotiated several basic principles set forth in a letter from EPA to all HPV participants (10/14/99), and reiterated in a Federal Register notice. The goal was to avoid check-the-box toxicology in fulfilling the basic SIDS data set, which if followed for each chemical, would result in well over a million animal deaths.

Laudable goals included the formation of chemical categories, the use of existing data to the greatest extent possible, and similar common sense approaches to spare animals and still meet the goals of the program. After more than five years experience and review of over 370 test plans, the success of this effort is disappointing. Many examples exist in which companies duplicated testing, for example, if the data were non GLP. In other instances, published data existed which were not used, either individually or in conjunction with other data (in a weight of evidence approach) to avoid new animal testing. Over time, however, some successful strategies were developed by reviewers in the animal welfare community and in collaboration with conscientious companies to reduce testing and still meet the SIDS requirements. Examples of these strategies will be provided and explored as they might apply to future testing programs.
Lecture

Access to obfuscation: Can rigid confidentiality and public accountability co-exist?

Troy Seidle
People for the Ethical Treatment of Animals, Research and Investigations Dept., Toronto, Canada

In many industrialised countries, it can be nearly impossible for a member of the public to obtain current and specific information regarding animal experiments. In Canada, research protocols specifying the number and species of animals used in an experiment, the procedures to which they were subjected, and the associated level of invasiveness, are considered “confidential,” as are meeting minutes of institutional animal care committees and reports of government inspections and private accreditations. The public does not even have access to a complete list of laboratories that conduct animal experiments. British policy is even more restrictive, such that it is a criminal offense to release such information publicly. Relatively greater transparency exists under U.S. “freedom of information” legislation, notwithstanding gaps in federal oversight and documentation for the most commonly used, yet “unregulated,” species. In contrast, the Swedish constitution guarantees the right of every citizen to have access to documents held by public authorities, which many Swedes consider to be an indispensable part of the democratic process. Legislated and other mechanisms enabling public access to official documents concerning animal experiments in these four countries will be examined, as will arguments both for and against preserving the confidential nature of certain types of research information. Overarching policy questions that will be explored include: “What level and type of information does the public need to make an informed decision about the need for and acceptability of animal experiments?” and, “Can rigid confidentiality and public accountability truly co-exist in a democratic society?”

Lecture

LD50 Testing of Botox® Cosmetic

Martin Stephens1 and Michael Balls2
1 The Humane Society of the United States, Animal Research Issues, Washington, DC, USA;
2 Fund for the Replacement of Animals in Medical Experiments, Nottingham, UK

Using animals for testing products with a cosmetic purpose has been controversial for decades. As revealed in a 2003 exposé by FRAME, the potency testing of Botox® Cosmetic, the popular wrinkle smoother, currently entails not only animal use for a cosmetic purpose, but also uses one of the most heavily criticised animal-based tests, namely, the LD50 test. Consequently, we argue that the manufacturer of Botox Cosmetic – Allergan, Inc. – has an ethical obligation to expeditiously replace the LD50 test with a non-animal method and, in the meantime, institute any appropriate reduction and refinement alternatives. The Humane Society of the United States (HSUS) approached the US-based company in 2004 to request disclosure of the details of its potency testing of Botox Cosmetic and its efforts at replacement, reduction, and refinement. Allergan revealed few details, and also declined an HSUS offer to work with the company to move this issue forward. The competent US authorities, the Food and Drug Administration (FDA), claim that they “encourage” alternative methods of assessing the potency of Botox Cosmetic, but do not appear to be doing anything concrete to move the issue forward. Some form of potency testing of Botox Cosmetic is essential, given that its active ingredient, botulinum toxin, is one of the deadliest substances known to man. Potential Three Rs alternatives have been identified by the European Pharmacopeia. Whatever alternatives are developed and validated for Botox Cosmetic will also apply to its sister product, Botox, which has several therapeutic applications.
Session 3.5
Ethical review – good practice and outputs

Lecture
Moral issues of animals, alternatives and public policy

David Bayvel
Biosecurity New Zealand, Ministry of Agriculture and Forestry, Wellington, New Zealand

In the New Zealand national animal welfare infrastructure, the National Animal Ethics Advisory Committee (NAEAC), the National Animal Welfare Advisory Committee (NAWAC) and the Australian and New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART NZ) all play important and discrete roles in ensuring that the government of the day received independent, broadly-based advice regarding the use of animals in science, in agriculture and for other purposes.

This paper covers the following:
- The genesis of NZ AEC/NAEAC system, including role of scientific community, Government, NGOs etc. with reference also to NAWAC and ACCART/ANZCCART.
- The legal status of committees, evolution of statutory role and importance of independent advice.
- Committee membership balance and succession and importance of consensus decision-making and leadership.
- The importance of formal management of committee activities, including strategic and operational planning and performance review including external feedback.
- The importance of monitoring international developments and building international networks, including reference to CCAC, ICLAS, AALAS and the nascent role of the OIE.
- The importance of policy, legal and ethical development re-use of animals in research, testing and teaching to use of animals in agriculture, and other areas, and vice versa.
- The NAEAC role in relation to independent monitoring of AECs and code holders.
Lecture
Documents and guidelines to help scientists provide material suitable for ethical review

Derek Fry
UK Home Office, Shrewsbury, Shropshire, UK

Scientists skilled at presenting results for publication and research proposals for peer group review by grant-awarding bodies often have difficulty with the different approach needed when presenting proposed work for ethical review or public scrutiny. Dialogue with researchers in the UK has provided some principles for documents and guidance that should help scientists in bringing out the points useful for ethical analysis and in developing a style for presenting the work to the lay reader.

Key elements in the documentation are:
1. a clear and logical layout that follows through from the intention of the work, to the plan for carrying it out and the experimental design principles to be followed, to the animal use itself, with a separate section on the costs to the animals and how these would be minimised.
2. prompts that scientists can readily understand for the main issues to be covered in ethical analysis.
3. easily-accessed notes and examples.

With help from journalists a set of guidelines for the style for lay summaries has also been developed.

This talk will give examples of the documents and guidelines and present some of the feedback from those who have used them and those who have been involved in ethical review of the material produced with their assistance.

Poster
Developing lay input into ethical review

Maggy Jennings and Jane A. Smith
Royal Society for the Prevention of Cruelty to Animals (RSPCA), Research Animals Department, Horsham, UK

Lay (sometimes known as “citizen”) involvement in the review of animal experiments is now a requirement – or at least recommended practice – in many countries. The involvement of lay participants is important because they widen the perspectives brought to bear on the issues surrounding the use of animals, and in particular on the assessment and weighing of harms and benefits. Although lay representation is most commonly thought of at the level of local and national ethics committees, lay people can make valuable contributions at other stages in ethical review, for example with respect to decisions on research directions, priorities and/or funding, and at the other end of the process when publishing research.

There are, however, many different interpretations of who qualifies as “lay”. It is important to recognise these differences when interpreting the role, because the individual’s background, expertise and affiliations will clearly influence the nature and extent of their input. Members from different backgrounds also vary in respect of their confidence in contributing to the review process and the resources that they need to help them feel comfortable and be effective in the role.

This presentation draws on the experiences of the wide range of lay members who attend the RSPCA’s Forum for Lay Members of Ethical Review Processes (ERPs), in the UK. It will review their role, describe where they feel they have made the most positive contributions, and explore issues that they find difficult. It will also introduce a number of lay members resources developed by the RSPCA at their request.
In Germany, according to the German Animal Welfare Act, scientists must provide prior to undertaking an animal experiment an ethical and scientific justification in their applications to the licensing authority. In such justifications reference is made to lacking knowledge with regard to development of human diseases or the need of better and even new therapies for humans.

The present study is based on applications of biomedical study groups of three universities in Bavaria (Germany) between 1991 and 1993. These applications have been classified according to their publications as successful in the animal model (Lindl et al., ALTEX 18, 171-178, 2001).

We investigated the frequency of citations, the course of citations and the question in which type of research the primary citations have been taken up: in subsequent animal studies, in vitro studies, in review articles or in clinical studies. The criterion we applied was whether the scientists succeeded to reach the goal in their applications: to contribute to new therapies or to gain results of direct clinical impact.

The outcome was unambiguous: even though 86 clinically orientated publications in which the above mentioned publications were cited could be tracked (7.1% of all citations), only in 2 publications a direct correlation between the results from animal experiments and observations in humans could be noted (0.16%). But even in these 2 cases the hypotheses that had been verified successfully in the animal experiment failed in any respect.

The implications of our findings may lead to demands concerning improvement of the licensing practice in Germany.

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**Poster**

**Literature survey of 51 approved legal proposals for animal experimentation purposes: No evidence for any human therapy after 10 years is apparent**

*Toni Lindl¹, Manfred Voelkel² and Roman Kolar³*

¹ Inst. Applied Cell Culture Ltd., Munich, Germany; ² Ethical Commission of Northern Bavaria, Wuerzburg, Germany; ³ Animal Welfare Academy, Neubiberg, Germany

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**Poster**

**Animal ethics and the question of killing**

*Joerg Luy*

Institute of Animal Welfare and Behaviour, Veterinary Departement/FU Berlin, Berlin, Germany

Introduction: About 25 years ago the Australian philosopher Peter Singer replaced the traditional question whether humans are allowed to kill animals by two new questions: the question of killing and the question of suffering. He is convinced that this approach, to consider the issue of killing animals in isolation from the infliction of suffering, is necessary for a clear philosophical understanding of the separate issues involved.

Methods: The ideas of recent philosophers – Peter Singer, Tom Regan, Paul Taylor and others – concerning the question of killing are compared with each other and with ideas of historic philosophers – René Descartes, Thomas Hobbes, Immanuel Kant, Jeremy Bentham and others – touching the question of killing. All arguments are analysed down to their underlying postulates. Because postulates are finally unprovable their plausibility was compared.

Results: The result is a paradox. The most plausible postulates are found in ancient arguments, although the question of killing was not understood in the modern way. The arguments however published since Singer raised the question of killing are build on postulates of low plausibility.

Discussion: Evidence suggests that the philosophical uncertainty about the question of killing follows from an inadequacy of the human ability to make moral evaluations for the problem in question. The killing of animals without any signs of fear or suffering (shown by the animals involved) seems to be neither moral nor immoral but unexpectedly without a moral status.
Lecture

Processes and policies for ethical evaluation in Nordic Countries and Europe

Timo Nevalainen
National Laboratory Animal Center, University of Kuopio, Kuopio, Finland

Revised Directive (86/609/EEC) shall require detailed and harmonised ethical evaluation of animal studies, and will be based on cost-benefit analysis. In this analysis the likely benefits of the study are weighed against the cost – i.e. harms like pain, suffering and distress – to the animal. It can be foreseen that both commodities to be weighed have to be broken down to smaller elements in order to weigh or attach an ethical value judgement to each and then these elements can be used in the overall assessment of an animal study. What is perhaps even more important is improving all relevant areas of concern, but particularly so that both animal welfare and good science are promoted. Whenever replacement alternatives cannot be used, ethical evaluation can and must focus on the two other alternatives, refinement and reduction and these are also fundamental elements of any harm in a cost/harm-benefit analysis. Processes and policies of ethical evaluation vary considerably in Europe, and need in many cases to be modified. Evaluation of the 6th Framework Programme applications is an example of truly European process: Benefit assessment is carried out first by scientific evaluators, and applications with high scores go to ethical panel. A similar assessment of benefits should be done in all cost-benefit analyses, but the local ethics committees may lack the needed expertise. Nordic Forum for Ethical Evaluation suggested a Cost-Benefit-Means approach for evaluation. In this model, the means are available methods either to improve the benefits or to decrease the costs.

Poster

Improving the effectiveness of research ethics committees

Catherine Schuppli and David Fraser
University of British Columbia, Vancouver, Canada

Animal Ethics Committees (AECs) play a key role in research governance, but there has been little study of the factors influencing their effectiveness. In-depth interviews with 28 AEC members from four Canadian universities were used to examine how committee effectiveness is influenced by committee composition and dynamics, recruitment of members, workload, participation level and member turnover. We found that a bias toward institutional/research interests versus animal interests may result from a preponderance of institutional and scientist members, an intimidating atmosphere for community and other minority members, and recruitment of community members who are affiliated with the institution and of members who joined for reasons other than to fulfil the committee mandate. Thoroughness of protocol review may be influenced by heavy workloads, type of review process, and lack of full committee participation. The introduction of new ideas may be limited by low member turnover. We suggest potential solutions to the problems identified so that AECs can improve their effectiveness. Institutional/research bias may be reduced by increasing numbers of community members, training chairpersons to provide a respectful committee atmosphere that encourages participation, and recruiting community members through advertisements followed by interviews. However, policies about the role of community members are often unclear, thus solutions may require further discussion. Ensuring thorough protocol review may be solved by ascertaining that all new members agree to fulfil the mandate of the committee and agree to the workload. Solutions are also discussed in relation to evidence for similar problems in Research Ethics Committees for human subjects.
Lecture

Criteria for expert review by animal experiments committees

Jan van der Valk and Sandra Swart

The Dutch Act on Animal Experimentation (1996) regulates the protection of laboratory animals. The Act imposes that animal experiments committees (AEC’s) should review animal experiments and balance the interest of the experiments against the suffering of the experimental animals. AEC’s advice the license holder of the organisation where the experiments are to be performed about the acceptability of each animal experiment. According to the Dutch regulations the AEC’s have to be composed of at least seven members which equally represent expertises on experimental animals, on alternatives to laboratory animals, on ethics and lastly on animal welfare and protection. Criteria that persons have to meet in order to be regarded as expert in one or more of these areas have not been described. The expertise of the AEC members can, therefore, not be guaranteed.

This study proposes criteria for each of the four expertises in the AEC. Representatives of the four expertises were consulted in order to draft criteria in a way that both adequate knowledge and expertise can be expected and that a sufficient number of people would qualify to complete the composition of the AEC’s.

Furthermore, it is proposed that in order to maintain the knowledge of each expert, compulsory continuing education of AEC members is required. The education should cover general items like statistics, alternatives, ethics, legislation, discussion techniques, importance of consensus, etc. In addition, it is proposed that compulsory courses should be established for each expertise to be regularly updated on developments in their respective areas.

Lecture

Assessing and reporting the impact of animal procedures – a fresh look at severity scales

Virginia Williams1, David Mellor2 and John Marbrook3
1 Unitec Institute of Technology, School of Natural Sciences, Auckland, New Zealand; 2 Massey University, Animal Welfare Science and Bioethics Centre, Palmerston North, New Zealand; 3 Retired, Auckland, New Zealand

In 1997, a severity scale to assess and record the level of welfare compromise to animals used in research, testing and teaching was introduced in New Zealand. Under this scale, the severity of procedures was expressed in terms of different categories of suffering based on numerous examples at the five levels outlined in a paper by Mellor and Reid (Mellor and Reid, 1994). The present paper reports on a review into the operation and effectiveness of that scale and the extent to which it fulfils the purposes for which it was devised. Key features of the scale are described along with its strengths and limitations, and comparisons with other scales operating internationally are made. Modification of the scale based on the review is outlined and key steps in its implementation are described.

Mellor and Reid (1994). Concepts of animal well-being and predicting the impact of procedures on experimental animals. Improving the Wellbeing of Animals in the Research Environment, Sydney, ANZCCART.
Lecture

**A wider interpretation of the 3Rs model**

*Flavia Zucco*

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The 3Rs model, proposed by Russell and Burch, fifty years ago, and specifically addressed to the scientific world, is nowadays inadequate to cope with more taxing questions posed by a more aware society, increasingly searching for a better equilibrium between the human species and the environment. In an effort to analyse those aspects and to implement a more advanced version of the 3Rs model, the project Anim.Al.See, has explored in details, both from the philosophical and the scientific point of view, alternatives to animal experimentation. For this purpose, concepts and languages involved in specific case-studies, related to each R, have been analysed, both by scientists and philosophers: for replacement, the case of cosmetic testing; for reduction, the single-dose approach in vaccines production, and the use of telemetry; for refinement, the housing of non-human primates and the welfare evaluation. On this basis, new definitions of alternatives, animal experiments, and animal welfare have been worked out, together with more precise definitions of replacement, reduction, and refinement. Recommendations are provided which would be of help to institutions, regulators, ethical bodies. The study performed, by widening the framework of reference, should improve the dialogue between science and society by promoting the awareness of the complexity of the problem, the research for alternative procedures, and the responsibilities of the different subjects involved.

The project “Alternative methods in animal experimentation: evaluating scientific, ethical and social issues in the 3Rs context” has been financed by the European Commission contract QLG6-2001-00028.
There have been a number of attempts to develop non-animal alternative methods for skin sensitisation testing, and one of the major approaches is to evaluate phenotypic and functional changes of dendritic cells (DCs) derived from human peripheral blood or cord blood. However, the effects of chemicals on the surface phenotype of DCs differ depending on the source of the cells, which is undesirable for a routine test. To overcome this problem, we have evaluated several human cell lines (e.g. THP-1; monocytic leukemia cell line). The aims of this study are as follows: 1. to optimise each step of the test, including culture time, antibody selection and effect of FcR blocking, and 2. to conduct an inter-laboratory validation study in order to confirm the robustness of the test. Based on the findings, the protocol for this assay was optimised. We used the optimised protocol to evaluate nine chemicals in an inter-laboratory validation study. Expression of CD86 and CD54 on the cells was measured after 24 h and 48 h exposure to six known allergens (e.g. DNCB, pPD, NiSO₄) and three non-allergens (e.g. SLS, Tween 80). The results indicate that a battery test involving measurement of CD86 and CD54 expression on THP-1 cells treated with test chemical for 24 h would be a useful in vitro skin sensitisation model.
Validation committee in the Japanese Society for Alternatives to Animal Experiments (JSAAE) has validated skin toxicity studies using a 3-dimensional cultured human skin model (skin model) commercially available in Japan. We used this material to screen the skin toxicity of various agents to anticipate the result of human patch testing, although this use differs from hazard identification of chemicals in the ECVAM validation study.

Since 2000, we have performed these pre-tests using 3 models (TESTSKIN™: TOYOB Co. Ltd. and Vitrolife-Skin™: Gunze Co. Ltd. and EPI-200 (EpiDerm™): Kurabo Industries Ltd.). In this pre-validation study, 19 laboratories participated excluding the kit suppliers. Three chemicals were selected, coded and supplied to each laboratory. These results were presented at WC4 in New Orleans and contributed to the Alternative to Animal Testing and Experimentation (AATEX).

Furthermore, we performed a validation study using skin model. At first, we validated an alternative test to corrosivity with the ECVAM protocol using Vitrolife-Skin™ and EPI-200. In this validation, 6 laboratories participated and tested 12 chemicals. As the next step, we validated an alternative to skin irritation test with ET50 protocol using TESTSKIN™ and with ET50 and PI (Post-Incubation) protocol using Vitrolife-Skin™. In these validation studies, each of 9 laboratories participated and, tested 41 and 14 chemicals. These models have been evaluated in JaCVAM in order.

These results show that the skin model is adequate for evaluating corrosivity and may be useful for evaluating skin irritation, the reliability of which was similar to that of animal testing.

The scientist in Japan begins also to recognise the importance of 3Rs in the biomedical education as a social concern for laboratory animals welfare rises.

Science Council of Japan proposes that it is necessary to educate the 3Rs about the animal experiment as part of the bioethics education at the university involved in the biomedical education and research. Moreover, to strengthen the animal welfare further, it was proposed to take 3Rs to the law though the guideline for animal experiment was established at each university, and an independent restriction by the institutional animal care and use committee was done.

Therefore, 3Rs will be taken to animal protection act of Japan, Law For The Humane Treatment and Management of Animals, which the revision will shortly be scheduled.

In this presentation current state of education about animal experiment involved in 3Rs will be introduced based on questionnaires from medical school in Japan.
Lecture

Establishment of JaCVAM and welcome to WC6 in 2007/Tokyo

Yasuo Ohno
National Institute of Health Sciences, Tokyo, Japan

Japanese Society for Alternatives and Animal Experiments (JSAAE) was established in 1982 by Tsutomu Sugawara et al. and has been contributing to the research on alternative methods (AM), validation of AM, education about 3R, and communication with the public. By co-operation with research group supported by MHLW and Japanese Cosmetic Industry Association (JCIA), JSAAE conducted validations of \textit{in vitro} cytotoxicity tests, eye irritation tests, skin irritation tests, skin corrosivity tests, and phototoxicity tests. Based on these efforts, Japanese government approved the establishment of new section in National Institute of Health Sciences for the management and evaluation of AM and for the international co-operation in this field. This section is small. However, we consider that it is a good start. We named the section, Japanese Center for the Validation of Alternative Methods (JaCVAM). It will open in this Autumn.

We are very pleased to host the 6th World Congress on Alternatives and Animal Use in the Life Sciences (WC6) in 21-25 August, 2007 in Tokyo. WC6 is also sponsored by ACT and Japanese Society of Toxicology, Japanese Society of Laboratory Animal Sciences, The Japanese Environmental Mutagen Society, Japanese Society for Laboratory Animal and Environment, and International Society of Toxicology and International Society on Toxicology. We are expecting the meeting will contribute to the developments and acceptance of new AM and activate further research on AM.

Lecture

Phototoxicity: 3T3 NRU PT and proposing a new battery system

Yuko Okamoto
Fundamental Research Center, KOSÉ Corporation, Tokyo, Japan

Several \textit{in vitro} phototoxicity methods have been developed to assess the phototoxic potentials of substances. These can be classified into two groups: the methods for screening purposes and tests focusing on the specific mechanisms of phototoxic reactions. Among these methods 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) was accepted as an established alternative method by ECVAM. In the year 2004, 3T3 NRU PT was adopted as OECD guideline for chemicals (OECD Test Guideline TG432). In Japan, Evaluation Committee of Japanese Society of Alternatives to Animal Experimentation in co-operation with NIHs carried out the peer review of 3T3 NRU PT in order to verify the propriety as a method for regulatory acceptance. We confirmed that the 3T3 NRU PT is a good screening method to predict phototoxicity potentials. However there are some limitations to adopt the substances in this assay. It is difficult to evaluate water insoluble substances clearly because of the cell culture system. In order to improve this problem, a new battery system with the yeast growth inhibition phototoxicity assay (Yeast assay) and the red blood cell photohemolysis assay (RBC assay) was proposed from a Japanese industry. The inter-laboratory validation study of this assay was performed using nine test substances. The correlation with \textit{in vivo} data of the battery system was better than those of the single use of each assay. The other results and the future investigation of this assay and our recent studies using 3T3 NRU PT will be discussed.
Several in vitro skin sensitisation methods using human cell lines have been reported. In our previous study, we optimised our human cell line activation test (h-CLAT) using THP-1 cells (monocytic leukaemia cell line) and conducted an inter-laboratory study. We found that measuring CD86/CD54 expression may be useful for predicting skin sensitisation at the concentration with a certain level of cytotoxicity. The aim of this study was to confirm the relationship between CD86/CD54 expression and viability of THP-1 cells in the h-CLAT. In this study, twenty allergens (e.g. DNCB) and nine non-allergens (e.g. SLS) were evaluated. For each chemical, more than 10 concentrations that gave a predicted cell viability range of 20-95% were used. The data showed that:

1. Expression patterns of CD86/CD54 differed depending on chemical. For the most allergens, some cytotoxicity (70-90% cell viability) was needed for enhancement of CD86/CD54 expression.
2. The criteria “CD86≥150 or CD54≥200” resulted in an accuracy of 93% which confirms appropriate cut-off criteria for h-CLAT.
3. A dose setting of serial 1.2-fold dilutions based on CV75 (estimated dose of 75% cell viability) may be provide a good prediction of allergens.
4. A good correlation was observed between EC3 of LLNA and EC150 (CD86) or EC200 (CD54) of h-CLAT. So EC150 or EC200 may be used as an estimate of allergic potency (EC150/200: Estimated Concentration required to induce 150% or 200% CD86/CD54 expression).

These findings suggested that h-CLAT would be a more robust in vitro skin sensitisation test.

The Japanese Society of Alternatives to Animal Experiments (JSAAE) was established in 1982 as an academic society, it now has more than 300 memberships and 13 supporting organisations. The main objective of the JSAAE is to promote alternative research and development, and education in alternatives to animal experimentation based on the Three Rs concept. The outcome of such activities is presented in annual meeting and official journal of Alternatives to Animal Experimentation (AATEX) in the JSAAE. In the last two years, we have performed and planed several validation studies such as skin irrita-tion assay using three-dimensional skin model, battery system of phototoxicity assay using erythrocytes and yeast, and an alternative of the LLNA assay without radioactive compound. The JSAAE encourages young scientists by presentation of awards in annual meeting and excellent articles contributed in journal of AATEX. As part of JSAAE activities, we are responsible for promoting a global view of the Three Rs concept to other Asian partners and we are trying to exchange information and technical transfer to South Korea and China.
Animal Ethics Infolink (www.animalethics.org.au) was developed by the NSW Animal Research Review Panel to (1) facilitate communication with key stakeholders to raise awareness of and rapidly respond to emerging issues and (2) enable ready access to web-based publications and other on-line resources to the widest possible audience. The scope and detail of the information has been designed to meet both specific and general enquiries and covers a broad range of activities. Utilising the resources of the NSW Department of Primary Industries, the website provides researchers, animal house managers, technicians, institutional Animal Ethics Committees (AECs), administrators, students and the public with information about legislation and codes of practice governing the use of animals in research and teaching, policies covering a range of subjects, such as, the operation of AECs and guidelines on topics such as housing and husbandry of particular species and specific research procedures. The website has extensive links to information on the 3Rs and to organisations and other useful websites. All listings are annotated to facilitate searching for specific topics. The website also includes a regularly updated newsletter.
Lecture

Database on animal experiments in Germany

Corina Gericke
People for Animal Rights Germany, Doctors Against Animal Experiments, Braunschweig, Germany

Animal experiments are widely funded by tax payers money, but still the broad public has got no access to information on what is really happening in animal research. By establishing an internet database we aim to reveal the conditions of animal experiments in German laboratories and to initiate a public discussion on this subject. In addition to this we offer solutions by providing information on a number of non-animal research methods.

Many animal experiments carried out at research institutes are published in English-speaking specialist journals. In general, these publications are not accessible or, due to technical jargon, not understandable to the layman. By summarising published papers on animal experiments and translating them into German we make these information accessible to the public.

The database currently provides descriptions of more than 3,000 experiments which have been conducted in Germany during the past 10 years. Users of the database can easily find out about experiments on certain animal species, in which cities they are carried out, which research area they belong to and which research institutes conducted them. The database on in vitro methods currently includes 90 methods.

Animal researchers often claim animal experiments are not harmful and antivivisectionists would exaggerate their extent. By providing access not to a secret world, but to a world which is hidden to the public, people can make up their own minds. The uncommented and plain descriptions of the database speak for themselves.

Lecture

AnimAlt-ZEBET – an internet database on alternatives to animal experiments

Barbara Grune, Rainer J. Box, Antje Dörendahl, Susanne Skolik and Horst Spielmann
Federal Institute of Risk Assessment (BfR), Centre for the Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

Background: According to current legislation scientists have to explore the use of alternatives to animal experiments (cf. German Animal Protection Law and EU Directive). For this reason the German Centre for the Documentation and Validation of Alternative Methods (ZEBET) at the German Federal Institute for Risk Assessment (BfR) has made available its database on the internet. The main aim is to provide information on alternatives both to scientists and the representatives of regulatory authorities.

Basic concept: The ZEBET Database “AnimAlt-ZEBET” contains reports which have been evaluated by ZEBET’s scientific staff according to the concept of the 3Rs (refinement, reduction, replacement) established by Russel and Burch in 1959. AnimAlt-ZEBET also provides an assessment of the current stages of development, validation and acceptance of a method for either scientific or regulatory purposes. AnimAlt-ZEBET is a full-text database in English. It covers alternative methods in many fields of the biomedical sciences and related disciplines. Each document consists of several data fields, e.g. title of method, keywords, evaluation according to the 3Rs principle, abstract and bibliographic references.

Access and AnimAlt-ZEBET usage: AnimAlt-ZEBET can be accessed free of charge on the Internet via the German Institute for Medical Documentation and Information (DIMDI), http://www.dimdi.de. Searches in AnimAlt-ZEBET may be combined with searches in well-established databases such as MEDLINE. As of April 2005, AnimAlt-ZEBET contained 117 documents. The database contents are updated on a regular basis and new reports are added. ZEBET’s Activity Report shows an average of about 23,000 visits per annum.
Lecture

Altweb, the alternatives to animal testing web site: A global clearinghouse of information about the Three Rs

Carol Howard
Johns Hopkins Center for Alternatives to Animal Testing (CAAT), Baltimore, Maryland, USA

In 1997, the Johns Hopkins Center for Alternatives to Animal Testing (CAAT) launched Altweb, the Alternatives to Animal Testing Web Site (http://altweb.jhsph.edu). Altweb was created to serve as a central reference point or gateway to alternatives information, resources, and news. It is international in scope and freely available to all users.

Altweb is intended to serve diverse audiences, including biomedical researchers, industry, regulatory agencies, the international alternatives community, animal care and use/animal ethics committees, the animal welfare community, veterinarians, lab technicians, educators, students, and the general public.

This session will present an overview of the resources available on Altweb, highlighting the latest developments. We have redesigned the site, with several goals in mind: To facilitate easier, faster, and more logical access to data; to make the site more aesthetically appealing; to take advantage of new Web standards (XHTML, CSS); to and to be adhere as closely as possible to accessibility standards.

An important new addition to the site is a guide to searching for alternatives – a web-based resource aimed at assisting scientists with both compliance and relevancy. We also are adding a Spanish-language version of several general interest sections (FAQs, history of alternatives, and glossary). A new special section on refinement issues is in progress.

Other resources available on Altweb include: Specialised databases; abstracts from major alternatives journals; relevant books, reports, proceedings, articles and newsletters; news updates in the alternatives field; a meetings calendar; and a special section on monoclonal antibody production.

Lecture

Special resources supporting the 3Rs at the U.S. national library of medicine

Vera Hudson
National Library of Medicine, National Institutes of Health, US Department of Health and Human Services, Bethesda, USA

Resources at the U.S. National Library of Medicine (NLM) have greatly increased since 1992 when the Bibliography on Alternatives to the Use of Live Vertebrates in Biomedical Research and Testing was first distributed to the scientists. With the advent of Internet technology, NLM has extended its services to the general public in providing health and toxicology information. This presentation will focus on features and NLM services which can assist and educate users looking for alternatives to animal experiments in PubMed, ALTBIB, TOXLINE, ChemIDplus, GenBank and PubChem. The National Center for Biotechnology Information (NCBI), an NLM component, maintains the GenBank of DNA sequences. It contains sequences from over 140,000 species in a centralised location which is integrated with information for all major research organisms. A new development under NCBI is the PubChem suite of databases. PubChem was developed as part of the NIH Small Molecule Repository which will eventually assist in the study of cell functions and metabolic pathways. This in turn will reduce animal experimentation.

The content of some of the specialised NLM and other NIH databases and linkages between them will be presented.
Lecture

ECVAMs database service-online

Annett Janusch Roi
European Commission-Joint Research Centre, Institute for Health and Consumer Protection, ECVAM, Ispra, Italy

ECVAM’s database service was established to achieve a principal objective of ECVAM as required by the European Commission and Parliament, namely, “to establish, maintain and manage a database on alternative procedures”.

The year 2005 will see the public access to the entirely revised Internet version of ECVAM’s DataBase service on Alternative Methods (DB-ALM), formerly known as “SIS”. In addition to the already available INVITTOX protocol collection, method-summary descriptions will be included, as well as details on formal validation studies and test results. A new sector on (Q)SARs is under development. DB-ALM provides its information as evaluated data sheets and is based on extensive literature reviews including ECVAM in-house information. So far, information is available for 21 topics mainly in the area of toxicity testing of chemicals. The current online version of SIS can refer to 3,000 registered users from 65 countries being the USA (22%), UK (15%) and India (13%) the major customers followed by Germany and Italy (7% each) and by France and Spain (6% each) in addition to others.

Poster

ASPCA Animal Poison Control Center (APCC) clinical data can support 3R initiatives

Safdar Khan1, Mindy Bough1, Steven Hansen1, Harold Trammel1 and Stephen Zawistowski2
1 ASPCA, Animal Poison Control Center, Urbana, IL, USA; 2 ASPCA, National Programs, New York City, NY, USA

The ASPCA Animal Poison Control Center (APCC) provides 24-hour consulting services to animal owners, practicing veterinarians, and pharmaceutical and chemical industry personnel. APCC staff obtains an extensive history, offers diagnostic recommendations, directs sample submission and offers detailed therapeutic recommendations for each case. In 2004, the APCC managed more than 95,000 cases, involving over 100,000 animals.

In 2001, the APCC developed a sophisticated veterinary database, AnTox™, to support management of animal poisoning cases and to collect detailed incident data. AnTox™ data have helped characterise sensitivities and syndromes and identified minimum toxic and lethal doses for several pharmaceutical and chemical agents. AnTox™ data analyses led to identification of clinical problems involving substances previously considered safe. For example, data retrieved from AnTox™ showed that ingestion of calcipotriene, a vitamin D3 analogue, causes hypercalcemia, renal failure, and death in dogs. Similarly, AnTox™ data indicated that cats are extremely sensitive to concentrated permethrin-containing flea and tick products. Data relating to permethrin exposure were used to suggest revisions to product labels. AnTox™ data summarising exposures to human non-steroidal anti-inflammatory drugs in animals were utilised by the FDA Center for Veterinary Medicine. AnTox™ data have also been used to verify consumer product safety in animals when products have been rumored to be unsafe. AnTox™ data can be valuable to regulatory agencies and product manufacturers when a product’s safety is in question or when new formulations are under consideration. The data can help pharmaceutical and chemical companies reduce, refine and replace animal studies when investigating product safety.
The quantity and diversity of xenobiotics creates a high demand for reliable and efficient predictive test systems to evaluate possible health risks. Although the incidence of renal tumours is on the rise in Western countries, no suitable test systems exist for the prediction of the carcinogenic potential in the human kidney so far – except for costly and protracted animal studies. The aim of our project is to establish an in vitro test system by identifying marker genes characteristic for responses to carcinogenic substances.

This study conducted by three research groups in different locations will generate large amounts of heterogenous data in the course of several years. Thus, a centralised and secure, easily accessible database solution is required.

With the system presented, data integrity is enforced, records of all experimental details are kept and thus documented in a standardised way (in compliance with OECD’s GLP Guidelines). In spite of internal complexity, data is displayed via web-interface in a user-friendly fashion. Data can easily be added and changed with dynamically generated forms and viewed using specialised datasheets. Handling of complex data is eased by sophisticated search options and contextual filtering techniques.

The server application built gives secure access to the experimental and result data, thus helping all co-operation partners to keep the big picture in focus. For each result, all relevant events in the course of the experiment can be displayed. In vitro and in vivo results are structured in a way which allows for direct comparison.

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**Poster**

**A database system for managing experimental data generated by parallel in vitro/in vivo renal carcinogenesis studies aimed at animal experiment replacement**

*Florian Odronitz, Tanja Lampertsdörfer*, Evelyn O'Brien and Daniel Dietrich

University of Konstanz, AG Umwelttoxikologie, Konstanz, Germany

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**Lecture**

**Acubase – database and online service of in vitro tests and methods for predicting human acute toxicity**

*Radoslaw Rzepka¹, Robert Rudowski¹, Dariusz Sladowski² and Sandra Coecke³*

¹ Medical University of Warsaw, Department of Medical Informatics and Telemedicine, Warsaw, Poland;
² Medical University of Warsaw, Department of Transplantology, Warsaw, Poland;
³ ECVAM, JRC, Institute for Health and Consumer Protection, Ispra, Italy

Acubase is a part of A-Cute-Tox – FP6 EU Research Project for Alternative Testing. Large European integrated projects, involving many partners require an effective and user friendly systems for data acquisition, management and analysis. Data generated in participating laboratories have to be integrated into one manageable database which enables to use raw data for statistical evaluation and construction/evaluation of prediction models. It also facilitates GLP compliance of conducted research which is now a prerequisite for successful validation and implementation of new toxicological methods. Due to a large number of participating centres, diversity of experimental design and type of generated data, creation of such an integrated data management environment is a very demanding task. Acubase is written as a multi-layered and multi-module web-hosted application that facilitates data transfer and analysis of in vivo and in vitro toxicity tests. The Acubase works on client-server architecture and any modern web browser on client-side is needed to get access. The database is already available on Internet https://acubase.amwaw.edu.pl and contains 16 selected chemicals which will be tested first. In the next stage protocols will be put into the database to standardise and optimise experiments. In summary, Acubase will serve as a central element of the project with regard to reporting, management and analysis of data in order to improve predictions of human acute toxicity and substantially reduce animal experiments. In future it can be modified and used for the data management of other similar multicentre projects.
Many new alternatives to conventional search methods have been developed. Some of these have examples potentially applicable to Alternatives and Animal Use in Life Science.

Meta-search and clustering techniques have been developed using natural language processing methodologies. Target databases or Web information resources can be designated for a custom search. The search results are analysed, and concepts of interests are aggregated and clustered. Users may then refine or modify their queries. For example, at the National Library of Medicine, a meta-search and clustering engine has been developed to perform topic specific search in areas of AIDS/HIV, toxicology, and environmental health information.

Information portals are collaborations among organisations in developing cross-organisation websites (portals) to provide the breadth of information and services available about a particular topic or audience group. An example of this work is a cross-agency portal on environmental health and toxicology for consumers. Several US government agencies collaborated to bring together environmental health information and services.

Another way of approaching information is the use of specialised tools. Among these, the Quantitative Structure Activity Relationship Tool, a new added feature in ChemIDplus, has potential interest. Chemical structures, physicochemical properties, and acute toxicity data have been added to the existing chemical profile database.

These new methods to consolidate information may be helpful to the community of Alternative and Animal Use in Life Science. Traditional and new search engine methodologies and examples will be presented.
Lecture

Search strategies for detecting alternative methods articles: A pilot study

Barbara Grune, Amrei-Christin Riemann, Antje Dörendahl, Susanne Skolik and Horst Spielmann
Federal Institute of Risk Assessment (BfR), Centre for the Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

Background: As a consequence of the increasing regulatory acceptance of the 3Rs concept of Russel and Burch (1959) on using alternative methods to animal experiments, it is essential that scientists, animal welfare officers, public policy-makers be able to retrieve relevant, high-quality alternative methods reports. Efficiently retrieved scientific literature supports decision-making for application of alternative methods. However, the volume of the literature is overwhelming for both scientific and regulators. The literature on alternative methods is spread over a large number of journals and consequently a large number of literature databases. Users of bibliographic databases are faced with retrieving the most important references.

Objectives: In a pilot study we endeavoured to answer the question: “What are effective search strategies for articles on alternative methods to acute oral toxicity testing?”

Free text searches (n=28) were performed in databases offered by DIMDI, the German Institute of Medical Documentation and Information. The retrieval performance of selected search terms (n=14) were evaluated. We determined the relevance of the articles retrieved.

Results: We identified a first ranking of search terms and databases for searching articles on alternative methods to acute oral toxicity testing. More research will be needed to address additional topics.

Conclusion: Recommendations for search strategies to improve the success of searching for articles on alternative methods should include appropriate search terms, phrases and recommendations for databases. Recommendations should be as specific as possible and applicable to any user-defined search conditions.

Lecture

The US Department of Agriculture, Animal Welfare Information Center – a source for meeting information requirements of the 3Rs

Jean Larson
National Agricultural Library, U. S. Department of Agriculture, Beltsville, Maryland, USA

The presentation will include a review of carefully selected information resources such as the following: Understanding with the Searching for Alternatives Worksheet; resources available for a workshop on searching for alternatives; documents on issues in animal welfare; what you can find in the AGRICOLA database; and the National Agricultural Library Thesaurus subset of terms useful in searching AGRICOLA, other databases or for using as keywords by authors of alternative research.
The search for alternatives appears to be a simple endeavour, yet causes a great deal of resistance and scepticism. The reasoning behind the development of animal welfare and alternatives legislation is usually clear; in the US, the alternatives search requirement was added to the Animal Welfare Act in order to assure the general public that no animal used in research undergoes unnecessary pain or distress. While meeting compliance is not difficult (list databases, dates and search terms), locating relevant information and giving thoughtful consideration to the breadth of alternatives is not the usual result. However, when a comprehensive search is performed by searching all of the scientific literature published in a specific area of study, the information retrieved will be more relevant, and attention to animal welfare assured.

Already familiar with locating scientific material for their research, scientists can readily learn to expand their literature searches to include additional databases. At the same time, the subject of alternatives can be more broadly defined to include the terms replacement, reduction and refinement. Equally important is expanding the search to include new ideas and technologies, as well as aspects of husbandry and care such as housing, blood collection, analgesia, anaesthesia, and humane endpoints. The web-based resources located at http://www.vetmed.ucdavis.edu/Animal_Alternatives/databaseapproach.html are designed to assist with a comprehensive search. Arranged by both animal model and topic, the tables prompt the user to consider additional concepts and additional databases.

Lecture

Considering alternatives and welfare via a comprehensive search of the scientific literature

Mary Wood and Lynette Hart
UC Center for Animal Alternatives, School of Veterinary Medicine, University of California, Davis, USA
Workshop 4.3
Search strategies – user requirements

Lecture
Overview of the regulatory requirements for the consideration of alternatives

Jodie Kulpa-Eddy
USDA-APHIS-Animal Care, Riverdale, Maryland, USA

This presentation will cover the development in the United States of the requirement for the principal investigator to consider alternatives to procedures likely to cause pain and/or distress in animals. Regulations and requirements in other countries to consider the 3Rs of Russell and Burch (replacement, reduction and refinement) will also be presented.

A review of a survey conducted by USDA in 2000 to assess the impact of our Institutional Animal Care and Use Committee regulations, as well as a review of the current inspection report citations involving the consideration of alternatives in animal use proposals will demonstrate the types of problems associated with enforcing this regulation.

A suggested list of “best practices” that may be utilised to prevent or control these types of problems will lead to an audience discussion.

Topics for discussion may include:
1) Ways to measure the impact of the researchers’ consideration of alternatives.
2) Best methods for distribution of information on potential alternatives to researchers and Committees.
3) How to encourage the sharing within industry of information on alternatives to animal use in regulatory testing.
Lecture

Effective alternatives search strategies: International perspective for compliance

Mary Wood¹, Vera Hudson² and Lynette Hart¹

¹UC Centre for Animal Alternatives, School of Veterinary Medicine, University of California, Davis, CA, USA; ²Division of Specialised Information Services, National Library of Medicine, Bethesda, MD, USA

In the member states of the EU, animal welfare legislation allows scientists to conduct an animal experiment only if no other scientifically satisfactory method is reasonably and practically available. The Animal Welfare Act in the US requires that scientists search for and consider alternatives to any procedures that may cause pain or distress. While these legislative requirements appear straightforward, compliance with a sincere effort to meet the intent is challenging. The research scientists and the institutional review committees endeavour to meet regulations without always fully understanding the best and most productive approach.

Specific strategies and techniques to be used when searching for alternatives and welfare information will be presented, together with a discussion of database selection and access. Resources and strategies available to researchers having only limited library support will be emphasised. Guidelines and suggestions on how to search for all Three Rs, with examples and templates, will be presented in an effort to support efficient and effective compliance. Resources covered will include those organised by the UC Centre for Animal Alternatives, http://www.vetmed.ucdavis.edu/Animal_Alternatives/main.htm and indexed by the National Library of Medicine, http://www.nlm.nih.gov/.
Exposure of humans and wildlife populations to increasing levels of endocrine disrupting chemicals (EDC’s) in the environment has focused attention of the US EPA on standardisation and validation of in vivo screens for EDC’s. In this study a comparison of sensitive, rapid and cost effective in vitro alternative methods for detection of EDC’s was conducted using high throughput yeast and mammalian cell reporter gene assays and results were evaluated against in vivo uterotrophic and Hershberger assays conducted in rats. The yeast assay (developed by GlaxoSmithKline) employed Saccharomyces cerevisiae containing the DNA sequence of the human oestrogen receptor (hER) and an oestrogen-responsive ERE/lac-Z reporter gene plasmid in a 96 well-plate chromogenic assay measuring absorbance at 540 nm. The mammalian cell assay used human ovarian carcinoma cells containing an oestrogen-responsive ERE/luc 7 reporter gene plasmid with measurement of luciferase expression. A range of oestrogenic and androgenic steroids, plasticisers, organochlorine pesticides and surfactants were screened and assessment of agonist and antagonist actions made. The results reported show a high degree of sensitivity and reproducibility between the in vitro reporter gene assays and compare favourably to the in vivo endocrine disrupter assays. It is considered that Regulatory acceptance of standardised and validated in vitro alternative assays for EDC’s, such as described here, will reduce the need for animal testing and provide sensitive, high throughput capacity to facilitate broad and cost effective uptake of tier I screening methods for endocrine disrupters worldwide.
**Poster**

**A novel non-radioactive method for measuring in vitro aromatase activity using the H295R, a human adrenocortical cell line**

Pascale Berckmans, Geert Verheyen, An Van Rompay and Hilda Witters  
VITO, Environmental Toxicology, Mol, Belgium

Certain environmental contaminants are suggested to disrupt endocrine processes with potential effects on reproduction, sexual differentiation, growth and development. Current research has mainly focused on interactions with the sex hormone receptors. However, other mechanisms such as effects on steroid synthesis and metabolism should be considered. Some chemicals have been shown to interact with aromatase (CYP 19) and consequently have a profound effect on hormone function and homeostasis. Aromatase is of particular interest because it is the rate limiting catalyst in the formation of estrogens. It is of importance, not only in cells involved in the de novo synthesis of estrogens, but also in brain and adipose tissue, which utilise circulating levels of androstenedione or testosterone as precursors. A novel non-radioactive method for measuring aromatase activity using H295R cells (human adrenocortical carcinoma) was developed through the optimisation and application of an ELISA-method for estrone in cell culture conditions. The aromatase assay was first tested using pure chemical compounds, such as atrazine, prochloraz, tributyltin, forsykolin. Both induction and inhibition of aromatase could be detected. The induction responses were accompanied by increases in CYP19 RNA levels, determined by real-time RT-PCR. In order to apply this in vitro aromatase assay for assessment of environmental exposure, selected chemical compounds were spiked in water and a solid phase extraction method (SPE) was optimised. With an adequate sample treatment method, real environmental water samples are ready to be analysed and the environmental load of chemicals with potential effects on steroid metabolism can be estimated.

**Lecture**

**REACH – CEFIC’s conception of a feasible, information- and priority-based approach**

Rüdiger Bias  
BASF Aktiengesellschaft, GUP/C – Z 570, Ludwigshafen, Germany

The European Commission’s proposal for a new Chemicals Legislation, REACH, presented as of 29 October 2003, is currently under strong discussion in the processes of the first readings in the EU-Council and in parallel in the EU-Parliament. It foresees in a very formal and systematic manner obligations to industry to register all substances and submit volume dependent information packages on each substance manufactured or imported above 1 ton/year. It further requires comprehensive documentation of the properties, hazard studies and profiles. Furthermore it stipulates substantial analysis of exposure through extensive surveys of uses. Routinely, starting with substances above 10 tons/year a Chemical Safety Assessment is needed. For dangerous substances a Chemical Safety Report has to be submitted.

The European Chemical Industry as associated within CEFIC has engaged itself in the search and development of proposals to improve the present European regulatory schemes on substances. Specifically CEFIC proposes to achieve the objectives of REACH in a less bureaucratic and thus in the long run less burdensome way.

In doing so the CEFIC proposal focuses on defining the appropriate scope of REACH, on priority setting according to risks likely going along with substances’ uses, and on improving the work flow between all actors.

The paper will highlight the key elements of the industry proposal. The prerequisites for a more efficient system shall be explored.
In 1998, the U.S. EPA’s Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended the standardisation and validation of several assays for identifying possible endocrine-disrupting (ED) substances. Included among these assays are estrogen (ER) and androgen receptor (AR) binding and/or transcriptional activation (TA) assays. NICEATM and the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) convened an independent scientific expert panel to evaluate the status of these assays and proposed reference chemicals for validation studies. Based on the expert panel’s recommendations and public comments on a draft list of substances, ICCVAM prepared a final list of 78 substances for use in future ER/AR binding/TA validation studies. ICCVAM recommends testing a minimum of 53 substances for ER-based assays and 44 substances for AR-based assays; each set includes at least 25% negative or presumed negative substances. The use of this standard list of reference substances in future validation studies will facilitate determination of the acceptability of in vitro and in vivo assays and test batteries for inclusion in screening programs for ED substances. However, to comprehensively assess the usefulness of ER/AR binding/TA assays as individual components of the EDSTAC Tier I screening battery, and to facilitate development of more predictive in vitro ED assays, ICCVAM recommends that all 78 substances be tested in the four types of assays. This will generate a high quality in vitro database to facilitate future validation efforts and comparison of performance among different test methods and protocols.

Poster

ICCVAM recommended reference chemicals for validation of in vitro estrogen and androgen receptor binding and transcriptional activation assays

Bradley Blackard1, Christina Inhof1, Raymond Tice1, William Stokes2, Marilyn Wind3, David Hattan4 and Leonard Schechtman4

1 Integrated Laboratory System, Inc./NICEATM, Research Triangle Park, North Carolina, USA; 2 National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA; 3 US Consumer Product Safety Commission, Bethesda, Maryland, USA; 4 US Food and Drug Administration, Rockville, Maryland, USA

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Poster

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Lecture

Intelligent testing strategies for REACH

Robert Combes, Nirmala Bhogal, Christina Grindon and Michael Balls

FRAME, Nottingham, UK

The proposed REACH system for chemicals risk assessment has prompted several proposals for intelligent testing strategies (ITS) which comprise (Q)SAR modelling, read-across (chemical) and other non-biological approaches. The potential advantages and limitations of such ITS will be reviewed. It will be argued that it would be premature to base a testing strategy mainly on chemical approaches and computational modelling, at least until such time as criteria to validate them for their reliability and relevance by using independent and transparent procedures have been agreed. This is due to the inherent problems with validating (Q)SARS for regulatory acceptance and using procedures that have been developed and applied to the validation of in vitro tests. Until this issue has been resolved, it is recommended that testing strategies should be developed and applied in a cautious and judicious way, incorporating computational and read-across approaches, along with information from available in vitro tissue culture methods and metabolism and biokinetic studies. Such strategies should be intelligently applied by being driven by exposure information (based on bioavailability and not just production volume) and specific hazard information needs, in preference to a generalised tick-box approach. In the meantime, there should be increased efforts to develop improved (Q)SARS, expert systems and new in vitro methods. Ways to expedite their validation and acceptance should also be found, and prospectively agreed with all major stakeholders.
The EU REACH (Registration, Evaluation and Authorisation of Chemicals) system aims to combine existing regulations covering chemical safety into one policy, and to pass the “burden of proof” from the regulators to industry, so that companies must evaluate the safety of substances and satisfactorily manage risks to humans and the environment. It is estimated that of all substances produced and marketed in the EU, 30,000 lack sufficient safety data to fulfil these new requirements and will therefore require further assessment. The number of animals which will be involved in this extra testing is currently estimated to be in the region of 2-4 million over the 11 year implementation period. FRAME is reviewing how the practical implementation of the Three Rs could minimise the number of animals required by REACH. The project focuses on the major endpoints within REACH, and includes a review of in vitro alternatives, their organisation into testing schemes for each major toxicity endpoint, and their potential inclusion into overall intelligent testing strategies for risk assessment. Where alternatives will not be available, either now or in the foreseeable future, we indicate how existing animal testing protocols could be improved both scientifically and from an animal welfare perspective. Our findings and recommendations will be presented. This work is linked to a DEFRA-funded project in collaboration with Liverpool John Moores University that includes an assessment of the potential for using (Q)SAR modelling and expert systems in REACH.

**Poster**

**A practical implementation of Three Rs approaches to REACH**

*Christina Grindon, Robert Combes and Michael Balls*

FRAME, Nottingham, UK

The potential effects of so-called “endocrine disrupting chemicals” on environment and man are controversially discussed by scientists and the public. We established in vitro screening systems based on genetically modified yeast cells to detect the (anti-) estrogenic potential (YES) and the (anti-) androgenic potential (YAS) of test substances. Genes, encoding for the human estrogen receptor alpha, and the human androgen receptor, respectively, have been integrated into the yeast genome of the strains used. Additionally, the cells contain a plasmid carrying the lac Z gene, which is receptor-dependently expressed and used as reporter gene (1, 2). The YES and YAS assay is conducted on 96 well plates using a standard protocol to determine agonistic as well as antagonistic effects over a concentration range of seven magnitudes on the same plate. Positive control substances used for estrogen and androgen agonistic activity were Estradiol and Dihydrotestosterone, for antagonistic activity Hydroxymestranol and Hydroxyflutamide, respectively. Both assays have been validated with more than 60 literature known substances, covering synthetic and natural agonists and antagonists, industrial chemicals, pesticides as well as substances expected to be cytotoxic and hormonally inactive. The results show very high reproducibility and good concordance with the literature data. As shown for different substances, an advantage of these assays is to detect cytotoxicity simultaneously with the endocrine modulating activity, to avoid artifacts due to cell death. In conclusion, the YES and YAS turned out to be robust systems, easy to handle and satisfying the requirements for screening systems.

**Literature:**
Lecture

**ToxCast: A strategy for the categorisation of chemicals**

*Robert Kavlock*
National Center for Computational Toxicology, Office of Research and Development, US Environmental Protection Agency, USA

The US EPA is often faced with assessing the hazards and risks of large numbers of chemicals (e.g. High Production Volume Chemicals, potential endocrine disrupting chemicals, pesticidal inerts, the Candidate Contaminant List) without necessarily having the full context of toxicological information desired for analysis. Tools are therefore needed to help the Agency prioritise and categorise chemicals for evaluation. Partly in response to this need, the EPA initiated a computational toxicology program (see [www.epa.gov/comptox](http://www.epa.gov/comptox)) and established the National Center for Computational Toxicology (NCCT) to carry out supporting research. “ToxCast” is a concept being developed by the NCCT to aid in the prioritisation process. It is based on the assumption that toxicological hazard is a result of chemical-biological interactions, and that information pertinent to such interactions can be derived from a number of domains. These information domains include physical-chemical properties, predictions of reactivity by structure-activity analyses, interactions with specific cellular macromolecules, reactions of cellular based assays, and “omic” information derived from cells in culture or whole animals. These domains would be populated to the extent of economic and technological feasibility with data for individual chemicals. Informatic tools would then be applied within and across these information domains to cluster chemicals exhibiting similar properties or patterns of activity. Using an initial set of chemicals whose toxicological profile is well characterised will allow a proof of concept demonstration of the ability to categorise chemicals based on potential biological activity. Examples from the areas of proteomics and genomics suggest that the approach is feasible. This is an abstract of a proposed presentation, and does not necessarily reflect agency policy.

Poster

**Endocrine disruptors – new challenges on phytoestrogens**

*Franz-Josef Klausdeinken*
Harlan Teklad Europe, Borchen, Germany

Plant substances with oestrogenic-like characteristics are called “Phytoestrogens”. The isoflavones contained in the soy plant are among the most important because they are often found at high, and variable, levels in most “traditional laboratory diet formulas”. Phytoestrogens will influence any body functions that involve estradiol. They will influence many research projects.

Because of their low estrogenic activity, dietary phytoestrogens have strong agonistic and antagonistic effects on estrogen receptors. Phytoestrogens can be thus considered selective estrogenic receptor modulators (SERM). They have effects not only on the reproductive tract but many, and perhaps more significant, effects on non-reproductive functions.

Phytoestrogens in GLP studies: Phytoestrogens may play a significant role in GLP studies. The “rodent uterotrophic bioassay” is a well-known test procedure. In an extensive validation study of the procedure by the OECD, the effect of the most important soya phytoestrogens genistein, daidzein and coumestrol has been examined. “Traditional standard diets” with a level of TGE (total genistein equivalent) between 99 and 513 mg/kg were evaluated. The study confirmed that the phytoestrogens had a uterotrophic effect in rats and might mask a mild endocrine disruptor at the higher end of the concentration range.

Solution for research: Not only may traditional laboratory diets contain high levels of phytoestrogens, but the levels may vary significantly from batch to batch. The phytoestrogen content of laboratory diets can be sustainable reduced by substitution of soy components. This low level should be monitored regularly.
Lecture

Animal testing will be minimised. REACH – the new EU chemicals policy and the German position

Uwe Lahl
German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety, Bonn, Germany

The decisions taken to date on the modernisation of EU chemicals legislation are outlined, with a particularly thorough discussion of the policy objectives being pursued with the REACH regulation. REACH will require a considerable amount of data on the intrinsic properties of substances and current levels of exposure to be gathered. This will succeed in ensuring that human beings and the environment are better protected against chemical risks. It also means that REACH will contribute to the conservation of wildlife and the restriction or prevention of the unintended animal experiments taking place today due to the ubiquitous application of xenobiotics.

REACH will lead to a temporary increase in the number of animal tests for a period of around 10 years. Scientists cannot dispense with data from animal testing when the risks of complex effects, such as long-term toxicity, are being evaluated. Nevertheless, the Commission’s draft regulation contains various measures to limit the number of animal tests. These measures are set out individually in detail:

The regulation itself and the proposals concerning it put forward by the member states will make data sharing obligatory in relation to animal testing. This will prevent duplicate or multiple animal tests having to be carried out.

Researchers will also be allowed to make use of older data that were not obtained with current standard methods or in compliance with Good Laboratory Practice, provided these data are valid.

Alternative methods that do not involve animal testing will also be deployed wherever available. An account is given, in particular, of the scientific efforts undertaken in Germany over recent years to develop alternative methods of this kind.

The introduction of structural activity relationship analysis techniques (SAR, QSAR) into risk analysis raises the prospect of a further minimisation of animal experiments.

Finally, there is still a defined time window before REACH enters into force during which ongoing research projects on alternative methods can be completed and further options for cutting down on animal testing developed.

Lecture

ECVAM activities for alternative methods to animal tests for the detection of chemicals with (anti)-estrogenic and (anti)-androgenic activity. ECVAM validation study

Patricia Pazos
ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, Ispra, Italy

Over the last 40 years, there have been constant reports concerning synthetic chemicals that were introduced into the environment that might pose risks to human health and wildlife. Within these xenobiotics, there exists a particular type with hormone-like activity and putative interference with the endocrine system.

Scientific organisation and international regulatory bodies agreed to establish special activities to address the issue of endocrine disruption, promoting initiatives to develop screening programs for identifying chemicals with endocrine disrupting activities with the scope to develop new Test Guidelines.

The European Centre for the Validation of Alternative Methods (ECVAM), created for the protection of animals used for experimental and other scientific purposes, is leading a project to validate test methods tailored for rapid in vitro identification of compounds for their potential to induce hormone-related health effects.
The European Commission is currently revising its chemicals policy, aiming to cover both existing and new chemicals under a common system, called REACH (Registration, Evaluation and Authorisation of Chemicals). According to official estimates, this will result in an increase of up to 45 million toxicological and ecotoxicological animal tests. From the point of view of animal welfare, animal testing should be prevented both for ethical and scientific reasons, and this goal can be met without impeding human health and environmental protection. Apart from the prerequisite to intensify the development and validation of new non-animal test methods and to include all available non-animal test methods in the REACH Regulation, mandatory data sharing and flexible testing strategies can play significant roles in preventing animal testing. Nevertheless, the draft Regulation does not go far enough to ensure that these requirements will be met. Concrete proposals are presented how to amend the provisions to ensure that all existing information on the effects of chemical substances is made available and shared without exception so that for every single substance only one dossier is submitted. The significance of mandatory data sharing is underlined by the results of a survey performed by the German Animal Welfare Federation in Germany, where such an obligation has been implemented in the national Chemicals Act. Additionally, a concept for a step by step testing strategy is discussed that ensures that only such data is collected on a given substance that is necessary for its safe handling.

**Poster**

**Mandatory data sharing and flexible testing strategies to prevent animal testing under the new EU Chemicals Policy**

*Ursula G. Sauer*

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The European Commission is currently revising its chemicals policy, aiming to cover both existing and new chemicals under a common system, called REACH (Registration, Evaluation and Authorisation of Chemicals). According to official estimates, this will result in an increase of up to 45 million toxicological and ecotoxicological animal tests. From the point of view of animal welfare, animal testing should be prevented both for ethical and scientific reasons, and this goal can be met without impeding human health and environmental protection. Apart from the prerequisite to intensify the development and validation of new non-animal test methods and to include all available non-animal test methods in the REACH Regulation, mandatory data sharing and flexible testing strategies can play significant roles in preventing animal testing. Nevertheless, the draft Regulation does not go far enough to ensure that these requirements will be met. Concrete proposals are presented how to amend the provisions to ensure that all existing information on the effects of chemical substances is made available and shared without exception so that for every single substance only one dossier is submitted. The significance of mandatory data sharing is underlined by the results of a survey performed by the German Animal Welfare Federation in Germany, where such an obligation has been implemented in the national Chemicals Act. Additionally, a concept for a step by step testing strategy is discussed that ensures that only such data is collected on a given substance that is necessary for its safe handling.

**Lecture**

**Data availability and needs for a precautionary assessment of chemicals for endocrine-mediated toxicity**

*Troy Seidle*

People for the Ethical Treatment of Animals, Research and Investigations Dept., Toronto, Canada

Various frameworks have been proposed for the testing and assessment of chemicals for possible endocrine-mediated effects, including a two-tier testing battery by the US, a “toolbox” model by the OECD, and entirely animal-free approaches by alternatives and animal advocacy organisations. The former two models identify an “enhanced” two-generation reproduction study in rats (OECD 416) as the “definitive” test for adverse, endocrine-mediated effects in humans. This raises a number of substantial science-policy considerations, including whether the addition of new endocrine endpoints to the current OECD 416 will enable the detection of adverse chemical effects at lower doses; the approach to verifying this empirically (i.e., validation process); and if the enhanced protocol does prove to be more sensitive, the implications for use of existing data from OECD 416 and other sub/chronic toxicity studies in a risk assessment. Some officials in government and industry have asserted that existing evidence of adverse reproductive effects may be insufficient to classify a substance as an endocrine disruptor, and that such a classification would depend on an OECD 416 study being repeated using an “enhanced” protocol. The animal welfare, economic, and regulatory implications of this position will be examined in the context of standard data requirements and availability for different substance classes (e.g., pesticides, pharmaceuticals, food additives, and new/existing chemicals), as well as strategies for avoiding new and/or duplicative animal testing (e.g., chemical grouping, read-across, and mining existing data from relevant toxicological studies).
Poster
Animal welfare implications of proposed data requirements under REACH: Opportunities for the 3Rs
Troy Seidle
People for the Ethical Treatment of Animals, Research and Investigations Dept., Toronto, Canada

Extravagant data requirements outlined in the European Commission’s proposal for a new regulatory regime for chemicals (REACH) could overshadow the laudable aim of the legislation – to protect human health and the environment from harmful substances – and undermine its sustainable implementation. According to the current REACH proposal, a new high production volume (HPV) chemical could be required to undergo up to six times more animal testing than is required elsewhere in the world in order to be marketed in Europe. Data requirements outlined in Annexes V-VIII of the Commission’s proposal could likewise trigger a dramatic increase in animal testing for all existing substances manufactured or imported into Europe in volumes greater than 10 tonnes. Some of the tests proposed consume as many as 2,500 animals and cost up to €1 million per chemical, yet most have never been properly validated according to modern standards. Substantial amendments to the REACH testing annexes have been proposed by animal protection and other stakeholders in order to promote maximum use of validated alternative methods and testing strategies available now or in the foreseeable future. Sustainable REACH implementation can be achieved, but only if industry, regulators, and Europe’s political leadership fully embrace the cost- and animal-saving efficiencies offered by modern computing and cell-based techniques, together with a more precautionary approach to chemical regulation.

Poster
Integration of the 3Rs in regulatory toxicology testing at Huntingdon Life Sciences
Susan Wilkins, Lynn Waterson, Peter Rees and Mark Wing
Huntingdon Life Sciences Ltd, Alconbury, Cambridgeshire, UK

Under the mammalian EU VIIA toxicology package, it is necessary to evaluate chemicals for their potential to cause harm as part of the registration process. At HLS we have embraced the 3Rs making full use of the increasing acceptance of in vitro data by the regulators.

A Weight-of-Evidence (WoE) analysis is performed before doing any in vivo skin and eye work, which relies on client information, together with relevant database searches. This together with test substance physical attributes will determine whether an animal exposure is necessary. If the above analysis suggests that the test substance may be a strong irritant or corrosive, then an in vitro alternative assay is performed, such as the EpiDerm corrosivity assay. In the absence of such an alert, sentient animals are used before exposing the main study animals. Similarly for eye irritation, no animal exposures are performed if there is known potential for eye irritation from the WoE or prior knowledge of the skin irritating potential.

In addition, the 3Rs are applied throughout the regulatory package. For instance, the data from the skin irritation and the acute dermal studies is used to decide whether there is a need to evaluate dose tolerability in a preliminary LLNA study and to assist with setting dose levels. Together, these approaches have resulted in a reduction both in the number of animals exposed and the severity of the findings.
Evidence linking exposure to natural and man-made substances in the environment to adverse effects on the endocrine and reproductive systems of a variety of animal species resulted in concerns about the possibility of similar adverse health effects in humans. The U.S. Congress enacted provisions to safeguard public health from exposure to pesticides in foods and drinking water and required the EPA to develop and validate a screening and testing program to identify substances with endocrine disrupting activity. There are an estimated 87,000 chemicals produced today which have insufficient scientific data to allow evaluation of their potential for endocrine disruption. The EPA is developing a two-tiered screening and testing process. Estrogen receptor (ER) and androgen receptor (AR) binding assays and transcriptional activation assays (TA) have been proposed as part of the Tier 1 screening battery. ICCVAM comprehensively reviewed all the in vitro ER and AR binding and TA assays and concluded that none were adequately validated. Minimum procedural standards such as dose selection criteria, number of replicates per test, appropriate positive and negative controls, and criteria for an acceptable test were proposed that should be incorporated into standardised protocols for each of the four types of assays evaluated.

*This abstract reflects the views of the author, has not been reviewed or approved by, and may not necessarily reflect the views of the Commission. This abstract was prepared as part of the author’s official duties and is in the public domain and may be freely copied. Supported by NIEHS Contract N01-ES-35504.
While conventional toxicology testing focuses on single chemicals, human exposures are usually to more than one chemical. Most regulatory agencies use the default assumption that the risk of exposures to more than one chemical is treated in an additive manner. This approach may under- or over-estimate the risk of chemicals depending on their different modes of action or interaction. Therefore, one objective of combination toxicology is to establish whether exposure to a mixture of chemicals will result in an effect similar to that predicted on the basis of additivity. In this study, human skin fibroblasts were used in the colourimetric MTS (tetrazolium salt; Promega®) and the NRU (Neutral Red Uptake; Sigma) assay to investigate combination toxicology phenomena. Individual IC₅₀ toxicity values for 24 chemicals whose in vivo toxicity was spread over the five Globally Harmonised System (GHS) categories for acute oral toxicity were used to create 18 binary and 6 ternary chemical mixtures. Concentrations of individual chemicals in mixtures were chosen based on an estimation of equitoxicity by applying the concentration addition concept, ensuring that no chemical contributed disproportionately to the overall combination effect. Both MTS and NRU assays were similar and consistent in estimating the interactive effect. The toxicity of the total mixture (IC₅₀(tot)), was then compared with the calculated value (IC₅₀(calc)). 37% of studied chemical combinations resulted in additive (IC₅₀(tot)=IC₅₀(calc)), 45% antagonistic (IC₅₀(tot)<IC₅₀(calc)) and 18% in synergistic interactions. These results suggest that while additivity covered some of the studied interactions, both antagonism and synergism cannot be excluded from chemical risk assessments.
### Poster

**Prevalidation study on testing percutaneous absorption via reconstructed human epidermis**

Udo Bock¹, Eleonore Haltner¹, Monika Kaca¹, Manfred Kietzmann², Hans Christian Korting³, Claus-Michael Lehr⁴, Manfred Liebsch⁵, Frank Netzloff⁴, Frank Niedorf², Maria K. Rübbelke³, Ulfried F. Schäfer⁴, Elisabeth Schmidt⁵, Sylvia Schreiber⁶, Alexander Gabriel Vuia*⁶ and Monika Schäfer-Korting⁶

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Introduction: Toxicological testing is of increasing importance for risk analysis. The recently approved OECD guideline 428 and guidance document 28 standardise experiments on in vitro percutaneous absorption using human and animal skin. Five laboratories plus the ZEBET run a prevalidation study funded by the German Ministry of Education and Research (BMBF) to qualify Reconstructed Human Epidermis (RHE) for in vitro testing of percutaneous absorption.

Methods: A test protocol was set up, thoroughly tested and refined in the partner laboratories. OECD standard substances caffeine and testosterone were applied to human epidermis sheets, pig skin and RHE (EpiDerm™, EPISKIN® and SkinEthic®) mounted into Franz cells.

Results and Discussion: Epidermis sheets and pig split skin are clearly less permeable compared to RHE. With respect to permeability the order of RHE was as follows: testosterone: SkinEthic®>EpiDerm™, EPISKIN®; caffeine: SkinEthic®, EPISKIN®>EpiDerm™. This, however, needs to be verified by a broader validation study including more substances of widely varying physicochemical characteristics.

### Lecture

**Possibilities for assessing risk to humans from chemical exposure by using non-animal test data**

Robert Combes

FRAME, Russell and Burch House, Nottingham, UK

The use of data from non-animal toxicity methods in risk assessment has mainly been limited to hazard identification and for elucidating mechanisms of toxicity. However, there is a need to extend the use of in vitro tests to hazard characterisation and risk assessment. For a test to be useful for HC it must: a) be mechanistically based with a biologically plausible relationship between the endpoint measured and the phenomenon being modelled; b) have been validated against human data (ideally); c) have a relevant endpoint (one occurring in the target species); and d) have a prediction model related to toxicity in the target species. This might be realised by: a) increasing the use of human cells; b) better maintenance of differentiated cells; c) use of genetically-engineered cells; d) development of organotypic cell systems; e) use of co-cultures of different cell types; and f) development of techniques for long term culturing, repeat dosing and assessment of recovery. Also, it will be necessary to obtain more information on the differences between cells in culture and in situ in tissues, and on the effects of dosing in vitro and in vivo, to develop realistic and meaningful uncertainty factors to allow in vitro information to be used for risk assessment in its own right, and in conjunction with animal data. These issues and a suggested proposal for using in vitro data in risk assessment by implementing the above strategies to facilitate the extrapolation from tissue culture to the whole animal, are discussed.
Toxicity of the most antiviral preparations is a major problem of their usage. We studied the cytotoxicity of two derivatives of adamantan – Rimantadine®: 1-(1-adamantyl) ethylamine hydrochloride and Polyrem®: the ionic complex of Rimantadine with the copolymer of vinylic alcohol and vinylamidosuccinic acid in the cell cultures – A-549 and MDCK. Cytotoxicity was assessed by: 1) reduction of MTT or resazurine; 2) leakage of LDH; 3) neutral red uptake.

The results of the short (2 h) exposure with the tested compounds were close to mammalian acute LD₅₀. A study of the metabolic indices after 48 h exposure revealed a higher susceptibility to tested antivirals of the system of endocytosis (NR uptake) in comparison to the other indices. Binding of the active compound to the polymeric complex significantly diminished a degree of its cytotoxicity: the toxicity of Polyrem was approximately twice lower comparing to the equimolar concentration of Rimantadine and the mixture of Rimantadine with the polymeric carrier.

The assessment of possible cytoprotective effect on Rimantadine-induced cytotoxicity of the reduced and oxidized glutathione and Hypoxene® (derivative of ubiquinone) applied in pharmacologically adequate concentrations revealed a significant effect only of the later compound – the enhancement of IC₅₀ 2.3 times in comparison to pure Rimantadine. Hypoxene exhibited its inherent significant antiviral activity diminishing the cytopathic effect on the cells of influenza A (H3N2) virus.

Our results demonstrate the perspective of the application of at least some antioxidants to diminish the toxic effect of antivirals and to boost their specific activity.
Percutaneous studies are usually performed on human skin samples set up on Franz® Cell device but they depend on the availability of skin samples. Reconstructed skin is an interesting alternative to overcome such limitations but it cannot be easily mounted on diffusion cell. However previous studies showed that Episkin® model could be set up on Permgear Cell device and provide a highly performing model, yet time consuming and unsuitable for screening purposes. Then the use of the insert of Episkin® model per se had to be reconsidered. The goal of this study was to compare cutaneous penetration of compounds when applied onto Episkin® samples either on Permgear cell or in their own insert.

Seven compounds having widely different chemical structure and penetration potential were applied in the same vehicle and evaluated in triplicate on two Episkin® batches in both devices (Permgear Cell vs. insert). After 4 hour exposure time, receptor fluids were analysed by LC/MS or LC/UV. No leak was detected in both experimental conditions. For six compounds, the penetrated dose was similar in both devices. For the last one, the penetrated dose was decreased by a factor of two using Episkin® samples still in their insert as compared to sample in Permgear cells.

It was demonstrated that percutaneous study on Episkin® samples could be performed easily using insert. Episkin® model has been greatly improved in the recent years. It is now possible to develop screening tests for evaluating skin penetration with a higher reliability.

Poster

**Improvement of experimental setup for cutaneous penetration screening studies with reconstructed skin**

*Sebastien Gregoire, Catherine Noe, Claire Patouillet, Florence Benech-Kieffer and Christele Ribaud*

L’Oréal Recherche, Life Sciences, Aulnay sous Bois, France

Percutaneous studies are usually performed on human skin samples set up on Franz® Cell device but they depend on the availability of skin samples. Reconstructed skin is an interesting alternative to overcome such limitations but it cannot be easily mounted on diffusion cell. However previous studies showed that Episkin® model could be set up on Permgear Cell device and provide a highly performing model, yet time consuming and unsuitable for screening purposes. Then the use of the insert of Episkin® model per se had to be reconsidered. The goal of this study was to compare cutaneous penetration of compounds when applied onto Episkin® samples either on Permgear cell or in their own insert.

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Poster

**Percutaneous absorption test on reconstructed skin: Validation for hydrophilic compounds**

*Sebastien Gregoire, Catherine Noe, Claire Patouillet, Florence Benech-Kieffer and Christele Ribaud*

L’Oréal Recherche, Life Sciences, Aulnay sous Bois, France

Previous studies demonstrated that human reconstructed skin models could be very useful to evaluate percutaneous absorption. They however involved 3 test chemicals only. The present study extended such validation to 10 hydrophilic compounds. 

*Ex vivo* human skin data were obtained for all compounds in the same laboratory under similar experimental setup using various conditions: whole or dermatomed human skin, two types of cosmetic vehicle (oil/water emulsion or hydroalcoolic gel), with 16 or 24 h exposition time. Despite these differences, data collected could be considered as homogeneous. Two groups of compounds could be distinguished: the first one includes 6 compounds with permeated dose higher than 3% of applied dose and the second one 4 compounds showing permeated doses lower than 0.5%. Reconstructed skin (Episkin® 0.38) model was then used. Each compound was tested on at least 3 batches. The intra- and inter batch variability was generally lower than 30%. The ranking was not modified over these batches. Appropriate experimental setup was used: an application time lowered to 4 hours and an applied dose concentration adapted to the compound solubility in the simplex vehicle. All compounds were studied in one set. To reach such throughput, Episkin® model was used in insert. The Episkin® 0.38 model was able to discriminate the two groups of compounds. The different ranking inside the two groups could be explained by the imperfect barrier function of reconstructed skin model and/or the variability of *ex vivo* data. These results validate reconstructed skin model as an efficient tool for estimating percutaneous absorption.
The aim of toxicological hazard assessment is to characterise the dangerous properties of chemicals for man and the environment. Information on both (a) the toxic potential, i.e. the spectrum of toxic effects a chemical can produce, and (b) the toxic potency, i.e. the quantitative relationship between dose/concentration and toxicity, are essential to characterise the toxic hazard. Toxicological risk assessment comprises hazard assessment and is aimed to characterise likelihood and severity of adverse effects occurring to man or the environment following exposure under defined conditions to a chemical.

Two fundamental problems hamper the application of in vitro assays for hazard assessment: Firstly, the endpoints of toxic action detectable in vitro are less complex and importantly mostly different from those assessed in vivo (toxicodynamic problem). Secondly, toxic concentrations determined in vitro are not equivalent to toxic doses or concentrations in vivo. This is due to important differences in biokinetics and bioavailability of chemicals in vitro and in vivo (toxicokinetic problem).

This contribution is focussed on the second aspect. It will be demonstrated, how it is possible to make improved predictions of toxic concentrations in human serum and the aquatic environment, respectively, that are equivalent to cytotoxic concentrations in vitro. This can be achieved by the application of a recently developed quantitative extrapolation model taking into account substance and system specific parameters important for the bioavailability of chemicals. It appears that this approach represents a real progress in solving part of the "toxicokinetic problem".

Lecture

**In vitro – in vivo extrapolation of toxic potencies for hazard and risk assessment – problems and new developments**

*Michael Gülden and Hasso Seibert*

University Medical School Schleswig-Holstein, Institute of Toxicology and Pharmacology for Natural Scientists, Kiel, Germany

The aim of toxicological hazard assessment is to characterise the dangerous properties of chemicals for man and the environment. Information on both (a) the toxic potential, i.e. the spectrum of toxic effects a chemical can produce, and (b) the toxic potency, i.e. the quantitative relationship between dose/concentration and toxicity, are essential to characterise the toxic hazard. Toxicological risk assessment comprises hazard assessment and is aimed to characterise likelihood and severity of adverse effects occurring to man or the environment following exposure under defined conditions to a chemical.

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Poster

**Application of decision theory to interpretation of in vitro tests battery results**

*Joanna Jaworska¹, Robert McDowell² and Marilyn Aardema³*

¹ Procter and Gamble, Central Product Safety, Brussels, Belgium; ² USDA, APHIS, Washington, USA; ³ Procter and Gamble, Central Product Safety, Cincinnati, USA

Different in vitro tests can give conflicting results. Bayesian decision theory incorporates all, including conflicting, results into one mathematical framework, and formally generates one result. The method allows for a science-based, fully transparent and objective consensus building. It combines strengths of individual tests and minimises influence of weak tests. Bayesian approach allows the framework to function in a tier mode. In addition, Bayesian approach allows to explicitly quantify improvement (reduction of uncertainty). If the target predictivity of a tier is preset the methods allows determining optimal number of tests needed and their minimum predictivity. Battery of genotoxicity in vitro tests will be used as a working case study.
Poster

Assessment of the performance of in vitro photogenotoxicity assays: Results of a collaborative study with 13 coded test chemicals

Peter Kasper¹, Pierre Aeby², Susanne Brendler-Schwaab³, Bernd Epe⁴, Roland Frötschl¹, Cynthia Hertel⁵, Stephan Kirchner⁶, Manfred Liebsch⁶, Krista Meurer⁶, Ulla Plappert-Helbig⁸ and Elisabeth Schmidt⁶

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A collaborative study with seven participating laboratories was conducted to evaluate the performance of previously developed test protocols for the photo micronucleus test (PMNT) and the photo Comet assay (PCA) with Chinese hamster V79 cells. Thirteen coded test chemicals were selected based on their ability to absorb UV light of which eight were classified as photogenotoxic and five as non-photogenotoxic (three phototoxic, two non-phototoxic) according to published data. Each compound was tested in two independent runs in both assays by 3-5 different investigators.

Results obtained showed a good reproducibility, both within and between laboratories. Sensitivity in detecting the photogenotoxic compounds (8-methoxypsoralen, chlorpromazine, lomefloxacin, ciprofloxacin, methylene blue, proflavine, dacarbazine, doxycycline) was higher in the PMNT (98%) than in the PCA (77%). Specificity of both models appears to be low as the three phototoxic compounds assumed to be non-photogenotoxic based on literature data (promazine, ketoprofene, acridine) showed predominantly positive findings. However, these results most likely suggest that the available published data were inadequate for a correct pre-study classification.

In summary, the data provide a secure foundation for future evaluations of both assays and for their eventual validation as models for the prediction of photogenotoxicity and potential photocarcinogenicity. An agreed standard list of calibration chemicals is considered key for any further evaluation/validation studies.

This work was supported by the German Federal Ministry of Education and Research, BMBF-project No. 0312916A/B/C/D.

Lecture

In vitro strategies to investigate the potential pro-arrhythmic effects of compounds during the drug discovery and development process

Rainer Netzer and Mark Slack

Senior Vice President, BD, Evotec OAI AG, Hamburg, Germany

Interaction of compounds with the hERG potassium channel have been detected as the main source of cardiotoxic events, in particular QT-prolongation and torsade-de-pointes. Therefore, pharmaceutical companies test their compounds at different stages of the discovery and development process on this ion channel. Evotec uses and combines a variety of technologies to detect potential hERG interaction. During discovery and early development, large numbers of compounds (1000-100,000) are tested using a fluorescence assay based on detection of changes in the membrane potential. This assay gives a first assessment of hERG liability. Later during the lead optimisation process measurements using automated patch-clamp are performed with reduced numbers of compounds (10-1000). Selected compounds are investigated using the manual patch-clamp methods, either under GLP or non-GLP to obtain relevant information and documentation for the legal approval of the compounds.

The activities of the compounds on hERG have to be discussed in relation to several factors including active concentration, onset and reversibility of a potential block. Information on other ion channel targets like SCN5A, the sodium channel of the heart, and the L-type calcium channel may be beneficial for a complete evaluation of the side-effect potential of the compounds.

In this presentation an overview of the cardiac ion channels and the combination of in vitro screening technologies will be given.
Evidence that infants and children may be differentially susceptible to chemical insult during critical periods of development has led to a marked increase in animal testing for reproductive and developmental endpoints. The US EPA has recently proposed amendments to its data requirements for conventional pesticide chemicals that could make 2-generation reproduction and two-species developmental toxicity studies absolute, rather than conditional, requirements for both food- and non-food-use chemicals. Developmental neurotoxicity testing (DNT) would also become a newly codified, conditional requirement. Although a variety of DNT protocols have existed for many years, no standardised study design has ever been subjected to formal validation according to modern standards, as evidenced by the many published studies that report profound differences in species sensitivity for this endpoint – up to 10,000-fold in some cases. From a regulatory perspective, an EPA retrospective analysis revealed that DNT studies rarely produce lower “no-effect levels” than studies within the existing database for a pesticide. DNT data have yet to be used as a basis for lowering any pre-existing reference dose; in fact, the EPA has done the opposite, by removing a statutory 10x “children’s health” safety factor for 30 organophosphate pesticides, replacing it with factors of 1x and 3x, respectively. Evidence to date suggests that DNT testing is significantly less protective of sensitive sub-populations than the application of a precautionary safety factor. Thus, in the interests of children’s health, DNT testing should be discontinued, and associated regulations and test guidelines should be repealed.

Lecture

DNT testing in the name of children’s health: A case for precautionary safety factors

Troy Seidle
People for the Ethical Treatment of Animals, Research and Investigations Dept., Toronto, Canada

The German Centre for the Documentation and Evaluation of Alternative Methods to Animal Experiments, ZEBET, reviewed the toxicological data on pesticides obtained in studies using the dog as test species. The study was sponsored by the German Foundation for the Promotion of Research on Replacement and Complementary Methods to Reduce Animal Testing, SET. SET initiated a retrospective analysis of toxicological data on pesticides from the files of the German Agency for the Regulation of Pesticides, which was conducted by the German Federal Institute of Risk Assessment, BfR, and published in two parts in 1998 and 2001. The outcome of this study has been compared with the results of other published retrospective analyses as well as preliminary results of the Agricultural Chemicals Safety Assessment project initiated in 2001 by the International Life Sciences Institute, ILSI, in which data from the database at U.S. EPA’s Office of Pesticide Programs are used. All studies support the conclusions of the SET study that toxicological safety testing in dogs can be restricted to sub-chronic studies of 13 weeks and that studies of longer duration do not provide additional essential information. Similar conclusions were drawn from retrospective analyses evaluating data on therapeutic drugs and incorporated in the recommendations of the International Conference on Harmonisation of Registration of Pharmaceuticals, ICH. The present survey thus shows that, the routine conduct of 12-month studies in dogs is no longer required for agricultural chemicals. Changing international regulations accordingly would be beneficial both in economical and animal welfare terms.

Poster

The dog as test species for the toxicological evaluation of pesticides – present status

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Federal Institute of Risk Assessment (BfR), Centre for the Documentation and Evaluation of Alternative Methods to Animal Experiments, Berlin, Germany
SC Johnson’s consumer product hazard evaluation program using alternative assays

Judith Swanson1, Nicole Cuellar*1 and John Harbell2

1 SC Johnson and Son, Inc., Product Safety, Racine, USA; 2 Institute for In Vitro Sciences, Gaithersburg, USA

SC Johnson is a global consumer product company that manufactures a variety of household products that must be evaluated for human health hazards. For over 10 years, SC Johnson has conducted the necessary research to spearhead efforts to reduce the use of animals in the hazard assessment process. We routinely use alternative approaches such as the eye and skin irritation assays in a weight of evidence approach for hazard classification and labelling purposes for a variety of products. Assay choice and protocol considerations are defined so as to address possible modes of action on the target tissues. Specific benchmark formulations have been employed with each study to facilitate interpretation of the results. The Bovine Corneal Opacity and Permeability Assay (BCOP) has been used to assess ingredient synergies and the impact of various formulation components on the irritancy potential of the end-use products. The overall safety evaluation approach will be illustrated using two case studies. Alternative assays, especially the BCOP, are indispensable tools for assessing the potential irritancy of our products distributed worldwide while reducing the use of animals.

Skin irritation testing: A correlation study between in vitro and in vivo

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1 VTB-SHP, Henkel KGaA, Düsseldorf, Germany; 2 VTD-BioServices, Henkel KGaA, Düsseldorf, Germany

Skin irritation is the most common adverse reaction in humans. For reasons of human risk assessment, formulations have to be assessed for putative irritant side effects. Therefore, the human patch test is an appropriate method. But, individual differences in the analyses of the read-out parameters and the test panels make a distinct assessment often difficult.

Aim of this study was to investigate the feasibility of an in vitro approach for human risk assessment concerning irritancy of surfactants. Test samples were kindly provided by the German Society of Cosmetic Chemists (DGK), which conducted a human patch test study with the same set of samples in parallel. By this, we had the unique chance to correlate in vitro and in vivo data.

In order to assess irritant effects in vitro, reconstructed human epidermis was exposed to 8 coded test samples, consisting of individual anionic surfactants, blends of surfactants and controls. A Multiple Endpoint Analysis was established to comprise the viability, cytotoxicity, histology, cytokine release and differential gene expression. As results, a high level of correlation was determined for our in vitro assessment of skin irritancy to an in theory ranking and the human patch test data.

Here, we presented the good predictability of the Multiple Endpoint Analysis for assessing irritant potentials of formulated surfactants. Further investigations are necessary to evaluate the potential of this in vitro method to assess also other classes of irritants and more complex formulations.
Lecture

Replacing animal testing for consumer safety – is it feasible?

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Unilever, SEAC, Sharnbrook, UK

The proposed EU Chemicals Regulation (REACH) and the 7th Amendment to the EU Cosmetics Directive challenge the use of animal tests for evaluating the safety of chemicals and consumer products. Unfortunately today much of the data derived from the existing alternative (non-animal) tests cannot be used for human health risk assessment.

The approach we suggest is based on the concept that consumer safety aims at preventing harm and disease in man. Animal tests served their purpose since technologies used in the animal studies to generate data for risk assessment are similar to the technologies used in clinical medicine. This allows interpretation of the animal data in terms of harm and disease in man.

New molecular biology and informatics technologies are continually being introduced in science and medicine. Using “systems biology” approaches in both experimental biology and medicine should support the integration and interpretation of the large amounts of complex data now being generated, by providing better understanding of the underlying biological complexity. We postulate that this will enable new experimental models and risk assessment paradigms to be developed that do not require the use of animals.

This new approach underpins our experimental projects in skin allergy and inflammation. The overall objective is to be able to make consumer safety decisions for these effects, with an acceptable level of confidence, without the use of new data generated in animal tests.
Non-animal test methods are currently developed for the identification of skin sensitisation hazard. In this context, we focused on U-937, a human myelomonocytic cell line, which exhibits a specific induction of CD86 expression following exposure to contact sensitisers in a dose-dependent manner but not to non-sensitisers. Some contact sensitisers like NiSO₄ and DNCB induce CD86 expression concomitantly with a decrease in cell viability. The purpose of this investigation was to determine if CD86 expression and programmed cell death are linked. We first monitored biochemical apoptotic changes after treatment with chemicals. The results showed that NiSO₄ and DNCB but not SDS induced externalisation of phosphatidylserine and activation of caspase-3. Z-V AD-fmk, a pan-caspase inhibitor, inhibited DNCB- but not NiSO₄-induced apoptosis indicating that DNCB, an organic sensitiser, but not NiSO₄, a non-organic sensitiser, induces apoptosis in a caspase-dependent pathway 48 hours after treatment. When apoptosis was inhibited by Z-VAD-fmk in DNCB-treated cells, CD86 expression remained unchanged. Moreover, by flow cytometric monitoring, we showed that the major U-937 cell subset that expressed CD86 following exposure to contact sensitisers is Annexin-V negative cells confirming that CD86 is induced mainly on non-apoptotic cells. In conclusion: 1) apoptosis is not a confusing factor for the evaluation of the skin sensitisation potential of a chemical on U-937 cells and 2) phosphatidylserine externalisation measurement through Annexin-V/propidium iodide co-staining is suitable for use in the development of an in vitro assay for the discrimination of contact sensitisers and irritants (including tensioactive agents that disrupt the membrane integrity).
In vitro analysis of the maturation of dendritic-like cells (DCs) induced by chemicals is becoming a useful tool for the elucidation of the sensitising properties of chemicals. We have used this new approach to analyse the different sensitising properties of p-phenylenediamine (PPD), its oxidation products including Bandrowski’s base (BB), and of p-toluylendiamine (PTD). Since acetylation is known to be a major pathway in human, mouse and rat hepatocytes as well as in human keratinocytes, mono- and di-acetylated PPD and PTD were synthesised and evaluated. Our in vitro test protocol uses human peripheral blood monocytes derived DCs that are exposed for 3 to 30 hours to the test chemicals. DC maturation is evaluated by flow cytometric measurement of the percentage of CD86 positive cells and quantitative measurement of the mRNA expression of interleukin-1β, interleukin 8 and aquaporin P3 using the Lightcycler® real time PCR system. Fresh PPD induces only a slight DC maturation whereas oxidised PPD and BB are much more potent inducers. On the other hand, fresh PTD is a potent inducer and does not need a prior oxidisation step. We could also show for the first time that mono- and di-acetylated PPD or PTD do not induce any relevant DC maturation.

In vivo results obtained with the same chemicals in the murine local lymph node assay confirmed the in vitro findings. We conclude that the described in vitro test system allows a refined analysis of the sensitising properties of chemicals and will further improve product safety.

Poster

In vitro activation of dendritic-like cells: A new tool for the elucidation of the sensitising properties of chemicals

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In vitro analysis of the maturation of dendritic-like cells (DCs) induced by chemicals is becoming a useful tool for the elucidation of the sensitising properties of chemicals. We have used this new approach to analyse the different sensitising properties of p-phenylenediamine (PPD), its oxidation products including Bandrowski’s base (BB), and of p-toluylendiamine (PTD). Since acetylation is known to be a major pathway in human, mouse and rat hepatocytes as well as in human keratinocytes, mono- and di-acetylated PPD and PTD were synthesised and evaluated. Our in vitro test protocol uses human peripheral blood monocytes derived DCs that are exposed for 3 to 30 hours to the test chemicals. DC maturation is evaluated by flow cytometric measurement of the percentage of CD86 positive cells and quantitative measurement of the mRNA expression of interleukin-1β, interleukin 8 and aquaporin P3 using the Lightcycler® real time PCR system. Fresh PPD induces only a slight DC maturation whereas oxidised PPD and BB are much more potent inducers. On the other hand, fresh PTD is a potent inducer and does not need a prior oxidisation step. We could also show for the first time that mono- and di-acetylated PPD or PTD do not induce any relevant DC maturation. In vivo results obtained with the same chemicals in the murine local lymph node assay confirmed the in vitro findings. We conclude that the described in vitro test system allows a refined analysis of the sensitising properties of chemicals and will further improve product safety.
The predictive identification of chemicals which possess the intrinsic ability to cause skin sensitisation represents only the very first step in the safety assessment of this toxicity endpoint. Once a sensitiser has been identified it is then necessary to determine whether there are conditions under which skin exposure may occur without the risk of inducing skin sensitisation. This determination is made by a risk assessment process, which historically involved a comparative approach, largely based on assessments of similarity of allergens in predictive guinea pig tests. More recently, a Quantitative Risk Assessment (QRA) approach has been promulgated, which depends on the characterisation of the relative potency of a sensitiser in the Local Lymph Node Assay (LLNA). In this assay, an objective quantitative measure of potency is used as the basis for the calculation of safe exposure levels in different product settings. Thus, a key challenge for the in vitro methods which will replace assays such as the LLNA is how the data derived from them can be utilised not only to identify a skin sensitiser, but also to characterise its relative potency. Thus as we develop in vitro alternatives for skin sensitisation, we must examine how to use their data output for risk assessment in addition to simple hazard identification.

Skin sensitisation represents the allergic activation of the immune system in response to repeated exposure to a subset of chemicals with the ability to behave as haptens, i.e. they can covalently bind to skin proteins. The clinical expression of skin sensitisation is allergic contact dermatitis, whose symptoms (e.g. erythema, oedema) are often similar to those of skin irritation. Historically, guinea pig models allowed the identification of potential skin sensitising chemicals. More recently, a refined, reduced method, the murine Local Lymph Node Assay (LLNA) has been employed. Our present aim is to undertake the work necessary to ensure the final step, replacement of animal testing, can be achieved. To this end, the COLIPA Skin Tolerance Task Force (STTF) has undertaken a range of research projects, from aspects of chemistry/peptide binding/skin metabolism, through evaluation of intracellular signalling pathways induced by allergens, to allergen induced changes in dendritic/Langerhans cells measured at genomic and protein level. The knowledge gained from this work aims to develop and pre-validate in vitro predictive assays. The current challenge is developing an appreciation of how to use their data output for risk assessment in addition to simple hazard identification.

Lecture

**Use of in vitro data for sensitisation risk assessment**

David Basketter

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The predictive identification of chemicals which possess the intrinsic ability to cause skin sensitisation represents only the very first step in the safety assessment of this toxicity endpoint. Once a sensitiser has been identified it is then necessary to determine whether there are conditions under which skin exposure may occur without the risk of inducing skin sensitisation. This determination is made by a risk assessment process, which historically involved a comparative approach, largely based on assessments of similarity of allergens in predictive guinea pig tests. More recently, a Quantitative Risk Assessment (QRA) approach has been promulgated, which depends on the characterisation of the relative potency of a sensitiser in the Local Lymph Node Assay (LLNA). In this assay, an objective quantitative measure of potency is used as the basis for the calculation of safe exposure levels in different product settings. Thus, a key challenge for the in vitro methods which will replace assays such as the LLNA is how the data derived from them can be utilised not only to identify a skin sensitiser, but also to characterise its relative potency. Thus as we develop in vitro alternatives for skin sensitisation, we must examine how to quantify the output from such methods and determine how to integrate multiple endpoints in a consistent and transparent manner such that a prediction of human skin sensitisation potency can be achieved.
The search for alternatives to in vivo testing of chemicals for skin sensitisation hazard and potency is dependent on improving our understanding of the molecular events. The sensitising potential of a chemical is directly linked to its reactivity towards proteins. Known sensitisers are either directly capable of covalently modifying proteins or are metabolised into protein reactive species. Additionally, small organic compounds are unable to induce a significant immune response and thus need to form a macromolecular immunogen. A protein reactive chemical could covalently modify any available nucleophiles given the suitable conditions but the immunological relevance of the particular modifications is unknown. Therefore the use of model proteins/peptides in such investigations is necessary. The data obtained from a limited number of studies demonstrate that the covalent protein binding is a characteristic of tested sensitisers. The three dimensional protein environment may restrict chemical modifications as only some of numerous reactive side chains are modified. Model peptide studies often produce different results with the same chemicals. Whilst such investigations further aid our understanding of the protein–hapten interactions, the knowledge about immunogenic relevance of observed modifications is sparse. Better model proteins/peptides could be chosen and better predictive assays designed if particular types of modifications were shown to be more relevant than others. Understanding protein–hapten binding mechanisms will increase the confidence in prediction of sensitisation hazard using in silico tools, as well as provide the opportunities to develop simple, accurate and cost effective predictive in vitro assays for skin sensitisation hazard and potency.

Lecture

Hapten-protein binding – what do we know?

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Assessing the sensitising potential of substances represents one of the major issues in the context of toxicological evaluation for the protection of human health, especially in view of the recent increase in the prevalence of skin and respiratory allergies. Although a number of promising in vitro and in silico systems are under investigation, animal tests are still currently used for the identification of chemical allergens. The primary objective of this key area is to develop and validate alternative testing strategies to replace animal experimentation. A first skin sensitisation Task Force meeting was organised in 2003 (chair D. Basketter) to advise ECVAM on future activities to be undertaken in this field to complement, to support and to harmonise ongoing efforts in the area. Following the recommendations of the Task Force, in 2004 a workshop was organised to review the state of the art of the use of cultured dendritic cells for the identification of skin sensitisation hazard (ATLA, Casati et al., 2005). Subsequently ECVAM took the lead together with COLIPA in the conception of an Integrated Project “Sens-it-iv” which has recently been evaluated and accepted by DG RTD. The overall goal of Sens-it-iv is to develop, with different partners, over a period of five years, strategies to replace animal experimentation with in vitro assays for identifying skin and respiratory sensitisers for chemical, cosmetic and pharmaceutical substances. The in vitro assays to be developed will allow the testing of the sensitising potency for classification and labelling and for the purpose of risk assessment.

Poster

ECVAM key area on sensitisation: Summary of ongoing activities

Silvia Casati
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Assessing the sensitising potential of substances represents one of the major issues in the context of toxicological evaluation for the protection of human health, especially in view of the recent increase in the prevalence of skin and respiratory allergies. Although a number of promising in vitro and in silico systems are under investigation, animal tests are still currently used for the identification of chemical allergens. The primary objective of this key area is to develop and validate alternative testing strategies to replace animal experimentation. A first skin sensitisation Task Force meeting was organised in 2003 (chair D. Basketter) to advise ECVAM on future activities to be undertaken in this field to complement, to support and to harmonise ongoing efforts in the area. Following the recommendations of the Task Force, in 2004 a workshop was organised to review the state of the art of the use of cultured dendritic cells for the identification of skin sensitisation hazard (ATLA, Casati et al., 2005). Subsequently ECVAM took the lead together with COLIPA in the conception of an Integrated Project “Sens-it-iv” which has recently been evaluated and accepted by DG RTD. The overall goal of Sens-it-iv is to develop, with different partners, over a period of five years, strategies to replace animal experimentation with in vitro assays for identifying skin and respiratory sensitisers for chemical, cosmetic and pharmaceutical substances. The in vitro assays to be developed will allow the testing of the sensitising potency for classification and labelling and for the purpose of risk assessment.
Lecture

Designing practical protein binding assays for screening skin allergens

Frank G. Gerberick
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There are a variety of characteristics that determine whether a chemical can function as a skin allergen including the ability to penetrate into the skin, react with protein, and be recognised as antigenic by immune cells. Since protein reactivity is a key step in the induction of skin sensitisation it is hypothesised that reactivity could be used to screen for the sensitisation potential of chemicals. Chemical allergens are electrophilic and as such react with nucleophilic amino acids like cysteine or lysine. The research aim is to develop an in vitro peptide reactivity method that allows for quantitative analysis of a chemical’s reactivity potential. In order to determine if reactivity correlates with sensitisation potential, work is underway in various laboratories to evaluate peptides containing the best nucleophiles for evaluating the reactivity of allergens and non-allergens. To date, excellent progress has been made demonstrating a significant correlation between a chemical’s skin sensitisation potency and its ability to react with peptides containing nucleophilic amino acids such as cysteine and lysine. Current work is focused on incorporating a metabolism system in the assay so that pro-haptens can also be identified and categorised as well as adapting the method to a high-throughput format for screening large numbers of chemicals. With the development of a robust chemical reactivity assay it is hoped that it will be possible to screen new chemicals in vitro and thus reduce the reliance on animal test methods.

This research is supported by the European Cosmetic, Toiletry and Perfumery Association (COLIPA).

Poster

Identification of potential dendritic cell markers for the prediction of skin sensitisation using real-time PCR

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Changes that occur within epidermal dendritic cells after allergen exposure represent potential endpoints for predictive in vitro skin sensitisation methods. Previous microarray analysis of human Peripheral Blood-derived Dendritic Cells (PBMC-DC) revealed changes in gene expression following dinitrobenzene sulfonic acid (DNBS)-allergen treatment. Using a select group of chemicals, the sensitivity, selectivity, and dynamic range of those genes changes were evaluated by quantitative PCR to determine their usefulness as markers for contact allergy. From that work a focused candidate gene list was selected. Subsequent validation of the target genes was performed using an expanded chemical dataset. PBMC-DC were treated for 24 hours with various doses of chemicals. RNA was extracted and used in real-time PCR reactions using specific primers. Mean Relative Fluorescence Units (RFU) were calculated and then converted to mean fold changes comparing mean RFU in control (vehicle-treated) samples versus mean RFU in treated samples. The dynamic range and sensitivity of these genes were evaluated by testing multiple doses of sensitisers of varying allergenic potencies, such as hydroxycitronellal and hydroquinone, as well as chemical irritants. As a result of that work, approximately 20 potential genes have been identified that meet the selection criteria for sensitivity, dynamic range, and reproducibility. Some of the allergen-induced changes were in the expression of genes associated with immune function, such as CCL2, CCL4, CCL23, SLAM, and the Lectin Receptor. These genes will be analysed further for their usefulness as an endpoint measure for the prediction of contact allergy.

This work was funded in part by COLIPA.
**Poster**

**Cytokine secretion profiles of mouse dendritic cells (DC): Influence of cell injury**

**Ian Kimber**, **Catherine Betts**, **Gemma Beckwith**, **Laura Bailey**, **Cindy Ryan**, **Frank Gerberick** and **Rebecca Dearman**

1 Syngenta CTL, Macclesfield, UK; 2 Proctor and Gamble, Cincinnati, USA

Cutaneous immune responses such as chemical contact allergy are regulated by cytokines expressed by keratinocytes and Langerhans cells (LC). Bone marrow derived LC-like DC were generated from BALB/c strain mice and cytokine secretion profiles measured following 24 h culture with the water soluble allergen dinitrobenzene sulfonic acid (DNBS) and the non-sensitising analogue, benzene sulfonic acid (BSA). Concentrations of DNBS or BSA were utilised that were without effect on cell viability, or that provoked significant cytotoxicity (measured by trypan blue exclusion). Control cells were cultured with medium alone, or with 1 µg/ml bacterial lipopolysaccharide (LPS), a potent stimulator of DC. DC expressed constitutively interleukin (IL)-1β, IL-1β, IL-6 and IL-12. Culture of DC with 1µg/ml LPS resulted in very marked (20 to 70fold) increases in expression of all cytokines. Culture with concentrations of DNBS (5 and 1 mM) that resulted in greater that 50% cytotoxicity reduced cytokine expression. Treatment with 0.5 mM DNBS, a non-toxic dose, stimulated modest increases in IL-6 and IL-12 secretion. In contrast, culture with cytotoxic doses of BSA (100 and 50 mM) provoked low level increases in IL-1β, IL-1β and IL-6, with a concomitant decrease in IL-12 production. These data suggest that it may be possible to distinguish chemical allergens from irritants on the basis of differential cytokine secretion under conditions of equivalent cell injury.

**Poster**

**Differences in surface marker expression on monocyte derived dendritic cells induced by LPS, irritants, and contact allergens**

**Ludger Kolbe, Jochem Spieker, Silke Gerlach, Horst Wenck and Walter Diembeck**

Beiersdorf AG, R&D, Hamburg, Germany

Dendritic cells play a pivotal role in the induction of skin allergies. After contact with sensitisers these cells undergo rapid maturation and migrate to the local lymph node in order to activate allergen specific T cells. Dendritic cell maturation is characterised by changes in cell surface marker expression and increased cytokine production. These changes represent potential endpoints for predictive in vitro skin sensitisation assays. In recent years much progress has been made in developing in vitro methods to study allergen induced changes in dendritic cells. However, it will still take some effort to develop a reliable in vitro method for the identification of potential skin sensitisers. Robust markers are required to distinguish potential allergens from irritants and innocuous substances. For this purpose we studied the time course and dose response relationship of allergen induced surface marker expression on monocyte derived dendritic cells. We determined changes in CD80, CD86, HLA-DR, CD83, and CD54 expression on dendritic cells after incubation with allergens of varying potency by flow cytometry. Lipopolysaccharide, Triton-x100, and SDS were used as irritant control substances. Our test design allows to discriminate between weak sensitisers and irritants and our data also demonstrate that test concentrations must be chosen carefully. High concentrations of sensitisers may result in low cell viability and therefore may give false negative test results.
The chemistry of skin allergy

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Allergic Contact Dermatitis (ACD) is a very common disease resulting from epidermal proteins being chemically modified by haptens. The processing of such modified proteins by Langerhans cells, the main antigen-presenting cells in the epidermis, generates altered peptides that are subsequently presented, in association with MHC molecules, to naïve T-lymphocytes in the lymph nodes. The whole process results in the selection and activation of T-lymphocyte sub-populations with T-cell Receptors (TcR) specific for the chemical modification. Haptens are usually low molecular weight molecules, lipophilic enough to penetrate the epidermis through the stratum corneum, and with a potent chemical reactivity allowing the formation of a covalent link with nucleophilic residues on protein amino acid side chains. For some time it has been considered that the more a molecule was able to modify proteins, the better a sensitiser was and that a direct relation could be established between the overall chemical reactivity of a molecule and its sensitising potential. Today, it is hypothesised that the sensitising potential of a molecule is related to its chemical reactivity towards a few specific amino acids relevant to the sensitisation process. Haptens can modify proteins through many different mechanisms, from classical nucleophilic-electrophilic reaction to radical reactions. The knowledge of how haptens can modify proteins is the base for the development of predictive alternative tests aimed at the identification of hazard and potency such as Structure Activity Relationships (SAR), Quantitative Structure Activity Relationships (QSAR) and peptide reactivity tests.

Poster

Predicting the classification of skin sensitisation potency using 4D-fingerprints and logistic regression with and without partial least-squares

Yi Li¹, Anton J. Hopfinger¹, Petra S. Kern²* and Frank G. Gerberick³
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A set of 132 structurally diverse compounds whose skin sensitisation potencies are none or weak (class 1), and strong or extreme (class 2) formed a training set to build two-state categorical QSAR models. A test set of 15 compounds spanning the structural diversity of the training set was used to evaluate the categorical QSAR models. Skin sensitisation potency measures were taken from historic local lymph nodes assay data. 4D-fingerprint descriptors were employed to construct the models. Model fitting was performed using Logistic Regression (LR) with, and without, Partial Least-Squares (PLS). The training set cross-validated accuracy of prediction of the LR models without PLS ranges from 77.3% to 78.0%. For PLS-LR models the accuracy spans 87.1 to 89.4%. The corresponding test set prediction accuracies range from 87.1 to 89.4% for LR and 73.3 to 80.0% for PLS-LR models. The categorical QSAR models are composed of descriptor terms related to aromatic atoms, hydrogen bond acceptor groups and negatively partially charged atoms. The descriptors of the models also define molecular size and shape features for skin sensitisation potency. The 4D-fingerprint LR, and PLS-LR, models developed in this study are superior in both data fit and predictivity to alternate models developed by ourselves, and models proposed by others, which use different descriptors, including classic 2D QSAR descriptors, and/or other data fitting methods.

Poster

Investigation of co-stimulatory molecule expression and cytokine production on THP-1 human monocyte cell line treated with skin sensitisers

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Dendritic Cells (DCs) play a critical role in the induction phase of sensitisation by presenting antigens. It has been demonstrated that the application of chemical hapten to DCs induces the production of some inflammatory cytokines and up-regulates the expression of class II major histocompatibility complex (MHC class II) and co-stimulatory molecules. Therefore, DCs have been used for the development of in vitro sensitisation test method. Recently, various methods using human monocytic cell lines as a substitute for DCs have been progressed. In this study, we cultivated THP-1, a human monocyte cell line, with skin sensitisers or non-sensitisers, and evaluated the production of cytokines and the expression of co-stimulatory molecules on THP-1 to develop in vitro skin sensitisation test method. The culture supernatants were collected and the production of cytokines was measured by ELISA. The expression of CD86 and CD54 on THP-1 was measured by flow cytometry. Our results indicated that treatment with sensitisers such as 2,4-dinitrochlorobenzene or nickel sulfate, but not non-sensitisers, induces the production of TNF-α and IL-1β and up-regulates the expression of CD86 and CD54. This study suggests that measuring cytokine production and co-stimulatory molecule expression on THP-1 is a useful in vitro method to predict skin sensitisation potential of chemicals.

Poster

Evaluation of the peptide-binding assay by using LC-mass spectrometry as a skin sensitisation test

Yosuke Nakamura¹, Kazuko Yamashita², Takashi Morimoto¹, Tamaki Higaki¹, Hiroshi Kato¹, Masahiko Okamoto², Mika Ota¹ and Satoshi Kawamura¹
¹ Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory, Osaka, Japan; ² Sumitomo Chemical Co., Ltd., Organic Synthesis Research Laboratory, Osaka, Japan

Allergic contact dermatitis is a common occupational health hazard for workers handling a lot of chemicals. To protect the workers from this hazard, it is necessary to assess the skin sensitisation potential of not only the final product but also the intermediates in a manufacturing process. But the number of them is so large that it is practically impossible to check the sensitisation potential of all of them by using a local lymph node assay. Thus, it is important to develop the short-term alternative methods for evaluation of the skin sensitising potential of the chemicals. As the majority of the skin sensitisers bind covalently to amino acids such as cysteine or lysine to provoke the immunologic reactions, it may be useful to check the binding ability of the chemicals to a peptide such as glutathione. To evaluate the correlation between the results of the peptide-binding assay and the animal tests, the peptide-conjugate formation of 82 chemicals, including 61 sensitisers and 21 non-sensitisers, was analysed by LC-mass spectrometry. Based on the structural analysis, it was concluded that 30 out of 61 sensitisers showed the peptide-conjugate formation but 19 out of 21 non-sensitisers did not in this study. Thus, the total concordance rate was 60% and the predicted performance for the in vivo test was 94%. Our data show the peptide-binding assay by using LC-mass spectrometry is a good alternative method as a screening test for the evaluation of the skin sensitisation potential of the chemicals.
Lecture

Collaborative study for the preparation of a test protocol based on the U937 human cell line for predicting skin sensitisation potential

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One of the alternatives to animal testing for the prediction of skin sensitisation is in vitro methods based on the use of human monocytic cell lines as surrogate dendritic cells. The European Cosmetic, Toiletry and Perfumery Association (COLIPA) is supporting ring trials to shed light on the practicability of this approach. Among the different human cell lines tested, the pro-monocytic cell line U937 has been identified as a good candidate responding to chemical sensitisers. The following U937/CD86 test protocol based on the up-regulation of CD86 expression, a co-stimulatory molecule, by U937 cells after 48 h exposure to chemicals has been proposed for pre-evaluation and further optimisation. Expression of CD86 is assessed by flow cytometry and a test item is declared positive if: i) viability (PI exclusion) is at least 70%, ii) CD86 expression reaches ≥ 120% of control cells (EC120, the concentration at which this happens, is calculated by linear regression), iii) the chemical induces CD86 in a dose dependent manner, iv) there are 2 out of 3 concordant experiments. Similar preliminary results were obtained by three independent laboratories (L’Oréal, Cosmital and LVMH) with two sensitisers (nickel sulfate and dinitrochlorobenzene) and one irritant (sodium dodecyl sulfate) indicating that upon further improvement of the culture conditions and test parameters, the U937/CD86 test system could be the basis for the development of an in vitro test system. A ring trial co-ordinated by the COLIPA should follow with a panel of reference sensitisers and irritants to evaluate the U937/CD86 test.

Poster

In vitro prediction of the skin sensitisation potential of chemicals using the U937/CD86 test

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Among the alternatives to animal testing for the prediction of skin sensitisation is in vitro methods based on the use of human monocytic cell lines as surrogate dendritic cells. One of the different human cell lines tested, the pro-monocytic cell line U937 has been identified as a good candidate responding to chemical sensitisers.

L’Oréal has developed the U937/CD86 test based on the up-regulation of CD86 expression, a co-stimulatory molecule, by U937 cells after 48 h exposure to chemicals. Expression of CD86 is assessed by flow cytometry and a positive result is recorded if: i) viability (PI exclusion) is at least 70%, ii) CD86 expression reaches 120% of control cells, iii) the chemical induces CD86 in a dose dependent manner, iv) there are 2 out of 3 concordant experiments. A wide concentration range is first tested and then refined based on cell toxicity and CD86 positivity. When all criteria are fulfilled, EC120, the concentration at which this happens, is calculated by linear regression.

With a panel of ~70 reference chemicals (sensitisers and non-sensitisers), a correlation of 95% with the human clinic was observed for the prediction of the sensitising potential using the U937/CD86 test.

A ring study for inter-laboratory evaluation of the U937/CD86 test is about to start (see theme 5, session 3) with the support of the European Cosmetic, Toiletry and Perfumery Association (COLIPA).
Lecture

Results of a ring trial of a human Cell Line Activation Test (h-CLAT) for predicting skin sensitisation potential

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In vitro methods based upon changes in cell surface marker expression induced in DC following exposure to contact allergens represent one approach for developing non-animal test methods for skin sensitisation. Donor to donor variability in response to allergen exposure has been observed in DC derived from human peripheral blood. Therefore, methods which utilise human monocytic cell lines as surrogate DC are being explored. The human Cell Line Activation Test (h-CLAT) examines the level of expression of CD86, a co-stimulatory molecule, and CD54, an inter-cellular adhesion molecule, on the surface of THP-1 cells (monocytic leukemia cell line) using flow cytometry following 24 hours of chemical exposure. The h-CLAT was evaluated by five independent laboratories in a ring trial co-ordinated by the European Cosmetic, Toiletry and Perfumery Association (COLIPA). A total of ten chemicals were evaluated in two separate trials and included seven sensitisers covering a range of allergenic potencies and three irritants. All laboratories correctly identified six of the seven known allergens as sensitisers and two of the three irritants as non-sensitisers. α-Hexylcinnamic aldehyde, an allergen of moderate potency, was classified as a sensiser by only two of the five labs. False positive results were obtained with the irritant benzalkonium chloride by two labs. As a consequence of the inter-laboratory differences observed in this trial, opportunities for refinement of the h-CLAT protocol and prediction model were identified. Additional work is needed to support the feasibility of utilising cell lines as surrogate DC in development of in vitro skin sensitisation methods.

Poster

Use of historical local lymph node data in the development of alternative test methods for skin sensitisation

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In developing new alternative test methods, the availability of high quality, relevant and reliable in vivo data for the endpoint of interest is essential. Data derived in humans would be the most appropriate as test methods attempt to predict a toxicological effect in man. However, a sufficient quantity of such data is most likely not available, so data derived from animal studies usually serves as the basis for comparison. Recently, the Local Lymph Node Assay (LLNA) has emerged as a practical option for assessing the skin sensitisation potential of chemicals. In addition to accurately identifying sensitisers, the LLNA has also been shown to provide a reliable measure of relative sensitisation potency; information critical to the successful management of human health risks. Therefore, for use in evaluating new test methods for skin sensitisation and the development of quantitative structure-activity relationship models, a database of historical LLNA data for 232 different chemicals has been created. This extensive dataset encompasses the biological and chemical diversity of known skin allergens. The range of relative allergenic potencies are represented in the dataset by 18 extreme, 31 strong, 76 moderate, and 65 weak contact allergens as well as 42 non-sensitising chemicals. Aldehydes, ketones, aromatic amines, quinones and acrylates are among the diverse chemical classes represented in the dataset. In addition to 2D chemical structures, the physicochemical parameters included are logKp, logK and molecular weight. It is hoped that this database will accelerate the development, evaluation and eventual validation of new approaches to skin sensitisation testing.
**Poster**

**Evaluation of the MUTZ-3 cell line as a model system for in vitro testing of sensitisation**

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The early detection of the sensitising potential of chemicals is of great importance to the industry. The number of animals that are currently used for these experiments has to be reduced, so in vitro alternatives are necessary. A promising alternative is an in vitro test system based on a model of human Dendritic Cells (DC), cultured from CD34+ progenitors. However, this DC model has several drawbacks. For instance, human cord blood has to be collected/available to obtain CD34+ stem cells. Furthermore, primary DC cultures are time-consuming, and experiments are hampered by the inter-individual differences that exist between donors. MUTZ-3 is a human, acute myeloid leukemia cell line with the potential to differentiate into immature DC and may therefore be used to overcome many of these difficulties.

**Poster**

**Contact sensitisers induce phosphorylation of p38 MAPK in U937 cells; a possible marker to discriminate between sensitising potential and biological activity?**

*Silvia Teissier, Denis Verda, Jean-Marc Ovigne and Françoise Rousset*

L’Oréal, Life Sciences Research, Aulnay sous Bois, France

Contact sensitisers induce several phenotypic and functional changes of dendritic cells (DC) in vivo and in vitro. One of these changes, the induction of CD86, is the most frequently analysed endpoint for the in vitro prediction of contact sensitisers using different cellular models based on DC or human myeloid cell lines. This marker has proven its relevance to evaluate the sensitising potential of chemicals. In contrast, in the context of pharmacophores, the evaluation of the sensitising potential may be problematical, since these bioactive molecules may interfere with the cellular processes involved in the induction of cell maturation and differentiation measured by the CD86 expression. In order to anticipate on this issue, we decided to explore very early events of the signal transduction activated by contact sensitisers in our U937 based model, which finally results in the induction of CD86. In this study we analysed the phosphorylation of p38 MAPK induced in U937 cells after treatment with several sensitisers, irritants and bioactive molecules. As already showed for DC, we confirmed in our U937 model, that contact sensitisers specifically activate p38 MAPK. Moreover, preliminary results indicate that phosphorylated p38 is a promising marker for the discrimination between biological activity and sensitising potential of a molecule.
In order to evaluate the immunoreaction on human skin, we attempted to create a three-dimensional human skin model consisting of three different cells such as dendritic cells, keratinocytes and fibroblasts. Checking the histological cross-section of the human skin model, we stained the human skin model with hematoxylin and eosin. After 11-day incubation the horny layer was initially observed and then 14-day incubation three-dimensional human skin model was completely formed. Due to non-cytotoxic dose of DNCB, NiSO₄ and compound48/80, the dendritic cells in the human skin model released IL-4, IL-2 and IL-1α into the incubating medium and expressed CD86. The results suggest that the three-dimensional human skin model with dendritic cells should be able to apply for studying the effect of immune-sensitising compounds.

**Poster**

**NF-κB, PI3K and JNK are essential signalling pathways implicated in human dendritic cells maturation after contact with allergens**

**Sandra Trompezinski¹, Julie Denoeud², Béatrice Zabawski², Claude Vincent², Daniel Schmitt², Pascal Courtellemont², Béatrice Le Varlet³ and Mireille Serres³**

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A critical step in the initiation of allergic reaction is the activation of naive T cells by Dendritic Cells (DC). In vitro, various agents are well-known to induce DC maturation, such as LPS, TNF, and contact sensitisers. This process is essentially characterised by an increase in cell surface expression of CD86, HLA-DR and CD54 and by cytokine production. However, only few studies have been reported on the intracellular mechanisms by which human DC respond to allergen treatments. A better knowledge of the kinases activated in this process is required for the understanding of immune response induced by allergens. For this purpose, our studies were performed on DC derived from human monocytes after 5 days of culture with GM-CSF and IL-4. Then, immature DC were induced to mature by nickel or LPS and TNFα (positive controls) for 48 h in X-VIVO medium without serum. To study the main kinases implicated in MAPKs (Erk 1/2, p38 MAPK and JNK), PI3K, PKC, PKA and NF-κB pathways, specific inhibitors at each step of phosphorylation cascades were used.

Our data demonstrated that CD86, CD54 and HLA-DR expression induced by DC maturation was essentially suppressed by inhibitors of JNK, PI3K, PKCσ and NF-κB pathways, suggesting a major role of these kinases. PKA does not seem to be directly implicated and the role of Erk1/2, p38 MAPK and other PKC isoforms remain to be specified. These studies will be extended to other allergens and irritants in perspective to develop alternative methods for contact dermatitis prediction.

**Poster**

**Construction of three-dimensional human skin model consisting of dendritic cells, keratinocytes and fibroblasts**

**Tadashi Uchino and Hiroshi Tokunaga**

National Institute of Health Sciences, Kamiyoga, Setagaya-ku, Tokyo, Japan

In order to evaluate the immunoreaction on human skin, we attempted to create the three-dimensional human skin model consisting of three different cells such as dendritic cells, keratinocytes and fibroblasts. Checking the histological cross-section of the human skin model, we stained the human skin model with hematoxylin and eosin. After 11-day incubation the horny layer was initially observed and then 14-day incubation three-dimensional human skin model was completely formed. Due to non-cytotoxic dose of DNCB, NiSO₄ and compound48/80, the dendritic cells in the human skin model released IL-4, IL-2 and IL-1α into the incubating medium and expressed CD86. The results suggest that the three-dimensional human skin model with dendritic cells should be able to apply for studying the effect of immune-sensitising compounds.
In order for a chemical to function as a contact allergen it must penetrate the skin, react with protein and be recognised as antigenic by immune cells. It is well established that protein reactivity is associated with skin sensitisation potential. An in vitro assay using peptides containing cysteine or lysine, or glutathione as surrogate nucleophiles was recently developed and used to measure the reactivity of more than sixty chemicals at various concentrations with sensitisation potencies ranging from weak to strong, and non-sensitisers. Using peptide reactivity data, in addition to LLNA potency data, a prediction model based on classification tree methodology was developed. Following reactivity determination, a compound is classified as a strong sensitiser if it reacts with more than 19% of the glutathione. A second test identifies a compound as a non-sensitiser if it reacts with less than 6% of the 1:10 cysteine peptide. The remaining compounds are classified as weak or moderate depending upon whether they react with less than or more than 76% of the 1:50 cysteine peptide, respectively. Using these model predictors, the potency of additional allergens was determined following reactivity testing. Model predicted potencies were within one category of the LLNA determined potency for all chemicals tested and the model identified correctly all of the non-sensitisers. Collectively, these data indicate that this model may be useful in screening the potency of skin allergens.

This research was supported by the European Cosmetic Toiletry and Perfumery Association (COLIPA).
Many types of chemicals have been reported to induce phototoxic effects. They all absorb radiation in the wavelength range of 290-700 nm and are distributed in sun-exposed tissues. The in vitro 3T3 Neutral Red Uptake (NRU) phototoxicity test is based on a comparison of the cytotoxicity of a compound when tested in the presence and absence of exposure to UV A light. This assay was recently adopted by the European Agency for the Evaluation of Medicinal Products (EAMA/CPMP, 2002) and the Food and Drug Administration (FDA/CDER, 2003) for guidance on photosafety testing. The 3T3-NRU phototoxicity test was shown to be predictive of acute effects in animals and in human for UVA/visible absorbers (Spielmann et al. 1998). Positive chemicals in this test are highly likely to be phototoxic in vivo following systemic or topical applications (OECD, 2002). In order to determine the reliability and relevance of this assay for strict UVB absorbers, 8 chemicals that absorb in UVB range only were tested in which 6/8 have been reported to be photo-irritants in vivo. The correlation between in vivo published data and those obtained with the 3T3-NRU in vitro assay will be shown.
Skin irritation is a multi-endpoint non-immune response of skin to insult, e.g. by substances or preparations. It covers both clinical (e.g. erythema, dryness) and sensory (e.g. burning, itching, stinging) reactions. In the past, the only accepted model for the predictive identification of agents which could cause skin irritation was the Draize rabbit skin test. More recently, \textit{in vitro} methods allowing the identification of corrosive substances have been adopted, allowing the complete replacement of animals for the assessment of corrosivity, as well as a refinement and reduction in animal usage in the general strategy for skin irritation. Our present aim is to undertake the work necessary to ensure the final step, replacement of animal testing, can be achieved. To this end, the COLIPA Skin Tolerance Task Force (STTF) has funded work on genomic analysis of the early phase of the response of skin to insult with a range of irritant substances. In addition, it has supported the analysis of the response to irritants of 3D skin models at the protein level. This work has yielded valuable insights, but also demonstrates the complexity of the response to irritants at the molecular level. Related to this is the concern about how to interpret \textit{in vitro} data in relation to skin irritation risk assessment. Ultimately, the ability of skin to tolerate a novel cosmetic formulation is assessed by carefully controlled human testing. The role of \textit{in vitro} skin irritation alternatives remains to be fully characterised.

\textbf{Lecture}

\textbf{The COLIPA strategy for the development of \textit{in vitro} alternatives: Skin irritation}

\textit{David Basketter\textsuperscript{1}, Pierre Aeby\textsuperscript{2}, Walter Diembeck\textsuperscript{3}, Frank Gerberick\textsuperscript{4}, Hiroshi Itagaki\textsuperscript{5}, Ian Kimber\textsuperscript{6}, Béatrice Le Varlet\textsuperscript{7}, Irene Manou\textsuperscript{8}, Marc Paye\textsuperscript{9}, Françoise Rousset\textsuperscript{10}, Joanna Rowland\textsuperscript{11} and Hitoshi Sakaguchi\textsuperscript{12}}

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\textsuperscript{1}In 1998, the European Centre for the Validation of Alternative Methods (ECVAM) commissioned a pre-validation study of five \textit{in vitro} methods for identifying skin irritants. The predictive ability of two reconstituted human skin methods (EpiDerm and EPISKIN) and one animal skin model (the mouse skin integrity function test) was found to be inadequate in this study but, following refinements to the protocols or the methodology for statistical analysis, ECVAM concluded that all three methods could proceed to a full validation study. This was conducted in two phases; in phase I, 20 coded chemicals (9 irritant and 11 non-irritant, as defined by the EU classification system) were tested in a single (lead) laboratory. The overall predictive ability was 75\% for EpiDerm, 80\% for EPISKIN but only 45\% for SIFT; for the human skin models, incorrect predictions were restricted to chemicals which were close to the classification borderline (mean \textit{in vivo} erythema scores between 1.7 and 2.4). EpiDerm and EPISKIN progressed to phase II in which 60 coded chemicals (26 irritant and 34 non-irritant) were tested in three laboratories. Interleukin-1-alpha release was evaluated as a test endpoint for both models in addition to the usual MTT cell viability assay to determine whether this would improve the predictive ability for chemicals on the borderline of classification. ECVAM will review the data from phase II in July 2005 and the results will be presented at this meeting.

\textbf{Lecture}

\textbf{The ECVAM skin irritation validation study}

\textit{Philip A. Botham}

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Poster  
**Effects of THz radiation on human keratinocytes in vitro**

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Terahertz frequencies are beginning to be employed in medical imaging. It is a non-ionising energy capable of producing images of skin tumours and teeth, speculatively at no damage to the body. In this laboratory primary human keratinocytes exposed in vitro to between 0.1 and 3 THz are still able to differentiate and commence production of the skin's natural barrier functions. The sensory innervation of the skin and the corneal epithelium are known to affect differentiation and therefore barrier functionality. A sensory nerve cell line, ND7/23, can be maintained in an undifferentiated state, and then driven to differentiate and develop dendrite-like projections. Our studies using human keratinocytes gave no adverse cell reactions or affected their ability to differentiate and form cornified envelopes. Hence more extensive exposure periods of up to 24 hrs, for the NHK and ND7/23 cells, were applied.

Exposed to THz radiation for 10 to 60 minutes both cell types alone or in co-culture converted reazurin, (a viability assay), and the fluorescein leakage assay (barrier function assay) gave no observed adverse effects, c.f. controls; 16 hours (ND7/23) and 24 hours (NHK), exposure in culture medium or HBSS, at room temperature or 37°C in a CO2 cabinet, for up to 16 hours, also showed no statistical loss of function compared to unexposed cells. Hence there is no indication that this level of THz results in cell damage. Repeat experiments are underway along with exposure to a more powerful source under construction.

Funded from the THz-BRIDGE EU grant (QLG1-2000-4.2).  

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Lecture  
**Optimisation of the EpiSkin protocol combining a tiered strategy in the framework of the ECVAM skin irritation validation**

José Cotovio, Marie-Hélène Grandidier, Pascal Portes and Roland Roguet  
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In recent years there has been considerable interest in the development of *in vitro* model substitutes for animals in cutaneous pharmaco-toxicology. Particularly suited to test chemicals with wide and diverse physical properties, the reconstructed epidermis model EpiSkin allows current use in fundamental and screening studies. Able to mimic *in vivo* situation, 3D models were included in the ongoing ECVAM-funded validation for acute skin irritation. *In vitro* selected tests should be capable of discriminating between irritant chemicals (EU risk R38) and non-irritant chemicals (EU risk “no classification”). In order to meet this specific need, an optimisation of the already published (Portes et al., 2002) EpiSkin protocol, based on a specific extended post treatment incubation period (42 hours) was applied to a set of 48 chemicals. Sensitivity, specificity and accuracy of the MTT-based PM were, 85%, 78.6% and 81.3% (respectively) with low false negative rate (12%). Stronger performances of this optimised protocol were sustained by robustness properties and an efficient separation between I and NI classes. IL-1α, IL-8 and the adenylate kinase were also investigated. Combining selected end points in a simple tiered strategy (TSTS), MTT being the first sort followed by IL-1α determination, resulted in a clear improvement of predictive capacities (95% sensitivity, drop of false negatives (4.3%)).

The results demonstrated the usefulness of the TSTS as a decision-making tool able to strengthen the PM performances. Furthermore, the transferability of this final optimised protocol 15 min - 42 hours to other skin models (Kandarova et al., 2004) was a great advantage shared in the ongoing ECVAM skin irritation test validation.
Lecture

7-ethoxycoumarin metabolism in a viable pig ear skin model: New alternative model for absorption and metabolism studies

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The skin is the largest organ and most accessible drug metabolising organ. Skin expresses many cytochromes P450 that have critical roles in exogenous and endogenous substrates metabolism. The role of absorption rate and enzyme activity on cutaneous metabolism of topically applied xenobiotics was assessed by determining simultaneously percutaneous penetration/metabolism of 7-ethoxycoumarin (7-EC) in a newly developed skin organ culture model, coming from ear of domestic pigs.

Six doses of 7-EC (from 10 to 400 nmol/cm²) in two vehicles (PBS/ethanol 2:1 solution and myritol/ethanol solutions 2:1) were applied on the viable pig ear skin model. Diffusion and metabolism of 7-EC was assessed by Radio-HPLC during a time period of 72 h. All the experiments were carried out in triplicate.

More than 65% and 40% of the applied dose of 7-EC with the hydrophilic vehicle and with the lipophilic vehicle respectively were recovered in the receptor medium. Metabolism occurred with both vehicles and remained active during 72 h. About 15% of the applied 7-EC dose were metabolised for the low doses (10-50 nmol/cm²) and 5% for the highest doses (100-400 nmol/cm²) suggesting a saturation of the enzymes. More than 95% of the metabolites was the glucuronide form and found in the receptor medium. The other 5% was hydroxy-7-EC, located in the skin.

This viable pig ear skin model exhibits cytochromes P450 dependant phase I and phase II activities. This model may provide a suitable, relevant and alternative model to animal and human studies for cutaneous uptake and detoxification metabolism.

Poster

In vitro dermal penetration studies with excised pig skin and reconstructed skin

Hans-Juergen Duesing, Mehdi Akhiani, Andreas Schepky, Horst Wenck and Walter Diembeck
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The OECD Test Guideline 428 “Skin Absorption: in vitro method” recommends the use of excised human or pig skin for the in vitro investigation of dermal absorption and penetration. Reconstructed skin models can be acceptable for this purpose as well, if their suitability is proven by appropriate correlation studies with both the respective skin model and excised skin.

Our studies with pig skin and reconstructed skin (Epiderm™, MatTek, USA) with cosmetic formulations (finite dose) confirmed the distinctly higher penetration rates through the reconstructed skin barrier as compared to pig skin for substances with lower octanol-water partition coefficients.

After topical application of e.g. Sodium-Dodecyl-Sulphate solutions (infinite dose) in a wide range of concentrations (0.05 mmol/l to 100 mmol/l including 14C-SDS as a radio-labelled tracer) we found an approximately 100 times higher permeability with Epiderm™ in comparison to split thickness pig skin (750±50 µm). Results for substances with higher octanol-water partition coefficients (log P<sub>ow</sub> >8) were more comparable in both models.
Poster

ECVAM Key Area Topical Toxicity: Summary of ongoing activities

Chantra Eskes¹, Laurie Scott² and Valérie Zuang²
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The area of topical toxicity managed and co-ordinated at ECVAM includes eye and skin irritation/corrosion, phototoxicity and percutaneous absorption. Validated alternative tests for skin corrosion, phototoxicity and percutaneous absorption have obtained regulatory acceptance at both the EU and OECD level. In contrast, alternative methods for eye and skin irritation are urgently needed, especially in light of the 7th Amendment to the Cosmetic Directive and REACH. Since topical application represents a main route of exposure to cosmetics, eye and skin irritation data are essential. As a base set requirement, around 30,000 substances produced or imported in quantities greater than 1 tonne/annum will require eye and skin irritation data under REACH.

An update on key activities to validate the most promising in vitro tests for skin and eye irritation will be provided. Highlights include: an update on the ECVAM validation study on the EPISKIN and EpiDerm assays for acute skin irritation, which is in its final phase and involves 6 laboratories. A review of ICC-VAM/ECVAM joint activities to develop a tiered testing strategy for eye irritation are provided. Lastly, technical support to DG Enterprise and DG Health and Consumer Protection in relation to Directive 76/768/EEC and to the Scientific Committee on Consumer Products (SCCP) are highlighted.

Poster

Skin irritation: Prevalence, variability and regulatory classification of existing in vivo data from industrial chemicals

Sebastian Hoffmann¹, Thomas Cole² and Thomas Hartung¹
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In vivo rabbit data for skin irritation registered in the European New Chemicals Database (NCD) and an ECETOC Database were evaluated to characterise the distribution of irritation potential among chemicals and to assess the variability of the animal test. These databases could be used to determine experimental and rudimentarily within-laboratory variability, but not between-laboratory variability. Our evaluation suggests that experimental variability is small. Using two classification systems – the system currently used in Europe and the Globally Harmonised System (GHS) – the prevalence of skin irritation data obtained from NCD was analysed. This analysis revealed that out of 3121 chemicals tested, less than 10% showed an irritation potential in rabbits which would require an appropriate hazard label and 64% did not cause any irritation. Furthermore, it appears that in practical use the European classification system introduces bias towards over-classification. Based on these findings, we conclude, that the classification systems should be refined taking prevalence into account. Additionally, prevalence should be incorporated into the design and analysis of validation studies for in vitro test methods and in the definition of testing strategies.
There are various \emph{in vitro} assay methods for evaluating the phototoxicity of chemicals. One of these techniques is \emph{in vitro} 3T3 cell Neutral Red Uptake Phototoxicity (3T3 NRU PT) Test, which had been scientifically validated by ECVAM between 1992 and 1996. In this assay, Balb/c 3T3 mouse fibroblast is recommended and a doped mercury metal halide lamp (SOL 500; Dr Honle, Martinsried, Germany), which artificially simulates the spectrum distribution of natural sunlight, is used as the UV light source.

The purpose of this study is to investigate the flexibility of this assay. We used some different cell lines in addition to Balb/c 3T3 and two light sources, SOL 500 (UV A plus visible light) and xenon lump with a filter which extracts only UV A. The assay was carried out according to the method described by EU/COLIPA (Spielmann et al., 1994, \textit{ATLA}, 314-348). Fourteen substances (nine phototoxic chemicals, two photosensitisers and three non-phototoxic chemicals) are assayed in this study.

Our results indicate that the application of different cell lines to 3T3 NRU PT test does not largely influence the sensitivity of this assay, however, the spectrum of light sources or the condition of irradiation affect that. Furthermore, we reveal that the exposure dose of UVA influences the sensitivity of this assay more strongly than the intensity of irradiation.

Human tissue models represent the most promising developments \emph{in vitro} toxicology. The production of such models requires specialist expertise, not available in all laboratories, and therefore commercial availability is required. Our laboratory sources cultures from several suppliers. In this report, the usefulness of human epithelial cultures in the selection and ranking of ingredients or formulations using a benchmark approach is illustrated by examples of irritation testing using MatTek EpiDerm™ skin and EpiOcular™ corneal, and SkinEthic™ buccal mucosa models. All models were multilayered, 3D tissues prepared from human cell lines or primary human keratinocytes grown on filter inserts at the air/liquid interface. Protocols involved topical application of test materials, at concentrations and exposures as recommended by manufacturers and/or from previous experience. Cytotoxicity was measured using MTT reduction. The cytotoxicity rank order of test materials was compared including benchmarks of known or acceptable irritation potential. In general, the most irritant materials were the most cytotoxic and differences/similarities between test materials and benchmarks could be identified. The conclusions of these studies were that 3D human tissue models are useful for screening for irritation potential to a variety of tissues, to identify more irritant materials and prioritise development of those potentially less irritant. Other advantages of the use of this type of model are that they enable the testing of water-insoluble materials and of both liquids and powders. These models will provide a useful tool for further investigation of the most appropriate protocols and more specific predictive endpoints/markers than general cytotoxicity.
Lecture

Skin irritation in vitro: EpiDerm™ test protocol developed and optimised for an ECVAM validation study on skin irritation testing of chemicals

Helena Kandárová, Manfred Liebsch, Ingrid Gerner, Elisabeth Schmidt, Elke Genschow, Dieter Traue and Horst Spielmann

Several validation studies have been conducted on in vitro methods, to discriminate between skin irritating and non-irritating chemicals. Most promising results were obtained until now with human reconstructed epidermal models EpiDerm™ and EPISKIN®. Based on experience of similar performance of the two skin models, it was suggested that a common test protocol and prediction model should be developed for the prediction of skin irritation potential with the two models (Fentem et al., 2002).

When the EPISKIN protocol (Portes et al., 2003) was applied to the EpiDerm model, an acceptable specificity was achieved, whereas sensitivity was low. In 2003, the EPISKIN protocol was further refined (Cotovio et al., 2005) by extending the post-incubation period after exposure to test chemicals. This refinement was as well evaluated on EpiDerm. With the new test design and additional technical refinements, high sensitivity (80%) and specificity (78%) were obtained (Kandarova et al., 2004).

Since all optimisation steps had been conducted always with the same test chemicals, it was decided to verify the protocol with a new set of chemicals. In the second study 26 additional chemicals (10 rabbit irritants and 16 non-irritants) were evaluated on EpiDerm. With this unbalanced testing set a specificity of 94%, and a sensitivity of 60% were obtained. Positive and negative predictivity and accuracy remained almost unchanged (around 80%).

Overall, 45 chemicals were tested in the final protocol. The resulting high positive (86%) and negative predictive values (79%) confirm the reliability of the improved test protocol (accuracy of 80).

Poster

ECVAM feasibility study: Can the pre-validated in vitro skin model phototoxicity assay be upgraded to quantify phototoxic potency of topical phototoxins?

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The determination of phototoxicity in the 3T3 NRU-PT according to OECD Test Guideline 432 is often the first step in the sequential phototoxicity testing strategy. If the chemical provides a negative result in the 3T3 NRU-PT, in most instances no further testing is required. However, if the result is positive, the chemical may be still applied topically at safe concentrations, depending on the absorption and accumulation of the chemical in the skin.

Thus, in addition to the information on inherent phototoxicity potential assessed by the 3T3NRU-PT, additional testing may be required to obtain combined information on the phototoxicity and bioavailability of the chemical in the skin.

Ideally, confirmatory tests should be performed in vivo on human volunteers, but for ethical reasons, this is not acceptable, if the 3T3 NRU-PT has provided a positive result. Thus, to avoid confirmatory testing in vivo in animals, reconstituted human 3D skin models are offering an attractive in vitro alternative for testing, since such models are characterised by both skin barrier function and viable primary skin cells.

In the current study, several substances (mostly cosmetic ingredients) which are known to be safely used in humans, and which provided positive results in the 3T3 NRU PT were evaluated on the reconstructed human skin model EpiDerm and, if the result was negative, tested in a limited group of human volunteers. First results we obtained show, that the human skin model phototoxicity test represents a useful step in the sequential strategy for phototoxicity testing.
**Poster**

**Skin corrosion in vitro: Assessment of the SkinEthic® reconstituted human epidermal (RHE) model for in vitro skin corrosion testing according to OECD TG 431**

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In 2002 the National Co-ordinators of OECD Test Guideline Programme (WNT) endorsed a New Draft Test Guideline TG 431 (Human Skin Model) for In Vitro Skin Corrosion Testing which was finally adopted in April 2004. This guideline specifies general functional and performance criteria that have to be met, if a new skin or epidermal model is intended to be used for the skin corrosion testing of chemicals in vitro.

In 2003 ZEBET tested several chemicals with known corrosive potential on the SkinEthic reconstituted human epidermal (RHE) model using the validated EpiDerm test protocol and prediction model. After minor technical adaptations, classifications obtained were comparable to those obtained previously with the validated human skin models EPISKIN and EpiDerm.

From December 2003 to February 2004 ZEBET, SafePharm and BASF conducted an inter-laboratory trial with 12 coded reference chemicals proposed by the OECD TG 431 in order to confirm the performance of the SkinEthic skin corrosion assay.

In each laboratory, for each of the test chemicals, three independent tests were performed. Results obtained with the SkinEthic epidermal model were reproducible, both within and between laboratories, and over time. Concordance between the in vitro predictions of skin corrosivity potential obtained with the SkinEthic epidermal model and the predictions obtained with the accepted skin models was very good. The new test was able to correctly distinguish between corrosive and non-corrosive reference chemicals and can be regarded as valid method for the use in context with OECD TG 431.

**Poster**

**Assessment of the SkinEthic® reconstituted human epidermal model for the prediction of the dermal irritation potential of chemicals**

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During the past ten years, a lot of attention has been paid to the development of in vitro tests that could substitute irritation studies employing animals. The best results were until now obtained with 3D reconstructed human skin equivalents, for which several test protocols have been developed.

In areas of skin corrosion and phototoxicity, it has been proven that similarities between well developed 3D skin models allow to use common test protocols, obtaining similar results. Recently, efforts have been undertaken to develop a common protocol for the assessment of skin irritation of chemicals using skin models.

This “common skin irritation protocol” for EPISKIN and EpiDerm™ reconstructed epidermal models is currently evaluated in an ECVM validation study.

Concurrently with the ECVM skin irritation validation study, ZEBET (Berlin, Germany) performed a small scale study applying the “common skin irritation protocol” on SkinEthic Reconstructed Human Epidermis (RHE) to verify whether this protocol can be successfully transferred to another epidermal model. Twenty substances from the ECVM pre-validation study on skin irritation were tested on SkinEthic RHE. After minimal model specific adaptations, almost identical results (when compared to results of EpiDerm and EPISKIN models) were obtained.

Then the protocol’s transferability was evaluated at ZEBET and Schering AG (Berlin, Germany) by testing six coded chemicals in three independent runs, obtaining very good results. In addition, an analysis of IL-1α release was performed, with the aim to investigate if a second endpoint could add valuable information for the prediction of skin irritation potential of chemicals.
Normal human epidermal keratinocytes (EK) and dermal fibroblasts (FB) were cultured to produce full-thickness skin equivalent (EFT-200) consisting of FB-containing dermal and stratified epidermal components with a fully developed basement membrane at the dermal/epidermal junction. The wound healing response of EFT-200 and excised human skin after solar UV-irradiation were compared. H&E stained, EFT-200 histology sections displayed a dose-dependent increase in apoptotic sunburn cells 24 h post-irradiation. After 72 h, sunburn cells persisted in mid dose samples (40 J/cm², metal halide lamp), which were thinner than controls (indicating a major decrease in EK proliferation) but without major epidermal damage. However, after 48 and 72 h, high dose (61 J/cm²) samples showed extensive epidermal and dermal damage. Nonetheless, viable basal cells remained in some areas, with signs of proliferation and epidermal regeneration. A 50% increase in MMP-1 released into the culture media was observed at 24 h and at 48 h, mid and high dose samples showed 100% and 150% increases, respectively. At 72 h, mid dose MMP-1 release was equivalent to control, but high dose release remained elevated at 125%. Similar experiments with excised human skin showed increased sunburn cell formation, tissue thinning at 40 J/cm² and extensive damage at 61 J/cm², and basal cell proliferation/epidermal regeneration at 72 h in high dose samples. These results show that EFT-200 behaves similarly to excised human skin in terms of UV induced damage and wound healing. The model will prove useful for additional applications in wound healing and other dermal/epidermal phenomena.
Skin irritancy of a cosmetic product or topical medicament has been evaluated by a visual assessment of 24 or 48 hours human patch testing in Japan. We consider that patch testing is useful and achieving high accuracy is important to develop alternative to skin irritation testing. Since patch testing involves visual assessment, the results of such visual approaches can be subjective, and therefore variable between observers and institutions.

1. Visual assessment of human skin irritation might not be reliable and reproducible: According to visual assessment based on the Japanese patch-test reading-criteria (JPTRC: Kawamura et al., 1970), there was a significant variance in evaluating erythema among the readers. It is difficult to evaluate erythema from weak to moderate. The experience of the readers was not always associated with reliable and reproducible evaluation.

2. Newly proposed criteria to evaluate skin irritation on patch testing: Education and improvement of evaluation criteria seem important for standardising the evaluation of skin irritation. We have tried to develop new criteria for judging and colour atlas to education in the use of patch test to provide reliable and reproducible results. Seven grades of erythema were established according to the intensity, distribution of erythema, and presence or absence of edema or surface change.

3. Establishment of an education system: Using the newly proposed criteria, we provided seminars and lectures on judging skin irritation. Before and after lecture, all participants performed examination. As a result, reliability and reproducibility of responses from all members were increased.

As a validation trial for an alternative to skin irritation testing in Japan, we performed a validation study of TESTSKIN™, a 3-dimensional cultured skin model. In this validation study, 7 laboratories participated, including the kit suppliers (TOYOBO Co., Ltd.). Participants performed the pre-test and main trial and obtained ET50 values (time to 50% reduction in MTT compared to the untreated control value) over a five month period from May to October 2002. Forty cosmetic ingredients in addition to 1% sodium lauryl sulphate solution (positive control), distilled water and olive oil (solvent) were selected, coded and supplied to the laboratories.

As a result, most chemicals did not show marked great differences in ET50 scores on tests repeated at each laboratory, using the estimation method as shown in the second paper. The feasibility of TESTSKIN™ was suggested through the experiment, although inter-laboratory variation was significant for some chemicals.

Furthermore, we compared the validation testing data and in vivo data in this study. In vivo primary skin irritation testing was performed using rabbits and humans. Comparing animal and human data, the consistency rate in humans was 66% of that in rabbits. The false positive rates were 32% and false negative rates were 40% in rabbits.

We compared human data with 200 data obtained from several laboratories in this validation study. The consistency rate of those data was 69%, the false positive rate was 38% and the false negative rate was 6%. These data show that the reliability of this method was similar to that of animal testing.
As a validation trial for an alternative to skin irritation testing in Japan, we performed a validation study of Vitrolife-Skin™, a 3-dimensional cultured skin model. In this validation study, 9 laboratories participated, excluding the kit suppliers (Gunze Co., Ltd.). Participants performed the pre-test and main trial and obtained ET50 values (time to 50% reduction in MTT compared to the untreated control value): ET50 protocol over a four month period from June to September 2004. Fourteen cosmetic ingredients, distilled water and olive oil (solvent) were selected, coded and supplied to the laboratories.

As a result, most chemicals did not show marked differences in ET50 scores on tests repeated at each laboratory, using the estimation method. The feasibility of Vitrolife-Skin™ using the ET50 protocol was suggested through the experiment, although inter-laboratory variation was significant for some chemicals.

Furthermore, we compared the validation testing data and in vivo data in this study. In vivo primary skin irritation testing was performed using humans. Compared to in vivo human data, the consistency rate of those data was 68.9%, the false positive rate was 54.5 % and the false negative rate was 3.6%. These data show the reliability of this method was similar to that obtained by animal testing such as using rabbits.

Poster

Validation study for Vitrolife-Skin™, a three-dimensional cultured human skin model as an alternative to skin irritation testing using ET50 protocol

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Poster

Validation study for Vitrolife-Skin™, a three-dimensional cultured human skin model as an alternative to skin irritation testing using Post-Incubation (PI) protocol

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As a validation trial for an alternative to skin irritation testing in Japan, we performed a validation study of Vitrolife-Skin™, a 3-dimensional cultured skin model. In this validation study, 9 laboratories participated, excluding the kit suppliers (Gunze Co., Ltd.). Participants performed the pre-test and main trial and obtained cytotoxicity using Post-Incubation (PI) protocol: treatment for 10 min after washing, incubated for a further 18 hrs (percent MTT viability) over a four month period from June to September 2004. Fourteen cosmetic ingredients, distilled water and olive oil (solvent) were selected, coded and supplied to the laboratories.

As a result, most chemicals showed marked differences in cytotoxicity on tests repeated at each laboratory. The low feasibility of the PI protocol was suggested through the experiment and inter-laboratory variation was significant for many chemicals.

Furthermore, we compared the validation testing data and in vivo data in this study. In vivo primary skin irritation testing was performed using humans. Compared to in vivo human data, the consistency rate of those data was 44.4%, the false positive rate was 41.2% and the false negative rate was 72.4%. These data show the reliability of PI protocol was lower than that obtained by animal testing such as using rabbits.
The human 21-day Cumulative Irritation Patch Test (21-CIPT) (Lanman et al., 1968) is a standard test of new products intended for repeated dermal contact. However, the duration and cost limit its use to screen multiple formulas in early phase of the product development. Thus, there is a need for a rapid, relatively inexpensive test that predicts performance on the 21-CIPT. The Cytosensor Microphysiometer (µϕ) (Molecular Devices Corp., Menlo Park, CA) was investigated as a screening tool. It measures metabolic changes in L929 cells as a function of test article dose in a cycle of exposure/wash/metabolism measurement. The dose producing 50% reduction in metabolic rate (MRD50), relative to the baseline, is used as a measure of toxicity. It is quick and effective in predicting potential irritation of surfactants. The acute toxicity of the µϕ assay can be compared to the chronic toxicity of the 21-CIPT, which is based largely on the exposure of wetting agents (surfactants) to the epidermal cells. Twenty wet wipe formulas were tested via the µϕ and 21-CIPT. One material was a product with over five years of successful market experience. Samples with MRD50 greater than 50 mg/ml provided 21-CIPT scores consistent with a product that performs satisfactorily in the market. When the MRD50 was greater than 78 mg/ml, the 21-CIPT score was usually zero. The µϕ assay showed greater sensitivity than the 21-CIPT for ranking materials with minimal irritancy. The µϕ assay is useful as a screen for predicting the performance of wet wipe formulas on the 21-CIPT.

Poster

Use of the cytosensor microphysiometer to predict results of a 21-day cumulative irritation patch test in humans

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The human 21-day Cumulative Irritation Patch Test (21-CIPT) (Lanman et al., 1968) is a standard test of new products intended for repeated dermal contact. However, the duration and cost limit its use to screen multiple formulas in early phase of the product development. Thus, there is a need for a rapid, relatively inexpensive test that predicts performance on the 21-CIPT. The Cytosensor Microphysiometer (µϕ) (Molecular Devices Corp., Menlo Park, CA) was investigated as a screening tool. It measures metabolic changes in L929 cells as a function of test article dose in a cycle of exposure/wash/metabolism measurement. The dose producing 50% reduction in metabolic rate (MRD50), relative to the baseline, is used as a measure of toxicity. It is quick and effective in predicting potential irritation of surfactants. The acute toxicity of the µϕ assay can be compared to the chronic toxicity of the 21-CIPT, which is based largely on the exposure of wetting agents (surfactants) to the epidermal cells. Twenty wet wipe formulas were tested via the µϕ and 21-CIPT. One material was a product with over five years of successful market experience. Samples with MRD50 greater than 50 mg/ml provided 21-CIPT scores consistent with a product that performs satisfactorily in the market. When the MRD50 was greater than 78 mg/ml, the 21-CIPT score was usually zero. The µϕ assay showed greater sensitivity than the 21-CIPT for ranking materials with minimal irritancy. The µϕ assay is useful as a screen for predicting the performance of wet wipe formulas on the 21-CIPT.

Poster

In vitro phototoxic potency assessment of chemicals using the human reconstructed epidermis EpiSkin

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The aim of the study was to evaluate the phototoxic potency of chemicals using the human reconstructed epidermis model EpiSkin. 8 phototoxic (ofloxacin, lomefloxacin, promethazine, chlorpromazine, menadione, demeclocycline, bergamot oil, 5MOP and 8MOP) and 3 non phototoxic (coumarin, histidine, penicillin G salt) compounds were evaluated. Chemicals were applied topically or systemically at non cytotoxic concentrations for 2 h. Following treatment, tissues were exposed to non cytotoxic concentrations of UVA. Viability and pro-inflammatory mediators (IL-1α, IL-8 and PGE2) were investigated 22 h after exposure. 2 criteria were used to evaluate phototoxicity: PIF>1 (Cmax/UV/CL50+UV) or a significant decrease in viability (25%) on exposed tissues. The results showed that, excepting lomefloxacin and ofloxacin, all known phototoxic chemicals induced a significant decrease in tissue viability (>25%) and a significant release of IL-1α after UVA exposure. Lomefloxacin and ofloxacin, described as systemic phototoxic chemicals in vivo, were found phototoxic after systemic treatment. Furthermore, viability decrease and IL-1α released levels were closely related. A significant release of PGE2 and IL-8 was detected with 8-MOP and 5-MOP after UVA exposure. These effects could be linked to specific furocoumarin phototoxic mechanisms. All tested non phototoxic chemicals, topically or systemically applied, were correctly identified.

Our results suggest that:
1. The phototoxic potential of chemicals can be determined using viability endpoints combined with inflammatory mediator measurements in a 3D epidermis model.
2. EpiSkin can be a relevant tool to predict the phototoxic potency of topically or systemically applied chemicals, bioavailability of the tested chemical depending on the administration route.
**Poster**

**In vitro skin irritation screening of cosmetic products on reconstituted epidermis**

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The *in vitro* skin irritation study is still under development to establish a standardised protocol to evaluate the irritation potential of developed cosmetic products. By using the human reconstituted epidermis model Skinethic, various cosmetic products such as surfactant and alcohol containing formulations, body and face creams or lotion, were tested by applying 20 µl of the test item on the epidermis of 0.5 cm². ELISA assays of IL-1 and IL-8 inflammation cytokines were performed on supernatants after 6, 24, and 72 hours. The cellular viability was assessed with a MTT coloration and a histological study was conducted at the same time. A positive control was systematically performed with a SDS 0.2% treatment, and compared with an untreated epidermis. An increase of IL-1 and IL-8 synthesis combined with a decrease in cellular viability was observed with the SDS treatment, whereas no cytokine was synthesised and cellular viability was high without treatment. Cytokine fluctuations induced by cosmetic products were analysed with histology features and the cytotoxicity by an analysis of variance (multifactor Anova). Human patch tests are currently being carried out with some of cosmetic products to correlate *in vitro* data with human skin reactivity. The results of this study should contribute to the determination of relevant endpoints for screening the *in vitro* skin irritation potential of different types of cosmetic products.

**Poster**

**Use of the yeast *Saccharomyces cerevisiae* as a pre-screening approach for assessment of chemical-induced phototoxicity**

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Photoreactive chemicals can induce dermatological reactions when present in the skin exposed to sunlight. Thus, new chemicals absorbing above 290 nm should have their potential phototoxicity tested. In order to screen a large number of molecules with various physico-chemical properties, a microbiological method is helpful. To this end, the yeast *Saccharomyces cerevisiae* was evaluated for its ability to detect phototoxic compounds. Twelve products known to be phototoxic *in vivo* and previously used as standards for validating the regulatory test 3T3 NRU were used in this work. Eleven of them could be detected in the yeast assay and, among them, 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), angelicin and, to a lower extend, tiaprofenic acid induced genetic alterations. Interestingly, a pre-incubation with yeast cells in the dark before exposure decreased the phototoxicity of 5-MOP and 8-MOP but had no effect on this of chlorpromazine and ketoprofen. *Saccharomyces cerevisiae* and *Salmonella thyphimurium* (strains TA100 and TA102) were compared for the evaluation of 5-MOP and 8-MOP photogenotoxicity; only the yeast assay allowed performing experiments in exposure conditions close to those encountered in environmental situations. Finally, an application of this experimental approach to the detection of traces of furcoumarins in fragrance materials was developed.
Lecture

Validation of human skin models for skin corrosivity tests in Japan

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As a validation trial of alternative for skin corrosivity testing in Japan, we performed a validation study of EPI-200 (EpiDerm™), a 3-dimensional cultured epidermal model and Vitrolife-Skin™, a 3-dimensional cultured skin model. In this validation study, 6 laboratories took part excluding the kit suppliers (Kurabo Industries Ltd. and Gunze Co., Ltd.). Participants performed the pre-test and main trial and obtained cut-off percentage cell viability values (viability after 3 minutes or 1 hour exposure) over a three month period from February to April 2004. Twelve chemicals were selected and coded, then 10 chemicals were supplied to each laboratory.

As a result, most chemicals did not show any great differences in scores on tests repeated at each laboratory. Inter-laboratory variation was significant in sulfuric acid alone, and the feasibility of using EPI-200 and Vitrolife-Skin™ was suggested through the experiment.

Furthermore, we compared the validation testing data and in vivo database in ECVAM. Comparing corrosivity data, the consistency rate of tests using EPI-200 was 81.7%. The chemicals showing false positives were 5% potassium hydroxide and lactic acid but sulfuric acid alone showed a false negative response. On comparison of the consistency rate of tests using Vitrolife-Skin™ showed 83.3%, the chemicals showing false positives were 5% potassium hydroxide and lactic acid whereas none of the chemical showed a false negative response. These data showed that the reliability of these two models was similar to the results obtained on the ECVAM validation.

Poster

Screening of skin irritation potential of surfactants with the Red Blood Cell Test. Comparison to EpiDerm™ skin model and human patch test data

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The assessment of the skin irritation potential of cosmetic formulations is normally carried out in vivo with the human epicutaneous patch test. However, test materials containing ingredients with an uncertain toxicity profile should be tested in vitro before testing on humans. Furthermore, time and cost reduction could be arguments for the use of in vitro tests. Since several years 3D skin models (e.g. EpiDerm™, MatTek) are routinely used for this purpose.

The Red Blood Cell Test (INVITTOX protocol no. 37) was originally developed to screen the mucous membrane irritation potential of surfactants and surfactant-based formulations. Twenty years of experience have demonstrated that the RBC test is a valuable tool for the assessment of eye irritation potential. Endpoints are haemolysis (damage of erythrocyte membranes) and haemoglobin denaturation. Over the years we have found that the haemoglobin denaturation correlates well also with the skin irritation potential of diluted surfactants and surfactant-based formulations.

In 2004 the DGK (Deutsche Gesellschaft für Wissenschaftliche und Angewandte Kosmetik) initiated a ring study for the validation of the human patch test. Eight diluted surfactants and mixtures of surfactants were tested in several laboratories. At Beiersdorf these test samples were additionally tested with the RBC test and EpiDerm™. A high correlation was found between the haemoglobin denaturation index of the RBC test compared to the results of EpiDerm™ and the patch test. Therefore, the RBC test is a valuable tool for the assessment of both eye and skin irritation potentials.
The Institute for In Vitro Sciences (IIVS), Gaithersburg, Maryland, USA, hosted a workshop on in vitro percutaneous absorption (PA) methods for a small group of international stakeholders in July 2005. The purpose of the workshop was to provide a forum where stakeholders and method experts could come together to discuss the various OECD approved guidance on in vitro PA methods (OECD Test Guideline 428, April 2004 and Guidance Document for the Conduct of Skin Absorption Studies, March 2004) and how this guidance may be practically applied to the protocols in current use. The workshop participants compared and contrasted specific components of different in vitro protocols, and made recommendations on protocol components that are essential for obtaining useful toxicological data from the in vitro PA methods. A major goal of this workshop was to provide industry, contract research laboratories and the regulatory community with practical information to facilitate successful, wider, and earlier use of in vitro PA data in regulatory submissions. Detailed conclusions and recommendations from the workshop will be presented.

**Poster**

**Report from an in vitro dermal absorption assay workshop**

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The Institute for In Vitro Sciences (IIVS), Gaithersburg, Maryland, USA, hosted a workshop on in vitro protocols for a small group of international stakeholders in July 2005. The purpose of the workshop was to provide a forum where stakeholders and method experts could come together to discuss the various OECD approved guidance on in vitro PA methods (OECD Test Guideline 428, April 2004 and Guidance Document for the Conduct of Skin Absorption Studies, March 2004) and how this guidance may be practically applied to the protocols in current use. The workshop participants compared and contrasted specific components of different in vitro protocols, and made recommendations on protocol components that are essential for obtaining useful toxicological data from the in vitro PA methods. A major goal of this workshop was to provide industry, contract research laboratories and the regulatory community with practical information to facilitate successful, wider, and earlier use of in vitro PA data in regulatory submissions. Detailed conclusions and recommendations from the workshop will be presented.

**Poster**

**In vitro modelling of chromium and zinc interactions in human dermal fibroblasts**

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One of the main goals of toxicology of heavy metals is to understand all adverse effects induced by individual members of this group of toxins. The studies of these effects are complicated by many factors, for example by ambivalent nature of many metals (toxicity vs. essentiality) and by the fact that in real environment heavy metals do not occur individually but in mixtures. Therefore, to investigate interactions of hexavalent chromium whose occupational exposure has been linked to various skin pathologies including skin ulcerations and allergic dermatitis and zinc (a bioelement implicated in many important cellular processes, with recognised cytoprotective effects in skin cells) in human dermal fibroblasts, a series of in vitro tests have been used during 48 hours. The followed markers included cell viability (WST assay), cell motility, cytoskeletal organisation, oxidative stress as well as measurement of cell death. Exposure to potassium chromate produced concentration and time dependent loss of cell viability, reorganisation of cytoskeleton and cessation of motile activity in fibroblasts. Cytoskeletal perturbation was accompanied by increased oxidative stress and activation of cell death. These changes were further enhanced in zinc-deprived fibroblasts. On the other hand, administration of zinc prevented chromium induced toxicity and, also, was able to reduce significantly cell death. These results demonstrate the important role of zinc in protection of skin homeostasis.

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Poster

A statistical method for estimating ET$_{50}$ under the condition of small volume of data

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The median effective time (ET$_{50}$) is usually used as an index of skin irritancy of chemicals in a three dimensional human skin model such as Vitrolife-Skin or EpiDerm. Due to restrictions on time setting, cost and labor, at most 5 time points were available for each chemical in the validation study in Japan using Vitrolife-Skin. Assuming such a restricted experimental condition, we investigated to improve the estimating method for confidence intervals of ET$_{50}$ (CI).

The least squares method is standard methods for estimating ET$_{50}$ to fit a logistic curve or a linear regression line with time on horizontal axis and viability on vertical axis. However, simple application of these methods sometimes fails to get reasonable CI. We, consequently, propose the combining use of the non-linear least squares method assuming a logistic regression curve, with the least squares method assuming a linear curve with log(time) as independent variable.

We examined the performance of the proposed method using a Monte-Carlo method assuming logistic or linear model. According to the result of simulation, more reasonable estimate of ET$_{50}$ was obtained in the proposed method compared with the simple use of respective method. However, the coverage probabilities of CI were less than the nominal confidence level 95% by 10% or more. The improvement of the proposed method is considered to be required in future studies.

Poster

Statistical considerations for positioning time points in ET$_{50}$ estimation using three dimensional human skin model

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Appropriate experimental design is essential to precisely estimate effective time 50 (ET$_{50}$), which is an index of skin irritancy of chemicals in a three dimensional human skin model such as Vitrolife-Skin or EpiDerm. One of the most important issues in the design of experiment to obtain estimates of ET$_{50}$ using as small number of kits as possible is appropriate positioning of time points within 24-hour interval, keeping the time points in a certain range, which is desirable for saving office hours of experimenters. It is also useful to reduce troublesome cases where reasonable confidence interval cannot be obtained, which frequently appeared in the validation study in Japan.

We conducted a Monte-Carlo simulation study using virtual data produced by a simulation model with a logistic curve on the time-response to examine the optimality of time positioning. In the simulation study, we used a logistic regression method to obtain estimates and confidence intervals of ET$_{50}$. We compared several cases of positioning of time points under the assumption that a preliminary experiment was performed in advance and a rough estimate of ET$_{50}$ was given, keeping the number of time points at 3, 4, or 5. Based on the result of simulation, we concluded that the choice of time point within 2 hours around the preliminary estimate of ET$_{50}$ was effective for obtaining reasonable estimates of ET$_{50}$ and that at least five time points was necessary when preliminary estimate was not reliable, whereas four time points was sufficient when it was.
Poster

**Occupational safety assessment of skin corrosion/irritation using human reconstituted epidermis models**

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The assessment of skin corrosion/irritation is an essential part of the safety evaluation of chemicals and is therefore demanded by international regulatory requirements. Because of ethical considerations and sometimes limited availability of compound for *in vivo* tests, alternative methods, including human reconstituted epidermis (hREP) models, gain more and more importance.

In an in house validation study with hREP models we used known corrosive or irritating chemicals and determined cell viability (MTT-assay) as the basis for classification of the respective hazard. The results of viability testing provided a good prediction for a wide spectrum of chemicals (e.g. organic acids and bases, anorganics, phenols). In addition, hematoxylin and eosin stained sections of the epidermis at the end of the study and the release of IL-1α in the assay medium were evaluated in order to investigate different endpoints for classification. In a comparability study for skin irritation performed together with ZEBET a good intra- and interlaboratory reproducibility of MTT-results could be shown over time and with separate lots of tissues. Therefore, *in vitro* skin corrosion and irritation assays using hREP models have now been implemented in the sequential testing strategy according to OECD test guideline 404 in our laboratory along with the evaluation of structure-activity-relationship (SAR) data and measurement of pH-value.

In conclusion, we have successfully established hREP models for the prediction of skin corrosion and irritation using cell viability as primary endpoint. These tests are integrated in a sequential test strategy for occupational safety assessment and replace the use of laboratory animals.

Poster

**Validation study on the battery system for prediction of phototoxicity in Japan: The overview of the results**

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The interlaboratory reliability of the battery system with the yeast growth inhibition phototoxicity assay (Yeast assay) and the red blood cell photohemolysis assay (RBC assay) developed in Shiseido Co. Ltd. was examined under the initiative of the Validation Committee of Japanese Society of Alternatives to Animal Experimentation. Six laboratories participated in the validation study for conducting experiments to evaluate the irritancy of nine chemicals, i.e. anthracene, amiodarone, chlorhexidine, chlorpromazine, bithionol, SLS, acridine, 6-methylcoumarin, and 4-t-butyl-4-methoxydibenzoylmethane. Different set of six chemicals out of nine was allocated to each laboratory and tested according to the SOP provided by Shiseido Co. Ltd. during the period from January to April, 2004. The irritancy of each chemical was evaluated whether it was positive, negative, or equivocal. All laboratories excluding one laboratory yielded consistent results, in the sense that both positive and negative judgements appeared in the same chemical. The combination of two assays yielded different results from single use of each assay. *In vivo* judgements could not always be reproduced by this battery, probably because sufficient data could not be obtained on the toxicity in *in vivo* assays. The consistency of judgement among laboratories was better when absorbance in 525 nm was used than when 540 nm was used.
The interlaboratory validation study of the battery evaluation system consisting of the yeast growth inhibition phototoxicity assay (Yeast assay) and the Red Blood Cell photohemolysis assay (RBC assay) was conducted under the charge of the Validation Committee of Japanese Society of Alternatives to Animal Experimentation. Six laboratories participated in the validation study for conducting experiments to evaluate phototoxic potential of nine chemicals, i.e. anthracene, amiodarone, chlorhexidine, chlorpromazine, bithionol, sodium lauryl sulfate, acridine, 6-methylcoumarin, and 4-t-butyl-4-methoxydibenzoylmethane. Each laboratory received six out of these nine chemicals under blinding so that each of the chemicals was evaluated in four laboratories. The experiments were finished by April with the start of January, 2004. The common SOP and work sheets were used in order to efficiently manage the experimental records. The phototoxicity of each chemical was classified into positive, negative, or equivocal by the battery system on the basis of both results of the Yeast assay and RBC assay. The intralaboratory reproducibility was good except for one laboratory. The difference in the results among laboratories was small, except for the data of amiodarone and sodium lauryl sulfate obtained from one laboratory. The results correlated with in vivo data when equivocal data were treated as positive although chlorhexidine and bithionol showed false positive. These results suggest that this battery evaluation system is effective in the prediction of the phototoxic potential of chemicals.
Ethical (animal welfare), economic (development of higher throughput testing), and scientific (development of mechanistic studies) concerns have led researchers to develop in vitro alternatives for the current in vivo rabbit eye test. NICEATM evaluated four in vitro test methods for their ability to identify substances that cause ocular corrosion or severe irritation. One of these test methods, ICE, is an organotypic model that provides short-term maintenance of the chicken eye in an isolated system. The ability of ICE to correctly identify ocular corrosives and severe irritants using available ICE and corresponding in vivo rabbit eye test data was evaluated according to current hazard classification schemes for the U.S. Environmental Protection Agency, the European Union, and the UN Globally Harmonized System of Classification and Labeling of Chemicals. Based on an interim analysis, ICE appears useful (with the exception of testing alcohols, surfactants, and solids) in a weight-of-evidence tiered testing strategy. Accordingly, positive results could be used to classify and label a substance, while substances with negative results would undergo additional testing. This approach would reduce the number of animals used for eye irritation testing and the number of animals experiencing pain and distress. A proposed standardised test method protocol and a proposed list of reference substances have been developed for use in future validation and/or testing studies to further characterise the accuracy, the reliability, and the applicability domain of ICE for the detection of ocular corrosives and severe irritants.

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NICEATM evaluated four in vitro ocular test methods (BCOP, ICE, IRE, and HET-CAM) for their ability to identify substances that cause severe irritation or corrosion. During these evaluations, a proposed list of reference substances for future optimisation and validation studies of these and other alternative test methods intended to detect ocular corrosives/severe irritants was developed. Based on the ICCVAM Submission Guidelines (ICCVAM 2003; http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm), substances included in this list are intended to: 1) represent the range of ocular responses (i.e., corrosive, severe irritant, non-severe irritant, non-corrosive) that is expected to be detected; 2) represent the classes of chemicals that are expected to be tested; 3) have produced high quality results in the Draize in vivo rabbit eye test and/or in humans; 4) have well-defined chemical composition; 5) be readily available; and 6) not be associated with excessive hazard or costs (purchase and/or disposal). Following completion of any optimisation and validation studies for each test method, reference substances from this list could be selected for inclusion in performance standards and for proficiency testing. This proposed list of substances is intended to represent the minimum number of substances that should be used to evaluate the accuracy and reliability of an in vitro ocular test method proposed for the detection of ocular corrosives and severe irritants. Testing the complete list of all reference substances will facilitate future validation efforts and comparison of performance among different test methods and protocols.

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Poster

Proposed reference substances for optimisation and validation studies with in vitro ocular test methods

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We have developed an in vitro ocular test model comprising human corneal epithelial cells in a physiologically relevant environment. Primary corneal epithelial cells were isolated from human corneas and transfected with Human Papilloma Virus (HPV) type 16 E6 and E7 genes. These cells were seeded onto microporous membrane inserts and grown to confluence under standard submerged culture conditions. The inserts were then cultured at the air-liquid interface in Corneal Epithelial Model Differentiation Medium to induce stratification and differentiation of the cells. Cell growth and culture parameters were tightly regulated to ensure normal cell behaviour and morphology. Routine quality control characterisation included Trans Epithelial Electrical Resistance (TEER), histological analysis, and time-to-toxicity (ET50) for reference compounds using the MTT cytotoxicity assay. The culture models exhibited a histological profile similar to that observed with in vivo corneal epithelium. Furthermore, the cytotoxic response of the system to known irritants shows good correlation with in vivo Draize data for several different irritant categories. Based on these results, the human corneal epithelial culture model described here presents a promising in vitro system for the assessment of ocular toxicity and irritancy.

Poster

A human corneal epithelial cell-based tissue model for assessment of ocular irritancy

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The ability of the EpiOcular™ construct to predict the eye irritation potential of surfactants has been the subject of a formal validation program. EpiOcular™ correlates a test article’s potential for ocular irritation with the time it takes to reduce tissue viability by 50% (ET50) as measured by MTT. This study investigated whether the histological changes following exposure to the surfactants are in agreement with the MTT results. Eight surfactants were selected and applied to the EpiOcular tissue using exposure times that bracketed the ET50 values established in the validation study. Upon completion of the exposure half of the tissues (2/timepoint) were set aside for histological examination while the viability of the remaining tissues was assessed. For all surfactants, the results showed a good relationship between the degree of histological damage with changes in tissue viability. An increase in the depth and severity of tissue damage was associated with a decrease in tissue viability. Histological changes ranged from subtle cellular changes such as vacuolisation and punctuate chromatin condensation to overt tissue loss and cell necrosis. Loss of or damage to the surface squamous epithelium was associated with <20% decrease in viability, while the degree of damage to the central squamous epithelium was directly related to a 20-80% decrease in viability. In conclusion, the nature and severity of the histological changes were in agreement with the MTT results. Understanding the progression and types of cellular changes associated with tissue damage may be able to help distinguish the degrees of ocular irritation.

Poster
Comparison of tissue viability and histological changes in EpiOcular™ human cell construct following exposure to surfactants

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Ethical (animal welfare), economic (development of higher throughput testing), and scientific (development of mechanistic studies) concerns have led researchers to develop in vitro alternatives for the current in vivo rabbit eye test. NICEATM evaluated four in vitro test methods for their ability to identify substances that cause ocular corrosion or severe irritation. One of these test methods, HET-CAM, was developed to mimic the mucosal eye tissues. The ability of HET-CAM to correctly identify ocular corrosives and severe irritants using available HET-CAM and corresponding in vivo rabbit eye test data was evaluated according to current hazard classification schemes for the U.S. Environmental Protection Agency, the European Union, and the UN Globally Harmonized System. Results from an interim analysis suggest that HET-CAM has a high false positive rate. However, the assay may still be useful in a weight-of-evidence tiered testing strategy, where a positive substance could be either re-tested with a modified method to confirm the result or used to classify and label a substance. Substances with negative results would undergo additional testing. This approach would reduce the number of animals used for eye irritation testing and the number of animals experiencing pain and distress. A proposed standardised test method protocol and a proposed list of reference substances have been developed for use in future validation and/or testing studies to further characterise the accuracy, the reliability, and the applicability domain of HET-CAM for the detection of ocular corrosives and severe irritants.

Poster
Performance of the Hen’s Egg Test – Chorioallantoic Membrane (HET-CAM) test method in detecting ocular corrosives and severe irritants

Neepa Choksi¹, David Allen¹, Bradley Blackard¹, Christina Inhof¹, Raymond Tice¹, James Truax¹ and William Stokes²

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The use of rabbits for the eye irritation test has been severely criticised since the end of 1970’s. Brazil is still using animals for assessing the safety of shampoos. In order to be updated with world tendency, we are studying some in vitro assays to be applied to the regulatory quality control. Since literature data do not present a cut off to establish when a product is considered irritant or not, we compared the results of Neutral Red Uptake assay (NRU) with those obtained from the routine rabbit assays.

Twenty-three shampoos and eight surfactants were studied. a) In vivo: Five rabbits were used for each product. 100 ml were instilled in one eye and the alterations were graded using the Draize scale. b) In vitro: SIRC cells were used. Products were kept in contact during 24 hours and the cells stained with Neutral Red. The stain was removed from cells and read by photocolorimetry.

The IC50 was calculated and the results compared with those from rabbits. Comparison between rabbits and SIRC presented good linear correlation (r=0.81 for shampoos and r=0.97 for surfactants). We found that products dilution that present IC50 value below 1 mg/ml were considered irritant by rabbit test, while IC50 values greater than 1 mg/ml referred to non irritant products.

Conclusion: The NRU assay may be used for classifying products. When IC50 value is lower than 1 mg/ml, it means that the product is irritant, otherwise, it should be considered as non irritant.

European legislation calls for alternatives to animal testing, especially in the area of cosmetics where animal replacement is required. Major validation and evaluation studies took place in the 90’s to replace the Draize test for eye irritation. The studies showed good reproducibility and reliability of some alternative methods, but no single method was able to replace the Draize rabbit eye test for regulatory purposes.

In order to advance towards validation of in vitro alternatives, ECVAM is working on 3 parallel fronts. First, existing data on the Draize rabbit eye test are being reviewed to gain a better understanding of its current uses and limitations. Secondly, the most promising in vitro models for identifying mild and moderate ocular irritants are being evaluated, based on an in-depth review of the existing data and on the application of the Modular Approach to Validation. These test methods comprise organotypic models, reconstituted human tissue models, cell cytotoxicity assays and cell function assays. Finally, the potential use of testing strategies that utilise the strengths of particular in vitro assay systems to address required ranges of irritation potential and/or chemical classes is being assessed. The latest progress of these on-going evaluations will be presented with focus for test methods for mild and moderate ocular irritants.
**Poster**

**Tissue engineering: HET-CAM test evaluation at day 10**

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Aim: The HET-CAM (Hen Egg Test – Chorioallantoic Membrane) angiogenesis test system, originally conceived as alternative method for toxicity and irritation studies, has for some time been suggested for tissue engineering tasks, biocompatibility testings and also as cell transplantation model. Published works show that in most of these cases incubation of the eggs was performed for up to 15 days. This time-point is way past neural tube fusion at ~day 11, resulting in possibility of embryo pain conduction. Therefore rating as actual animal testing has been discussed previously. Aim of the work was to show the feasibility of altering existing CAM-test protocols by cancelling experiments at incubation day 10 for reasons of achieving satisfactory data quality while following animal welfare considerations.

Methods: Example of published testing schemes of biomaterial testing and hetero/autologous cell transplantation using the CAM angiogenesis onset were performed but truncated at day 10. Histological/elektronmicroscopical analysis was conducted. Results: The authors believe resulting data matched that from original experiments in means of quality and reproducibility sufficiently, proofing the suitability of the altered time-schedule. Conclusion(s): The CAM-angiogenesis-assay can be used also for innovative experiments in the fields of biomedical engineering, even with a total duration of experiments of just 10 days. Thereby its quality as alternative method to animal testing remains intact without undue minor quality of results.

**Poster**

**Comparative evaluation of benzalkonium chloride on in vitro rabbit and human corneas**

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Purpose: To develop an in vitro technique for comparison of corneal toxicity of surfactants in isolated rabbit and human corneas.

Methods: Rabbit eyes were obtained from Pel-Freeze and human corneas, stored in Optisol GS™, were obtained from the Georgia Eye Bank. Corneas were isolated and mounted for endothelial perfusion in an in vitro specular microscope. Benzalkonium Chloride (BAC) was applied to corneas with and without an intact epithelium during which time the corneal endothelium was continuously perfused with a balanced salt solution. The effects of 0.005%, 0.01%, 0.1% and 1% BAC on the rabbit corneal tissue were evaluated after 3, 9 and 18 min exposures and on the human corneal tissue after 3 min. Following exposure, BAC was removed and the corneas were perfused with the balanced salt solution, the corneal thickness was measured half hourly for 3 hours. The corneas were fixed for histological evaluation by light and electron microscopy.

Results: In the rabbit cornea, BAC showed time and concentration dependent corneal swelling and structural changes with associated damage to the endothelial layer. Human corneas exposed to the BAC for 3 min showed the similar corneal alterations as observed in the rabbit corneas.

Conclusion: This study demonstrated that this technique can use commercially available rabbit corneas to examine early BAC-related changes in histopathology. Furthermore, the pattern of damage seen in the human cornea was similar to that of the rabbit.
ICCVAM is evaluating four in vitro assays for their ability to detect ocular corrosives/severe irritants in a weight-of-evidence tiered testing strategy. Ideally, this analysis would evaluate the ability of alternative assays and the Draize eye test to each correctly predict human ocular toxicity. However, the lack of appropriate human data only allows for a determination of how well alternative assays predict the rabbit response. In assessing the performance of alternative assays, information on the Draize eye test reliability would be useful but the paucity of repeat test data precludes an accurate estimate of inter- and intra-laboratory reproducibility. However, Draize eye test results can be used to estimate the likelihood of under-classifying a positive substance or over-classifying a negative substance using the current 1-3 rabbit sequential test. Data from Draize eye testing using 3-6 animals was obtained for ~900 substances from U.S. Federal regulatory agencies, published studies, and scientists and organisations. Ocular irritation categories were assigned based on the 2003 UN Globally Harmonized System of Classification and Labeling of Chemicals. Assuming either a homogeneous or a heterogeneous response among rabbits within a classification category, the distribution of individual rabbit responses was used to estimate the likelihood of under- and over-classification for a 1-3 rabbit sequential testing strategy. The estimated under-classification rate for corrosives/severe irritants that induced any severe response was <10%; rates were higher when this classification was based on only lesion persistence at day 21. Estimated over-classification rates will also be presented.

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Poster

**Estimated under- and over-classification rates for a 1-3 rabbit sequential Draize rabbit eye test**

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Relatively few alternative studies have been conducted with products of interest to the petrochemical industry. Therefore, an in-house program was implemented to develop/validate new alternative in vitro test methodologies for the prediction of ocular irritation and acute systemic toxicity of petrochemical products.

For the eye irritation evaluation, the Bovine Corneal Opacity and Permeability (BCOP) assay was used to differentiate 16 petrochemical products (e.g. lubricant additive packages, base stocks, cutting fluids, solvents). When compared to the 10-minute exposure results, the BCOP assay correctly classified the 14 out of 16 products and produced 2 false negatives. For the assessment of potential acute systemic toxicity, the same 16 test articles were examined in the 3T3 Neutral Red Uptake (NRU) bioassay. The low solubility of the petrochemical products resulting in the inability of cells to be exposed to concentrations below desired concentrations was reflected in a poor predictability of the 3T3 NRU assay (less than 60%). Despite the poor predictability of the 3T3 NRU assay (vs. in vivo data), results did indicate its potential if the procedure is modified to suit this class of chemicals. These results suggested a good potential of the in vitro BCOP assay in predicting eye irritation for certain petrochemicals: it may be a screening tool before in vivo testing (refinement) or an alternative stand alone in vitro assay (replacement) for assessing hazard. Ultimately, the validation and acceptance of alternative testing methodologies will benefit animal welfare through the reduction, replacement, and more-humane use of laboratory animals.

Poster

**Validation of the BCOP assay for the evaluation of ocular irritation of various petrochemical products**

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Lecture

An industry perspective – needs, strategies and development programmes for ocular irritancy

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The standard regulatory approved test to evaluate eye irritation is the Draize test. Success in fully replacing it with in vitro methods has not occurred. It has been concluded (COLIPA Workshop 1997, ECV AM Workshop, 1998) that reasons for this are multiple and include lack of understanding for the physiological mechanisms of eye irritation, variability of in vivo Draize test data and ability of the Draize test to reliably predict the human response.

Though not formally validated, some of the currently available in vitro/ex vivo methods are used in industry by raw material suppliers and cosmetic companies for in-house routine screening purposes in the development of new products or in integrated testing strategies. Although reduction/refinement methods/approaches are available today, there remains a clearly identified need to define in vitro methods that reliably predict the human eye response to chemicals exposure and can replace the in vivo test. As such, a fundamental understanding of what is needed to fill the knowledge gaps is essential to continued progress. Certainly, the key focus area for current/future research that emerged is the need for mechanistic understanding of eye injury resulting from chemical exposure.

This presentation provides an overview of: 1) why eye irritation evaluation is needed; 2) current industry use of in vitro assays; 3) previous development/validation efforts and what was lacking; 4) what is new today (e.g. new approaches, technology advances, changes in regulatory environment e.g. 7th Amendment, REACH) and 5) current industry efforts/development programmes/collaborations that will aid success.

Lecture

ICCVAM progress in evaluating in vitro test methods for identifying severe ocular irritants/corrosives

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ICCVAM, with the assistance of NICEATM and in response to a nomination by the US Environmental Protection Agency, recently initiated a review of the validation status of four in vitro ocular toxicity test methods with potential for use in screening chemicals for severe eye irritation or corrosion. The four test methods are the Bovine Corneal Opacity and Permeability (BCOP) assay, the Hen’s Egg Test – Chorioallantoic Membrane (HET-CAM) assay, the Isolated Chicken Eye (ICE) assay, and the Isolated Rabbit Eye (IRE) assay. NICEATM compiled available data and information for the four methods and prepared a Background Review Document (BRD) for each. An expert panel was then convened to evaluate the usefulness and limitations of the in vitro tests based on information in the BRDs. The conclusion of the panel was that each of the four test methods could be used in a tiered testing strategy to identify severe eye irritants and corrosives, with specific limitations and caveats. Several modifications to optimise the standardised protocols were proposed by the panel and it was also recommended that additional data be requested from test method users. Subsequently, additional data received in response to a Federal Register Notice were included in accuracy and reliability analyses for the ICE, IRE and HET-CAM assays. The panel also recommended the inclusion of known human severe eye irritants as reference chemicals. Collaborative interactions between ICCVAM, NICEATM, and ECVAM to review the validation status of other available methods for assessing ocular corrosion or irritancy will also be discussed.
Introduction: In recent years, various approaches for assessing eye irritation potential by using in vitro test systems have been developed for replacing the Draize rabbit eye irritation test. The purpose of this study was to compare the HET-CAM and RBC tests for predicting the ocular irritancy of hair-care products, with a view to determine which alternative method may represent a suitable alternative to the in vivo assay.

Methods: Twenty-two shampoos and eight conditioner formulations were selected for this study which covered a range of irritant categories (non-irritant/mild/moderate/severe). The Red Blood Cell assay was a modification of that described by Pape et al. (1987) and HET-CAM procedure was as described by Luepke (1985).

Results: Results of each assay were compared with MAS of the Draize test. The frequency of agreement of the HET-CAM test and MAS was 91% sensitivity, 86% specificity and a precision value of 90%. The performance of the RBC assay presented 87% sensitivity, 100% specificity and 90% precision. The rank correlation coefficients for HET-CAM and RBC assays in relation to Draize test were 0.799 and 0.814 respectively.

Discussion: Observations of the performance of the HET-CAM test for the assessment of ocular irritancy of shampoos presented an over-prediction of in vivo effects and appears less reliable to identify mild irritant. In contrast, the Red Blood Cell presented an under-prediction of the ocular irritancy, but was able to identify mild irritants. These results indicate that both assays should be used together in order to assess the eye irritancy.

Poster
Evaluation of two alternative methods for assessing the ocular irritancy of hair-care products

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Poster
Optimisation of an in vitro long term corneal culture assay

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The long-term culture of corneas has been proposed as an in vitro model to evaluate potential eye irritation and post-treatment recovery following chemical exposures. Porcine eyes were obtained within 24 hours of sacrifice and disinfected prior to excising corneas. Corneas were filled with an agar/gelatin gel in M199 medium to support the corneas, and were cultured at 37°C, 5% CO₂, 90% RH in M199 medium to the limbus. The corneas were moistened by brief immersion in medium every 2.7 hours using a modified plate rocker. Corneas were treated with either SLS, EtOH, or H₂O (controls). The corneas were rinsed with PBS, cultured for a pre-determined post exposure time, and fixed in buffered formalin. H&E-stained control corneas showed normal morphology after 4 days, similar to excised/immediately fixed corneas. Controls were characterised by an intact epithelium with viable squamous, wing, and basal cells. The stroma showed minimal swelling with frequent viable keratocytes. The endothelium was typically intact. Some stromal swelling near the sclera was noted after 5 to 7 days. Corneas treated with 3% SLS or EtOH showed complete epithelial cell damage or loss 24 hours after treatment, as well as loss of viable keratocytes in the upper stroma. After 48 hours, epithelial cell sheet migration was observed into the damaged zone. After 120 hours, the regeneration of a stratified epithelium was observed. These results confirm the ability to culture porcine corneas for at least 120 hours, as well as demonstrate the potential for further optimisation of evaluating recovery of damaged corneas.
Poster

**In vitro methods for assessing ocular irritancy of cosmetics**

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Varieties of *in vitro* test protocols serve as replacement for the Draize test to assess ocular irritancy. Five protocols (BCOP, HET/CAM, ICE, CEET and IRE) are validated and accepted by legal authorities. For the EpiOcular assay validation is under its way. Additionally, the RBC test is a widespread method to assess the irritating potential of surfactants.

We compared the feasibility of common *in vitro* methods to assess ocular irritancy of cosmetics. Also, the RBC assay is often used; it is only suitable for anionic surfactants and susceptible to interfering elements from the test material. For cell culture tests a high enough solubility of test materials in the watery test system is a basic prerequisite. Therefore, testing of ready formulations is limited. The HET/CAM test proved to be practicable for cosmetics and shows good correlation with the recognition of highly irritant and non-irritant substances. Nevertheless, it is based on a subjective rating and thus missing an objective endpoint. Similarly, the EpiOcular assay is suitable for various test products, but has the advantage that test materials can be analysed directly on human tissues. It proved to be a valuable tool in the routine analysis of formulations and single substances. Simultaneously with measuring tissue viability additionally parameters e.g. LDH, PGE-2 and IL-1α can be quantified to obtain further information about the irritation potential and possible inflammatory processes.

In conclusion, the HET/CAM test is a cost-effective alternative whereas testing on human tissues equivalents enables an objective distinctive classification for assessing ocular irritancy of cosmetics.

Lecture

**Use of in vitro data for classifying eye irritating chemicals in the EU – experience at the BfR**

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ZEBET (National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments) and Dep. of Chemical Safety at the BfR (Federal Institute for Risk Assessment), Berlin, Germany

The New REACH Chemicals Policy of the EU suggests to replace animal tests for the assessment of acute human health hazards by using both information based on (Q)SARs and *in vitro* test results to reduce costs and duration of testing and the number of test animals employed.

At the BfR the authors have compiled a regulatory database using data on chemicals with a purity >95%, which were submitted within the current notification procedure for New Chemicals of the EU. We have used this database to develop (Q)SAR rules for the prediction of acute local lesions on eye and skin within the new REACH system of the EU.

From these data, (Q)SARs for the prediction of local irritation/corrosion were developed and published. These (Q)SARs and an expert system supporting their use was submitted for official validation and application within regulatory hazard assessment strategies to European Chemicals Bureau ECB.

Main features of the BfR database are: a decision support system (DSS) for the prediction of skin and/or eye lesion potential built from information extracted from our database. This DSS combines SARs defining reactive chemical substructures relevant for local lesions to be classified, and (Q)SARs for the prediction of the absence of such a potential.

The impact of the use of (Q)SARs and physico-chemical data on current EU testing strategies for eye irritation testing of chemicals will be discussed.
Poster

Safety assessment of ocular contact lens solutions

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Introduction: Today there are millions of contact lens wearers. One out of every 20 contact lens wearers develops a lens-related complication each year.

Methods: The safety of ocular contact lens solution MULTISON (M) (Latvia) has been tested on a monolayer culture of NIH 3T3 cells (ECACC), i.e. embryonic murine fibroblasts. Solution M, the solution in which the lenses are stored (K- (USA)), and the positive control 50 mg/ml phenol (K+) were used.

The testing was performed according to the Guidance Document from ICCVAM and NICEATM. The irritation effect of M was also tested on 3 albino rabbits (EN ISO 10993-10). The cornea, area of cornea involved, iris, conjunctiva and chemosis discharge were assessed.

Results and discussion: Solution M displayed a slight toxicity with an IC\textsubscript{50} of 37 µl. When solution M was diluted 20-fold, it lost its toxic effect. Solution M (50.0 and 15.8 µl) caused the death of cells, but did not change their morphology. The toxicity of M corresponds to its disinfectant feature. The solution’s leftovers on the lenses cannot do any harm to the eye, as the solution is naturally diluted in the eye fluid. These results were confirmed with the rabbit experiment – during the experiment there was no state of lacrimation, photophobia, swelling of lids, sap and pain in the eyes, opacity of cornea, or other inflammatory signs of the eyes or the conjunctiva.

Solution K- was not toxic. The IC\textsubscript{50} for K+ was 0.6 mg/ml and K+ causes the death of cells and changes their morphology.

Lecture

Minimising pain and distress in ocular safety testing: Current best practices and research needs

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Rabbit eye testing has been used effectively for over 60 years to safeguard human health by identifying and classifying chemicals and products that have the potential to cause temporary or permanent eye damage. However, pain and distress may occur in such testing from the initial application of the test substance and from subsequent chemically-induced damage to ocular tissues. Current test guidelines attempt to avoid and minimise animal pain and distress by allowing ocular hazard decisions to be made in some situations without the use of animals, and requiring only 1-3 animals in most situations where animals must be used. Current guidelines also seek to minimise pain and distress by allowing the use of pre-application treatment with topical anaesthetics. However, regulatory guidelines state that such agents should not interfere with the outcome of the study. The lack of information about potential interference has precluded the routine use of pre- and post-application analgesics and topical anaesthetics. Current guidelines also seek to reduce the duration of pain and distress by allowing for humane euthanasia of animals that develop severe ocular lesions or that exhibit severe and enduring signs of pain and distress. If identified, predictive biomarkers could serve as humane endpoints for terminating studies in order to avoid potential pain and distress. Additional research is needed to support the identification and use of humane endpoints, analgesics, and topical anaesthetics that will further minimise or eliminate pain and distress in routine ocular toxicity testing. Related recommendations from a recent ICCVAM-NICEATM-ECVAM symposium will be discussed.
Ethical (animal welfare), economic (development of higher throughput testing), and scientific (development of mechanistic studies) concerns have led researchers to develop in vitro alternatives for the current in vivo rabbit eye test. NICEATM evaluated four in vitro test methods for their ability to identify substances that cause ocular corrosion or severe irritation. One of these test methods, BCOP, is an organotypic model that provides short-term maintenance of the cornea in an isolated system. The ability of BCOP to correctly and reproducibly identify ocular corrosives/severe irritants using available BCOP and corresponding in vivo eye irritation data was evaluated according to current hazard classification schemes for the U.S. Environmental Protection Agency, the European Union, and the UN Globally Harmonized System of Classification and Labeling of Chemicals. Based on an interim analysis, BCOP appears useful (with the exception of testing alcohols, ketones, and solids) in a weight-of-evidence tiered testing strategy. Accordingly, positive results could be used to classify and label a substance, while substances with negative results would undergo additional testing. This approach would reduce the number of animals used for eye irritation testing and the number of animals experiencing pain and distress. A proposed standardised test method protocol and a proposed list of reference substances have been developed for use in future validation and/or testing studies to further characterise the accuracy, the reliability, and the applicability domain of BCOP for the detection of ocular corrosives and severe irritants.

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Recently, the in vitro Bovine Corneal Opacity and Permeability (BCOP) assay was considered acceptable by the EU National Regulatory Authorities in order to identify and label severe eye irritants. In addition, the BCOP assay was part of ICCVAM’s evaluation on the current status of in vitro test methods for detecting ocular corrosives and severe irritants.

Although bovine eyes are widely used in ocular irritancy testing, limited studies are available that address potential sources of variability. For example, in the present literature no detail is provided on the specific age of cattle used as the source of the bovine eyes. Consequently, it is recommended to perform additional studies in order to specify an age range for donor animals.

In order to evaluate the impact of age on the performance of the BCOP test, this study compared the experimental outcome when 20 chemicals were tested both on corneas from young animals (6-8 months) and adult animals (>24 months). Corneas were treated for 10 minutes followed by a 2-hour post-exposure period. Opacity and permeability were determined and the calculated in vitro scores for both cornea types were compared with the in vivo (EU and GHS) classification.

Results clearly showed that age can impact the outcome of the assay. Although no important differences in opacity were observed, permeability values assessed in calf corneas were clearly decreased (especially for alcohols) when compared with adult corneas. The possible advantages related to the use of corneas from young animals will be discussed.
THEME 5, SESSION 5.5

**Poster**

**The concepts of systems biology to validate an in vitro ocular test battery: Why new Draize eye data is not relevant**

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Validation has been defined as “the process of determining how well one system replicates properties of some other system.” However, a predictive model can only be validated by judgement, since a model may fit past data without being predictive. The use of judgement is the critical criterion missing from current validation guidelines. Historical experience with the Draize test has shown human eye hazard can be estimated by clinical signs of chemical injury to the rabbit eye. The large variability in historical Draize data confirms the futility in using these data in rigorous in vitro/in vivo comparisons. Even when new animal testing has been conducted, correlative results have not fit well within 95% confidence intervals – a stringent comparator for biological assays. Ophthalmic experts have agreed that: 1) human data should be the gold standard, and 2) ocular chemical injuries can almost exclusively be evaluated as corneal responses with the corneal epithelium being the first tissue injured, and the overall degree of epithelial/corneal injury correlating well with severity and recovery. The identification of the appropriate biologically-relevant in vitro models, and a battery of mechanistically-based endpoints in each model that accurately assesses the degree of injury from different chemical and product classes will provide predictive data for human hazard assessment. This “perfect battery” will not pass the scrutiny of current validation criteria without the concurrent use of “judgement.” The concepts of systems biology will be used to explain how to identify and validate this test battery.

**Poster**

**Evaluation of the effects on viability and barrier function prolonged surfactant exposure has on a human corneal epithelial cell line**

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In this study we aim to generate an in vitro model to investigate chronic corneal damage. Initially, the viability and barrier function of SV40 human corneal epithelial cells (donated by Dr Araki-Sasaki, Japan, J-HCET) were monitored during prolonged exposure to subcytotoxic concentrations of representative surfactants.

J-HCET were grown to confluency (n=4) on 24 well plate culture inserts in a defined media containing subcytotoxic concentrations of tween 20 (T20: 25 µg/ml-1), sodium dodecyl sulphate (SDS: 4 µg/ml-1), benzalkonium chloride (BAK: 0.0025 µg/ml-1) or cocamidopropylbetaine (CAPB: 3 µg/ml-1). Cell viability and barrier function was assayed repeatedly from prior to chronic exposure and subsequently at 72 hour intervals over 504 hours, using the combined resazurin/fluorescein leakage assay. Chronic exposure effects were determined and compared statistically (repeat measures ANOVA) with non-chronically exposed cultures. Total cell number was assessed by the Kenacid Blue protein assay. The location of adhesion molecules Zonula Occludins 1 (ZO-1) and E-Cadherin were assessed using immunostaining.

At all time-points the level of fluorescein leakage across the J-HCET monolayer, the production of resorufin (µgm-1) and the total protein content (µg/ml-1) were unaltered in surfactant exposed cultures compared to the unexposed control. ZO-1 localisation was disrupted in BAK pre-exposed cultures, whilst E-Cadherin expression remained unaltered during exposure to all test surfactants. Modulation of J-HCET viability and barrier function was not detected compared to control cultures. Assessment of ZO-1 and E-cadherin expression indicted surfactant specific effects. This work was funded by the sponsors of the FRAME research programme.
Poster

A tissue engineered human corneal model for the prediction of ocular irritation

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Introduction: Over the last 60 years the Draize rabbit eye test has been used in regulatory safety testing. Apart from ethical considerations, this test has often been criticised for its lack of objectivity, reproducibility and for over-predicting human responses. Despite intensive efforts to replace the Draize test by alternative methods, acute local eye irritation is still tested on animals, because existing non-animal methods are considered to be only suitable for the prediction of severe lesions. Since organotypic models are supposed to give rise to several advantages, it was the aim of our study to develop a cornea equivalent model composed of immortalised cell types derived from the natural human tissue.

Methods: Human corneal keratocytes were immortalised via SV40-transfection. The cytotoxic response towards different surfactants was measured by MTT test. Cornea equivalents were constructed in cell culture inserts using immortalised human epithelial and endothelial cells and keratocytes embedded in collagen. For morphological estimation sections were stained and analysed by light microscopy.

Results and discussion: We established a new human keratocyte cell line with cytotoxic sensitivities towards different surfactants comparable to primary keratocytes. Therefore, the new cell line represents an appropriate model for the prediction of keratocyte-specific toxicity. A stromal matrix, built up with these cells, displayed morphological accordance to the natural human stroma and serves as a biomatrix for corneal epithelial and endothelial cells. Consequently, we report a case of construction of a whole corneal equivalent from immortalised cells. Application of this model may be useful in regulatory eye irritation testing.
The occurrence of asthma and Chronic Obstructive Pulmonary Disease (COPD) in industrialised countries has been increasing for the past 30 years. Local respiratory tolerance is a critical issue since lung irritation is a common acute side effect for inhaled drug. An in vitro airway epithelium model was implemented to determine irritancy potential of new chemical entities when given by the inhalation route to humans.

The Calu-3 model consists of human bronchial epithelial cells of the airway tissue of the respiratory tract. Calu-3 cells when placed at the air-liquid interface form a 3-dimensional epithelium model, that develop tight junctions and functional barrier properties. It also induces ciliogenesis and produces mucus in our culture conditions.

To determine the relevance and the reliability of this in vitro model, 8 chemicals intended for the treatment of COPD/asthma were tested in the Calu-3 model. A multiple endpoint analysis approach was used by measuring cell viability (MTT), inflammatory responses (interleukines and chemokines), tight junction disruption (trans-epithelial resistance) and production of mucin secretion on the apical side of the model. In vitro and in vivo animal data correlated suggesting that the Calu-3 model could be used to address lung irritancy potential of new chemical entities.
Poster

**Relationship between total antioxidant activity of plasma and parameters related to oxidative stress induced by hepatotoxic drugs in rats**

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Introduction: Antioxidant defense system encompasses the enzymatic and non-enzymatic factors some of which are often measured in tissues. Measurement of all these parameters is not feasible. Total Antioxidant Capacity (TAC) of plasma is a single assay that represents the balance between pro- and antioxidants factors. In this study the reliability of TAC of plasma as an index of oxidative stress was assessed in relation to formation of lipid peroxidation and changes in individual antioxidants.

Methods: Rats were treated with different doses of acetaminophen or menadione, blood was collected and ferric reducing ability of plasma (FRAP) was determined as a measure of TAC. The rate of lipid peroxidation products were measured in plasma. The relationship between FRAP and antioxidants such as blood glutathione, plasma bilirubin, plasma uric acid and total protein together with catalase and superoxide dismutase (SOD) activities in erythrocytes were assessed.

Results: FRAP was markedly increased (5-6 fold) in rats following administration of a single i.p dose of APAP to rats. Elevation of FRAP was observed to be highest, 4-12 h after APAP injection. FRAP was increased depending on APAP dose given. Elevation in FRAP was inversely related to the rate of lipid peroxidation in liver. Interestingly, in growing rats among the enzymatic and non-enzymatic factors measured, plasma bilirubin and erythrocyte’s superoxide dismutase (SOD) were correlated with changes in FRAP.

Discussion: FRAP is a simple and reliable assay for assessment of whole body antioxidant capacity. FRAP changes due to hepatotoxins is correlated with certain antioxidant factors namely bilirubin and SOD.

Poster

**Novel in vitro exposure techniques for toxicity testing and biomonitoring of airborne contaminants**

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Exposure to air toxicants is a major contributor to human health problems. The aim of this study was to develop practical and reproducible *in vitro* techniques for assessing the toxicity of airborne contaminants. Two methods were developed based on the physiochemical properties of test chemicals: static and dynamic direct exposure techniques at the air/liquid interface. Xylene, Toluene and Nitrogen dioxide were chosen as a model test compounds. Human cells including A549 (lung derived), HepG2 (liver derived) and skin fibroblasts were grown in porous membranes. For the static method, test atmospheres of volatile organic solvents were generated in glass chambers (322 ml) and cells were exposed to airborne concentrations for 1 hour at 37°C. For the dynamic method, cells on membranes were placed in horizontal diffusion chambers and exposed to dynamic flow (25 ml/m) of test gas for 1 hour at 37°C. Cytotoxicity was investigated using the MTS (tetrazolium salt; Promega), NRU (neutral red uptake; Sigma) and ATP (adenosine three phosphate, Promega) assays. Xylene (e.g. IC₅₀ = 5,350 ± 328 ppm, NRU; IC₅₀ = 5,750 ± 433 ppm, MTS in fibroblasts) was found to be more toxic than Toluene (e.g. IC₅₀ = 10,500 ± 527 ppm, NRU; IC₅₀ = 11,200 ± 1044 ppm, MTS in fibroblasts) in all cells tested. Dose dependant effects of NO₂ were observed in human cells tested. Our findings suggest that the static direct exposure is a practical technique for assessing the toxicity of volatile compounds. Further, dynamic direct exposure offers the potential for respiratory toxicity studies and as an advanced technology for biomonitoring of airborne contaminants.
Poster
The third FRAME Toxicity Committee: Alternatives in toxicity testing

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Substantial progress has been made since the first FRAME Toxicity Committee was established in 1979, particularly with regards the successful validation and regulatory acceptance of non-animal replacement methods. A third FRAME Toxicity Committee (FTC) was established in 1999 and comprised 18 experts from industry, academia, the legislative, regulatory bodies and animal welfare. The primary objective of the FTC is to review and make recommendations about the use of Three Rs approaches in the research, development and safety evaluation of medicines, biological products, cosmetics and chemicals. The FTC has more recently been restructured in the form of a Standing Committee and a smaller Steering Group which together will guide the activities of five working parties. The first of these working parties specifically addresses issues relating to risk assessment and, later this year, will hold a focused scientific workshop entitled ‘Toward a better way to assess risk of toxic exposure’. The workshop will address, among other issues, how the risk assessment process can be improved to cope with the demands of legislation such as the REACH system. A separate working party is looking at the practicalities of data-sharing with particular reference to the REACH system and assessing the reliability of read-across within (Q)SAR-driven intelligent testing strategies. Two further working parties are addressing more specific areas namely, carcinogenicity testing and endocrine disruption, whilst a final working party is more directly involved in organising workshops. This presentation summarises the main activities and recommendations of the third FTC.

Lecture
The role of biokinetic information in the interpretation of in vitro cytotoxicity data: An essential part of estimating acute toxicity in the ACuteTox programme

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In previous programmes, the feasibility of the use of in vitro cytotoxicity data for the prediction of in vivo lethal doses was tested. In these studies it was shown that basal cytotoxicity gave good estimates for about 70% of the compounds, i.e. these chemicals could be classified in the appropriate LD_{50} classes.

An important drawback of the use of cytotoxicity data is the difficulty of extrapolating a toxic concentration in the in vitro system to a toxic dose in the in vivo situation. Deviations from a simple linear relationship between effective concentrations in vitro and toxic doses in vivo can be the result of the fact that the effective concentrations in vitro are irrelevant for the concentrations that may cause toxicity in target organs in vivo. These deviations may be caused by the processes in the biokinetics of the compound under study. For instance, the absorption of the compound may be minimal, thus leading to low systemic concentrations. Moreover, the processes of distribution, metabolism and elimination may lead to lower or higher concentrations in target organs than could be expected from an even distribution of the compound over the body.

Efficient tools to estimate the biokinetic processes can be found in biokinetic modelling. Therefore, one of the work packages in the ACuteTox programme is focusing on biokinetic processes: in vitro determination of metabolism and transport and biokinetic modelling.

In conclusion: the incorporation of biokinetic information will highly improve the possibilities of estimating the in vivo toxic dose on the basis of in vitro basal cytotoxicity data.
Several studies have shown that in vitro cell systems can predict acute toxic effects in vivo. NICEATM and ECVAM recently conducted a multi-laboratory validation study to assess the predictive capacity of two in vitro basal cytotoxicity assays primarily for predicting rodent, but also human acute toxicity. Seventy-two coded chemicals were tested in mouse 3T3 fibroblasts and normal Human Epidermal Keratinocytes (NHK) using the Neutral Red Uptake (NRU) assay. Forty-one chemicals used in the study are MEMO chemicals, i.e., chemicals for which relevant human toxicity data exists. The collection of human toxicity data for four other chemicals, for which these data were lacking, was commissioned by ECVAM. A preliminary analysis conducted with twelve chemicals showed a good correlation for both cell types (3T3 cells: R²=0.787, NHK: R²=0.886) between in vitro IC₅₀ values (i.e., the concentration of a chemical that inhibits cell growth by 50%) and peak serum concentration in humans derived from time-related lethal and sub-lethal concentrations curves. However, the in vitro IC₅₀ values for cadmium chloride and ethylene glycol under- and over-predicted human toxicity by more than 10-fold respectively. The evaluation of the correlation between human data and the IC₅₀ values for the complete set of chemicals is ongoing.

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**Poster**

**High-resolution respirometry – an important tool to evaluate energy metabolism of isolated hepatocytes**

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Mitochondrial system of energy provision plays important role in many basic biological functions, including cell death and cell proliferation. It is also a target of many toxic substances. Therefore detailed information about energy metabolism is essential to elucidate mechanism of toxic action of xenobiotics and to evaluate stimulatory or inhibitory effect of various substrates. Recently new method to assess energy status of mitochondria is available.

High-resolution respirometry (Oxygraph 2K, OROBOROS, Austria) is very sensitive electroanalytical method, which allows measuring oxygen consumption in isolated cells or mitochondria. Specific substrates and inhibitors of respiratory chain enzymes and ATP formation enable to evaluate function of individual complexes of oxidative phosphorylation. In this study we compared respiratory rate of intact and digitonin-permeabilised hepatocytes. Permeabilisation allows better accessibility of energy substrates for mitochondria and offers conditions more close to the situation in vivo. Hepatocytes were isolated from male albino Wistar rats (220-250 g) by collagenase perfusion of the liver. Oxygen consumption was measured in suspension of intact hepatocytes incubated in Krebs-Henseleit medium, digitonin-permeabilised hepatocytes were incubated in potassium-medium.

Our results indicate that succinate-dependent respiration is twofold increased after the addition of ADP (p<0.001) and there was a fivefold increase of glutamate+malate-dependent respiration in permeabilised hepatocytes (p<0.001). These findings are additional evidence that mitochondria in permeabilised hepatocytes are tightly coupled.

High-resolution respirometry enables monitoring of oxygen consumption in small amounts of biological material (pmolO₂/s/10⁶ cells) and is thus suitable method for evaluation of energy metabolism.

This work was supported by grant MSM 0021620820.

**Poster**

**Use of alternatives to animal methods in risk prognostication for hazardous effects of cosmetics preservatives**

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Depending on their chemical structure, preservatives have been shown to increase or decrease barrier function of cell membrane and stratum corneum lamella. Alternatives to animal methods were used to investigate the effects of cosmetics preservatives (Benzoic acid, Phenoxyethanol, Triclosan, Kathon CG, Dimol, maximum authorised concentrations and lowest concentrations) on the membrane permeability and barrier function of epidermis using a modification of the BRC-test and a biphasic water-lipid model of the epidermis. Membrane permeability in the BRC-test was evaluated by measurement of the leakage of haemoglobin. The barrier function of the biphasic water-lipid model was evaluated by TEWL measurement.

Triclosan, Benzoic Acid and Phenoxyethanol caused a concentration-dependent increase of membrane and stratum corneum lamella permeability. Dimol and Kathon CG increased the cells’ resistance to cold-induced stress (t=4°C). Dimol and Kathon CG can be used in cold protective cosmetic products. In the BRC-test Benzoic Acid, Phenoxyethanol and Triclosan caused concentration-dependent membrane alterations when applied under normal conditions of use (maximum authorised concentration, t=37°C). Exposure of erythrocytes to Benzoic Acid (0.1%) and Triclosan (0.01and 0.3%) induced more than 13-fold (Benzoic Acid) and 3-fold and 120-fold (Triclosan) higher levels of erythrolysis than solvent control. Phenoxyethanol (0.5%) caused a 2-fold increase of the membrane permeability. Treatment of erythrocytes with Phenoxyethanol (0.25%) did not elicit any alteration of membrane integrity. Topical application of Benzoic Acid and Phenoxyethanol on the lipid layer of the water-lipid model caused an increase of the level TEWL by 29 and 40%, respectively. Benzoic Acid and Phenoxyethanol increased the cold-dependent changes in membrane permeability.
**Lecture**

**AcuteTox – Optimisation and pre-validation of an *in vitro* test strategy for predicting human acute toxicity**

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Validated alternative test methods are urgently required for safety toxicology testing of drugs, chemicals and cosmetics. ACuteTox is a 5-years integrated project under the EU 6FP with the aim to develop a simple and robust *in vitro* testing strategy for prediction of human acute systemic toxicity, which could replace the animal acute toxicity tests used today for regulatory purposes.

The extensive amount of work performed since the 70s has led to a great number of existing *in vitro* models. Many studies have shown good correlation (about 70%) between *in vitro* basal cytotoxicity data and *in vivo* LD₅₀ values or human lethal blood concentrations. However, this correlation means that a certain number of misclassifications have to be faced when using the existing tests. ACuteTox aims to improvement of this correlation to a level sufficient enough to ensure a valid prediction of acute toxicity.

This will be performed by evaluating the existing outliers of the correlation in order to introduce further parameters, such as ADE, metabolism and organ specificity, which might improve the correlation. This would allow the integration of alerts and correctors in the prediction algorithm, which together with robust implementation of medium throughput approaches, would allow the establishment of a new testing strategy with a better prediction of classification. In summary, A-Cute-Tox aims to improve the prediction of acute toxicity using *in vitro* methods and, at the same time, to signal, which compounds require further testing because their acute toxicity can not be properly predicted.

**Poster**

**Toxicity of environmental pollutants on GM-CFU: Comparison between species and genders**

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*In vitro* haematotoxicology provides the opportunity to study the effects of toxicants directly on relevant human target tissues. Exposure to environmental pollutants as inorganic Arsenic, Atrazine and Naphthalene in drinking water has emerged as a public health concern since they can be easily transferred to the fetus.

Umbilical cord blood is part of the fetal tissues, and it is possible to estimate the effects of chemicals that can affect the future development of fetus by analysing the main feature of these cells, which is their capability to clone.

In this study we evaluated the effect of Arsenic, Atrazine and Naphthalene on the clonogenic capability of blood progenitors (cord blood cells and bone marrow) belonging to different species (human and murine), different genders (male and female) and through different schedules of treatment.

Our data indicated that Arsenic has a relevant toxic effect, at the same level both in human and in mouse, without any difference between the genders (IC₅₀=0.81 uM in female mice and 0.80 uM in male mice; IC₅₀=0.87 in human). Atrazine treatment affects very poorly only at the maximal dose tested (50 uM) the clonogenic capability of both mouse bone marrow (84% male, 91% female) and human cord blood (85%). Naphthalene treatment relieved a different sensitivity in the two species, being human CBC totally unaffected by the treatment, while the murine bone marrow clonogenicity is reduced of 40%, in both the male and female gender.
Novel approaches to assessing the toxicity of chemicals or formulations often follow a tortuous path from initial reflections on scientific plausibility to the dream of regulatory acceptance. Currently, many barriers (both real and perceived) to industry’s full implementation of a new toxicity method are in this path. Examples include: 1) Liability concerns – Will courts find the new method to be “state-of-the-art” in providing assurance of safety? 2) Cost concerns – Will the new method take longer to perform, be more expensive, or might submission of data from the method to a regulatory agency not prepared to receive or evaluate the information result in longer approval times for products? 3) Public relations concerns – Will submission of paired data from traditional animal tests and non-animal tests (generally necessary to establish the validity of the new method) draw the attention of the animal protection community to the amount of animal testing done in the past by the company? 4) Utility – Does the new method provide information sufficient to address the specific safety concern? and 5) Regulatory acceptance – Will regulatory bodies approach the method with a proactive attitude, or will there be an uphill battle to bring about movement from the status quo? It is important that solutions to these potential barriers (and many others besides) be understood and addressed by all stakeholders interested in the implementation of any non-animal test method. Progress, or lack of it, in the implementation of in vitro cytotoxicity testing to estimate acute systemic toxicity will be presented.

Poster

Barriers and potential solutions to the implementation of in vitro cytotoxicity testing for acute systemic toxicity

Rodger Curren
Institute for In Vitro Sciences, Inc., Gaithersburg, USA

Lecture

Is progress being made in replacing traditional rodent studies with in vitro approaches for determining acute systemic toxicity?

Rodger Curren and John Harbell
Institute for In Vitro Sciences, Inc., Gaithersburg, USA

Investigations of a potential correlation between a chemical’s toxicity to animals (including humans) and its toxicity to cells in culture have been conducted for over 50 years. It has seemed reasonable to many scientists that chemicals are toxic in animals because they cause the failure of one or more organ systems. Since interference with cell function (via cytotoxicity, for instance) in the organ system is the likely cause of organ failure, levels of chemical which might cause such organ failure should be determinable from in vitro experiments. Thus in vitro measurements alone might provide sufficient information on systemic toxicity. This hypothesis has been constructively explored recently by the publication of the Registry of Cytotoxicity (RC) by Willie Halle and collaborators from ZEBET, and in the MEIC program, championed by Bjorn Eckwall. The RC demonstrated a positive correlation between in vitro cytotoxicity values from published manuscripts and rodent LD₅₀ values from a standard reference source (347 chemicals). These results have prompted Spielmann et al. (1999) to propose using in vitro cytotoxicity results to estimate starting doses for animal studies, and an international study is ongoing to validate this proposal. The MEIC program showed that cytotoxic concentrations of selected chemicals in vitro correlated well with lethal blood concentrations in humans; actually better than rodent LD₅₀ values did. Recent efforts have begun to add necessary ADME components to the cytotoxicity predictions, hopefully resulting in the construction of a fully in vitro prediction system for human acute systemic toxicity.
The most widely used pharmacological approach for Alzheimer's disease (AD) is the enhancement of cholinergic transmission with acetylcholinesterase inhibitors (AChEI). Tacrine (THA) was the first AChEI in the market but it is now hardly ever used because of its hepatotoxicity and gastrointestinal side effects.

Neuropharma is working on the development of new drugs for AD treatment and has synthesised a series of dual AChEI with a very potent inhibition of both the esteric site of the enzyme and the peripheral site involved in the aggregation of b-amyloid. NP0361, which activity is about 2.5 × 10^{-11} M, reduced both plaque load and soluble Aβ peptides and improved cognitive functions in a transgenic h-APP mice model. Here, we present the experiments performed to explore whether NP0361 might present a better safety profile than THA exploring the cytotoxicity of these two compounds in cell models of hepatic origin.

We investigated the toxicity of styrene and styrene-7,8-oxide on HepG2 cells, evaluating different endpoints of toxicity such as metallothioneins, heat shock proteins, apoptosis related proteins, as well as the accumulation of styrene within the cells and the expression of two isoforms of cytochrome P450. Moreover, the potential activity of styrene and styrene-7,8-oxide in modulating gene expression has been investigated.

Our data revealed that in HepG2 cells there was an hsp70, metallothioneins, BclX/L, c-myc induction and a decrease in BAX expression after styrene and SO treatments, confirming that styrene and SO activated protective mechanisms and did not induce apoptosis.

In addition, we found an up-regulation of TGFβ2 and TGFβRIII in HepG2 after exposure to styrene, while in human normal hepatocytes these genes have been down-regulated after both treatments.

Finally, we found that the styrene and SO treatment induced CYP1A2 protein expression.

In conclusion, both the compounds caused toxic stress in HepG2 cells, being SO directly more toxic; in the meantime, we observed an opposite effects of the two compounds in carcinoma cells and normal hepatocytes regarding their activity in gene modulation.
Poster

Armenia: achievements and problems in development of alternative approach in vitro

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Our Department is an only institution in Armenia using animal cells in vitro (cultures of normal human and rat fibroblasts, transformed human and animal cell lines) as alternatives to predict acute lethality in vivo, to assess risk of new chemicals, to identify potential mammalian cell-based biopharmaceuticals. The techniques applied include acute (cell survival determination by vital dye exclusion and cell clonogenic activity) and chronic (induction of chromosome aberrations and micronuclei, comet-assay and comet-assay-FISH) toxicity evaluation. Earlier we have revealed radioprotective and antioxidant activity of two new Mn-chelates and cytotoxic action of two new metalloporphyrins and extracts of callus culture of medicinal plant Oleander. To introduce alternative approach in vitro into practice of research we collaborate with Armenian scientists in biology, organic chemistry and medicine in screening of new compounds’ biological and anticancer activities. To disseminate 3Rs’ ideas and principles we perform animal-free teaching at the Biological Faculty of the Yerevan State University and educating of researchers from the Yerevan Medical University in cell systems in vitro as animal alternatives. Alternative approach in vitro in Armenia is on the early steps of development and we strongly need contacts, collaboration with and support of global community to be integrated into international activity.

Poster

Assessment of the Halle Register for classifying and labelling new chemicals according to the EU toxicity classes

Elke Genschow, Dieter Traue, Willi Halle, Manfred Liebsch and Horst Spielmann
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To reduce the number of animals used in acute oral toxicity testing, cytotoxicity data (IC₅₀) can be used to predict the in vivo acute toxicity of chemicals by using statistical modelling approaches between the IC₅₀ values and acute oral LD₅₀ values in the Register of Cytotoxicity (RC).

Here, we propose strategies for using in vitro data as a basis for classifying and labelling new chemicals representative of all 3 EU toxicity classes as well as the unclassified category and evaluate the probabilities of correct classification depending on the true LD₅₀ and depending on the neighbourhood of class limits.
Lecture

Cell culture models of the air-blood barrier for the evaluation of aerosol medicines

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Introduction: The pulmonary route is of increasing interest for the development of new medicines, not only for the treatment of lung diseases (e.g. asthma, COPD) but also for the fast efficient delivery of drugs into the systemic blood circulation. Advanced drug carriers, such as nanoparticles or liposomes, however, require the use of polymers and other excipients, the effects of which on the airway and respiratory epithelia are still relatively unknown, especially with regard to their safety.

Methods: We have been evaluating the pulmonary epithelial cell lines, Calu-3, 16HBE14o- and A549, as well as primary cultures of human alveolar epithelial cells (HAEpC). Typically, cells are grown on permeable filter supports, allowing to form monolayers with functional tight junctions and pharmaceutically relevant transporter proteins. This setup can be used to perform transport, cytotoxicity and irritancy studies of drugs, excipients, particles and other xenobiotics.

Results: While the cell lines Calu-3 and 16HBE14o- appear useful to model the bronchial epithelium, the cell line A549 develops only week barrier properties. Therefore, it still appears necessary to use primary cultured cells to model the alveolar epithelium.

Discussion: Cell culture models of pulmonary epithelia offer excellent opportunities to study transport processes of drugs and other xenobiotics across the air-blood barrier, as well as to assess the inhalation safety of new polymers and other chemicals. After adequate characterisation and validation, such systems may be valuable alternatives to inhalation experiments on small rodents or dogs.

Poster

Evaluation of in vitro effects of 50 toxic reference chemicals using an electronic cell counting and sizing system versus MTT- and NRU-assay

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According to the guidance document on using in vitro data to estimate in vivo starting doses for acute toxicity (NHI publication No.: 01-4500 from 2001) the performance of the electrical current exclusion method (ECE) was studied for its suitability as an in vitro cytotoxicity test. In a comparative study two validated in vitro assays based on quantification of metabolic processes necessary for cell proliferation or organelle integrity (MTT/WST-8 assay and NRU-assay) and two cytoplasmic membrane integrity assays (trypan blue exclusion and electrical current exclusion) were performed.

IC₅₀ values were evaluated for 50 chemicals from low to high toxicity, 46 listed in Halle’s Registry of Cytotoxicity (RC, Halle and Goeres, 1988). High correlation between IC₅₀ values obtained in this study and the IC₅₀ data published in the RC was found. The sensitivity of the assays was highest for the electrical current exclusion method and decreased from MTT/WST-8 assay to NRU to trypan blue (TB) assay. The consistent results of the electrical current exclusion method (ECEM) are based on technical standardisation, high counting rate and the ability to combine cell viability and cell volume analysis for detection of first signs of cell necrosis and subsequent damage of the cytoplasmic membrane caused by cytotoxic agents.
Previous studies have identified a correlation between 
\textit{in vitro} cytotoxicity and acute oral toxicity. NICEATM and ECV AM subsequently initiated a three-phase multi-laboratory validation study to evaluate the usefulness of two standardised 
\textit{in vitro} basal cytotoxicity assays for estimating acute rodent toxicity and the extent that they may reduce animal use. Seventy-two coded chemicals (12 from each of five acute oral hazard categories and 12 unclassified/non-toxic chemicals) were tested in mouse 3T3 fibroblasts and in normal human epidermal keratinocytes (NHK) using neutral red (NR) uptake assays. Phase Ia established the historical databases for sodium laurel sulfate, the positive control, for each of three laboratories. Three chemicals were tested in Phase Ib and nine chemicals were tested in Phase II. Protocols were optimised after each of the first two phases to minimise intra- and inter-laboratory variation prior to testing 60 chemicals in Phase III. Technical challenges arose in Phases Ia/Ib (i.e., formation of NR dye crystals; uneven growth of NHK cells; slow growth of 3T3 cells) that were resolved with Phase II protocols. Significant variation in NHK growth in Phase II attributable to different lots of media and supplements required pre-qualification of medium. The optimised final protocols were used for Phase III testing. These studies demonstrate the value of using a phased approach during validation to optimise and standardise a final test method protocol that can then be used for the final validation phase.

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**Poster**

**Protocol optimisation during a validation study to evaluate \textit{in vitro} cytotoxicity assays for estimating rodent acute systemic toxicity**

Michael Paris\(^1\), Judy Strickland\(^1\), William Stokes\(^2\), Silvia Casati\(^3\), Raymond Tice\(^1\), Hans Raabe\(^4\), Cheng Cao\(^5\), Richard Clothier\(^6\), John Harbell\(^4\), Greg Mun\(^4\), Angela Szemere\(^4\), Greg Moyer\(^4\), Janna Madren-Whalley\(^5\), Chundakkadu Krishna\(^5\), Monika Owen\(^6\), Nicola Bourne\(^6\), Joseph Haseman\(^1\), Pat Crockett\(^7\), Martin Wenk\(^8\) and Molly Vallant\(^9\)

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The overall aim of this key area is to validate \textit{in vitro} tests relevant for target organ and target system-specific toxicities to be incorporated into optimal test batteries for the estimation of human systemic toxicity.

In the area of acute toxicity an ECVAM workshop report has recently been published. An ongoing international validation study aims to reduce the number of animals used in oral acute toxicity testing. An Integrated Project, A-Cute-Tox, aims to achieve full replacement for predicting human acute toxicity. In the area of haematotoxicology the main achievements are the peer review of the validation of the GM-CFU assay to predict \textit{in vivo} acute neutropenia, the pre-validation of the \textit{in vitro} CFU-MK assay to predict thrombocytopenia, and the refinement of the clonogenic assays for high throughput testing.

In the area of immunotoxicology two task force meetings and a workshop have been held. A multi-laboratory study is underway to evaluate the most promising endpoints for immune-suppression. At ECVAM, an intensive search for \textit{in vitro} models for developmental (DNT) and adult neurotoxic hazard assessment is ongoing. ECVAM in collaboration with CEFIC and CAAT organised a DNT workshop to assess the available models and endpoints relevant for validation of alternative approaches.

In the area of chronic \textit{in vitro} toxicity a task force has been created and a workshop was held. ECVAM is involved in two FP6 projects, Predictomics and Pulmonet. The final goal in the field of systemic toxicity is to provide cheaper, more ethical and more scientifically based testing strategies.
Lecture

A European pharmaceutical company initiative to challenge the requirement for conventional acute toxicity studies in rodents: Do these studies add value prior to first dose in man?

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Conventional acute toxicity studies in rodents are usually conducted to support the registration of any pharmaceutical intended for human use. The main objective of these studies is to estimate the minimum dose causing lethality. The information may be used to set the starting dose in the first studies in man and/or to give an indication of the likely effects of acute overdose. However, these studies do not usually include clinical pathology, histopathology or toxicokinetics and their clinical usefulness is questionable. In addition, data may be available from other study types that don’t have lethality as an endpoint.

A working party representing the pharmaceutical industry was formed in 2003. It aims to:
- Review how acute toxicity studies are conducted within the industry.
- Assess the value of acute toxicity data in a clinical setting and establish whether information from other study types could be used.
- Agree a short term harmonised industry approach focussing on reduction and refinement.
- Develop a strategy for challenging the guidelines on the requirement for conventional acute toxicity where lethality is a defined endpoint.

This presentation will describe the working party’s progress in assessing the value of conventional acute toxicity data prior to the first clinical trials in man. The results of a data sharing exercise will be described and a proposal that these studies should not be a mandatory requirement prior to first trials in man, leading to a significant reduction in the numbers of animals used due to compound attrition during the development process.
Alternative methods to animal experimentation were used to investigate the effect of heavy metal compounds (Zn(CH₃COO)₂, ZnSO₄, Pb(CH₃COO)₂, CoCl₂, CoSO₄, SrCl₂, Pyrithione zinc, concentration from 0.00625 to 0.1%) on the membrane permeability and barrier function of epidermis: modification BRC-test and biphasic water-lipid (liquid-crystal) model of the epidermis. Depending of chemical structure, heavy metal compounds have been shown to increase or decrease membrane permeability in BRC-test. Zn(CH₃COO)₂, ZnSO₄, Pb(CH₃COO)₂ caused a concentration-dependent membrane alterations at t= 37ºC. All of these compounds at highest concentration (0.1%) led to strong damage of cell’s membrane. Exposure erythrocytes to low concentration of these compounds (0.0125%) caused, respectively, 3.85-, 2.35-, and 1.5- fold higher levels of erythrolysis than control solvent. At minimum concentration (0.00625%) these compounds did not effect on membrane permeability. In all experiments, erythrocytes treated with CoCl₂, CoSO₄ did not differ significantly from control group in their membrane permeability. Pyrithione zinc have the ability to increase cells’ membrane permeability with high efficiency. Topical application of Pb(CH₃COO)₂ at concentration 0.1% on lipid layer of the biphasic water-lipid model of the epidermis did not effect on the level TEWL. At the similar conditions CoCl₂, SrCl₂, and ZnSO₄ demonstrated a tendency to protect water losing, respectively by 30, 19, and 18%. Our in vitro results suggest that both inorganic and organic heavy metals’ compounds such as Zn(CH₃COO)₂, ZnSO₄, Pb(CH₃COO)₂ and Pyrithione zinc can induce changes in barrier function of biological membrane.

### Lecture

**Progress in refinement:**

**The oral Acute Toxic Class method is a successful alternative to the oral LD₅₀ test**

_Eva Schlede¹, Wolfgang Diener¹, Elke Genschow², Horst Spielmann², Gisela Stropp³ and Detlev Kayser¹_

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The oral Acute Toxic Class method (ATC method) was developed as an alternative to replace the oral LD₅₀ test. The ATC method is a sequential testing procedure using only 3 animals of one sex per step at any of the defined dose levels (5, 50, 300, 2000 mg/kg b.w.). Depending on the mortality rate 3 but never more than 6 animals are used per dose level. The reduction of numbers of animals used in comparison to the LD₅₀ test is 40% to 70%. The oral ATC method is based on the probit model and biometric evaluatations were conducted before a national (6 participants) and subsequently an international (9 participants from 5 countries) ring study were conducted. An excellent agreement was demonstrated between the toxicity and the animal numbers predicted biometrically and observed in the validation studies. The oral ATC method was adopted as an official test guideline by OECD in 1996 and was slightly amended in 2001. The oral ATC method has been successfully used in Germany for many years and in 2004 >90% of all tests on acute oral toxicity testing was conducted as oral ATC tests. In member states of the European Union the ATC method is used in the range of >50% of all tests conducted. Since the deletion of the oral LD₅₀ Test by member states of OECD and EU the use of alternatives to the oral LD₅₀ Test is mandatory. So far, the oral ATC method is the most widely used alternative.
Introduction: Mercury, a common heavy metal is an environmental pollutant. Immunosuppression has been recorded in birds due to in vivo treatment with mercury. The present study was conducted to investigate apoptosis and T- and B-cell blastogenesis on avian lymphocytes treated with mercury in vitro.

Methods: Avian Lymphocytes isolated from the spleen of apparently healthy birds were treated with No Observable Effect Level (NOEL)x10-2, NOELx10-3, NOELx10-4, NOELx10-5, NOELx10-6 and NOELx10-7 concentrations of mercuric chloride for 30, 60, 90 and 120 min. Apoptosis was detected by transmission (TEM) and scanning electron microscopy (SEM), agarose gel electrophoresis of isolated DNA and immunoperoxidase staining. T- and B-cell blastogenesis was assessed by lymphocyte stimulation test using Concanavalin-A (Con-A) and Lipo-polysaccharide (LPS) as mitogens, respectively.

Results: TEM revealed shrunken cells, chromatin margination, karyorhexis, budding and phagocytised apoptotic bodies and SEM showed ultrastructural alterations on cell surface such as formation of apoptotic bodies and budding. Fragmentation of DNA was observed by agarose gel electrophoresis. Apoptotic lymphoid cells exhibited brown colour by immunoperoxidase staining. There was significant reduction in delta OD of the mitogen stimulated lymphocytic cultures. The alterations were maximum in cells treated with NOELx10-2 dose of mercuric chloride for 120 min while minimum at NOELx10-7 concentration for 30 min.

Discussion: The present in vitro investigation indicated that mercury exerts its deleterious effects on avian lymphocytes via apoptosis even at very minute concentrations and short exposure time. Therefore, dose and time dependent studies on immunotoxic effect of heavy metals can be done in vitro on lymphocyte cell culture system.

Posters

**Poster**

*In vitro* immunotoxicity induced by mercury on avian lymphocytes

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**Results:** TEM revealed shrunken cells, chromatin margination, karyorhexis, budding and phagocytised apoptotic bodies and SEM showed ultrastructural alterations on cell surface such as formation of apoptotic bodies and budding. Fragmentation of DNA was observed by agarose gel electrophoresis. Apoptotic lymphoid cells exhibited brown colour by immunoperoxidase staining. There was significant reduction in delta OD of the mitogen stimulated lymphocytic cultures. The alterations were maximum in cells treated with NOELx10-2 dose of mercuric chloride for 120 min while minimum at NOELx10-7 concentration for 30 min.

Discussion: The present *in vitro* investigation indicated that mercury exerts its deleterious effects on avian lymphocytes via apoptosis even at very minute concentrations and short exposure time. Therefore, dose and time dependent studies on immunotoxic effect of heavy metals can be done *in vitro* on lymphocyte cell culture system.

**Poster**

Results of the final phase of a validation study to evaluate *in vitro* cytotoxicity assays for estimating rodent acute systemic toxicity

William Stokes¹, Michael Paris², Judy Strickland¹, Silvia Casati¹, Raymond Tice², Hans Raabe⁴, Cheng Cao⁵, Richard Clothier⁶, John Harbell⁷, Greg Mun⁷, Angela Sizemore⁷, Greg Moyer⁷, Janna Madren-Whalley⁵, Chandrakakku Krishna⁵, Monika Owen⁶, Nicola Bourne⁷, Joseph Haseman², Pat Crockett⁷, Eric Harvey⁷, Robert Lee⁷, Martin Wenk⁸ and Molly Vallant⁹

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Recent studies have identified a correlation between *in vitro* basal cytotoxicity and *in vivo* acute oral toxicity. NICEATM and ECVAM subsequently initiated a three-phase multi-laboratory validation study to evaluate the usefulness of two standardised Neutral Red Uptake (NRU) assays for estimating acute rodent toxicity and to determine the extent that they may reduce animal use. Seventy-two coded chemicals (12 from each of five acute oral hazard categories and 12 unclassified/non-toxic chemicals) were tested using the NRU endpoint with mouse 3T3 fibroblasts and Normal Human Epidermal Keratinocytes (NHK). Three chemicals were tested in Phase Ib, nine chemicals in Phase II, and 60 chemicals in the final Phase III. Based upon preliminary analyses, the results for the positive control, sodium laurel sulfate, were reproducible over the entire study. IC₅₀ results from all phases were used with rodent oral LD₅₀ values to calculate linear regressions for each lab and assay. Although the NHK data were more reproducible than the 3T3 data, the 3T3 data yielded a better regression fit. Comparison of the regressions for both assays to the Registry of Cytotoxicity (RC) regression indicated that the new regressions were statistically different from the RC, but that their predictions of toxicity category were generally similar. The new regressions will be used in conjunction with computer simulations to determine animal savings that may result by using *in vitro* data as the basis for starting doses for acute toxicity studies. Supported by: N01-ES-35504, N01-ES-75408; EPA IAG DW-75-93893601-0; European Commission 19416-2002-04 F2ED ISP GB.
### Poster

**Data collection and analysis for an in vitro cytotoxicity validation study**

Judy Strickland, Michael Paris, Hans Raabe, Joseph Haseman, Silvia Casati, Richard Clothier, Cheng Cao, Pat Crockett, Raymond Tice and William Stokes

1 ILS, Inc., NIEHS/NICEATM, Research Triangle Park, USA; 2 IIVS, Gaithersburg, USA; 3 ECVAM, JRC, Ispra, Italy; 4 Univ. of Nottingham, Nottingham, United Kingdom; 5 US Army ECBC, Aberdeen Proving Ground, USA; 6 Constella Group, Durham, USA; 7 NIEHS, NICEATM, Research Triangle Park, USA

A multi-laboratory validation study designed by NICEATM and ECVAM evaluated two in vitro basal cytotoxicity test methods using 72 coded chemicals with a wide range of acute oral toxicity. The study was designed in three phases to allow for refinement of the protocols, and data collection and evaluation procedures. An Excel® template was distributed to the participating laboratories for entry of the raw data, identification of outliers among the six concentration replicates, documentation of materials and procedures, graphical analysis of dose-response, and formatting of data for further analysis. A Hill function analysis with GraphPad Prism® software was used to calculate IC20, IC50, and IC80 values and associated 95% confidence limits, and graph the data and fitted model. Initial criteria for an acceptable dose-response for individual tests included one data point between 10 and 50% viability, one data point between 50 and 90% viability, and r2 ≥ 0.8. A Prism® template was distributed to the laboratories to automate and provide uniformity of analysis. To increase the speed of data collection and evaluation by the Study Management Team (SMT) and consulting biostatisticians, the laboratories submitted the Excel® and Prism® files by e-mail. Results compiled by the SMT were returned to the originating laboratories for audit to ensure accurate transmission of data. Implementation of these procedures demonstrated that automated data collection in relatively common, easy-to-use electronic formats facilitates uniformity of data collection and analysis.

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### Poster

**Use of alternative to animal methods for prognostication the effect of benzalkonium chloride on membrane permeability**

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Alternative to animal methods were used to investigate the effect of preservatives on the membrane permeability and barrier function of epidermis: modification BRC-test, cells *Escherichia coli*, biphasic water-lipid (liquid-crystal) model of the epidermis, lyophilised pig skin Xenografs. Membrane permeability in BRC-test was value by means of the flow out of haemoglobin. Penetration of plasmatic membrane of the isolated cells of the *Escherichia coli* was value by means of the flow out of the intracellular components (E260). The barrier function of the biphasic water-lipid model and lyophilised pig skin Xenografs was value by means of TEWL. In BRC-test Benzalkonium Chloride at concentration 0.001 induced 8,3-fold higher levels of lysis of cells than control solvent. Additionally, quaternary ammonium compound caused inhibition of dehydrogenase activity of *Escherichia coli*. Inhibition observed with a 0.001-, 0.00075- or 0.0005% concentration of Benzalkonium chloride was 79, 68 or 34%, respectively, compared with control group. Topical application of Benzalkonium Chloride (concentration 0.1%) on the lipid layer of water-lipid model don’t cause the increasing the level TEWL. Topical application of Benzalkonium Chloride (concentration 0.1%) on the lyophilised pig skin Xenografs caused increasing the level TEWL by 24 %. Our experimental data indicate that Benzalkonium chloride caused a concentration-dependent increase of cells’ membrane and lyophilised pig skin Xenografs permeability.
The ICCVAM/ECVAM validation study employed 72 coded chemicals tested with Normal Human Keratinocytes or BALB/c 3T3 fibroblast cells, using the Neutral Red Uptake assay at 48 hours. Twelve chemicals from each of the six in vivo classes had human and rat in vivo data.

Neutral red uptake can be influenced by lysosomal pH without affecting cell number or viability, so a Total Protein assay was performed. The Neutral Red desorb solution is the fixative for the Kenacid Blue assay (Riddell et al., 1986) allowing the total protein assay to be conducted on the same cells.

NHKs were seeded onto 60 wells of a 96 well plate. Eight decreasing geometric concentrations of test chemical were applied to 6 replicate wells of the plates, (http://iccvam.niehs.nih.gov/methods/in vitro.htm). SLS was the positive control, and the results were required to meet acceptance criteria. The NHK results for the NRU and KB assay were assessed to determine comparability. Chemicals that, at high concentrations, fix the cells do give a different toxicity profile between the two assays.

This approach was also adopted with 11 coded anticancer drugs (see poster by Budworth et al.), which revealed comparability between the IC50 values obtained.

This work was funded by the FRAME research programme.

Poster

**Comparison of two cytotoxicity assays with the ICCVAM/ECVAM validation study chemicals**

*Sarah Watts, Rachel Budworth, Nicola Bourne, Monika Owen and Richard Clothier*

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Neurons and astrocytes form a functional unit in the nervous system. The NT2.D1 human embryonal cell line may be differentiated to yield post-mitotic NT2.N neurons, which are the closest model of human neurons currently in use. The CCF-STTG1 astrocytoma line also strongly resembles human astrocytes. n-Hexane is metabolised to 2,5-hexanediol (HD), which reacts with neural proteins to give pyrrole adducts and neurofilament cross-linking. Our group previously found the closely related 2,3-HD and 3,4-HD (used as food additives), also to be neurotoxic *in vitro*. The aim of this study was to investigate the sensitivity of NT2.N, CCF-STTG1 and NT2.D1 cells to toxic insult from 2,5-. 2,3-, and 3,4-HD, for 4 or 24 hours, using MTT turnover to measure cytotoxicity sustained. Comparison of IC50 values showed that 2,3- and 3,4-HD were significantly more toxic in all three cell lines, than 2,5-HD. All three cell lines were similarly sensitive to 2,3- and 3,4-HD toxicity and there was no significant difference between toxicity sustained after 4 or 24 hours exposure, whilst 2,5-HD was significantly more toxic following 24 hours than 4 hours exposure, in all three cell lines. After 4 hours 2,5-HD was significantly more toxic to NT2.D1 than NT2.N and CCF-STTG1 cells, which did not differ in their sensitivity. However, after 24 hours exposure, NT2.N cells were significantly more sensitive towards 2,5-HD than were the CCF-STTG1 cells. These data suggest the toxic profile of the 2,3-HD and 3,4-HD isomers differs from 2,5-HD, in rapidity of onset, acute severity and cellular specificity.

Poster

**Toxicity of hexanediones in human neuronal and astrocytic cell lines**

*Elizabeth Wright and Michael Coleman*

Aston University, Life and Health Sciences, Birmingham, UK
Poster

**Pyruvate uptake and lactate release by liver spheroids and their changes after exposure to toxicants**

*Jinsheng Xu and Wendy E. Purcell*
Faculty of Applied Sciences, University of the West of England, Bristol, UK

Pyruvate is a key intermediate in energy metabolism. It can be transported into mitochondria to generate ATP, transformed to lactic acid and used for gluconeogenesis to generate glucose. Mitochondrial injury can affect pyruvate metabolism and lactic acid production. Liver spheroid culture has been shown to be a useful model in functional and toxicological studies. This study investigated pyruvate uptake and lactate release by rat liver spheroids and their changes after exposure to toxicants. Rat liver spheroids were prepared by a gyrotatory method. Pyruvate uptake and lactate release were investigated over a period of 15 days. The results showed that liver spheroids took up pyruvate and released lactate across the culture period. After exposure to selected toxicants, diclofenac, isoniazid, paracetamol, m-dinitrobenzene and 3-nitroaniline, pyruvate uptake and lactate release were affected differentially. Diclofenac, isoniazid, paracetamol and m-dinitrobenzene which can cause mitochondria injury significantly (p<0.05) reduced pyruvate uptake. Diclofenac, isoniazid, paracetamol and galactosamine significantly (p<0.05) decreased lactate release but m-dinitrobenzene increased lactate release (p<0.01). The lesser toxic toicant 3-nitroaniline, a metabolite of m-dinitrobenzene, did not cause significant changes in either pyruvate uptake or lactate release. It is concluded that pyruvate uptake and lactate release are two functions of liver spheroids that should be suitable for use as endpoints in *in vitro* toxicology studies using liver spheroid model.

Poster

**Toxicity of hexanediones in a two-tier neurotoxicity system**

*Thomas Zilz, Helen Griffiths and Michael Coleman*
Aston University, Life and Health Sciences, Birmingham, UK

In order to develop an *in vitro* high throughput screening system for potential neurotoxins, two assays were evaluated using the SK-N-SH line, an established model of human neurotoxicity. MTT turnover was chosen as a first tier or triage with a simple endpoint (cell death), whilst a second more sophisticated assay utilised flow cytometry with Propidium Iodide dye (PI) to provide more detailed data on toxin-mediated alterations of cell cycle phases. The cells were exposed to 2,3- and 2,5-hexanedione for 48 hrs and assayed for viability using MTT and flow cytometry, with PI. IC$_{50}$ values for 2,3- and 2,5-hexanedione were 3.31±0.13 mM and 20.08±1.93 mM respectively. 2,3-Hexanedione (1.6 mM) caused a significant (p<0.01) increase in the percentage of cells in the G2/M phase with respect to control, but apoptosis was not significantly increased until 7.4 mM compared with control. 2,5-Hexanedione (3.4 mM) significantly increased the fraction of cells in the G2/M phase (p<0.001), whilst the percentage of cells undergoing apoptosis increased at 17 mM (p<0.01). The increases in the G2/M phase specifically occurred at concentrations where no cell death was observed with MTT and before any increase in apoptosis transpired. This suggests that G2/M checkpoint arrest is elicited in response to low concentrations of hexanediones. The MTT assay provides an indication of the broad cytotoxicity of toxins such as 2,3- and 2,5-hexanediones in SK-N-SH cells and is a good basis for an initial neurotoxicity screen. Flow cytometry with PI, offers more detailed information on the effects of 2,3- and 2,5-hexanedione on the cell cycle.
Angiogenesis, defined as sprouting of new capillaries from pre-existing ones, occurs in a cascade of migration, proliferation, differentiation and three-dimensional organisation of endothelial cells. Angiogenesis is a pre-requisite for growth and differentiation of organs and tissues and is involved in many pathological processes, for example growth and metastasis of tumours.

Pro- and anti-angiogenic factors are tested in numerous in vivo and in vitro models of angiogenesis. However, in these models, effects of the substances tested were quantified in only a few phases of angiogenesis.

The aim of this study was to establish and validate a method to quantify all stages of angiogenesis and anti-angiogenesis in vitro. Endothelial cells isolated from slaughtered cattle were incubated in specific medium. Angiogenesis up to the formation of lumenised capillary-like structures was examined by phase contrast and electron microscopy. Both morphological and ultrastructural changes of cells showed analogies to angiogenesis in vivo. By precise staging of the cellular alterations the entire angiogenic cascade was quantified. The reproducibility of quantitation of angiogenesis was verified by examination by different persons and in different culture dishes. Statistical evaluation showed that reproducible quantitation was possible by different persons and in a small sample size.

In conclusion, the present in vitro model allows a viable quantitation of angiogenesis and anti-angiogenesis in vitro. It can be employed either in trial studies of potential angiogenic and antiangiogenic substances, respectively, or in the investigation of their cellular mechanisms and may thus provide an efficient method to reduce animal testing.
Lecture

The Importance of Good Cell Culture Practice (GCCP)

Michael Balls, Sandra Coecke, Gerard Bowe, John Davis, Gerhard Gstraunthaler, Thomas Hartung, Robert Hay, Otto-Wilhelm Merten, Anna Price, Leonard Schechtman, Glyn Stacey and William Stokes

The maintenance of high standards is fundamental to all good scientific practice, and is essential for maximising the reproducibility, reliability, acceptability and proper application of results produced. Following the publication of outline guidelines for Good Cell Culture Practice (GCCP) after the 3rd World Congress on Alternatives and Animal Use in the Life Sciences (Bologna, Italy, 1999), a new task force was convened by ECVAM, with a broader range of expertise in cell and tissue culture, in order to produce an updated and more-detailed GCCP guidance document for practical use in the laboratory.

This GCCP Guidance, which will have been published in ATLA and made available elsewhere before the Berlin Congress, is based on the following six operational principles:

1. Establishment and maintenance of a sufficient understanding of the in vitro system and of the relevant factors which could affect it.
2. Assurance of the quality of all materials and methods, and of their use and application, in order to maintain the integrity, validity, and reproducibility of any work conducted.
3. Documentation of the information necessary to track the materials and methods used, to permit the repetition of the work, and to enable the target audience to understand and evaluate the work.
4. Establishment and maintenance of adequate measures to protect individuals and the environment from any potential hazards.
5. Compliance with relevant laws and regulations, and with ethical principles.
6. Provision of relevant and adequate education and training for all personnel, to promote high quality work and safety.

Lecture

Macroscopic evaluation of HET-CAM biomaterial testing: How reliable is macroscopical scoring without histology?

Claudia Eder, Erwin Falkner, Helmut Appl, Harald Schöffl, Udo M Losert and Stefan Nehrer

The high vascularity and the rapid development of connective tissue and vascular system of the chick chorionallantoic membrane offer an interesting environment for tissue reaction studies, which are often evaluated by macroscopical examination only.

Various biodegradable scaffolds were applied onto the CAM and maintained in ovo for 3 days prior to digital documentation, macroscopical biocompatibility evaluation and subsequent histological analysis. A collagen sponge, two different Collagen Type I/III scaffolds (Chondro-Gide®, Bio-Gide®) and a Collagen Type II membrane (Chondrocell®) were tested.

Collagen sponge: Macroscopic analysis demonstrated extreme rapid degradation, spontaneous bleedings in the surrounding of the implant and a vessel retraction from the implantation site. Histological analysis, in contrast, demonstrated an increase in blood vessel content. A foreign body tissue reaction was observed only histologically.

Chondro-Gide®: Macroscopic evaluation showed excellent integration and biocompatibility patterns which were confirmed by histology. Bio-Gide®: Macroscopic observation showed excellent integration and significant induction of angiogenesis, which was confirmed by histology. An inflammatory infiltrate was observed in histological sections only. Chondrocell®: Spontaneous bleedings at the implantation site as well as vessel retraction and altered vessel courses were observed macroscopically. Histological evaluation in contrast demonstrated good angiogenetic properties.

Macroscopical scoring only partially correlated to histological evaluation: The impact of spontaneous bleedings and vessel path alteration was often overestimated and a foreign body tissue reaction could not be detected after macroscopical evaluation. HET-CAM biomaterial testing should therefore be combined with histological evaluation to receive the full force of expression of the CAM model.
Lecture

Varioscope-head mounted microscopy for HET-CAM applications

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The HET-CAM (Hen Egg Test-Chorioallantoic Membrane) angiogenesis test system was standardised for validation by use of a head-mounted operating microscope for both experimental procedures and digital documentation. Today HET-CAM test procedures, documentation and interpretation are usually performed macroscopically with a common hand-held camera. This modus makes it difficult to recognise fine structures and risks generation of artefacts due to bleeding, membrane rips, contamination with bacteria/yeasts and cooling the in vivo test system below the critical incubation temperature of 37°C. Reproducibility of generated data is therefore often unsatisfactory.

Head-mounted microscopy systems offer improved resolution and can also be used in class I/II safety cabinets, minimising the risk of contamination and permitting testing of substances potentially hazardous for the staff. To establish a Standard Operating Procedure (SOP), CAM dissection and specimen application procedure protocols as well as the technical equipment must be standardised. Miniature head-mounted operating microscopes like the Varioscope M5 tested by the authors enables the user to analyse objects difficult to access with the required magnification and operating distance and to manipulate them precisely. Automatic sensors detect the object continuously and adjust the optics, documentation is digitally performed from the experimentor’s visual angle, zoom is stagelessly variable, the pivoting radius is 72°. This kind of optical vision enhancement is used in operating theatres/dental clinics and manufacture/quality control of precision components. Varioscope M5 allows standardisation of CAM experimental procedures following Good Laboratory Practice (GLP) rules.
Lecture

**Standardisation in cell and tissue culture – the need for specific GLP guidelines in the cell culture laboratory (Good Cell Culture Practice - GCCP)**

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The cultivation of eukaryotic cells has become a powerful technique in basic cell and molecular biological research, applied biotechnology, and *in vitro* alternatives. Before cell culture could be carried out successfully, two problems had to be overcome: (1) Populations of cells had to be established from single cells; and (2) these populations had to be maintained for many generations. In a successful propagation of cells *in vitro*, cells from various tissues should grow and proliferate under appropriate culture conditions, while preserving highly differentiated functions, which closely resemble their ancestor cells *in vivo*. Thus, cell proliferation and cell differentiation are two major, albeit opposing, end points in tissue culture. Which of these contrasting goals should be achieved depends on the aim of a selected cell culture study and thus, on the culture conditions applied: (i) the supplementation of culture media with growth factors or differentiation factors, (ii) the use of specific extracellular matrix components, (iii) the subcultivation intervals and seeding densities, (iv) the feeding cycles, and (v) stationary cultures versus dynamic media supply in perfusion reactors. In sum, a number of tissue culture parameters have to be defined and coordinated. However, despite the widespread use and broad applications of cell and tissue cultures, a significant number of basic questions and methodological protocols are still unsolved and are handled in various ways by tissue culture laboratories. Selected examples will be presented, on how culture medium composition, medium volumes, feeding cycles, serum supplementation, or use of extracellular matrix components will influence growth of cultured cells and the expression of differentiated functions, which represents a serious impact on the credibility, reliability, reproducibility, and comparability of *in vitro* alternatives.

In conclusion, a minimum set of standards has to be defined in order to establish reproducibility and interlaboratory comparability of results obtained with *in vitro* cell culture technologies. Therefore, in analogy to GLP, a Good Cell Culture Practice (GCCP), i.e. good laboratory practice in the cell culture laboratory, was initiated at the 3rd World Congress on Alternatives and Animal Use in the Life Sciences in Bologna, 1999. As a result, GCCP Guidelines were elaborated by an ECVAM Task Force and published 2002 in *ATLA* 30, 407-414. Following this publication, a new GCCP Task Force was convened at ECVAM, Ispra, Italy, in order to produce an updated GCCP Guidance document (*ATLA* 33, 261-287, 2005), which will be presented in an accompanying lecture.

Lecture

**The reference in *in vitro* studies: Quality assurance and assessment**

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To assess the relevance of any kind of test, it is usually linked to a reference by comparing the results obtained with it to those of a reference standard (test). Regarding *in vitro* tests, usually the routine test, i.e. most often an *in vivo* test, constitutes this reference standard. For the comparison, the reference standard results of substances to be tested in the alternative test are collected from various sources, i.e. retrospectively. Indeed, the availability of reference data is a critical and often limiting factor for the chemical selection. However, quality aspects of the *in vivo* data can thus often not be controlled. But as their quality might have a tremendous impact on the relevance assessment of the *in vitro* test, it is crucial to assure its evaluation. For example, if reference data are searched for, this search should be structured, complete and unbiased. In any case, the obtained reference data should be documented including all relevant quality information. For example, chemical identity, chemical properties, GLP-compliance, guideline-compliance, selected doses or number of animals is important information indicating quality. In the compilation and documentation of this information, the completeness and transparency, allowing a complete quality assessment, is most important. Once retrieved, the data should be inserted into databases simplifying their evaluation. Furthermore, chemicals with high quality data should be chosen to build training and calibration sets for the setup and performance checks of *in vitro* tests in order to assure their results’ quality.
**Lecture**

**Long term reproducibility of EpiOcular™, a 3-dimensional tissue culture model of the human corneal epithelium**

*Mitchell Klausner, Patrick Hayden, Joseph Kubilus and John Sheasgreen*
MatTek Corporation, R&D, Ashland, USA

The EpiOcular tissue model (OCL-200) is an organotypic model of the human corneal epithelium cultured from normal human keratinocytes. Since commercial introduction in 1995, personal care and household product companies have increasingly used EpiOcular to determine the ocular irritancy of their products without using animals. Currently, validation of the EpiOcular model as a replacement for the Draize rabbit eye test is underway in the US. In addition, a validation study sponsored by ECVAM is scheduled to begin in 2005.

For commercial and regulatory purposes, a model must be reproducible within a given lot and between lots, especially over extended periods. Regulators and end users need to be assured that these test methods will provide consistent, good quality data during the validation process and over time.

Quality control of weekly lots of EpiOcular is performed using the MTT assay, which historically has been the *in vitro* endpoint of choice for European and US regulators. The exposure time needed to reduce the viability to 50% (ET50) for a positive control is determined.

Yearly average ET50 values have ranged from 20.6 minutes (2000) to 25.0 minutes (1998). The coefficients of variation (CV) for tissue exposed to the negative control (ultrapure H2O) have averaged under 6% and the average CV for all tissues has never exceeded 6.5%.

These results over the past 8 years of commercial production show EpiOcular to be a highly reproducible, stable toxicological model that is ideally suited for industrial and regulatory ocular irritancy studies.

**Poster**

**Long term reproducibility of EpiDerm™, an epidermal model for dermal testing and research**

*Mitchell Klausner, Patrick Hayden, Joseph Kubilus and John Sheasgreen*
MatTek Corporation, R&D, Ashland, USA

An *in vitro* model of human epidermis, EpiDerm (EPI-200), cultured from normal human epidermal keratinocytes has been sold by MatTek Corporation since 1993. Weekly lots of EpiDerm are produced for dermal irritancy, product efficacy, percutaneous absorption, pharmacological, and basic skin research studies.

In 2000 and 2002, respectively, European and US regulators approved the use of EpiDerm to assess the skin corrosivity of chemicals. Validation studies utilising EpiDerm for phototoxicity and skin irritation are currently underway.

For commercial and regulatory purposes, models must be reproducible within a given lot and between lots, especially over extended periods. Regulators and end users need assurance that the *in vitro* models will provide consistent, good quality data during the validation process and over time.

To address tissue reproducibility, quality control (QC) testing of each EpiDerm lot involves both a positive (1% Triton X-100) and a negative control (water). Using the MTT assay, which historically has been the endpoint of choice for European and US regulators, a dose response curve is constructed and the exposure time that reduces the tissue viability to 50% (ET50) is interpolated.

The yearly average ET50 since 1996 has varied from 6.2 hr (2003) to 7.5 hr (1998). The coefficients of variation (CV) for tissue exposed to the negative control (ultrapure H2O) have averaged under 6% and the average CV for all tissues has never exceeded 12%.

Over the past 10+ years of commercial production, EpiDerm has remained a highly reproducible, stable toxicological model that is ideally suited for industrial and regulatory toxicology and other skin related studies.
Many people prefer natural drugs instead of synthetic because of their lower side-effect risk. Often the therapeutic use of natural drugs is based on traditional knowledge, never proven by modern scientific methods. We decided to evaluate the HET-CAM assay, utilising the well vascularised chorioallantois membrane (CAM) of fertile hen eggs. This assay is useful to detect a number of important pharmacological actions close to the in vivo situation.

The HET-CAM assay was evaluated regarding irritative, anti-inflammatory and anti-angiogenic effects of several common essential oils. The irritative activity was determined by identifying the “irritation threshold”. Anti-inflammatory potential was detected ranking the phenomenons “star like vascularisation” and “granuloma formation”, the supposed endpoints of inflammation. Anti-angiogenic action was identified by detecting a vessel free area around a drug containing carrier.

Detecting the irritation potential was very successful. It was possible to rank essential oils by their irritation threshold. Evaluating the HET-CAM assay as an anti-inflammatory test system by ranking the noted endpoints failed. No reproducible results could be obtained, even for the established anti-inflammatory drug hydrocortisone. It also wasn’t possible to identify any other specific endpoint for inflammation inhibition on the CAM. The studies to evaluate the anti-angiogenic HET-CAM assay have just started. First results indicate that creating a vessel free area around the carrier is possible. We decided to determine this action by a “yes/no” decision. We will further evaluate the HET-CAM assay to serve as a detection model for several pharmacological activities of different natural compounds without using animal tests.
Poster

Applying Good Laboratory Practices (GLPs) to in vitro studies, one laboratory’s perspective

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The steady increase in industry use and regulatory acceptance of in vitro test methods has resulted in an increased need to apply Good Laboratory Practice (GLP) regulations to these systems. The original GLP regulations, developed to address the conduct of animal studies, are concerned with many special conditions that apply to animal housing and care, and the relatively long duration of animal studies that are not present in the shorter in vitro studies. In animal studies, for example, emphasis is placed on the isolation of species and periodic analysis of feed and water, whereas in non-animal studies there is increased importance on the justification of the test system. Recently the OECD has published advisories (No. 7, The Application of the GLP Principles to Short-term Studies, 1999; No. 14 The Application of the Principles of GLP to in vitro Studies, 2004) to clarify the application of the GLP principles to both short term and in vitro studies. This poster outlines the approach applied at the Institute for In Vitro Sciences, Inc. (IVS) to the conduct of in vitro GLP-compliant studies. We describe the translation of the OECD guidance documents into a framework for conducting assays which use ex vivo tissues, monolayer cell cultures, reconstructed skin constructs, and manufactured test kits. We are grateful to auditors from numerous study sponsors and regulatory agencies who have helped us develop what we feel is a best practices approach.
Although in Europe the problem of marine toxins is slightly different than in other parts of the world, the international trade requires a control for any toxin from around the world. Those toxins currently being detected by animal sacrifice methods are: hydrophilic compounds (saxitoxin and analogs), and lipophilic compounds (yessotoxins, pectenotoxins, ostreocins, maitotoxins, ciguatoxins, cyclic imines, okadaic acid analogs, azaspiracids).

The situation created in the field of marine biotoxins with current decision EU/2002/225, that requires all lipophilic toxins to be detected by the mouse bioassay, has put a great pressure on having alternative methods readily available to replace the bioassay, and backed up by an international validation study. With the chemical diversity of marine toxins, the bioassay is not a reliable system to safely control all the lipophilic toxins.

Although there are many technical possibilities to develop alternative methods, the authors will elaborate on those that have the highest chances of success: optical biosensors, functional (biochemical based) assays, and chemical (separation, mass spectrometry) methods. Antibody-based assays will be discussed as not good candidates for this field.
**Lecture**

**Challenges in the use of transgenic mouse models in the toxicity testing of food additives**

*David Hattan*

FDA Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, USA

Introduction: The assessment of carcinogenicity potential for many types of regulated chemicals with significant human exposures currently relies on the use of two lifetime rodent bioassays. Problems with these bioassays are: numbers of animals used, long duration, high expense, histopathological complexities and potentially inconsistent results. Selected transgenic mouse models are discussed to provide a review of advantages and limitations of these methods.

Methods: The transgenic mouse models to be discussed will be: Tg.rasH2, Tg.AC and p53+/- as replacements for a mouse bioassay.

Results: A large-scale, multinational collaborative study that was co-ordinated by the Health and Environmental Sciences Institute of the International Life Sciences Institute and evaluated 5 different *in vivo* and *in vitro* assays will be briefly reviewed. Twenty-one well-characterised chemicals were submitted for testing with outcomes that were predicted and others that were surprising. In addition the recent regulatory experience of the US Food and Drug Administration with these methods will be presented.

Discussion: Three regional authorities (US, Europe, Japan) regulating pharmaceutical testing for carcinogenicity potential allow the use of transgenic mouse models in substitution for a mouse bioassay. The FDA policy for assessing the potential carcinogenicity of food additives still relies upon the completion of rat and mouse bioassays. Some of the reasons for this difference in testing policy will be discussed. Possible uses of these models in food additive testing will be presented.

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**Lecture**

**Summary and recommendations of the ECVAM/DG SANCO workshop on Three Rs approaches in marine toxin testing**

*Philipp Hess*, *Barbara Grune*, *Paolo Caricato* and *Marlies Halder*

1 Marine Institute, Galway Technology Park, Parkmore, Galway, Ireland; 2 Federal Institute for Risk Assessment, ZEBET, Berlin, Germany; 3 European Commission, DG SANCO, Brussels, Belgium; 4 European Commission, Joint Research Centre, Institute for Health and Consumer Protection, ECVAM, Ispra, Italy

Taking into account ongoing discussions on discrepancies between Council Directive 86/609/EEC on the protection of laboratory animals and the EU legislation on shellfish toxin testing, ECVAM and its Task Force on Shellfish Toxin Testing organised with DG SANCO a workshop held in January 2005 at ECVAM, which was attended by experts from national and international control laboratories and institutions as well as academia.

The objectives of the workshop were to: a) discuss the state of art of available methods and testing strategies; b) consider immediate possibilities to reduce and refine the currently required animal tests; and c) evaluate the status of non-animal methods regarding development, validation and regulatory acceptance for monitoring purposes and/or reference methods replacing the current animal tests. The outcome of the discussion and the recommendations will be presented.
The concern on the use of the animals, in particular for experimental purposes in the European Community, is laid down in the Council Directive 86/609/EEC of 24 November 1986 on the approximations of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. This Directive is currently being revised. The principles about animal welfare found in this Directive are also found in the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. In the founding Regulation of EFSA (Regulation No. 178/2002) it is stated that the mission of EFSA includes that “The Authority shall contribute to a high level of protection of human life and health, and in this respect take account of animal health and welfare …” (Article 22).

Methods: The EFSA Management Board at its meeting of 22 June 2004 supported EFSA’s willingness to develop a proactive animal welfare policy provided that this policy would be based on sound scientific principles. The scope of the task will be restricted to vertebrates used as experimental animals. The implementation of such a policy should stimulate the development of new food and feed assessment approaches that would not only minimise the numbers of experimental animals and their suffering, but also work towards their replacement through the use of alternative techniques (replacement, reduction and refinement, i.e. the Three Rs approach).

Results and Discussion: It is recognised that the implementation of fundamental changes to improve the welfare of experimental animals in relation to EFSA’s activities would take several years and can be achieved only step-by-step. The European Food Safety Authority requested its Scientific Committee to develop a stepwise approach to incorporate animal welfare approaches into EFSA’s activities without compromising the quality of the safety evaluations. The Scientific Committee focuses initially on tasks which can be completed within a reasonable time frame such as:

- Development of a comprehensive overview of all current EU legislative and guidance documents that address the experimental animals and their welfare. It should also address methodologies officially accepted or in use in the EU or some countries although not formally validated;
- Identification of guidance documents and procedures, currently applied by the EFSA Panels that could have an impact on experimental animals and their welfare;
- Making an inventory of all current activities of the Panels and Scientific Committee that relate to animal welfare, e.g. implementation of the Qualified Presumption of Safety approach, voluntary data sharing;
- Proposing ways to harmonise the application of guidance and legislative elements across EFSA Panels;
- Advising on how Panels could be kept informed of the latest scientific developments related to alternative methods to animal testing, and to internationally available, alternative approaches for hazard characterisation;
- Advising on how to improve sharing of information with organisations active in the area of animal welfare, e.g. bodies involved in the development and validation of methodologies for safety assessment, regulatory bodies requiring animal testing; and
- Advising on how to stimulate new research activities and new approaches in the field of risk assessment which would work towards the Three Rs policy.

Longer-term achievements could include proposals for the application or evaluation of new methods for risk assessment purposes, as well as new concepts in the risk assessment process that would take better account of the Three Rs.
The development of methods in modern biotechnology allows selected individual genes to be transferred from one organism into another and also between non-related species in a way that does not occur naturally. Foods produced using modern biotechnologies are known as genetically modified (GM) foods and widespread concerns have been expressed regarding their safety for human consumption. New challenges regarding safety assessment of GM foods are now being posed to both the food industry and food regulators. New foods are not traditionally subjected to extensive safety testing but rely on the fact that the parent varieties have a long history of safe use as food. Different approaches are required when assessing the safety of whole foods compared to chemical or microbial contaminants. Conventional risk assessment procedures that are used to determine the safety of discrete chemical entities are not particularly useful when applied to whole foods. Animal studies for assessing the toxicological endpoints of chemicals in the diet are a major element of conventional risk assessment. However animal studies cannot be applied in the same way to whole foods. This presentation will discuss the development of the concept of substantial equivalence which is used to structure the safety assessment of GM foods and will also address the limitations of animal testing when applied to whole foods.

**Lecture**

**Safety assessment of genetically modified foods**

*Alan Reilly*
Food Safety Authority of Ireland, Dublin, Ireland

The finding of acrylamide in food has raised concern about potentially toxic or carcinogenic compounds formed during heat processing in the Maillard reaction. In order to anticipate similar issues, we developed an approach to model the formation and human exposure to Maillard products formed through the same mechanism. The health significance of these molecules is difficult to assess since little or no toxicological information exists. Because of the number of molecules and exposure levels involved, it appears neither feasible nor necessary to characterise them all in detail.

In the absence of toxicological data, computational toxicology was used to predict chronic toxicity of the compounds identified, and the comparison to estimated exposure levels allowed to rank the compounds according to safety concern. Data obtained until today revealed that acrylamide is the compound of most concern and others are unlikely to raise significant safety concern.

The probability of mutagenic or carcinogenic activity of both the contaminants and potential metabolites formed was predicted in a computational approach. In order to verify results, *in vitro* mechanistic studies on primary hepatocytes were initiated to study the mechanism of toxic action, including gene expression and metabolomic profiling. Preliminary results of experimental data will be shown that confirm results of the modelling. We demonstrate in this study that under certain conditions, in the absence of information, a preliminary evaluation of safety concern is feasible, allowing priorisation of research needs and resources. An optimisation of animal use is also a consequence of the application of such an approach.

**Lecture**

**The use of chemical and computational modelling combined with *in vitro* toxicity testing to assess a safety concern of chemical contaminants in food**

*Gabriele Scholz and Benoît Schilter*
Nestlé Research Center, Quality and Safety, Lausanne, Switzerland
Poster

Using of an *in vitro* method for determination of toxic activity in food chain products

Alexey Solyakov and Per Häggblom
National Veterinary Institute (SVA), Department of Animal Feed, Uppsala, Sweden

Introduction: Mould growth in animal feeds, which form the basis of the food chain, can cause the production of mycotoxins. Intoxication by mycotoxins can cause suffering in animals, economic losses and the transfer of toxins to humans via foodstuffs. Therefore, there is a need to be able to identify feeds/foods of questionable quality. The objective of the study was to evaluate the possibility of using a cellular *in vitro* technique as a screening method for monitoring toxic components in mould-damaged silage.

Methods: A grass crop was ensiled in three ways: (1) aerobic storage, (2) aerobic storage plus a spore suspension containing *P. roqueforti* and *A. fumigatus*, and (3) anaerobic storage. Samples were taken after 45 and 90 days of ensiling, extracted, purified, applied to human neuroblastoma SH-SY5Y cells, and the general cytotoxicity was determined as described by Wenehed et al. (2003).

Results and discussion: After 45 and 90 days of ensiling, mould-damaged silage (methods 1 and 2) was more cytotoxic than control silage (method 3). The search for toxic secondary metabolites with standard chemical methods is possible but very time and labour consuming, as well as expensive. Thus the reported cell-based *in vitro* method can provide a practical and more realistic method of evaluating general toxicity in food chain products. The results of this study are important in the light of the requirements of EU legislation concerning the safety in the food chain.

References
In 1991 a contractual co-operation in harmonising medicines control started between the European Union and the European Directorate for the Quality of Medicines (EDQM)-Council of Europe (CoE): the EDQM has been charged of co-ordinating a network of national official medicines control laboratories (OMCL) and a research programme referred to as Biological Standardisation Programme (BSP). In line with the CoE convention on the protection of animals, the BSP establishes European Pharmacopoeia (Ph. Eur.) standards for biomedicines quality control with a special emphasis on alternative methods for the 3Rs. Sixteen projects on vaccines and one on blood products have been initiated in this field. The programme, run in the spirit of international harmonisation, involves the OMCL network, public and private sector medicines control laboratories in Europe, the Americas, Asia and Australia and non-European standardisation bodies. Completed projects on Newcastle disease and clostridial veterinary vaccines and on diphtheria and tetanus human vaccines led to new Ph. Eur. general methods and standards thus showing that the BSP promotes regulatory acceptance of alternatives. Further studies deal with botulinum toxin, vaccines for human use (inactivated poliomyelitis virus, hepatitis A, hepatitis B and pertussis) and tetanus immunoglobulin. For the future the programme hopes to benefit from synergies between fundamental, medical and pharmaceutical sciences experts for promoting animal welfare aspects in control whilst guaranteeing quality, safety and efficacy to biomedicines potential users. To prompt interactions, the development of a particular BSP project in the field of vaccines control will be presented in Berlin with a critical review of key steps emphasising all specific and global implications.
Poster
The use of Mono Mac 6 cells as indicators of endotoxin contamination in the quality control of injectable products
Cristiane Caldeira, Izabela da Costa Gimenes, João C. B. Rolim de Freitas and Octavio A. F. Presgrave*
National Institute of Quality Control in Health, Department of Pharmacology and Toxicology, Rio de Janeiro, Brazil

Introduction: The rabbit pyrogen test is used for detecting contaminations of injectable products. Although broadly accepted, both ethical and economic considerations call for a replacement by in vitro methods. Although LAL is to be considered as a possible replacement to rabbit test, it has the limitation of detecting only endotoxins. The test systems using human whole blood and cell line Mono Mac 6 (MM6) have been proposed for detecting pyrogenic contamination. Although the human whole blood assay has greater relevance to the in vivo situation, it is not yet widely accepted. The aim of this study was to use the MM6 as an indicator of the presence of endotoxin due to its feasibility of processing and low variability.

Methods: The MM6 was provided by Dr. Ziegler-Heitbrock and Dr. Stephen Poole. The cells were incubated in the presence of different LPS concentrations overnight. After this period supernatant was collected and interleukins 1b and 6 release were determined by ELISA.

Results: MM6 presented a good dose-response relationship with linear region between 0.06 and 1.0 EU/ml. Our results showed that MM6 was able to distinguish the threshold pyrogenic concentration (5 UE/ml) from the negative control. Linear regression was used (r=0.889).

Conclusion: These results suggest that this in vitro test can detect endotoxin with high sensitivity and it is able to detect different pyrogen levels. MM6 seems to be a good alternative for replacing the rabbit test in cases of ethical problems on using human beings as donors of whole blood.

Poster
A new method for determining in vitro potency of hepatitis B in combined vaccines
Mario L. Chovel Cuervo, Ivette Abreu Nicot, Ana L. Sterling, Mabel García Rodríguez and Orlando R. Pérez
Cuban Centre for Quality Control of Drugs, Cuban National Control Laboratory, Havana, Cuba

Although some manufacturers have developed in vitro potency tests for monitoring consistency of hepatitis B vaccines, the in vivo test remains as the most suitable for the evaluation of hepatitis B component in combined vaccines. Nonetheless, the Cuban National Control Laboratory has evaluated an in-house method that allows to get reliable hepatitis B potency results in vaccine combinations. The aim of this paper was to evaluate the potential interferences of the rest of components on hepatitis B vaccines and set up an in vitro potency test for lot release of combined vaccines. We evaluated combined vaccines from different manufacturers and compared the results regarding monovalent vaccines. At the same time, we prepared some experimental vaccine formulations in order to discriminate potential interferences on the hepatitis B component, including the adjuvant effect. In all cases we performed a neutralisation ELISA using Hepanostika anti-HbsAg kit. It was shown that there’s no significant interference on hepatitis B in combined vaccines, although the results were consistently lower than monovalent vaccines. This most likely arises from a complex antigen mimicking effect of Bordetella pertussis whole cells conforming combined vaccines. In spite of this, all combined vaccines successfully passed the specification defined for our in vitro test of hepatitis B vaccine. Besides, we got a relatively significant correlation between our in vitro and the in vivo potency test. Hence, we have available a reliable, fast and accurate test for lot release of hepatitis B in combined vaccines.
Traditionally the in vivo potency tests have had an important role in the quality control of vaccines. However, this tendency has dramatically changed in the world. Nowadays the main trend is the development of alternative methods based on the principle of 3R. There are several previous facts to the development of alternatives methods. One of them is the development of an in vitro potency assay for the recombinant hepatitis B vaccine, which was already recognised by WHO. In Cuba there have been some interesting approaches in alternative toxicology. The aim of this lecture is to show the current status of the development and implementation of alternative methods in Cuba for vaccines. In our country we have successfully implemented some alternative methods (in vitro) for routine quality control tests of vaccines like hepatitis B, DT, DTP, rabies and Haemophilus influenzae type b. Some of these methods have been developed in our laboratories (in house techniques) and all of them have been correlated in regard to the in vivo assays. At the present time, these methods are being used by the National Control Laboratory and the vaccine manufacturers.

The Humane Society of the United States (HSUS), as part of its Pain and Distress Campaign, held a workshop of international experts in August 2002 in order to develop recommendations for minimisation of pain and distress associated with polyclonal antibody (Pab) production. A group of twelve experts in the fields of antibody production, animal welfare, in vitro alternatives, animal protection and/or regulatory compliance participated in the roundtable discussion. Several aspects of Pab production were considered, including: determination of appropriate adjuvants; optimal volume of adjuvant, number of injection sites, and route of immunisation; use of booster injections; availability of alternatives; and measurement of animal welfare. Recommendations were made on each of these topics in regards to minimising pain and distress. General recommendations addressed outsourcing to reputable Pab suppliers; improving training of personnel; improving pain and distress assessment via score sheets; harmonising guidelines internationally; minimising the number of animals used when possible; and including relevant Pab production information in published papers. Specific recommendations included using the chicken egg yolk technique as a refinement and reduction procedure; choosing an adjuvant that produces high antibody yield while minimising pain and distress; using the smallest volume of adjuvant possible; determining appropriate use of booster injections; and discouraging use of intramuscular, intraperitoneal, intrasplenic, intravenous and footpad injections while allowing use of subcutaneous and intradermal injections. Finally, areas that require additional research were discussed; such as proper pain and distress assessment; formulation of new adjuvants; and determining the influence that pain, distress and environmental enrichment may have on Pab production.
The hepatitis B vaccines have been available since 1982 and billion doses have been used. Approximately 100 countries according to World Health Organization policy, have added hepatitis B vaccination to their routine immunisation program. The methodologies for production and control these vaccines are based on WHO’s recommendations and the European Pharmacopoeia. Historically, potency test to release vaccine consists of an immunological assay in animals. Frequently, these in vivo tests required a large numbers of animals for potency assays. This test involves the immunisation of groups of mice with diluted test and reference vaccines. The in vitro method based on the quantification of hepatitis B surface antigen. Dilutions of test and reference vaccines are assayed in a parallel line to quantify the antigen in each preparation. We tested vac- cines’ batch from four different manufacturers. The number of the mice used to in vivo assays was 9,000 approximately. After introduction of the in vitro assay at INCQS this number reduced gradually. In 2004 the in vivo assay was replaced completely. The development in alternatives methods to replace the use of laboratory animal improved animal welfare, economic, safety and scientific considerations.

Poster

Alternative method for potency test of hepatitis B vaccine

Catia Costa, Jaline Costa, Eduardo Pereira and Maria Paixão
FIOCRUZ-INCQS, Immunology, Rio de Janeiro, Brazil

Every batch of vaccines is tested for potency and safety, currently consuming approximately 10 million animals per year (an estimated 10% of all animal use in biomedical experimentation globally). Animal potency testing involves a pathogenic challenge, and thus causes much suffering. Animals can be entirely replaced in potency testing by physico-chemical methods of quantifying antigens (the part of the vaccine that stimulates the protective response) if they are known, but for most currently used vaccines which were developed empirically, the protective antigens are not known. This is problematic because poorly characterised vaccines are difficult to test in ways other than black-box animal studies, but animal potency tests are often unpredictable due to species differences as well as non-biological routes of exposure (e.g., rabies inoculation through intracerebral injection in the NIH test). Antigen quantification systems have been devised for some older vaccines, but resources may be better spent focusing on new vaccines and novel methods of vaccine development. As researchers deduce the sequences and structures of pathogenic proteins and develop a detailed knowledge of their roles, they can purposefully design vaccines with defined components in order to maximise effectiveness and minimise safety concerns. Computational immunology can help predict which epitopes are likely to be the best targets. Fortuitously, rational vaccine design by its nature goes hand-in-hand with non-animal testing since designing vaccines to contain defined antigens enables the direct measurement of antigenic content, thus rendering the use of animals in potency testing obsolete.

Poster

Rational vaccine design: Rendering black-box animal potency testing obsolete

Sadhana Dhruvakumar
People for the Ethical Treatment of Animals (PETA), Research and Investigations, Norfolk, VA, USA
The Key Area Biologicals covers ECV AM’s activities related to reduction, refinement and replacement of animal tests stipulated for the quality control of immunobiologicals, hormones, blood products and other related products, as well as for pyrogenicity and shellfish toxin testing. ECV AM has established a Steering Group on Biologicals and Task Forces for Pyrogenicity and Shellfish Toxin Testing.

Activities on immunobiologicals and hormones resulted in the regulatory acceptance of several alternative methods for the quality control of various products and deletion of animal tests for routine quality control. ECV AM continues its activities by funding validation studies, commenting on European Pharmacopoeia monographs/EU guidelines and organising workshops. Thus, one workshop held in 2005 was focused on biochemical methods for the quality control of toxoid vaccines and another is planned on consistency of production approach for less well-defined vaccines.

Regarding pyrogenicity testing, various in vitro methods based on fresh and cryopreserved human monocytoid cells were validated in a 5th Framework Programme project during 2000-2003 and in a catch-up validation study during 2004. They are currently peer reviewed by ECV AM’s Scientific Advisory Committee.

Co-operation with DG SANCO on replacement of the mouse bioassay for shellfish toxin testing started in 2004 and a workshop was held in early 2005 (see also poster/oral presentation Hess et al). ECV AM is currently collaborating with the Community Reference Laboratory (Vigo, Spain) on the validation of a functional assay for detection of diarrhetic shellfish toxins.

Poster

**ECVAM Key Area Biologicals: Summary of activities**

*Marlies Halder*
ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra, Italy

The Key Area Biologicals covers ECV AM’s activities related to reduction, refinement and replacement of animal tests stipulated for the quality control of immunobiologicals, hormones, blood products and other related products, as well as for pyrogenicity and shellfish toxin testing. ECV AM has established a Steering Group on Biologicals and Task Forces for Pyrogenicity and Shellfish Toxin Testing.

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**Poster**

**International validation of novel pyrogen tests based on human monocytoid cells**

*Thomas Hartung ¹ and Stefanie Schindler²*
¹ ECVAM, Institute for Health and Consumer Protection, Joint Research Center, Ispra, Italy; ² University of Konstanz, Konstanz, Germany

Parenteral medicines are required to be tested for pyrogens (fever-causing agents) in one of two animal-based tests: the rabbit pyrogen test and the bacterial endotoxin test. Understanding of the human fever reaction has led to novel non-animal alternative tests based on in vitro activation of human monocytoid cells in response to pyrogens. Using 13 prototypic drugs, clean or contaminated with pyrogens, we have validated blindly six novel pyrogen tests in ten laboratories. Compared with the rabbit test, the new tests have a lower limit of detection and are more accurate as well as cost and time efficient. In contrast to the bacterial endotoxin test, all tests are able to detect Gram-positive pyrogens. The validation process showed that at least four of the tests meet quality criteria for pyrogen detection. From two of these methods, the development of successful cryopreservation procedures led to the validation of fresh and cryopreserved human whole blood or isolated PBMCs in four laboratories using the same protocol. The tests reached >90% sensitivity and specificity.

The here validated in vitro pyrogen tests overcome several shortcomings of animal-based pyrogen tests. Our data suggest that animal testing could be completely replaced by these evidence-based pyrogen tests and highlight their potential to further improve drug safety.
**Poster**

**International validation of novel pyrogen tests based on human monocytoid cells**

Sebastian Hoffmann¹, Anja Peterbauer², Stefanie Schindler³, Stefan Fennrich³, Stephen Poole⁴, Yogesh Mistry⁴, Thomas Montag-Lessing⁴, Ingo Spreitzer³, Bettina Loeschner⁵, Mirjam van Aalderen⁶, Rogier Bos⁶, Martin Gommer⁶, Ria Nibbeling⁶, Gabriele Werner-Felmayer⁷, Petra Loitzl⁷, Thomas Jungi⁸, Marija Brcic⁸, Peter Bruegger⁹, Esther Frey⁹, Gerard Bowe¹, Juan Casado¹, Sandra Coecke¹, Jan de Lange¹, Bente Mogster¹⁰, Lisbeth Naess¹⁰, Ingeborg Aaberge¹⁰, Albrecht Wendel³ and Thomas Hartung³

¹ European Commission, JRC, ECVAM, Ispra, Italy; ² Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Linz, Austria; ³ University of Konstanz, Institute of Biochemical Pharmacology, Konstanz, Germany; ⁴ National Institute for Biological Standards and Control, Potters Bar, United Kingdom; ⁵ Paul-Ehrlich Institut, Langen, Germany; ⁶ RIVM, National Institute of Public Health and the Environment, Bilthoven, The Netherlands; ⁷ Institute of Medical Chemistry and Biochemistry, Innsbruck, Austria; ⁸ University of Bern, Institute of Veterinary Virology, Bern, Switzerland; ⁹ Novartis Pharma AG, Biological Analytics, Basel, Switzerland; ¹⁰ Norwegian Institute of Public Health, Division of Infectious Disease Control, Oslo, Norway

It is a requirement that parenteral medicines be tested for pyrogens (fever causing agents) using one of two animal-based tests: the rabbit pyrogen test and the bacterial endotoxin test. Understanding the human fever reaction has led to novel non-animal alternative tests based on in vitro activation of human monocytoid cells in response to pyrogens. Using 13 prototypic drugs, clean or contaminated with pyrogens, we have validated blindly six novel pyrogen tests in ten laboratories. Compared with the rabbit test, the new tests have a lower limit of detection and are more accurate as well as cost and time efficient. In contrast to the bacterial endotoxin test, all tests are able to detect Gram-positive pyrogens. The validation process showed that at least four of the tests meet quality criteria for pyrogen detection. These validated in vitro pyrogen tests overcome several shortcomings of animal-based pyrogen tests. Our data suggest that animal testing could be completely replaced by these evidence-based pyrogen tests and highlight their potential to further improve drug safety.

**Lecture**

**Achieving the 3Rs in the manufacture and testing of veterinary vaccines – opportunities and challenges**

Peter Johnson¹, Rosemarie Einstein*², Lynette Chave¹, Margaret Rose² and Ross Burton¹

¹ Animal Welfare Unit, NSW Department of Primary Industries, Sydney, Australia; ² Animal Research Review Panel, NSW Department of Primary Industries, Sydney, Australia

In New South Wales, in 2002, 18% of the laboratory animals used for research on human or animal biology and health were used in regulatory product testing where the procedures involved death as an endpoint. Testing is mandatory in the production of biological products, mainly livestock vaccines produced for local use and export. In New Zealand 20% of animals were used in research for commercial purposes including 6% subjected to manipulations in the severe and very severe categories, primarily for regulatory testing. In the Netherlands 15% of laboratory animals used in research were involved in vaccine production. Variations between countries will reflect the types of vaccines produced and the specific testing requirements for each. Before LD₅₀ and similar tests may proceed in New South Wales, the law requires Ministerial concurrence upon the recommendation of the NSW Animal Research Review Panel, which monitors legislation regulating the use of animals in research and teaching. Through consultation with industry, significant refinements and some reductions in the use of animals have been achieved. The development of in vitro alternatives and phase-out of in vivo tests may be difficult, but success will bring substantial animal welfare payoffs, together with improved efficiencies for this industry. However, where animal based tests are used, the requirements of regulatory testing are often at odds with implementation of the 3Rs and further reductions are unlikely without an international effort to identify opportunities and strategies for progressively replacing animals in the regulatory testing of veterinary and other biological products.
THEME 5, SESSION 5.9

Poster

**Development of an in vitro assay for testing the safety of tetanus vaccines**

Birgit Kegel, Ursula Bonifas, Heike Behrens-Nicol, Jolanta Klimek, Katja Silberbach, Karin Weisser and Beate Krämer
Paul-Ehrlich-Institut, Federal Agency for Sera and Vaccines, Langen, Germany

The characteristic spastic paralysis associated with tetanus infections is caused by a powerful neurotoxin (tetanus toxin) produced by *Clostridium tetani*. This toxin is a zinc-dependent metalloprotease which specifically cleaves synaptobrevin, a key molecule in neurotransmitter release.

Tetanus vaccines are prepared from purified tetanus toxin by chemical inactivation. According to the European Pharmacopoeia, safety testing is required for every batch of tetanus toxoid (inactivated tetanus toxin) to ensure the absence of residual toxin activity, and to exclude reversion to toxicity. At the present state, only *in vivo* methods exist for these safety tests. We are currently developing an *in vitro* assay as a fast and reliable alternative to these safety tests in animals.

Our method is based on the detection of the proteolytic activity of tetanus toxin in an ELISA format. For this purpose, recombinant synaptobrevin is immobilised and incubated with the test samples. If the samples contain any active toxin, cleavage of synaptobrevin occurs and can be quantified using an antibody which specifically recognises the cleavage product. With this assay, we are able to detect low amounts of purified tetanus toxin (current sensitivity in the range of the LD50). Furthermore, we show that the assay allows us to detect active toxin in tetanus toxoids (spiked samples).

Our present studies concentrate on further increasing the sensitivity of the test in order to achieve an even closer approximation to *in vivo* conditions. We are confident that this novel method will represent a useful tool for the safety testing of tetanus vaccines.

Poster

**The comparative effect between high diluted Calendula officinalis in 6CH dinamisation, placebo and culture medium on growth of cultured mammalian cells. Preliminary tests**

Maria F. G. Klingbeil¹, Monica B. Mathor¹ and Ida Caramico-Soares²
¹IPEN/CNEN-SP Instituto de Pesquisas Energéticas e Nucleares de São Paulo, CTR Centro Tecnológico das Radiações, São Paulo, Brazil; ²USP – Universidade de São Paulo, Pharmacy, São Paulo, Brazil

We have tested the *in vitro* effect from *Calendula officinalis* on duplication of 3T3 murine fibroblast compared with placebo and standardised normal culture medium. It is already known the antiseptic, tissue repair, antinflammatory and cicatrisation properties from *Calendula officinalis*. This herb belongs to the family from the vulnerable plants. Its constitution has the three most important groups: the flavonoids, the volatile oils and the triterpenos.

The utilisation of the cell culture technics has often been demonstrated to achieve similar results to the once compared *in vivo*, and allow us to explain the intrinsic potential from some drugs. Murine fibroblast 3T3 were cultivated with standard culture medium DMEM, supplemented with 10% fetal bovine serum (FBS). The medium was changed to DMEM without FBS, to get the G0 cell point. After 24 hours the medium was replaced with the medium to be tested: 1. normal culture medium supplemented with 10% fetal bovine serum, 2. the same medium with homeopathic *Calendula officinalis* 6CH, diluted in purified water added at the concentration 1/25, 3. the same medium with dinamised purified water added at the concentration 1/25. The cells were stained with Trypan Blue to exclude the died cells and counted in a haemocytometric chamber.

There was an increase in the cell quantity in the presence of *Calendula officinalis* 6CH and the placebo. Further investigations for a longer time period should be done, with higher dinamisations, higher concentrations, different medicaments and other cell types.

Financed for CNPq – Conselho Nacional para o Desenvolvimento da Pesquisa.
Lecture
United States Department of Agriculture
"3Rs" initiatives in veterinary biologics

Jodie Kulpa-Eddy
USDA-APHIS-Animal Care, Riverdale, Maryland, USA

This presentation will consist of a brief overview of the U.S. system of regulatory oversight of veterinary biologicals (vaccines, bacterins, and diagnostic test kits intended for use in animals). A historical perspective will be given on the impact of alternative test methods on the use of animals for regulatory test requirements.

Examples will be provided regarding the USDA’s current initiatives to fulfill its strategic goal of significantly refining, replacing and reducing animal testing of veterinary biologics. This will include updates on information presented at the Fourth World Congress on Alternatives and Animal Use in the Life Sciences, held in New Orleans, Louisiana (USA) in 2002.

Poster
In vitro models to study biological activity of toxoid vaccines

Marlies Leenaars 1, Sytse Piersma 1 and Coenraad Hendriksen 2
1 Netherlands Vaccine Institute (NVI), Bilthoven, The Netherlands;
2 Netherlands Centre Alternatives to Animal Use (NCA), Utrecht, The Netherlands

Although substantial progress has been achieved in reduction and refinement of the use of laboratory animals in quality control of conventional produced vaccines (like diphtheria and tetanus), still large numbers of animals are required, particularly for potency testing. Application of the “consistency approach” using in vitro tests, bring replacement of in vivo potency tests for toxoid vaccines within reach. Consistency in structure and conformation of the toxoid antigens can be monitored using (new) chemical techniques. However, additional to these chemical tests, functional tests will be needed to confirm the biological activity of the vaccine (antigen). The poster deals with a project at NVI in which the biological activity of vaccine antigens is studied by in vitro immune response models.

Tetanus is used as a model antigen. Tetanus toxoid batches of different quality were in vitro compared for their immunogenicity (cytokine production, cell proliferation, cell stimulation etc.) using murine spleen cells, porcine PBMC’s and human PBMC’s.

Based on the cytokine profiles that were induced after in vitro stimulation, tetanus toxoid batches of different quality could be distinguished. The in vitro immune response models are considered a promising tool to demonstrate consistency in biological activity of toxoid antigens. Application of these models, combined with chemical tests, will finally result in replacement of large scale in vivo potency tests of toxoid vaccines.
Lecture

Current approaches to animal testing in regulation of biologics and vaccines by U.S. FDA/CBER

Richard D. McFarland
United States Food and Drug Administration, Center for Biologics Evaluation and Research, Rockville, USA

Current laws administered by FDA – including the Federal Food, Drug and Cosmetic (FD&C) Act, and the Public Health Service (PHS) Act – are intended to ensure product safety and effectiveness, thereby protecting the health of the U.S. consumer. These laws place responsibility on FDA’s Center for Biologics Evaluation and Research (CBER) to ensure that the products it regulates, such as vaccines, blood products, tissues, and somatic cell and gene therapies, are safe and effective. Currently, animal testing by manufacturers seeking to market these products is often necessary to establish product safety prior to human exposure. CBER supports and adheres to the provisions of applicable laws, regulations, and policies governing animal testing, including the Animal Welfare Act, the ICCVAM Authorization Act of 2000, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Moreover, in all cases when animal testing is used, CBER advocates that research and testing using animals derive the maximum amount of useful scientific information from the minimum number of animals, while employing the most humane methods available. CBER recognises that emerging areas of biopharmaceuticals provide an opportunity to incorporate innovative testing strategies into the product development and manufacturing paradigms. While committed to its primary mission to protect the public health, CBER suggests and encourages the development, validation and use of alternative testing methods that refine, reduce or replace animal testing. CBER advocates the use of validated non-whole animal techniques, which may include in vitro methodologies (e.g. tissue culture, cell-based assays), biochemical assays, or emerging proteomic and genomic methodologies.

Lecture

Alternatives for potency testing of toxoid vaccines: A realistic option?

Bernard Metz¹, Wim Jiskoot², Coenraad Hendriksen¹ and Gideon Kersten¹
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²University Utrecht, Pharmaceutics, Utrecht, The Netherlands

Introduction: The most critical step in the production of diphtheria vaccines is the inactivation of the toxin by formaldehyde. Diphtheria toxoid is produced during this inactivation process through partly unknown, chemical modifications of the toxin. Consequently, diphtheria vaccines are difficult to characterise and the quality of the toxoids is routinely determined with potency and safety tests. We have developed a series of physicochemical and immunochemical tests for monitoring product quality.

Methods: Diphtheria toxin was treated with increasing formaldehyde concentrations resulting in toxoid products varying in potency and residual toxicity. Differences in the quality of the experimental toxoids were also assessed with the following in vitro techniques: electrophoresis, primary amino group determination, fluorescence spectroscopy, circular dichroism and biosensor analysis. Subsequently the methods were used to analyse routine toxoid samples from different manufacturers.

Results: The results obtained with experimental toxoids correlated well with the potency and safety tests. Further assessment of the methods with routine samples showed that one test (circular dichroism) needs further evaluation and for another assay (electrophoresis) adaptation of criteria were necessary.

Discussion: The methods developed are excellent for demonstration of comparability of toxoid batches. This opens the possibility to limit the number of animal tests to one per bulk instead of testing the bulk every time it is used to prepare a batch of final lot. In principle the set of in vitro analyses can replace the classical in vivo tests completely, provided that the validity of these tests is demonstrated in extensive validation studies and regulatory acceptance is obtained.
Poster

Using HET-CAM for testing mucosal irritation potential of vaccines and vaccine adjuvants

Gisela Antonia Murillo
Centre for Toxicology and Biomedicine (TOXIMED), Santiago de Cuba, Cuba

The Centre for Toxicology and Biomedicine (TOXIMED) in Santiago de Cuba has among its tasks the toxicity testing of vaccine components and vaccine formulations, using traditional methods (in vivo) as well as alternatives (in vitro). The vaccine application by mucosal via is of high-priority interest in the contemporary vaccinology; however, the pre-clinical study in vivo of the mucosal irritation potential of a vaccine component or its formulation is a difficult procedure, not only for the way done but also for the interpretation of the results. On the other hand, the assay may cause suffering to the laboratory animals and there is not a standardised or internationally validated method that allows its routinary use. The fact that any ocular irritating substance may be also irritating for other mucosa led to consider the possible usefulness of the hen’s egg test on chorioallantoic membrane (HET-CAM) to evaluate the mucosal irritation potential of vaccines and adjuvants. Seven substances used as adjuvants or candidates to adjuvants and four vaccine formulations were tested. The values obtained led to classify them as no irritating. New evidences of how useful the HET-CAM is to determine the mucosal irritation potential of vaccines and vaccine adjuvants are given in this work.

Poster

The use of cytokine release (whole blood assay) for detecting pyrogens in anti-venom sera

Octavio A. F. Presgrave¹, Fernanda Peres Sabagh¹, Luana Fortes Faria¹, Cristiane Caldeira¹, João C. B. Rolim de Freitas¹, Izabela da Costa Gimenes¹, Hugo C. de Faria Neto², Patrícia Torres Bozza², Isabel Dieterich³, Ilona Kindinger³ and Thomas Hartung³

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Introduction: Pyrogen is one of the most important problem of injectable products. The rabbit assay is still used, mainly for products like hyperimmune sera (e.g. anti-venom sera) and vaccines, since these products strongly interfere in the LAL reaction. The use of animal in experimentation has been severely criticised during the last years. In order to reduce the number of animals subjected to pain and distress, many alternative assays have been studied. Although LAL is to be considered as a possible replacement to rabbit test, it has the limitation of detecting only endotoxins. The whole blood assay was developed for replacing the in vivo pyrogen test since it can detect all types of pyrogens. Brazil has a large production of anti-venom sera and it represents about 70% of pyrogen tests performed at INCQS. The number of rabbits used reaches more than 1,000 per year. So, the cytokine release assay could be useful for replacing rabbits for detecting pyrogens in this kind of products.

Methods: Human whole blood was incubated with hyperimmune sera overnight. After this period supernatant was collected and cytokines (IL-1β and IL-6) release was measured by ELISA.

Results: When tested undiluted, hyperimmune sera showed strong cytotoxicity with no cytokine release. When diluted 1:10 in order to avoid cell death, cytokine release presented a good dose-response curve and distinguished the rabbit threshold pyrogenic dose (5 EU/ml) from a non-pyrogenic dose (2.5 EU/ml).

Conclusion: The whole blood assay can be used for detecting pyrogen contamination in hyperimmune sera diluted 1:10.
Poster

Program to reduce animal testing for EPA registrations of antimicrobial products – A novel approach to animal alternatives

Len Sauers¹, Karen Acuff¹, Dan Marsman¹, Usha Vedula², Nicole Cuellar², Rodger Curren³, John Harbell¹ and Pat Quinn¹

¹The Procter and Gamble Company, Cincinnati, Ohio, USA; ²The SC Johnson Company, Racine, Wisconsin, USA; ³Institute for In Vitro Sciences, Gaithersburg, Maryland, USA; ⁴The Accord Group, Washington, DC, USA

In the US, antimicrobial cleaning products are registered as pesticides with the US EPA. To support registration, an assessment of dermal and eye irritation must be completed. Normally required for such assessments are data from the Draize eye and Draize skin irritation tests. For antimicrobial cleaning products, much research has been done to develop non-animal alternatives to these tests, and today these alternatives are routinely used by many to make safety and labelling decisions. However, because these tests have not undergone formal validation, there are barriers to their acceptance by the EPA. Although the database is sufficient to support use of these alternatives for antimicrobial cleaning products, there are data gaps for some materials outside this specific category, which inhibits validation for all formulations. Therefore the companies that manufacture antimicrobial cleaning products have embarked on a novel, modular program with the US EPA to obtain acceptance of alternative methods specifically for this limited category of products – antimicrobial cleaning products. These companies have come together to define the specific formulation types that are to be included in this evaluation and to provide all the animal and non-animal data necessary to demonstrate the predictability of these assays for the purpose of determining appropriate cautionary labelling. This information will be assembled and submitted for a technical review by an expert panel organised by ICCVAM. A favourable review by this panel would lead to new EPA guidelines, allowing the use of these alternative approaches for these specific formulations.

Lecture

International validation of pyrogen tests based on fresh and cryopreserved human primary blood cells

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Pyrogens as fever-inducing agents can be a major health hazard in parenterally applied drugs. For the control of these contaminants, pyrogen testing for batch release is required by Pharmacopoeias. This has been done either by the in vivo rabbit pyrogen test (since 1942) or the limulus amoebocyte lysate test (LAL), since 1976. A new approach are cell-based assays employing in vitro cultivation of human immune cells which respond e.g. with cytokine production (IL-1, IL-6) upon contact to pyrogens. 6 variants of these assays have recently been validated in a collaborative international study. From two of these methods, the development of successful cryopreservation methods promises to make standardised immunoreactive primary human blood cells available for widespread use. Furthermore, the pre-testing of donors for infectious agents such as HIV or hepatitis has made it possible to develop a safe and standardised reagent for pyrogen testing. Using altogether 13 drugs, we have validated here two pyrogen tests based on fresh and cryopreserved human whole blood or isolated PBMCs in four laboratories. The tests reached >90% sensitivity and specificity. In contrast to the LAL, the tests are capable of detecting non-endotoxin pyrogens derived from Gram-positive bacteria or fungi.
Lecture

Animal usage in quality control tests for the release of immunological veterinary medicinal products in the United Kingdom

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Animals and animal derived starting materials are used routinely in the production and quality control of immunological veterinary medicinal products (IVMPs). Quality control (QC) tests are necessary to provide assurance that each batch of an IVMP is safe and efficacious before it is released onto the market. The Veterinary Medicines Directorate (VMD) has recently conducted a study to investigate the extent of animal usage in QC tests for the release of IVMPs onto the UK market in 2003 and 2004. This has identified a number of areas where efforts could be made in reducing or eliminating some animal tests on IVMPs.

The project investigated the number of batches of authorised IVMPs released onto the UK market, the in-process and final product QC tests performed on each batch for release purposes and the number of animals used to conduct these tests.

In 2003, the VMD released 1101 batches of IVMPs onto the UK market for 221 products. A total of 31,047 animals were used in QC tests in 2003 and 26,160 in 2004. The QC tests, in which the majority of animals are utilised, were the batch potency tests and safety tests, accounting for 52.1% (16.175) and 20.9% (6,480) respectively of animals in 2003. Other significant animal tests include extraneous agents testing of avian vaccines (10%, 3,111) and absence of toxicity of clostridial vaccines (15.4%, 4,773).

Under the new provisions of the European Pharmacopoeia it is now possible for manufacturers to remove the final product batch safety test, subject to agreement of the competent authority and providing data on a sufficient number of consecutive batches to support the safety of the product. The number of animals used in batch safety tests for IVMPs will continue to be monitored to assess the progress in reducing animal usage. Furthermore, the amended monograph for the final product test for extraneous agents in live avian vaccines should result in a similar reduction of animal usage.

To reduce animal usage in final product QC tests for potency, further research and investment is need to develop satisfactory in vitro alternatives to the in vivo tests used for most inactivated vaccines.

Poster

The 3Rs – A breeder’s perspective

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Reduction, refinement and replacement, as put forward by Russell and Burch, are partial solutions to finding alternatives to the use of laboratory animals in research, education and testing. As a commercial breeder we believe that harvesting body fluids and tissues from laboratory animals that no longer have a commercial value is an ethical way of achieving both reduction and replacement.

This presentation describes how as a commercial breeding and supplying establishment we have implemented a policy for the minimisation of overproduction and wastage of animals. It also describes how this policy has allowed us to successfully implement the 3Rs, by the use of unused and unsold animals for the harvesting of biological samples. To achieve this one of our main concerns was making use of the animals we had available from our own breeding colonies to supply biological samples such as blood products, body fluids, tissues and danders. In doing this we believed that it would lead to a reduction in the number of animals being bred for the same purpose at other establishments. Similarly, in the time that we have been supplying such products our expertise has improved so that we are now more efficient and are able to obtain the same amount of material from fewer animals. In addition, our techniques have been refined to improve not only the quality of the products, but also our efficiency and more importantly animal welfare.
Post

**In vitro evaluation of the release profile of a papain-incorporated non-cytotoxic polymeric matrix**

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Drug delivery systems for topical administration in therapeutics treatments have attracted attention due to advantages such as elimination of the first passage effect, reduction of adverse effects and ease of interruption in cases of intolerance. Papain is a proteolytic enzyme widely used in dermatology and cosmetology in wounds treatment, peelings and skin whitening. Toxicity studies of papain were done and are related in the literature. In addition, efficacy and assurance of the enzyme were evaluated in vitro by using keratinocytes cell cultures. Papain is usually incorporated in common base vehicles, such as emulsions and gels. Such vehicles, however, require more than one application a day, which reduces patient compliance and consequently reduces treatment efficacy. In this research we report the development of a biological system for controlled release of papain based on elastomer silicone polymeric matrix. The polymer was assessed non-cytotoxic by using in vitro Neutral Red method with NCTC cell cultures. The delivery properties of the papain-containing membranes were evaluated in vitro over 28 hours period by analysing the amount of released enzyme using Specific Substrate Dosage Test. Preliminary results indicate the possibility of this biological system control the release of papain during one day.
The bioaccumulation (“B”) of a chemical in aquatic organisms can be determined by exposing fish to a chemical via water and/or food, and monitoring its uptake and loss. The standard method requires numerous animals, excessive labor, and costly analytical methods. So, a tiered approach is proposed for the evaluation of bioaccumulation in fish. *In silico* methods may be used to predict bioaccumulation potential. The current “B” models are based solely on chemical lipophilicity, and several models ignore key parameters like metabolism. Methods are being explored to incorporate absorption, distribution, metabolism, and excretion (ADME) properties in “B” predictions. ADME parameters can be quantified using *in vitro* methods, although few fish data are now available. A recent bioaccumulation workshop in Cincinnati, USA focused on ideas to optimise and validate *in vitro* fish methods because the quantitative relationship of *in vitro* to *in vivo* results is lacking. A key action step is for a subgroup to develop methods and choose environmentally significant test materials to compare results of *in vitro* and *in vivo* tests from different labs. Planned action steps and preliminary methods from this subgroup will be presented. This effort is expected to reduce time, labor, and animal usage for bioaccumulation testing needed to meet developing global regulatory demands.
Acute ecotoxicity tests on vertebrates are required by law for hazard characterisation and for environmental risk assessment of chemicals, plant protection products (PPP), biocides and veterinary medicines. While acute toxicity data on mammals and birds are only required for the registration of the active substances of PPP, acute fish bioassays needs to be performed in all cited substances and chemicals, and in some EU countries, for Whole Effluent Assessment.

3R approaches for mammals in environmental risk assessment are benefitting from the advantages reached in the process of hazard characterisation for humans, on the contrary little attention has been paid to acute oral toxicity data on birds. Almost no advancements have been made in applying the 3Rs in this area.

Most progress over the last 5 years has been made concerning the acute fish bioassays: Reductions up to 75% in the number of fish was proposed by Hutchinson and co-workers in 2001 with the concept of the threshold approach and is on the way to be peer reviewed by ECVAM. Refining the acute bioassay on adult fish by adopting the fish embryo test (Nagel, 2002) was already proposed and in some countries is the way to be adopted for WEA. A very interesting approach combining the 3Rs: i.e. replacement by fish cells, reducing using the threshold approach and refining by means of a fish-embryo test is in the way to be proposed for the expert group of the ECVAM taskforce on Ecotoxicology. My presentation will analyse the present situation and outline future prospects.

The public discussion on the Three Rs in animal testing mainly focuses on mammalian testing in human toxicology. Rarely it is realised that a considerable number of non mammalian vertebrates are used in regulatory ecotoxicology. This number may even increase when the new European chemical legislation will become effective by the end of this decade and respective toxicity data will be required for more than 10,000 substances.

By analysing the number of animals used in regulatory ecotoxicology it becomes evident that most vertebrates are used for fish acute toxicity testing. This test is a basic ecotoxicological requirement in most environmental regulations that are triggered by chemical’s effects. The German Federal Environmental Agency has been given priority to the replacement of fish acute toxicity testing in regulatory ecotoxicology. The most promising and successful approach to replace fish acute testing is the fish egg test or fish embryo test. This test is using fish eggs instead of adult fish. This type of test has been standardised for waste water toxicity assessment and successfully introduced in the German legislation on waste water fees as an alternative test resulting in an overall reduction in the number of fish used for testing in Germany by more than 30 percent.

For the testing of chemicals the fish embryo test has been included into the current work plan of the OECD Test Guidelines Programme under the lead of Germany. This method is designed to make the traditional acute fish toxicity testing obsolete and will probably be one key element of the ecotoxicological hazard and risk assessment within the new European chemical legislation.
ECVAM’s activities in ecotoxicology started in 2001 with its workshop on “The use of fish cells in ecotoxicology” (Castaño et al., 2003; *ATLA* 31, 317-351) and was followed up with the establishment of the ECVAM Task Force Ecotoxicology (chaired by Peter Párt, JRC, Ispra, I) in 2003.

Current activities are focused on reduction and eventual replacement of the acute LC₅₀ test in fish. Thus, a new testing strategy based on the threshold (step-down) approach published by Hutchinson et al. (2003, *Environ. Toxicol. Chem.* 22, 3031-3036) for pharmaceuticals was used to retrospectively evaluate ecotoxicological data of new and existing chemical substances extracted from databases maintained at the European Chemicals Bureau and ecotoxicological data of active substances extracted from published reports for the plant protection products. The evaluation revealed that a reduction of at least 50% might be feasible (see also poster/oral presentation Jeram et al.). This new testing strategy is now reviewed by ECVAM’s Scientific Advisory Committee and the Competent Authorities of the EU Member States.

Regarding the complete replacement of the acute fish test, ECVAM and its Task Force are developing a testing strategy, which is based on the use of fish cells, fish embryos and QSARs. However, all of these possible replacement methods still need to be evaluated and/or validated.

Linked to ECVAM’s activities on high-throughput screening for human health effects, it is planned to establish cytotoxicity tests using various fish cell lines and determine in comparison the cytotoxic effects of the chemicals tested on mammalian cell lines.

**Poster**

**ECVAM Key Area Ecotoxicology:**

**Summary of ongoing activities**

*Marlies Halder and Sonja Jeram*

ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra, Italy

In this study, we developed statistical models to describe the distribution of herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) in the Scheldt estuary based on long-term using data. The influence of water quality and the characteristics of the fish assemblage were analysed using a range of regression techniques, including stepwise and linear regression. We considered fish density in response to 15 water quality factors during a 10-year period. The distribution of herring and sprat showed statistically significant correlations with the difference of water temperature in the river and at sea (DT). This illustrates the importance of temperature to distribution of herring. A connection between altering water thermal conditions and fish migratory patterns proves to be the best predictor of herring and sprat abundance in Scheldt estuary.
Variation in the energy density of herring and sprat during estuarine residency in the Scheldt

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Changes in whole body energy content of herring (Clupea harengus) and sprat (Sprattus sprattus) during estuarine residency were measured and compared in the marine part and the brackish water part of Scheldt estuary. Fish of between 4 and 10 cm were used for analysis. The average amount of energy of wet weight (EWWT) in herring and sprat in Borssele station was higher than those in Doel station in both species. Water content varied from 78 percent to 82 percent in herring, and from 77 percent to 81 percent in sprat. Energy density of wet weight (EWWT) herring ranged between 2754.1-6487.13 J/g and between 2473-6958.4 J/g in sprat during the estuarine residency. We found that the average EWWT was higher in sprat (4998.4 J/g) than in herring (4438.6 J/g). Dry mass energy densities varied between 22.5-33.7 KJ/g in herring and 23.7-33.1 KJ/g in sprat.

Biological and technological advances for ecotoxicity and human health risk assessment using zebrafish embryos

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Animal use for chemical testing and pollution assessment is an issue of serious social concern and represents an important cost-rising factor. These drawbacks are further enhanced with the application of the new chemicals testing policy (REACH) and its requirement of intensive testing and animal use reduction.

Assessment of toxicity in non-mammalian embryos is one of the most promising approaches for ecotoxicity testing, given that they provide the opportunity of testing chemicals in a real and complete developing organism in a short time, with reduced costs and avoiding many ethical constraints.

Experimentation in non-mammalian embryos is currently limited by the lack of knowledge of the biology of these species and the poor technological developments in this area. These facts result in poor embryo yields, bad embryo quality and technological handicaps that ultimately lead to difficult scalability and scarce reproducibility of the results.

ZF Biolabs (Tres Cantos, Madrid) has deepened the knowledge in the zebrafish biology to allow a high, constant and high quality embryo production. This milestone has been achieved improving key aspects of the zebrafish biology such as diet and embryo production. Besides, ZF Biolabs has developed and patented the first sorter specifically designed for zebrafish embryos.

These biological and technological advances contribute to reduce the variability associated with the use of model systems based on non-mammalian embryos, and thus support the development of reliable assays using the zebrafish embryos for ecotoxicity, and human health risk assessment of chemicals.
Lecture

**A strategy to reduce the number of fish for acute aquatic toxicity testing of chemical substances and plant protection products**

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ECV AM and ECB applied the threshold (step-down) approach, recently described by Hutchinson et al. (2003, *Environ. Toxicol. Chem.* 22, 3031-3036) for pharmaceuticals, to chemical substances and plant protection products.

According to the current EU regulatory requirements, acute aquatic toxicity is determined by three endpoints, namely algae EC₅₀ 72h, daphnia EC₅₀ 48h, and fish LC₅₀ 96h. The proposed testing strategy takes into consideration that only the lowest value of the three endpoints is considered for hazard and risk assessment, and that fish is often not the most sensitive species. Therefore, algae and daphnia tests are carried out first and then, with the lowest of the two EC₅₀ concentrations, a test using five test and five control fish is performed. When fish toxicity occurs at this concentration, further testing on fish at lower concentrations would be needed to determine the LC₅₀.

Ecotoxicity data of chemicals and plant production products from various data bases were retrospectively evaluated by selecting the lowest value from the reported daphnia and algae data and by subsequently calculating the step-down tests needed to reach the reported fish toxicity. Comparison of the numbers of fish needed in both testing strategies revealed a possible reduction of 55% to 70% when applying the threshold (step-down) approach. The evaluation report is now peer-reviewed by the ECV AM Scientific Advisory Committee.

The new testing strategy is a promising approach to reduce number of animals and costs for ecotoxicity testing not only in the current regulatory framework but also with regard to REACH.

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Lecture

**Ecotoxicological tests in non-ecotoxicological research: Contribution to 3Rs**

Anne Kahru

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Due to the increase of population and industrial development the role of chemicals in everyday life is constantly increasing. Some unforeseen properties of pollutants have already been discovered, e.g., endocrine-disrupting side effects.

The framework of “chemicals – human – environment” contains two opposite scientific tasks: 1) to discover new (toxic) chemicals/mixtures to cure diseases, fight pests and microbes with minimum unwanted side-effects and 2) how to live safely in this world of chemicals, i.e. to minimise the unwanted effects of xenobiotics already accumulated in the environment (e.g., PCBs) in hazardous amounts or going to be produced in high-production-volumes in the nearest future (e.g., nanoparticles). The growing awareness of hazard of chemicals is clearly shown by introduction of REACH project. The increasing need for toxicity testing creates a big challenge for scientists to work out relevant alternative methods to laboratory animals or fish. One not yet widely explored area is the use of simple prokaryotic and eukaryotic models. Bacteria, fungi, protozoa, insects, plants and invertebrate animals – normal test organisms for ecotoxicological studies – are well suited for implementation of 3Rs in scientific and regulatory research.

This talk will focus on use of invertebrate organisms (e.g., bacteria, crustaceans, protozoa) in toxicity screening as well as for mechanistic studies.

For the illustration, the toxicity data for 50 MEIC programme chemicals (MEIC – Multicenter Evaluation of In vitro Cytotoxicity) involving pharmaceuticals, solvents, pesticides etc as well as cationic polymers (nanoparticles) – potential targeted drug delivery agents – will be presented. The potential of genetically modified bacteria as models for mechanism-targeted toxicity will be discussed.
The EU notification process requires toxicological and eco-toxicological evaluation of new chemicals. Tests for environmental hazard assessment are carried out according to Council Directive 67/548/EEC Annex V, which involves fish acute toxicity (guideline OECD N°203), Daphnia acute toxicity, and Algal growth inhibition test. Fish, as any living vertebrate other than man, fall into the scope of European regulations for the protection of laboratory animals. This protection extends to immature forms including larval stages of development, but excluding embryonic stages before they become capable of independent feeding.

The Cosmetics Directive and the REACH proposal promote the use of in vitro methods for the hazard evaluation of chemicals. We present an ongoing tiered approach for acute ecotoxicity assessment of cosmetic ingredients, aimed at replacing the fish acute toxicity testing required by the European legislation on chemical safety: Algae and Daphnia acute toxicity assays are realised on tier 1, then selected chemicals are submitted on tier 2, to a fish embryo test in replacement of juvenile or adult fish required by OECD guideline N°203.

We present data, obtained with two different fish species (Medaka and Zebrafish), showing similar sensitivity in adult and embryonic stages of development, to 26 reference chemicals (surfactants and quaternary ammoniums). Sensitivity differences between embryonic stages, before and after hatch, are observed. With regard to the selective permeability of the fish eggs envelopes (chorion), especially to quaternary ammoniums, we recommend testing on fish embryonic stage after hatch (named Eleutheroembryo), which still relies on autochthonic vitellogenic supply.

Evaluation of the fish embryo test as a potential replacement for the standard acute fish toxicity test using juveniles

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The 96 hrs acute fish toxicity test is the standard early tier method used in prioritising chemicals and effluents for subsequent hazard and environmental risk assessment. Current and proposed European chemical legislation and ethical stands on the use of animals for the purpose of risk assessment has resulted in an increased desire to implement the Three Rs in regards to vertebrates, including fish. Present regulatory definitions of protected and unprotected life stages of fish have led us to evaluate fish eggs (embryos) and sac fry (eleutheroembryos) as potential replacements in acute toxicity test protocols for fish. Because several species are preferred in different parts of the world and our companies operate globally we are developing an understanding of the relationship between embryo, eleutheroembryo, and juvenile sensitivities with Japanese Medaka, Zebrafish, and fathead minnow. In this paper we review: (a) the first stages of results with Zebrafish and Medaka embryonic stages side-by-side with 96 hrs juvenile tests using 26 compounds on a nominal basis, and (b) Zebrafish egg (48 hrs), eleutheroembryo (48 hrs), and juvenile (96 hrs) tests (4 compounds) with detailed confirmatory analytical to explore issues with chemical sorption and loss when dealing with small volumes. Sensitivity of Medaka and Zebrafish embryonic stages appear promising for the purpose of an early tier replacement test. We will review additional high priority research needs. These early studies are being used to develop protocols that we believe can be useful for development of future robust validation plans.
Lecture

Practical applications of the principles of the 3Rs in environmental assessment

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Experimental bioassays are currently used in ecotoxicology and environmental toxicology to provide information for risk assessment evaluation of new chemicals and to investigate their effects and mechanisms of action; in addition, ecotoxicological models are used for the detection, control and monitoring of the presence of pollutants in the environment. As a single bioassay will never provide a full picture of the quality of the environment, a representative, cost-effective and quantitative test battery should be developed. In order to study the effects of chemicals in the environment, a test battery has been applied. Such a battery should represent a wide range of organisms belonging to different trophic levels. A number of ecotoxicological model systems with more than twenty endpoints were evaluated at different exposure time periods. The systems included the immobilisation of the cladoceran Daphnia magna (1st consumer), bioluminescence inhibition in the marine bacterium Vibrio fischeri (decomposer) and growth inhibition of the alga Chlorella vulgaris (producer). Total protein content, neutral red uptake, lactate dehydrogenase (LDH) activity and MTT metabolism were investigated in Vero monkey kidney cells (model of 2nd consumer). Neutral red uptake, total protein content, MTS metabolism, LDH activity, lysosomal function, succinate dehydrogenase activity, G6PDH activity, metallothionein levels, EROD activity, apoptosis induction and changes in morphology were studied in the RTG-2 cell line, derived from rainbow trout gonad (Oncorhynchus mykiss), and in the hepatoma fish cell line PLHC-1, derived from Poeciliopsis lucida (models of 1st consumer).

Poster

In vitro biotransformation and bioconcentration of surfactants – development of assays and strategies for improving the prediction of the bioconcentration potential

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Bioconcentration is the process of accumulation of chemicals from water into an organism. The occurrence of significant bioconcentration is a prerequisite for further accumulation in the food-chain and possible secondary poisoning effects. Hence, the bioconcentration potential is highly relevant for the environmental risk evaluation of chemicals. It is also one of the criteria triggering whether or not a chemical is assigned to the “persistent, bioaccumulative and toxic, PBT” or the “very persistent, very bioaccumulative, vPvB” type chemicals.

As a first approximation, the bioconcentration potential (BCF) is estimated on the basis of the octanol-water partitioning coefficient. This approach yields high values for the BCF of surfactants which are in contrast to experimental determination. The discrepancy between measured and estimated BCF is often caused by biotransformation reactions in the organisms, which counteract the buildup of elevated internal surfactant concentrations.

Refined non-animal methods for the assessment of bioconcentration of surfactants therefore require information about the rates of biotransformation. Such information might be obtained through in vitro biotransformation experiments or by comparison of the bioconcentration behaviour with that of a metabolically stable reference compound. Possible strategies for utilising this information are discussed with regard to replacing whole animal studies, reducing the use of test animals, and with regard to refining computational estimates of the bioconcentration potential.
Lecture

Application of in vitro alternative methods to ecotoxicology

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Detection of environmental pollutants is major problems in ecotoxicology. Usually chemical analyses have been applied for the testing of contaminated samples. Although chemical analyses are becoming accurate by advanced analytical technology, they detect only targeted chemicals among test sample mixed with various chemicals. Bioassay systems are also used to detect pollutants in the waters using green algae and fish etc. We applied mammalian cell culture systems using several in vitro methods to detect their toxicities because of high sensitivity and comparable with the results of chemical analyses. Liquid samples, river waters and cooling tower waters were used after filtration for cytotoxicity test with two cell lines. Solid samples, ashes from incinerators, river sediments or airborne particulates, were extracted with organic solvent. Extracts were examined with in vitro phototoxicity test which was recently adopted as OECD Test guideline 432. Some samples were also examined in the mutation assay with mouse lymphoma TK assay.

The results are as follows: The data from river waters obtained in the colony formation assay were highly correlated with that of chemical analyses, although samples showed different response between two cell lines. The results of phototoxicity and mutagenicity with extracts of airborne particulates collected from in the last 20 years showed that the air in the area has become cleaner recently which was supported by chemical analysis. Our results suggested that in vitro tests are useful to assess and screen environmental pollutants as well as chemical analysis and other bioassays using animals.

Poster

The use of in vitro estrogen-reporter assays to predict potential endocrine disrupting effects in fish

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Numerous chemicals of industrial, agricultural or domestic origin, which show estrogen-like properties and which can interfere with wildlife reproduction, do occur in the aquatic environment. It is not evident to identify and quantify all these target compounds and biological active metabolites by analytical techniques. Therefore a screening approach, based on two in vitro bioassays, was first applied to assess the overall exposure to pollutants with estrogenic activity. The estrogen-inducible screen with a recombinant yeast strain (YES-assay) and human breast cancer cells, stably transfected with pVit-tk-Luc (MVLN-assay), both based on estrogen receptor binding, were used. Both assays were previously compared with an in vivo test with Zebrafish for their performance characteristics (sensitivity, reproducibility) and response spectrum for known estrogenic compounds. As part of two environmental monitoring projects many samples from Flemish waters (rivers, effluents of municipal wastewater treatment plants and industry, drinking water resources) were analysed. Next, sites with different estrogenic potential were selected in order to evaluate the likelihood of in vivo adverse effects on reproduction success in fish. The in vivo model used was the Zebrafish, Danio rerio, which was exposed to environmental water samples for 3 weeks and biomarkers for estrogenic exposure (vitellogenine in blood plasma and the gonado-somatic index) were measured. It was investigated whether measured levels of estrogenic activity, as determined by in vitro assays could indicate potential hazardous effects in fish. This step-wise approach using in vitro screening has been proposed for future studies on natural fish populations in Flemish rivers.
Workshop 5.11
Mechanisms of chemically-induced ocular injury and recovery: Current understanding and research needs

Poster
The assessment of the oral irritation potency of dentifrices with and without sodium lauryl sulphate as evaluated with the Slug Mucosal Irritation assay

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Ingredients such as sodium lauryl sulfate often used in dentifrices can induce oral irritation. In this study the mucosal irritation potency of dentifrices with and without SLS was assessed using the Slug Mucosal Irritation test. The concentration-response effect of 5 OTC dentifrices (A, B: containing no SLS; C, D, E: containing SLS) on the mucosal tissue was evaluated by placing the slugs two times for 60 min each on the diluted dentifrice (1%, 3%, 10% and 30% w/v in PBS). The mucus produced during each 60 min treatment is a measure for irritation. After the 60 min treatments, the protein and enzyme release (LDH, ALP) from the slug mucosa was measured. Concentrations up to 10% of dentifrice A and B resulted in a mucus production (< 2%) and protein release (<50 µg/ml.g) that was comparable with the negative controls (PBS). However, the 30% dilutions resulted in significantly increased mucus production. Formulation B induced an increased protein and LDH release whereas formulation A induced no tissue damage. The SLS (C, D, E) containing dentifrices induced an increased mucus production already at 3% dilutions. Higher concentrations induced mild to moderate tissue damage as was detected by the increased protein (>100 µg/ml.g) and LDH release (>1 U/l.g). None of the dentifrices induced ALP release. According to the SMI assay the following rank order of increasing irritation potency was established: A<B<<C~D<E. These results are consistent with other in vitro data and confirm clinical inflammatory effects of SLS in oral care products reported in the literature.
Lecture

Ocular toxicology in vitro – cell based assays

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Background and aims: Interactions between the three cell types in the cornea control cell differentiation and responses to stimuli. We have sequentially added cell types in a 3-dimensional construct to assess the minimal requirements for a representative model of the human cornea to be used in toxicology tests.

Methods: We used single applications of toxicants from 3 different classes on cell cultures in defined medium. For a number of human corneal epithelial cell lines we assessed whether stratification modifies responses to toxicants. Primary corneal stromal cells were grown in collagen gels, keeping activation to a minimum. We assessed the influence of three-dimensional co-culture of the two cell types on cell differentiation, cytokine production and recovery from exposure to the chosen toxicants. This process was repeated after the addition of an endothelial layer.

Results: Stratification of epithelium, compared to monolayer cultures, did not modify responses to toxicants probed by classical toxicology assays. Co-cultured cell types displayed patterns of cytokines different from the single cell-type 3D models, suggesting interactions between the different cells of the construct. Following exposure to toxicants there were marked changes in cytokine profiles, that could be related to the toxicant used. These changes were, however, markedly influenced by the epithelial cell-line used.

Conclusions: To rationalise the choice of cell lines for complex corneal constructs, their steady-state immune signal molecule patterns should be compared to those observed in normal human preocular fluid.

Supported by Colipa.

Lecture

Can toxicogenomics be used to identify chemicals which cause ocular injury?

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Chemical injury to the eye can be severe, moderate or mild dependent on the type of chemical (e.g. acid, alkali, surfactant), concentration, duration of exposure and the ability of the eye to repair itself. Severe chemical injuries to the cornea can result in tissue coagulation, degradation of the stroma, acute inflammation, angiogenesis, fibrosis, and recurrent corneal ulcers. By contrast, mild chemical injury can present as irritation, pain, inflammation, red eye and cell loss, which usually subsides following treatment and tissue repair. The Draize test in rabbits is currently the “gold standard” for identifying ocular hazards associated with chemicals and a variety of household formulations (e.g. cleaners and cosmetics). However, political necessity and public concern require an alternative to animal testing. One option is the toxicogenomic approach, whereby a gene fingerprint directory can be used to identify mild/moderate chemical preparations, which are toxic to the eye. This can be achieved by gene expression profiling of bioengineered human corneas using microarray analysis. Proof of principle is confirmed by exposure to generic chemicals/preparations with well documented ocular injury characteristics. Clustering and pathway analysis of the gene profiles will lead to the development of diagnostic arrays to identify key genes/pathways differentially regulated by various chemicals. The diagnostic arrays can then be used for high throughput testing of new chemical preparations. Thus, this two-pronged approach; the combination of human bio-engineered corneas and microarray analysis will provide a cheap, effective and rigorous alternative to the Draize test.
Poster

Evaluating the eye irritancy of solvents in a simple fragrance mixture with the Bovine Corneal Opacity and Permeability (BCOP) assay

Nicole Cuellar¹, Paul Lloyd², Judith Swanson⁴, Greg Mun³, John Harbell³ and Kim Bonnette⁴

¹ SC Johnson & Son, Inc., Product Safety, Racine, USA; ² SCJ EURAFNE Ltd., Product Toxicology, Egham, UK; ³ Institute for In Vitro Sciences, Inc., Gaithersburg, USA; ⁴ Charles River Laboratories, Inc., Spencerville, USA

Fragrances are complex mixtures used in many consumer products. Organic solvents, such as ethanol, are major components of fragrance formulations functioning mainly as solubilisers and fragrance delivery mechanisms. The BCOP assay and primary eye irritation study (EPA-OPPTS 870.2400) were conducted using simple fragrance mixtures containing six commonly used solvents. The corneal depth of injury was assessed histologically both in vitro and in vivo. In the BCOP assay, corneas were exposed for 3 minutes, rinsed and incubated for 2, 4 and 20 hours before the opacity and permeability endpoints were assessed. Thus, the time course of lesion development was determined. The early lesions (2 and 4 hours after exposure) were compared to damage observed after 20+ hours in vitro and in vivo. In vivo, animals were scored at 1, 4, and 24 hours and then the eyes taken for histology. Individual solvents in both assays impacted the level of irritation of these formulations. In vivo, certain solvents increased the rate of lesion development but not the overall intensity or duration compared to the fragrance alone. Other solvents decreased the overall intensity and duration. The BCOP assay showed a generally similar pattern of lesion development as seen in vivo. Those combinations that showed opacity at 4 hours in vivo, showed epithelial and stromal lesion in the BCOP by 4 hours post-exposure. Fragrance alone was slower to develop opacity in vivo and required the 20 hour post-exposure to produce appreciable lesions in vitro. These data suggest that the standard post-exposure (2 hour) can be predictive of irritation potential of fragrance/solvent mixtures.

Lecture

In vitro models for ocular injury: Current and potential biomarkers

Chantra Eskes¹, David Allen², Raymond Tice², Neepa Choksi², James Truax², Wiley Chambers³ and Leonard Schechtman³

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Numerous in vitro/ex vivo methods for eye irritation have been developed and are currently being used within industry for specific purposes. In vitro model systems for eye irritation can be divided into four major categories: organotypic models, human corneal epithelium models, cell cytotoxicity assays and cell function assays. The biomarkers and mechanisms usually addressed range from simple cytotoxicity to more complex functional endpoints such as corneal light transmission and barrier functions. However, the range of criteria for injury, inflammation and reversibility covered by the Draize rabbit eye test was found to be unlikely to be replaced by a single in vitro test. One of the recommendations to achieve full animal replacement is to support the development of mechanistically-based models in order to address the mechanisms not currently covered by the existing assays. During the ICCVAM-NICEATM-ECVAM symposium on Mechanisms of Chemically-Induced Ocular Injury and Recovery (May 11-12, 2005), novel and existing biomarkers were identified that may allow further mechanistic insight into the ocular irritancy potential of a test substance. Discussions addressed the potential in vitro test systems and biomarkers that may allow adequate prediction of the mechanisms of chemically-induced ocular injury and lesion persistence versus reversibility. Finally, novel in vitro biomarkers or test systems were identified where further research and development is recommended to investigate the correlation with the in vivo test.

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The BCOP assay was developed by Pierre Gautheron and Joe Sina to address ocular irritation potential of pharmaceutical intermediates and is now widely applied across industries and chemical/formulation classes. For many, if not most, of these chemical/formulation classes, the mode of action(s) of the test material is known. Membrane lysis, protein coagulation, and saponification are common modes of action that lead to ocular irritation. In our experience, the opacity and permeability endpoints (generally combined into an "in vitro score") have been able to identify the epithelial and stromal changes associated with this type of damage. However, chemicals that react with nucleic acids, mitochondrial proteins, or other cellular targets, that do not lead to immediate loss of cellular integrity or protein precipitation, have proven more difficult to identify without the addition of histological evaluation of the treated corneas. Histological evaluation is performed on the epithelial, stromal and endothelial layers of the cornea and can identify lesions not revealed by opacity or permeability. It also provides a direct measure of the depth of injury which Maurer et al. (2002) have shown to be predictive of the degree and duration of eye irritation. Thus, understanding the depth of injury to the treated corneas (especially relative to the injury from known benchmark materials) through histological evaluation of the bovine corneal tissue, can be crucial to interpreting the actual ocular irritation potential of novel materials or formulations. Data from reference compounds will be used to illustrate the approach.

Poster

Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay

John Harbell and Rodger Curren
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The COLIPA eye irritation research programme for the development of in vitro methods for eye irritation incorporates integrated research projects and collaborative activities with external partners. The integrated research projects focus on understanding mechanisms of eye injury and identification of new in vitro endpoints that are more predictive of the in vivo human response to chemical injury. This is expected to result in new or improved in vitro methods that would proceed to formal validation. There are three projects: 1) investigation of whether kinetics/patterns of change in physiological function and signals of injury released from the cornea in vitro can predict a chemical’s potential to damage the eye with a focus on recovery; 2) identification of endpoints related to magnitude of injury and quality of repair in human immortalised cells and 3-dimensional human conjunctival and corneal constructs and 3) a genomics project using a pattern recognition approach to identify new endpoints for injury and repair that build on corneal models being evaluated in projects 1 and 2 for potential use in current/future in vitro assays. Equally important to achieve validated in vitro methods is collaboration of industry, academia, external scientific organisations and regulators. COLIPA is working with ECVAM by actively participating in its Eye Irritation Task Force and providing support for post-hoc statistical analysis of current in vitro methods. The presentation describes these different projects and activities and how they are combined into the overall COLIPA strategy to address the development of in vitro alternatives for eye irritation.
The standard regulatory approved test to evaluate eye irritation is the Draize Test. Success in fully replacing it with in vitro methods has not occurred. This is in part attributed to lack of understanding of underlying physiological mechanisms of eye irritation.

To address this, COLIPA research is focused on understanding mechanisms of eye injury and identification of new in vitro endpoints that are more predictive of the human response to chemical injury resulting in new or improved in vitro methods that would proceed to formal validation. The programme has three integrated projects: 1) investigation of whether kinetics/patterns of change in physiological function and signals of injury released from the cornea in vitro can predict a chemical’s potential to damage the eye with a focus on recovery; 2) identification of endpoints related to magnitude of injury and quality of repair in human immortalised cells and 3-dimensional human conjunctival and corneal constructs and 3) a genomics project using a pattern recognition approach to identify new endpoints for injury and repair that build on corneal models being evaluated in projects 1 and 2 for potential use in current/future in vitro assays.

Equally important to achieve validated in vitro methods is collaboration of industry, academia, external scientific organisations and regulators. COLIPA is working with ECVAM by actively participating in its Eye Irritation Task Force and providing support for post-hoc statistical analysis of current in vitro methods.

This poster provides a detailed overview of the integrated elements of the COLIPA eye irritation programme.

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**Poster**

**The COLIPA strategy for the development of in vitro alternatives: Eye irritation**

Pauline McNamee1, Dan Bagley2, Claudine Faller3, Penny Jones4, Beatrice Le Varlet5, Irene Manou6, Wolfgang Pape7, Christine Van den Berghe8 and Freddy Van Goethem9

1 The Procter & Gamble Company, Egham, Surrey, UK; 2 Colgate-Palmolive, Piscataway, NJ, USA; 3 Cosmital Wella, Marly, Switzerland; 4 Unilever, Safety and Environmental Assurance Centre, Sharnbrook, UK; 5 LVMH Recherche, Saint Jean de Braye, France; 6 COLIPA, Brussels, Belgium; 7 Beiersdorf, Hamburg, Germany; 8 L’Oreal, Aulnay Sous Bois, France; 9 Johnson & Johnson, Pharmaceutical Research and Development, Beerse, Belgium

**Background and aims:** Interactions between epithelial cells and keratocytes maintain a healthy cornea and its ability to respond to an insult. Following chemical insults, we assessed cytokine production, as an indication of intercellular communication, on addition of a stromal construct to stratified corneal epithelia.

**Methods:** Immortalised human corneal epithelial cell models were built and stratified at the air-liquid interface in a fully defined medium. Models were also built with the epithelium stratified on top of a collagen gel seeded with primary human keratocytes. The stratified constructs were treated for 10 min with NaOH, sodium dodecyl sulfate and Tomadol™ 45-7 at a concentration of 0.66%. Cytokine production was assessed as well as fluorescein leakage, LDH release, protein and metabolic assays.

Results: IL-8 and IL-6 were detected in the Araki-Sasaki cell line, whilst IL-10, 8, 6 and 12p70 was produced by the USA line. The cytokine responses were different with different toxicants. The stromal constructs did not produce any measurable cytokines. In co-cultures of stroma and USA epithelium IL-8 production increased ten-fold, whilst there was a four-fold increase in IL-6. On stratified epithelia the non-ionic surfactant caused an increase in IL8, while SLS and NaOH did not. In epithelium-stroma models it is the latter which cause an increase in IL8 and trigger IL6 production, whilst the non-ionic surfactant did not.

Conclusion: Upon addition of further layers to the constructs cytokine patterns altered, implying communication between the layers.

Supported by Colipa.

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**Poster**

**The cytokine response of a wounded corneal model**

Marcus Radburn-Smith and Monica Berry

University of Bristol, Academic Unit of Ophthalmology, Bristol, UK

Background and aims: Interactions between epithelial cells and keratocytes maintain a healthy cornea and its ability to respond to an insult. Following chemical insults, we assessed cytokine production, as an indication of intercellular communication, on addition of a stromal construct to stratified corneal epithelia.

Methods: Immortalised human corneal epithelial cell models were built and stratified at the air-liquid interface in a fully defined medium. Models were also built with the epithelium stratified on top of a collagen gel seeded with primary human keratocytes. The stratified constructs were treated for 10 min with NaOH, sodium dodecyl sulfate and Tomadol™ 45-7 at a concentration of 0.66%. Cytokine production was assessed as well as fluorescein leakage, LDH release, protein and metabolic assays.

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Supported by Colipa.
Background and aims: Keratocytes play a major role in wound healing, transforming from a quiescent state to an activated fibroblast or myofibroblast phenotype that acts to contract the wound and release inflammatory mediators. Cytokines are such mediators, allowing interactions to occur between all cell types in the cornea. Understanding the factors that influence this communication is essential for building a bioengineered model that can be used to study corneal physiology.

Methods: Primary fibroblasts, immortalised corneal epithelia and immortalised endothelial cells were grown in either serum enhanced or serum free media. Three dimensional cultures were grown within type I collagen gels.

Results: Production of IL-6 and IL-8 decreased with time after transfer to serum-free medium. IL-12p70, TNF, IL-10 and IL-1 were not detected. Ratios of IL-6/IL-8 are different in fibroblasts grown on different substrates. Constructs were stable for 34 days in KGM with some low production of IL-6. Addition of endothelium caused slight increase in IL-6 and production of IL-8. A marked increase in both cytokines was observed after epithelial addition. Continuity or contiguity of the two cell types influence the cytokine profile. Contraction of the gel was accompanied by a striking increase in IL6 and IL8.

Conclusion: Cytokine patterns were influenced by the culture medium, the substrate and the presence and contact with other cell types.

Supported by Colipa.

Poster

**Effect of environment on signaling profiles of corneal constructs**

**Marcus Radburn-Smith and Monica Berry**

University of Bristol, Academic Unit of Ophthalmology, Bristol, UK

Background and aims: Keratocytes play a major role in wound healing, transforming from a quiescent state to an activated fibroblast or myofibroblast phenotype that acts to contract the wound and release inflammatory mediators. Cytokines are such mediators, allowing interactions to occur between all cell types in the cornea. Understanding the factors that influence this communication is essential for building a bioengineered model that can be used to study corneal physiology.

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Conclusion: Cytokine patterns were influenced by the culture medium, the substrate and the presence and contact with other cell types.

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Poster

**Inflammation mediator detection in the ex vivo Eye Irritation Test (EVEIT)**

**Norbert Schrage¹, Markus Frentz², Marting Reim³, Brigitte Kondring ² and Jakob Becker³**

¹ Dept. of Ophthalmology Cologne Merheim, Ophthalmology, Cologne Merheim, Germany; ² Aachen Center of Technologytransfer in Ophthalmology (ACTO), Aachen, Germany; ³ Dept. of Ophthalmology University of Aachen, Aachen, Germany

Objective: Eye irritations can be simulated during cultivation of isolated rabbit corneas in the EVEIT. We found early epithelial healing of the cornea after abrasion. Irritaton was give proof by opacification and epithelial defect. We examine the immunological response of the isolated cornea on specific irritations.

Methods: In an ex vivo culture we subjected each 8 corneas to epithelial abrasio of 34 ±3% surface, a corneal burn with 2n NaOH in (LVET) and another 8 cultured corneas served as negative control. We examined at different time points perfusion medium and supernatants on the content of IL 1 alpha and beta, IL 8 and FGF by means of ELISA technique.

Results: We found high contents of FGF 6 h after exposition towards NaOH in supernatants but not in perfusates. FGF concentrations were elevated in supernatants of corneas with epithelial abrasion at day 1 significantly. The levels of FGF in negative controls remained low. In sodiumhydroxide exposure IL 8 decreased within the medium and a rise from 280 ± 220 pg/ml to 780 ± 270 pg/ml in supernatants. In abraded corneas 290 ± 30 pg/ml rose to 375 ± 50 pg/ml post expositionem. Negative controls remained low. IL-1 alpha and beta were measured high after sodiumhydroxide expositon and less in case of corneal abrasion.

Conclusions: We conclude that the EVEIT model gives reliable biochemical reactions in irritation and recovery. The mediators released from the isolated cornea are causative for inflammation, leucocyte recruitment and ulceration. There is strong evidence that the system might replace animal experiments in future.

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Poster

Replacement of the Draize test by a new system of ex vivo cornea culture

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¹Dept. of Ophthalmology, Cologne Merheim, Germany; ²Aachen Center of Technologytransfer in Ophthalmology (ACTO), Aachen, Germany; ³Dept. of Ophthalmology at the University Aachen, Aachen, Germany

Objective: By a new construction of ex vivo corneal culture we try to simulate irritation, recovery and healing on cultured animal corneas to improve predictive results on chemicals for human eyes.

Methods: Rabbit corneas freshly prepared from abattoir are mounted in a perfusion system and pre-incubated for 32 hours. After this time we expose the corneas with a modified Low Volume Eye Irritation Test (LVET) or to mechanical abrasion. We monitor the vitality of the corneas by means of continuous glucose lactate measurements in medium and supernatants, microscopic and macroscopic examination of the erosion, endothelial damage and opacification.

Results: Each 16 corneas were exposed to abrasion, no touch or 2n NaOH for 20 sec. Corneas without any touch showed stable epithelium with small rough zones of 2 +- 2% of fluorescein positive staining. Defined corneal abrasions of 34 +- 3% healed within 5 days completely and expositions to sodium hydroxid resulted in persistent corneal erosion of 45 +- 12%. All 36 corneas showed considerable consumption of glucose and production of lactate. The supernatants showed less lactate in case of epithelial damage.

Conclusions: With the presented system we are able to simulate the two main criteria of eye irritation in animal experiments, the acute damage and its regeneration or healing. The system is close to the natural cornea and is ophthalmological evaluated and proofed to replace eye irritation in animals. Additional parameters of tissue repair and inflammation are available in the sampled media.

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Lecture

Predictive eye irritation test ex vivo system on rabbit corneas

Norbert Schrage¹, Martin Reim², Markus Frentz², Brigitte Kondring², Anja Buckermann¹ and Azadeh Babaei²
¹ACTO, Aachen, Germany; ²University clinic of Aachen, Dept. of Ophthalmology, Aachen, Germany

The rabbit cornea is the mostly spread in vivo test system used in Draize test but also in ophthalmological experiments. Thereby the rabbit eye has been well described and parallel diseases and features especially in eye burns and irritation been found to be predictive for humans. This was the reason for us to search for an ex vivo rabbit eye irritation test. We derived knowledge from cornea banking and storage and built an ex vivo system for rabbit corneas. In several experiments we were able to maintain the cornea for more than 20 days in culture exposed to air with the epithelium. Perfusates of an artificial anterior chamber and supernatant fluids were incubated and analysed on Glucose and lactate and pH and proinflammatory factors and growth factors.

We were able to demonstrate stability and healing of epithelium of the cornea and found differential healing dependent on the amount and severity of irritative substances.

We found differential images of reactions concerning healing, expression of VEGF triggered by mechanical abrasion and hydrogen peroxide exposure as well as IL 8 releas in early phase of exposure.

We believe that the ex vivo rabbit cornea test provides the possibility to replace Draize test by an as close to nature system as possible based on the huge amount of existing data on humans and rabbits.
Significant efforts have been made during the past 25 years to develop and validate in vitro test methods to replace the rabbit ocular irritancy and corrosivity test. Despite these efforts, there still is no scientifically valid test method or battery of test methods capable of completely replacing the in vivo test for the assessment of novel substances. Critical reviews of this issue by various expert groups have generally concluded that greater emphasis on mechanism-based data from in vivo and in vitro test methods is essential for future meaningful progress. However, observations in the current rabbit eye test remain unchanged since the establishment of this test over 60 years ago. These consist of visual observations and subjective numerical scores for damage to the cornea, iris, and conjunctiva. In contrast, ophthalmological assessments of human ocular injuries have evolved to routinely include slit lamp biomicroscopy, confocal microscopy, fluorescein staining, and other measures of inflammation and injury. Accordingly, experts have suggested that animal ocular tests, when such tests are necessary, should include similar assessments as part of a new set of standard observations that can be used to maximise the comparability of animal and human data. The routine use of objective quantitative endpoints and biomarkers to assess human and animal chemically-induced ocular injuries is expected to provide mechanistic insights that will support the development and validation of more predictive in vitro methods, and improve the accuracy and reliability of ocular hazard assessments. Related recommendations from a recent ICCV AM-NICEATM-ECV AM scientific symposium will be discussed.
Poster

Comparative in vitro cytotoxicity of lens care products with three cell lines and two assay methods

Ann Wright¹, Alana Renaud¹, Mary McKee¹ and Ruy Tchao²

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Purpose: Compare cytotoxicity potential of contact lens care products (LCP) according to the Neutral Red Uptake and Release assay (NRUR), using immortalised human corneal epithelial (HCE-T) and murine fibroblastic cells (L929), with cytotoxicity using the Fluorescein Leakage assay with Madin-Darby canine kidney cells (MDCK).

Methods: NRUR assay: Serially dilute SOLOcare® AQUA and OPTI-FREE® Express® with Aldox™ solutions on 96 well plates to final test concentrations of 12.5%, 25% and 50% for the NRUR assay for 24 hours cell exposure. Positive control: 10 ppm BAC. Negative control: DPBS. MDCK assay: MDCK inserts exposed to neat solutions for 30 min and observed post 24 hour recovery for fluorescein leakage. Negative control: HBSS. Positive control: 300 µg/mL SDS.

Results: SOLOcare® AQUA was non-cytotoxic at all concentrations with both cell lines for the NRUR and for the MDCK assay. The following solutions were considered cytotoxic in comparison to the negative control for the NRUR assay: OPTI-FREE® Express® (50 & 25%) and BAC. The following solutions were significantly different in comparison to the negative control using the MDCK assay: OPTI-FREE® Express® and SDS. Cytotoxicity was determined by an ED5024 for NRUR.

Conclusions: The cytotoxicity results for LCPs determined using the NRUR assay are similar to results obtained using the MDCK fluorescein leakage assay. The LCP cytotoxic by the NRUR assay was cytotoxic by the MDCK assay and the LCP noncytotoxic by the NRUR assay was noncytotoxic by the MDCK assay. Both of these assays help differentiate subtle differences in LCPs.
A novel computational method based on a genetic algorithm was developed to study composite structure of promoters of co-expressed genes. Our method enabled an identification of combinations of multiple transcription factor binding sites regulating the concerted expression of genes. In my presentation, I report the study of genes whose expression is regulated by the aryl hydrocarbon receptor (AhR), that mediates responses to a variety of toxins. AhR-mediated change in expression of AhR target genes was measured by oligonucleotide microarrays and by reverse transcription-polymerase chain reaction in human and rat hepatocytes. Promoters and long-distance regulatory regions (>10 kb) of AhR-responsive genes were analysed by the genetic algorithm and a variety of other computational methods. Rules were established on the local oligonucleotide context in the flanks of the AhR binding sites, on the occurrence of clusters of AhR recognition elements, and on the presence in the promoters of specific combinations of multiple binding sites for the transcription factors cooperating in the AhR regulatory network. Our rules were applied to search for yet unknown Ah-receptor target genes. Experimental evidence is presented to demonstrate high fidelity of this novel in silico approach.
Lecture

Validation of toxicogenomics-based tests, a new generation of alternatives

Raffaella Corvi, Jens Linge and Thomas Hartung
ECVAM, Institute for Health and Consumer Protection, European Commission Joint Research Centre, Ispra (VA), Italy

Toxicogenomics-based methods are being widely applied in toxicology and biomedical research. Since data are already being generated using these technologies, it is both timely and important to address the critical validation issues now with the aim of establishing a foundation that will facilitate future regulatory acceptance of scientifically valid toxicogenomics-based test methods. Addressing such issues early on, will also facilitate early buy-in and confidence in the technologies by the regulatory arena in its quest for new and improved methods by which to help ensure human health, protect the environment, and demonstrate responsiveness to animal welfare issues.

For that reason, the European Centre for the Validation of Alternative Methods (ECVAM), the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) have started to investigate the specific considerations necessary for adequate validation of toxicogenomics-based test methods. Experience in validation of conventional alternative test methods has led to an understanding that the validation approach will have to be adapted to the evaluation of methods based on toxicogenomics. The toxicogenomics field is rapidly evolving; therefore the validation process should accommodate the anticipated changes in the technology and must not be at the expenses of innovation. Moreover, other international Organisation as the OECD and the WHO/IPCS are currently drafting activity programs related to the possible use of toxicogenomics-based test methods for hazard and risk assessment purposes.

Lecture

Achieving the potential of toxicogenomics

James C. Fuscoe
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Microarray technology is gaining widespread attention in toxicology studies. This technology allows the global assessment of gene expression profiles in tissues and purified cell subtypes, as well as tissue cultures. Thus, the effect of a test substance on the activity of nearly every gene can be determined in a single experiment. Although presently not a high throughput technology, the high information content of the assay may allow the elucidation of possible toxicity, as well as mechanisms of action. Microarrays, combined with other high information content technologies, such as metabolomics and proteomics, will provide a global picture of the effect of test substances on a biological system. With the understanding of underlying molecular events caused by a test substance, extrapolation between surrogate test systems and humans can be rationally performed. In order to fulfill the promise of such toxicogenomics approaches, standard reference materials are needed for quality control, standardisation, and performance proficiency. The Microarray Quality Control Project (MAQC) was initiated to provide such standards for the microarray community. Large reference datasets will be produced by different microarray platforms using common RNA samples that will be available to the microarray community. The derived QC metrics/thresholds will aid individual laboratories in better assessing performance and to avoid procedural failures. The recently issued U.S. FDA Guidance for Industry on Pharmacogenomic Data Submissions will help facilitate the use of this type of data in pharmaceutical drug development.
Lecture

“Percellome” and “Mille-Feuille data” system for toxicogenomics

Jun Kanno, Ken-ichi Aisaki, Katsuhide Igarashi, Noriyuki Nakatsu, Satoshi Kitajima and Yukio Kodama

National Institute of Health Sciences, Division of Cellular and Molecular Toxicology, Biological Safety Research Center, Tokyo, Japan

A systematic approach is proposed to normalise the mRNA expression values from DNA microarrays and quantitative-PCR in a “per one cell” basis, designated as “Percellome”. This Percellome system will enable us to plot all data on to one common linear scale. Transcriptome data between experimental groups, between studies, and even between different organs can be directly compared without further normalisation. This system, developed primarily for Affymetrix Gene Chips, can be expanded to other platforms and quantitative-PCR as long as they meet a few requirements. Direct data comparison between different laboratories would be possible in this respect.

Our Percellome toxicogenomics projects monitors, for example, the time- and dose-dependent alteration of gene expression induced by various chemicals in mouse (4 time points and 4 dose levels, total of 16 groups, 3 mice each). The percellome data can be visualised as a three-dimensional graph (X=time, Y=dose, Z=expression per one cell) containing 45,000 layers of surface corresponding to each of the probe sets in the Affymetrix MOE430v2 Gene Chip (“MilleFeuille” data). X and Y axes can be any experimental parameters. This MF data is biologist-friendly that it is helpful to extract biologically plausible alterations and to develop further methods for drawing the gene cascades.

The aim of this Percellome toxicogenomics project is to develop the predictive toxicology through the development of the gene cascade data based on the high-precision transcriptome database/informatics. We believe that this approach should lead to our ultimate goal of generating “virtual mouse” in silico in the future.

Poster

Toxicogenomic analysis of rat liver carcinogenesis: Mechanistic insights and implications for risk assessment

Axel Oberemm1, Kareen Tenz1, Eberhard Krause2, Gabriele Scholz3, Heidrun Ellinger-Ziegelbauer4, Hans-Juergen Ahr4, Carina Itrich5, Annette Kopp-Schneider5, Juergen Hellmann6, Kerstin Fella6, Michaela Kröger6, Peter-Juergen Kramer6, Hans-Bernhard Richter-Reichhelm1 and Ursula Gundert-Remy1

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Genomic and proteomic approaches are widely explored to evaluate their usefulness for detecting early toxicological endpoints and mechanisms. In order to relate changes of transcriptional and translational profiles to conventional endpoints of toxicity, a common animal model of chemical hepatocarcinogenesis was used. N-Nitrosomorpholine (NNM) was administered to adult male Wistar rats for 7 weeks to induce hepatocarcinogenesis. Specimens of each treatment group (vehicle-control + NNM low and high dose) were killed at different time points between 1 day and 50 weeks after the start of the study and left liver lobes from five animals were sampled for simultaneous biochemical and histopathological processing. Gene expression was analysed using the Affymetrix rat genome chip U34A. Proteomic analysis was based on 2D-electrophoresis and mass spectrometry. Results demonstrated characteristic deregulations of genes and proteins by complementary use of transcriptomics and proteomics, which can be related to mechanisms of carcinogenicity. It was shown that comparably few genes were deregulated at both levels of expression and each approach contributes different parts of information, which can be used to analyse mechanistic toxicity of genotoxic carcinogens and to identify early biomarkers of carcinogenesis. By this approach, adverse long-term effects could be detected in short-term bioassays and, in consequence, the number of long-term animal studies can be reduced. New approaches focus on the identification of predictive molecular profiles for non-genotoxic carcinogens.
Poster

Can the number of animal experiments be reduced by the application of transgenic models?
A case study for testing estrogenic effects using the ChgH-GFP medaka strain

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Reporter gene constructs of GFP (Green Fluorescent Protein) are ideal tools to visualise the gene expression in living animals. They are in particular useful for transparent, early life stages of fish and can be applied to analyse the individual response of transcript abundance to a certain stimulus, such as the exposure to toxic chemicals or hormones. In contrast to other techniques, that require a certain amount of RNA, the fluorescence of the non-toxic GFP can be analysed conveniently in early life stages of a single organism. In the present study we have developed a technique to quantify the GFP levels in living ChgH-GFP transgenic medaka by image analysis. The ChgH-GFP strain was developed by Kurauchi et al. (2005, EST, in press) and harbours a regulatory region of the estrogen-responsive choriogenin H gene fused to the GFP gene. A series of calibration measurements was performed to develop a technique that allows to calculate GFP fluorescence intensity independent of the photo exposure time. ChgH-GFP showed a strong induction in 14 day-old fish at exposure to \( \geq 183.5 \text{ pM} \) (50 ng/L) 17-beta-estradiol. Time course and recovery experiments indicated an accumulation of GFP in the liver. The ChgH-GFP medaka is a very useful tool to analyse water contamination and to identify environmental chemicals with estrogenic activity. However, the quantification of GFP could be easily adopted for any other GFP-reporter strain. If applied for the purpose of gene regulation studies in early life stages, it will contribute to the refinement and reduction of animal experiments.

Poster

A two-dimensional gel database of rat liver proteins useful for detection of toxic and carcinogenic effects of chemicals

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Although promising approaches during the past decade, the availability of publicly accessible databases of rat liver proteins became limited. Since emerging proteomic technologies still depend on data generated by two-dimensional electrophoresis (2-DE), we decided to establish a new 2-DE map of male Wistar rat liver proteins in the context of a molecular toxicological joint research project. This information is useful to quickly retrieve protein information from 2-DE experiments. 2-DE separation was performed using the common IPG technique. Spots were excised from 2-D gels using a spot picker (ProPic, Genomic Solutions) and the proteins were identified after in-gel digestion with trypsin by using peptide mass fingerprint (MALDI-TOF-MS) and Tandem MS (LC-ESI-Quad-TOF).

At present, the map contains about 621 proteins and could be the basis for a publicly accessible HTML-database, providing information to identify protein expression patterns of toxicological relevance. Protein spots are linked with detailed information, i.e. protein identity, EC-number, function, localisations and molecular weight. Among the annotated protein spots we found several of proteins with relevance to mechanistic toxicological endpoints and some of them are well known as toxicological marker proteins. Furthermore we could assign many of them to toxicological categories like DNA damage response, detoxification, stress response or apoptosis. Expression patterns of marker proteins could contribute mechanistic data to assess toxic and carcinogenic effects of chemicals in short-term rodent bioassays. In perspective, our data should be integrated in comprehensive, publicly available toxicogenomic databases.
Liver cancer is a public health concern in many parts of the world. We used albumin-SV40 transgenic rats, which spontaneously develop hepatic neoplasms within 6-9 months, as a model for the liver cancer study. Data from microarray analysis of liver tumours from these animals demonstrated an altered gene expression. A wide range of bioinformatics methods was used to determine the relevance of the findings in rat to human with respect to chromosomal aberration, pathways and functions (Gene Ontology). Using these bioinformatics tools, particularly ArrayTrack that is developed in house and available to the public, we found that genes related to cell cycle control, cell proliferation, apoptosis, transcriptional regulation, and protein metabolism were altered. We also closely examined gene expression in regions of previously identified chromosomal aberrations associated with early hepatic neoplasms in this transgenic rat model using a novel visualisation tool. The utility of ArrayTrack was demonstrated in this project for analysis of gene expression data derived from microarray experiment. Analysis indicates that the altered gene expression associated with rat liver tumour development may be useful in the analysis of human liver cancer.

Extrapolating the toxicogenomics data derived from rat to human – the roles of bioinformatics in liver cancer analysis using cross species mapping

Weida Tong
FDA’s National Center for Toxicological Research (NCTR), Division of Systems Toxicology, Jefferson, USA

DNA microarray is a key technology in pharmaco- and toxicogenomics, a field that has been identified in the U.S. FDA Critical Path document as a major opportunity for advancing medical product development and personalised medicine. It is expected that the regulation of microarray-based medical diagnostics and the review of microarray data submitted as part of an IND or NDA will become an essential regulatory responsibility for the FDA. A single microarray experiment generates a large volume of data. The management, analysis and interpretation of this vast amount of data are the most critical components in realising the value of the technology. Many solutions are available, but, unfortunately, most of them, if not all, deal with these components separately, posing a great challenge for reviewers to efficiently handle microarray data. ArrayTrack, developed at NCTR/FDA, integrates these three essential components into a single application with a user-friendly and intuitive interface, and thus provides a single solution for reviewers to analyse microarray data, interpret the information and verify the results submitted by sponsors. In addition, ArrayTrack provides the capability to develop an aggregate genomics knowledge base to support scientifically sound future regulatory policies. Currently, ArrayTrack is being integrated and further refined at the U.S. FDA as a review tool for pharmaco- and toxicogenomic data submission.

Review tool for pharmacogenomiccis data submission: ArrayTrack

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Poster

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The H295R adrenocortical cell line as alternative in vitro system for screening of endocrine disruptors:
A microarray based approach

Caroline Vanparys¹, Marleen Maras¹, Bart Naudts², Johan Robbens¹, Paul Vanhummele², Erwin Witter⁴, Harry Van Onckelen⁵, Eddy Esmans⁶, Ronny Blust¹ and Wim De Coen¹

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Developing screening methods is an important effort to bypass initial needs to identify individual endocrine disruptors (EDs). However, screening methods for EDs will have to accommodate a wider variety of diverse chemicals than ever been subjected to screening methods before. The urge for more comprehensive in vitro systems that make multiple endpoint detection possible, is however in contrast with the few hormonal tissues currently analysed. So far, testing strategies have omitted the adrenal gland and therefore do not adequately cover the process of steroidogenesis, critical in adrenocortical, testicular and ovarian function. The present study combines the advantage of a pluripotent adrenocortical cell line with the capacity of the microarray technique to analyse thousands of genes at ones. The H295R cell line covers the entire biochemical pathway responsible for steroidogenesis and therefore presents multiple molecular targets for toxicity, ranging from general effects on all steroidogenic tissues (e.g. aromatase) through to specific targets affecting only adrenocortical function. The idea of the project is to develop a cell line specific microarray, which allows classification of EDs according to their mode of action and is an important step in selecting potential biomarkers. In preparation of this custom array, gene expression profiles of H295R cells exposed to model chemicals will be analysed using an extensive human array, covering 21,000 genes. The group of differentially expressed genes form the basis of the custom array. Here, first results of gene expression profiles in preparation of the custom array are presented and discussed.

Differential gene expression patterns in the zebrafish (Danio rerio) embryo – development of a test system for the prediction of chronic toxicity

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Acute, prolonged and chronic fish tests are used for ecotoxicity assessment in the process of the registration of chemicals, biocides, pesticides and veterinary pharmaceuticals. As alternative for the acute fish test, the zebrafish embryo test (DarT – Danio rerio Toxicity test) is available. Until now, no alternative assay methods have been developed for prolonged and chronic fish tests. The objective of the research project Gen-DarT (gene expression Danio rerio Toxicity test) is to develop a gene expression-based test system for zebrafish embryos to predict toxic effects in chronic and prolonged fish tests. Sensitive marker genes were identified by analysis of selected, potential candidate genes by RT-PCR and by using a 14k-oligonucleotide-microarray with cDNA of embryos exposed for 48 h to the model substances cadmium chloride and 3,4-dichloroaniline. We identified 8 marker genes (cyp1a1, abr2, hsp70, fzr1, nrf2, muat, hmox1, mt2), of which the altered expression could be confirmed by quantitative RT-PCR in independent embryo tests. The lowest observed effect concentrations for differential gene expression and toxicity were compared in embryos (48 h) as well as 5 and 30 day-old fish from an early life stage test (ELST). Toxicity in the ELST was the most sensitive endpoint, followed by gene expression in 5 day-old (post fertilisation) fish larvae. Our studies are continued by the analysis of additional chemicals, the identification of further marker genes with higher sensitivity and by comparison of gene expression patterns in embryos and fish larvae.
Lecture

Application of toxicogenomics towards drug safety evaluation

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Introduction: Toxicity remains a major hurdle for the successful discovery and development of new pharmaceuticals. Drug safety studies are generally not conducted until the later stages of the drug discovery process. Ideally, the evaluation of the toxic properties of a compound should be conducted early before a significant amount of time or resources has been spent. However, the traditional *in vivo* toxicologic approach is not suited for an early evaluation, due to compound requirement. At Abbott, we are using gene expression signatures generated using isolated rat hepatocytes to evaluate compounds *in vitro* for their toxic properties.

Materials and methods: Isolated rat hepatocytes were treated with over 100 reference compounds at a TC20 concentration for 24 hours. RNA was harvested, amplified and analysed using Affymetrix microarray chips.

Results: Expression signatures were developed by profiling over 100 reference compounds in isolated rat hepatocytes. The signatures are being used to identify compounds with the potential to induce a variety of toxic changes, including mitochondrial damage, phospholipidosis, microvesicular steatosis and peroxisome proliferation with a high degree of sensitivity and specificity. These signatures were originally developed using microarray technology, but have since been transferred to a gene expression platform with higher throughput, lower cost, and amenable to customisation.

Summary: Identifying and characterising compounds with the potential to cause toxicity early in the drug discovery process leads to a more rationale selection of compounds in discovery, improved productivity of the research and development process, and ultimately should result in drugs with a better safety profile.
Workshop 5.13
Strategies for prioritising and streamlining the validation process

Lecture
Validation via weight-of-evidence approaches

*Michael Balls and Robert Combes*
FRAME, Nottingham, UK

It is not always possible, necessary, or even desirable, to evaluate the relevance and reliability of an *in vitro* or *in silico* test or testing strategy by comparing the predictions it provides with those obtained from an *in vivo* test or testing strategy. A weight-of-evidence approach aims to use all the available information that meets certain criteria, in a structured, systematic, independent and transparent review of relevance and reliability in relation to purpose, the outcome of which will be published in the peer-review literature. Crucial aspects include: The selection of the reviewers and the application of procedures to ensure independence and lack of bias; criteria for the selection of data; procedures for the collection of data and for data quality control; procedures for the differential weighing of various types of evidence, alone and in combination; criteria for test/strategy performance in terms of reliability and relevance in relation to purpose; and agreement on how the outcome should be expressed, published and otherwise made available. Examples will be given to illustrate where the weight-of-evidence approach has been used in the past and where it will need to be applied in the future.
Large numbers of animals are required for the regulatory testing of chemicals, cosmetics, biologicals, biomaterials, pharmaceuticals and other products. Alternative methods and new testing strategies, which could reduce, refine and replace the use of animals, are being developed and need to be validated and evaluated at European level.

The Key Area Strategic Developments relates to enabling technologies and activities (e.g. “omics” technology, high-throughput screening, GLP and GCCP, nanoparticle toxicology) and their use in development and validation of alternative methods for tackling new areas in toxicology and speeding up the validation process by introducing new technologies.

Omics technologies, fingerprints, pattern-based assessments and biomarker approaches are explored for use in regulatory toxicology in close co-operation with other international initiatives supported by in-house laboratory activities.

ECV AM is evaluating how high-throughput screening of substances with \textit{in vitro} cellular systems could assist the validation process by running its own automated facility. This work is also serving the A-Cute-Tox FP6 Integrated Project.

Nanotechnologies and nanoparticle toxicology are areas which are getting more on the political agenda. ECV AM is involved in the FP6 STREP “ToxDrop” focussed on high content analyses of cell cultures. Furthermore, ECV AM’s laboratory group is contributing to assessing the toxicity of organic and inorganic nanoparticles using \textit{in vitro} cell and tissue cultures.

ECV AM continues to steer and contribute actively to advisory and guidance documents on GLP and GCCP crucial for the validation process.

By investing into enabling technologies we hope to reshape hazard identification in European legislation.
Lecture

Optimising validation study designs by separating between-laboratory reproducibility and predictive capacity: The example of Skin Corrosion

Sebastian Hoffmann and Thomas Hartung
European Commission, JRC, ECVAM, Ispra, Italy

The modular approach to test validity assessment (Hartung et al. (2004), Altern Lab Anim, 32, 467-472) claims to be more flexible and efficient by defining validation by seven independent modules. In particular and in contrast to former approaches, the aspects of between-laboratory reproducibility and predictive capacity are formally separated. Potentially facilitating retrospective validation exercises, the main advantage of this separation, however, effects prospective validation by opening up opportunities for reduced and thus more time- and cost-efficient study designs. Taking the previous ECV AM validation study on in vitro methods for skin corrosivity as an example of a successful validation study – two of its methods resulted in OECD test guidelines adopted in 2004 – we analysed the feasibility of this separation. Study designs reducing the number of tests to be performed by up to 50% were simulated with the original validation data of the EPISKIN model. According to these designs, the data were re-sampled at least 10,000 times/design either randomly or stratified randomly, i.e. accounting for the potency in vivo in the chemical selection for reproducibility testing. We demonstrate the effects of the lean designs on the variability of several between-laboratory reproducibility measures and on the predictive capacities, in terms of sensitivity and specificity, in comparison to the original study. Overall, the study results were only little affected by the modelled lean designs. We conclude that the separation of the two modules is a promising way to speed-up prospective validation studies and to substantially reduce their costs without compromising study quality.

Lecture

Catch-up validation: Principles and case studies

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Federal Institute for Risk Assessment, Centre for Alternative Methods to Animal Experiments – ZEBET, Berlin, Germany

In several validation studies performed on in vitro alternative methods, experience has shown that developers of new test procedures tried to define their procedures very tightly in order to assure (i) the best possible outcome for their test systems in these studies, (ii) to be “primus inter pares” in case similar test systems were available, and (iii) to gain the recognition they seemed to deserve because of their intellectual and financial investment in a new technology. However, this “artificial competition” caused significant problems in validation trials of several methods, in particular those employing reconstructed human epidermis/full skin models.

In 1994, Advanced Tissue Sciences (ATS, USA) tried to address regulatory needs of the dangerous good transport system by proposing a skin corrosion assay with the human reconstituted skin model Skin2 that was “tuned” incredibly insensitive, just to be able to discriminate three different corrosivity transport classes. Almost expectedly, the protocol failed in the formal ECVAM skin corrosion validation study because of low sensitivity. At the same time, we had made the experience in the area of phototoxicity testing, that the Skin2 model did not differ with regard to sensitivity/specificity, if we applied an identical protocol to different skin/epidermis models. As a consequence, when the epidermal model EPISKIN (SADUC, France) in 1998 had performed very well in the ECVAM skin corrosion validation study, but then became temporarily unavailable, ECVAM and ZEBET successfully collaborated on the concept of a “catch-up” validation study. We used the model EpiDerm (MatTek, USA) with a similar protocol and prediction model. This study opened the door to a general use of reconstructed skin/epidermal models for skin corrosion testing provided they meet structural and performance requirements defined in OECD Test Guideline 431.

Examples of currently finalised and ongoing catch-up validation studies employing other human skin models will be presented. The necessary balance between formal requirements for such studies and investment of resources when an increasing knowledge has proved high similarity of the models will be addressed.
The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has developed and implemented a process for the nomination and submission of test methods and for their prioritisation for review and evaluation. Prioritisation of proposed test methods is a function of their regulatory applicability, anticipated multi-agency interest and use, responsiveness to the replacement, reduction, and refinement of animal use, potential for improved predictivity of adverse effects relative to currently employed methods, and efficiency and economic savings. The newly revised ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods (http://iccvam.niehs.nih.gov/docs/guidelines/sub-guide.htm) were developed to assist test method sponsors/nominators in organising the information needed to assess the validation status of test methods at any stage of the validation process and the extent to which the ICCVAM validation and acceptance criteria have been or will be addressed. The original guidelines, in use since 1998 to evaluate the scientific validity of test methods that have since achieved regulatory acceptance, have been updated to reflect experience gained and help to facilitate a more efficient process. Adherence to these revised guidelines will help ensure the sufficiency of data and information for independent peer review and for regulatory authorities to determine the scientific validity and regulatory acceptability of test methods. The elements comprising these guidelines have now been incorporated into international guidance for the evaluation of methods proposed for new test guidelines. The ICCVAM nomination, submission, and prioritisation process and the content and organisation of submissions or nominations will be described.

Lecture

The ICCVAM nomination and submission process and guidelines for new, revised, and alternative test methods

Leonard M. Schechtman 1, Marilyn L. Wind 2 and William S. Stokes 3

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Gold standard or FeS? Are non-validated reference tests obstructing the transition to non-animal approaches in toxicology?

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People for the Ethical Treatment of Animals, Research and Investigations Dept., Toronto, Canada

Modern validation standards stipulate that the accuracy and reliability of a new or revised toxicological test method should be evaluated relative to the study it is intended to replace. This proviso seems to take for granted that toxicity tests in widespread use today are valid themselves, even though only a handful of animal tests have ever been subjected to formal or rigorous validation according to ECVAM/ICCVAM/OECD criteria. Nonetheless, some insist that these tests have been “validated by convention”, and regard the data generated by these tests as the “gold standard” to be met by any prospective alternative method. The integrity of this perspective will be examined relative to published Draize and developmental toxicity data, which reveal high levels of intra- and inter-laboratory variability as well as marked species differences in chemical sensitivity. These factors have been closely linked to earlier, unsuccessful efforts to validate replacements to the Draize eye irritation test. Alternate sources of reference data (e.g., occupational exposure and biomonitoring, human clinical drug trials, poison control center data, etc.) will be identified, as will the strengths and limitations of each. This analysis will provide additional support for a recommendation that emerged from an OECD validation conference in 2002: That there is a pressing need for an international workshop on the acquisition and use of human data to better evaluate the relevance and accuracy of new, revised, and existing toxicological test methods relative to the species of regulatory interest.
Lecture

The use of test method performance standards to streamline the validation process

William Stokes¹, Leonard Schechtman², Marilyn Wind³, Amy Rispin⁴, Kalaish Gupta⁵ and Raymond R. Tice⁶

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Regulatory authorities are often required to communicate the basis on which new test methods have been determined to have sufficient accuracy and reliability for specific testing purposes. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently developed the concept of test method performance standards (PS) to address this need. PS are based on adequately validated proprietary and non-proprietary test methods that have been accepted by one or more regulatory agencies. PS can then be used to evaluate the performance of other mechanistically and functionally similar test methods that have been accepted by one or more regulatory agencies. PS can then be used to evaluate the performance of other mechanistically and functionally similar test methods that measure or predict the same biological or toxic effect. PS consist of three aspects: 1) essential test method components, which are the essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed similar test method; 2) a minimum list of reference chemicals selected from the chemicals used to demonstrate acceptable performance of the validated test method, which is used to assess the accuracy and reliability of a proposed similar test method; and 3) the accuracy and reliability values that should be achieved or exceeded by the proposed test method when evaluated using the minimum list of reference chemicals. Proposed PS are developed and undergo concurrent independent peer review during the technical evaluation of a test method. The development and use of PS is expected to significantly streamline the validation and acceptance process for test methods that are mechanistically and functionally similar to accepted test methods.
To meet the requirements of the EU 7th Amendment to the Cosmetics Directive, manufacturers of cosmetics products will need to ascertain the safety of ingredients using non-animal methods. Starting in 2009, *in vivo* genotoxicity tests for cosmetic ingredients will not be allowed. Skin is one of the target areas of interest for many cosmetic products because it is generally the tissue with the highest exposure. Therefore we have begun development of a micronucleus assay using a commercially available 3D engineered human skin model, EpiDerm™ (MatTek Corp, Ashland, MA, USA). We first evaluated whether a population of binucleated cells sufficient for a micronucleus assay could be obtained by exposing the tissue to 1-3 μg/ml cytochalasin B (Cyt B). The frequency of binucleated cells increased both with time and with increasing concentration of Cyt B. Cyt B at 3 μg/ml allowed us to reliably obtain 40-50% binucleated cells at 48 h and was used in future studies. The background frequency of micronuclei in this model is low (∼0.1%) and reproducible. Studies with model genotoxins including mitomycin C, vinblastine sulfate and methylmethane sulfonate demonstrated that micronuclei can be reproducibly induced in this 3D skin model. This is the first step in developing a routine “*in vivo*-like” assay for chromosomal damage in human tissue.
Poster

**Differential effects of irritants and allergens in an epidermoid cell line**

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Experience to date has suggested that dendritic cells are the most likely to respond in a differential manner to chemicals which are either irritant and/or allergenic to the skin. However, the mechanism underlying such a differential response is unknown. Furthermore, there have recently been observations suggesting epidermal keratinocytes may also display a differentiated response. Accordingly, we have measured the response in an epidermoid cell line (A431) to a range of irritants and allergens. The effect of exposure to two or more sub-cytotoxic concentrations of each chemical on the elevation of MHC class II expression (RT-PCR) and the release of interleukin-12 (IL-12) (ELISA) were investigated. The irritant sodium dodecyl sulphate had no effect on either MHC-II expression or IL-12 release. All the allergens tested showed at least 20% increase in IL-12. For MHC II, allergens induced a modest increase in mRNA expression, whereas several irritants failed to induce the MHC. However, these changes in MHC need to be confirmed by quantitative real time PCR. Further work is required to both test this assay and develop the prediction model, but it serves to remind us that in an in vitro test approach for the assessment of irritants and allergens, it is appropriate for us to remember that keratinocytes also play an important role.

Poster

**The COLIPA strategy for the development of in vitro alternatives**

Odile de Silva¹, Rolf Fautz², Stuart J. Freeman³, Carsten Goebel⁴, Irene Manou⁵, Pauline McNamee⁶, Thierry Oddos⁷, Wolfgang Pape⁸, Winfried Steiling⁹, Hidekazu Toyoda¹⁰ and Carl Westmoreland¹¹

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The cosmetics industry’s commitment to phase out animal tests is long standing: since 1992, SCAAT (Steering Committee on Alternatives to Animal Testing) is co-ordinating activities towards development of non animal alternative methods, contributing to their validation. In vitro tests are needed to identify relevant aspects of the complex interactions of a chemical with human skin, eye and other target tissues. COLIPA’s Task Forces (TF) undertake the work necessary to develop in vitro alternatives for toxicological endpoints of key interest, i.e. skin sensitisation, skin irritation, eye irritation and genotoxicity. The TF Skin Tolerance currently runs projects to develop in vitro test systems to identify potential allergens and irritants. The TF Eye Irritation is focussed on the underlying physiological mechanisms of eye irritation and recovery to identify in vitro endpoints more predictive of the in vivo human response to chemicals and is working in close collaboration with ECVAM. For all TFs the current challenge is developing an appreciation of how to use their data output for risk assessment in addition to hazard identification.

Risk assessment for chemicals used as ingredients also covers systemic exposure. The acceptance of a method to assess percutaneous absorption in vitro (OECD 428) was a major success for our TF. As a further step, the TF Genotoxicity plans work to adapt in vitro genotoxicity testing to dermal exposure. In co-operation with academia, industry, scientists and regulators, our strategy tries to combine the best scientific approaches resulting in alternative methods for the most appropriate safety assessment of cosmetics.
Lecture

The challenges of the 7th Amendment

Odile de Silva
L’Oréal, Paris, France

The cosmetic industry has a pro-active approach on the issue of alternatives to animal testing through its voluntary SCAAT research programme since 1992. It has recognised the specific challenges set in the 7th Amendment and responds with an increased engagement in R&D programmes. Domains of toxicity currently covered are eye irritation, skin irritation and allergy, mutagenicity. Research projects are investigating mechanisms of toxicity, however SCAAT teams are also helping the validation process through ECVAM and ICCVAM.

Fundamental scientific questions are raised: the appropriate basic knowledge of key biological mechanisms needs to be understood, what should be the balance between in vitro tests versus in silico or chemistry-based tests, how should the data be integrated and interpreted. This also leads to the fact that although industry is already working in partnership with academia, regulators, ECVAM, etc. What we really need is a critical number of scientists from academia attracted by the challenge of developing “non-animal alternatives” in view of replacing regulatory tests, and who would lead the research in new and bold areas.

Industry together with other stakeholders are currently investigating new and pragmatic thinking: read-across, TTC, analytical methods, etc. There is a need to come up with different approaches, stemming from the fact that we shall have to integrate all kinds of new data, new ways of combining, extrapolating, of dealing with data gaps. Some of these tools will be applicable to “known” chemistry, however new methods should be developed for future chemistry, where no references or similarities can be of help.

Poster

Strategy for in vitro alternatives to inhalation toxicology: Where do we begin?

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The 7th Amendment to EU Cosmetics Directive stipulates that alternatives to repeat-dose toxicity animal studies must be in place by 2013. Addressing the challenges of developing a suitable battery of tests to replace in vivo inhalation testing is no simple task. A multitude of questions must be answered, ranging from the technically challenging “how do we emulate representative inhalation exposure in vitro?” to the more ethereal “what exactly defines a NOAEL in vitro?”. In order to maximise the chances of developing a complete strategy by the EU deadline avoiding replication of effort, cross-industry co-operation is essential. Attempts are being made to formally establish an “International Partnership for Alternatives to Animal Testing” (IPAAT). Meanwhile, we (Unilever, Novozymes and GSK) have established a collaboration to undertake the necessary research and development in the area of respiratory toxicology.

We are currently investigating a range of approaches encompassing both “top down” bridging studies and “bottom up” investigative studies. These include the use of various models (i.e. lung slice, air-liquid interface and co-cultures), in silico systems (i.e. deposition, exposure) and leading edge technologies (i.e. ‘omic markers, stem cell derived models, raman spectroscopy).

Progress is being made – markers of specific endpoints are being characterised in vitro, and efficacy and limitations of different models are being assessed, along with their comparability to observed results in vivo. Research is being initiated into representative aerosol exposure in vitro and the development of mutually available databases.
Lecture

7th Amendment to the Cosmetics Directive

Thomas Hartung
ECVAM, Institute for Health and Consumer Protection, Joint Research Center, Ispra, Italy

The political expectations of the 7th Amendment to the Cosmetics Directive are high with regard to the phasing out of animal experiments: For two years already, a testing ban for finished products is in place. Testing bans for ingredients enforced by marketing bans are approaching in 4 and 8 years, notably, independent of the availability of validated alternative methods. This political pressure has resulted in the targeted development and validation of methods required in a new dimension. However, the question has to be raised, how realistic it is to meet the deadlines for the different animal tests? A review of the efforts and achievements in areas like topical and systemic toxicities, reproductive toxicology or carcinogenicity shall provide an interim analysis of the state of the art.

Poster

Bergamot oil intended for topical use – attempts for a risk assessment of phototoxicity

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Bergamot oil is a widely used aromatic ingredient, e.g. in food and cosmetics. Its use is often limited due to reported phototoxicity, usually attributed to bergapten content.

The aim of this study was to clarify the differences in the phototoxicity of several bergamot oils obtained from different suppliers. The phototoxicity of the samples was evaluated in vitro in the 3T3 NRU Phototoxicity Test (PT) and a phototoxicity test on reconstructed human skin model (EpiDerm™, Mattek). In addition, in case of non-phototoxic classification in the EpiDerm phototoxicity assay, photo-patch testing in a limited group of human volunteers was performed.

Amongst 4 different samples, two phototoxic and two non-phototoxic oils were classified by 3T3 NRU PT, however, only on the basis of borderline phototoxicity results. Surprisingly, even samples classified borderline proved to be clearly phototoxic in the EpiDerm test. In general, the skin model test and human patch test provided concordant results. In both cases, it was estimated that bergamot oils (classified as non-phototoxic by 3T3 NRU PT) were safe for use up to 1%. The skin model test therefore seems to be a useful tool in the risk assessment, since it enables to set a margin of safety before any testing in humans.

Analytical analysis (applying capillary GC/MS) enabled identification and quantification of photoactive compounds present in the test samples. Besides bergapten, differences in citropten, bergamottin, geranial and neral content were identified. We conclude, that the different phototoxic effect depends also on the amount of these components.
Lecture

**Animal testing and alternative methods relating to cosmetic products**

*Georgette Lalis*

European Commission, Directorate F – Consumer Goods, Brussels, Belgium

The European cosmetics industry is an example of an internationally competitive industry which has established itself as a market leader. With 35 billion €, the European cosmetics industry has an ex-factory output which is twice that of Japanese companies and one third higher than U.S. companies. It is estimated that around 5 billion cosmetic products are consumed by Europeans every year. The cosmetics industry is a dynamic industry, characterised by innovation and a high rate of product development. On average, major cosmetics companies replace or reformulate around 25% of their products each year.

The Cosmetics Directive 76/768/EEC has been adopted in order to ensure the free circulation of cosmetic products in the internal market and the safety of cosmetic products placed on it. It also establishes a prohibition to test finished cosmetic products and cosmetic ingredients on animals (testing ban), and a prohibition to market in the European Community, finished cosmetic products and ingredients used in cosmetic products which were tested on animals (marketing ban).

A number of initiatives have been launched at the European level to promote alternative methods to animal testing, such as funding under the 6th Framework Programme on Research and Development amounting to 39 Mio Euros. Research in the development of alternatives is not only beneficial for animals but also encourages the development of new markets for these methods.

DG ENTR and DG Research will hold a conference on animal tests and alternative methods, “Europe Goes Alternative”, on November 7, 2005, in Brussels to demonstrate that the European Commission keeps animal welfare high on the political agenda. Given that the 5th World Congress covers most of the scientific issues, we will pursue a more policy oriented approach for the conference in Brussels to identify further possibilities to improve development, validation and legal acceptance of alternative methods.

Lecture

**Japanese challenge to develop alternative methods for safety evaluation of cosmetics**

*Yasuo Ohno*

National Institute of Health Sciences, Setagaya, Tokyo, Japan

Research group supported by Japanese Ministry of Health, Labor, and Welfare have already evaluated *in vitro* eye irritation test (several cytotoxicity tests), 3T3-NRU phototoxicity tests by the co-operation of JSAAE and Japan Cosmetic Industry Association. These methods are useful for the toxicity evaluation of cosmetics ingredients if they are combined with *in vivo* method in case of ambiguous prediction or utilisation of positive chemicals. We have conducted validation of *in vitro* skin corrosivity tests (VitroLife Skin) and *in vitro* phototoxicity test battery using yeast and red blood cell. These methods are in the process of evaluation. We are going to conduct validation of modified LLNA that do not use radioisotope labelled compounds. The research group is now conducting research to develop alternative methods for *in vitro* acute toxicity tests with metabolic activation steps, *in vitro* skin sensitisation tests, *in vitro* photo-sensitisation tests, and appropriate data collecting and processing procedures for the evaluation of alternative methods. We are expecting our results will contribute to correspond to the 7th Amendment to the EU Cosmetics Directive.
Lecture

**Good science must be the key factor in the development and use of alternative methods for safety assessment of cosmetics**

*Vera Rogiers*

Department of Toxicology, Vrije Universiteit Brussel (VUB), Brussels, Belgium

Background/Aims: With the implementation of the 7th Amendment (2003/15/EC) into the EU cosmetic legislation a testing and marketing ban have been introduced. In practical terms this means that all toxicological testing on animals must be replaced by validated alternatives in a time span of 6 years, with the exception of repeated-dose toxicity, toxicokinetics and reproductive toxicity (10 years). The question arises now whether this is scientifically feasible.

Discussion: ECV AM provided an objective overview of the current status of alternative methods and strategies and the prospects for their validation and regulatory acceptance (30/4/2004). The SCCNFP was asked for its comments (SCC-NFP/0834/04). The clear message was given that total abolishment of animal tests within 10 years was considered to be not feasible from a scientific point of view, in particular, seen the fact that only replacement methods would be allowed. In a joint document, experts from three committees, advising the commission on toxicological matters, came to the same conclusion (CSTEE 2004). Nevertheless, the optimistic time frame was retained by the Commission. Recently, several EU research projects have been initiated, including ReProTect, AcuteTox, Predictomics, Sensitive, etc. which give hope for future new developments. However, seen the timeframe to scientifically elaborate such complex studies, to pre-validate and validate potential successes and to implement these into the EU legislation, it becomes evident that the deadlines cannot be met. It is high time to realise that science follows its own rules and cannot be driven by a political agenda.

Poster

**EU Cosmetics Directive: Failures and challenges from the animal welfare point of view**

*Irmela Ruhdel*

German Animal Welfare Federation, Animal Welfare Academy, Neubiberg, Germany

EU Directive 2003/15/EC lays down deadlines for bans in the cosmetics sector: an animal experimentation ban for 2009 and a sales ban on animal tested cosmetics for 2013. The Directive has to be regarded a minimum compromise, but even some of its basic provisions have not been implemented by the European Commission so far. This concerns for example an immediate ban on animal experiments where animal free methods are scientifically validated or accepted also when they are not included into Annex V of the EU Dangerous Substances Directive. The Commission failed to list several animal free methods that have been endorsed by ECVAM or that have been accepted by the OECD or individual EU Member States in the new Annex IX of the Directive. In its timetables of October 2004 the Commission also ignored the time-limits for the sales ban on three additional endpoints of the safety evaluation as given in the Cosmetics Directive. This is counterproductive as the deadlines for the bans were intended to give a fresh impetus for new animal free tests. The Commission must ensure optimal conditions for the replacement of animal experiments such as sufficient funding. Additionally, all types of industries should be involved actively in this process because all of them profit from new animal free tests and testing strategies. In any case the animal experimentation ban and at least part of the sales ban have to come into force 2009 and 2013 irrespective of the availability of animal free methods.
Lecture

Sound science: A prerequisite for advancing alternative methods and protecting public health

William S. Stokes
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Current laws and regulations protect human health by requiring the safety assessment of new products such as cosmetics prior to their marketing and subsequent human exposure. Such laws were enacted in response to public outrage following blindness, severe injuries and deaths caused by untested cosmetics and other products. Subsequent safety evaluations of cosmetic ingredients and products using animals and in vitro methods have now largely eliminated adverse health effects. Nevertheless, public pressures have led to recent adoption of the 7th Amendment to the European Union Cosmetics Directive, which now bans the use of animals for testing finished cosmetic products and will ban the use of animals for most testing for ingredients in 2009. In order for in vitro methods to gain acceptance as complete replacements for animals, there must be scientific evidence that the use of these methods will provide for equivalent or improved protection of human health. ICCVAM, which is charged by law with evaluating the scientific validity of new, revised, and alternative test methods, has evaluated several alternative test methods applicable to cosmetics testing that have now achieved regulatory acceptance and is currently evaluating several other applicable methods. These methods have or will significantly reduce animal numbers and animal pain and distress; however, none have been found to be scientifically valid as complete replacements for animals. Despite the legislated testing bans, alternative methods will only be able to fully replace animal use and ensure adequate protection of the public when supported by sound science.
In risk assessment of chemicals – due to the lack of human data – extrapolation from the results in animals to the human situation has to be performed. Qualitative and quantitative differences in metabolism play an important role for species extrapolation. CYP2E1 is an enzyme, which plays a critical role in the metabolism of many industrial chemicals. In order to determine species differences in CYP2E1 dependent metabolism of chemicals, a cell battery consisting of V79 cell lines stably expressing CYP2E1 from rat (V79r2E1), man (V79h2E1) and mouse (V79m2E1) has been established. The cell battery has been characterised with regard to enzyme kinetic properties towards the CYP2E1 model substrate chlorzoxazone (CLX) and towards the mutagenicity of N-nitrosodimethylamine (NDMA). Species differences in vmax values of CLX hydroxylation (V79r2E1: 130, V79h2E1: 60 and V79m2E1: 40 pmol/mg protein/min) and in the mutagenic effect of NDMA could be observed.

In order to extend the investigations on species differences in CYP2E1 dependent metabolism, acrylamide has been selected as a further compound. Acrylamide is activated by CYP2E1 to the ultimate genotoxic metabolite glycidamide.

Currently, a LC-MS-MS methodology for the quantitation of acrylamide and glycidamide in cell protein obtained after culture of the respective cell lines is being developed. After incubation of the cell lines expressing CYP2E1 from rat, mouse and human with acrylamide, the parent compound and glycidamide will be quantified and thus, information of probable species differences in CYP2E1 dependent metabolism of acrylamide will be obtained.

Lecture
The use of genetically modified V79 cell lines for the investigation of species differences in the metabolism of chemicals
Ulrike Bernauer¹, Barbara Heinrich-Hirsch¹, Fritz Sörgel² and Ursula Gundert-Remy¹
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Toxicokinetic information is crucial to extrapolate in vitro toxicology data to in vivo situations, and to select concentrations to be tested in vitro. An ECVAM workshop on toxicokinetic prediction using physiologically-based biokinetic models is in preparation. Following a workshop on blood-brain barrier (BBB) permeability, an ECVAM Task Force was set up and a feasibility study to evaluate various in vitro BBB models is planned. ECVAM is involved in the study of absorption barrier models: gastro intestinal barrier (in vitro model validation studies), skin barrier (alternative tests for percutaneous absorption have obtained regulatory acceptance at EU and OECD level in 2004).

Studying the fate of compounds in in vitro toxicology test systems (in vitro biokinetics) is most promising for their correct interpretation. An ECVAM Task Force on metabolism and toxicokinetics was established and a workshop on metabolism in in vitro tests was held in January 2004. A workshop on in vitro biokinetics practices is currently in preparation. ECVAM in vitro biokinetic research looks for correlations between cellular uptake, intracellular distribution, metabolic pathways, interaction with biomolecules of compounds, and their mechanisms of action and toxicological effects, using advanced spectrochemical and radioanalytical techniques.

Furthermore, in the context of the 7th Amendment to the Cosmetics Directive a detailed document was elaborated by expert consultation, including a chapter on toxicokinetics. This provides a basis for further work necessary to replace, reduce and refine the use of animals through alternative tests and strategies under development, before validation at the European Union level.

Poster
**ECVAM Key Area Toxicokinetics: Summary of ongoing activities**

Michel Bouvier d’Yvoire, Sandra Coecke, Massimo Farina, Salvador Fortaner, Siegfried Morath, Barbara Munaro, Nicholas Parissis, Jessica Ponti, Pilar Prieto and Enrico Sabbioni

ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra, Italy

A high-throughput system was developed for cytochromes P450 based on a 96-well-plate technology with a build in sensor for measuring oxygen consumption as a measure for metabolism of drugs. Several drugs were applied to check for stability of the system, reproducibility and reliability. The system can be applied particularly in the early phase of preclinical development to yield metabolism data relevant for humans, e.g. metabolic stability, enzyme profiling, and kinetics to support an efficient selection of lead compounds. The system is available on a non-exclusive licensing base.

Poster
**HTS for human cytochromes P450**

Johannes Doehmer and Juergen Scheuenpflug
GenPharmTox BioTech AG, Planegg/Martinsried, Germany

A high-throughput system was developed for cytochromes P450 based on a 96-well-plate technology with a build in sensor for measuring oxygen consumption as a measure for metabolism of drugs. Several drugs were applied to check for stability of the system, reproducibility and reliability. The system can be applied particularly in the early phase of preclinical development to yield metabolism data relevant for humans, e.g. metabolic stability, enzyme profiling, and kinetics to support an efficient selection of lead compounds. The system is available on a non-exclusive licensing base.
Lecture

**The drivers for in vitro technologies in drug development**

*Johannes Doehmer*
GenPharmTox BioTech AG, Martinsried, Germany

There is widespread concern about increasing cost and time in drug development due to several attrition factors causing poor pharmacokinetics, unforeseen toxicity, and lack of efficacy in humans. In order to improve the situation, a wide range of *in vitro* technologies are being implemented in the drug development process and substitute for animal studies from early stage of target identification, defining better lead compounds, up to preclinical studies, which yield data with higher predictive value for humans.

The *in vitro* technologies currently applied result from various areas, e.g. molecular biology, bioanalytical chemistry, computational chemistry, statistical sciences, biochemistry, and automation engineering.

Examples are given for checking on metabolism related problems with genetically engineered V79 cells expressing specific human cytochromes P450, and the implementation of a HighThroughPut system in the early screening phase of drug development to yield information on metabolic stability of drug candidates which may support the lead compound selection process.

Drug development is considered as the most promising area for installing alternative methods.

Lecture

**Metabolic activation of pro-teratogens in vitro**

*Angelika Langsch, Daniel Eikel, Alfonso Lampen and Heinz Nau*
Veterinary Medical University Hannover, Institute for Food Toxicology and Chemical Analytics, Hannover, Germany

Introduction: Many substances are not directly toxic but must be enzymatically activated to reactive metabolites. Our previous studies with valpromide (VPD) show teratogenic effects in mice that were not found *in vitro*. The development of metabolic systems which can be incorporated within *in vitro* techniques is, therefore, a critical step for the use of *in vitro* methods in toxicology. Ideally, the metabolic system should express all relevant enzymes, because during screening of many substances it is *a priori* not known which enzyme(s) may be involved.

Methods: S9-mix is an easy to handle metabolic system and standard method. Two approaches are tested: pre-incubation and extraction compared to direct cultivation of S9 with target cells. Target cells comprise F9-teratocarcinoma cells and D3 mouse embryonic stem cells, as both proved very useful for detecting teratogens. Their capacities for proliferation and differentiation are monitored.

Results: Known metabolic *in vivo* differences can also be observed *in vitro*: VPD is metabolised by human S9-fraction to teratogenic VPA, whereas with rat S9-fraction VPA is further metabolised. The treated target cells show effects depending on the concentration produced via metabolism. A major problem was encountered: direct product concentrations resulting from S9-metabolism are comparatively low, so a concentration step is needed.

Discussion: Although S9-mix could be used as a metabolic activation system, it is difficult to obtain physiologically relevant data. Enzymes and cofactors are not present in physiological concentrations. Further studies will be done with freshly isolated hepatocytes, which are considered to be the better model for metabolic activation.
Early assessment of absorption, distribution, metabolism, excretion, and pharmacokinetic (ADME/PK) properties of new chemical entities is nowadays occurring at nearly every stage of the discovery process, from lead identification to lead optimisation. A main reason for this is that biologist and medicinal chemists have recognised the value and importance of optimising on drug-like properties in addition to potency and selectivity. Although ADME screening is a necessity, there is more and more awareness that the ADME screening should be rational (“issue-driven”) and should be flexible enough to address the specific needs of each project. This approach requires a careful balance of in vivo studies and in vitro high throughput screening and a careful balance of screening versus prediction.

Despite the improved human-based in vitro ADME screens and the ever-growing evidence of significant species differences with respect to drug metabolism and excretion, animal models are still commonly used within drug metabolism departments of pharmaceutical industries. Area’s such as allometric scaling to predict human pharmacokinetics, enzyme induction and toxicity evaluation, still rely heavily on animal testing in spite of the numerous examples of misleading results.

Currently, one of the major challenges in drug discovery is to accurately predict drug-induced adverse reactions and the role of drug metabolism. This is emphasised by the several examples of drugs withdrawn from the market because of adverse reactions due to metabolic activation. Presently, many in vivo/in vitro tools are in the exploratory stage, including computation approaches and experimental assays to predict drug induced adverse reactions. At this moment, it is unlike to believe that a single assay will be able to predict the potential risk of reactive metabolites and (like for many ADME/PK parameters) a combination of several assays and an integrated approach is required to obtain the best prediction and to minimise the risk for late stage attrition.

### Lecture

**“Issue-driven” drug metabolism screening and the value of human based in vitro models**

*Mario Monshouwer*
Associate Director PKDM – Amgen Inc., San Francisco, USA

A significant portion of substances are not directly toxic, but must be activated to reactive metabolites which exert their toxic potential by reacting with constituents of the cell, especially proteins and nucleic acids. These compounds include polycyclic aromatic hydrocarbons (e.g. benzo(a)pyrene), cyclophosphamide, aflatoxin B1, Vitamin A and acrylamide, and their metabolic activation pathways are well established. For most compounds it is unknown if metabolic activation plays a role. In vitro systems presently can only determine the toxicological potential of the parent drugs, and not that of potentially toxic metabolites. The development of metabolic systems which can be incorporated within a particular in vitro technique, is therefore of high priority. Ideally, the in vitro system should express all the relevant enzymatic activities because during screening of large number of substances it is a priori not known which enzyme(s) are involved. Also, the metabolic system must be compatible with the in vitro assay. Different metabolic systems appear suitable for such a task, and main emphasis is placed on the liver preparations as liver exhibits in most cases the highest amount and complexity of metabolic enzymes:
- S9 (liver 9.000 x g) preparation as used in the Ames test
- Hepatocytes or liver slices
- Genetically engineered cells for expression of relevant metabolic enzymes

The possible advantages of using hepatocytes for activation are several fold: (1) human cells can be used to avoid species differences; (2) they contain enzymes and cofactors at physiological levels; (3) high activity of both phase I and phase II enzymes are present. These metabolic activation systems are now being developed for incorporation into in vitro systems for development of robust testing systems which can be transferred to other laboratories.

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Workshop 5.16
Reproductive toxicology – the EU ReProTect project

Lecture
The future of teratology is in vitro

Jarrod Bailey
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Birth defects induced by maternal exposure to exogenous agents during pregnancy are preventable, if the agents themselves can be identified and avoided. Billions of dollars and man-hours have been dedicated to animal-based discovery and characterisation methods over decades. We show here, via a comprehensive systematic review and analysis of this data, that these methods constitute questionable science and pose a hazard to humans. Mean positive and negative predictivities barely exceed 50%; discordance among the species used is substantial; reliable extrapolation from animal data to humans is impossible, and virtually all known human teratogens have so far been identified in spite of, rather than because of, animal-based methods. Despite strict validation criteria that animal-based teratology studies would fail to meet, three in vitro alternatives have done so. The Embryonic Stem Cell Test (EST) is the best of these. We argue that the poor performance of animal-based teratology alone warrants its cessation; it ought to be replaced by the easier, cheaper and more repeatable EST, and resources made available to improve this and other tests even further.
In addition to the development of batteries of in vitro assays for the effects on critical components of reproduction, a major aim of the ReProTect EU 6th Framework Integrated Project is to improve the accuracy and predictivity of existing and new assays through the implementation of innovative approaches. An interdisciplinary WorkPackage “Cross-cutting Technologies” is therefore under way and includes the following tasks: development of biosensors, currently targeting receptor interactions; development of QSARs to predict receptor binding as well as transfer of chemicals through blood-testis and placental barriers; implementation of metabolic activation systems to in vitro assays for embryotoxicity and endocrine disruption; development of microarray technology as a hazard identification tool for ER-alpha and AR interaction; and, finally, the validation of assays for ER-alpha and AR binding and transactivation, thus representing a major EU component of the OECD validation work. Endocrine disruption also represents a major topic within this WorkPackage, due to its relevance to all areas of the reproductive/developmental toxicology as well as to the overall progression of chemical testing strategies. The outcomes of this WorkPackage will support the other parts of the ReProTect project by providing approaches and experimental models. The achievements of this WorkPackage during its first year include preliminary deliverables in all major areas (e.g., protocols, list of genes for microarrays). Furthermore, two workshops on metabolic activation systems (November 2004, chaired by Prof. Heinz Nau) and sensor technologies (May 2005, chaired by Prof. David Cowell) have been organised.

Lecture

Cross-cutting technologies in the ReProTect project: Objectives and early achievements

Mark T. D. Cronin1, D. Cowell2, D. Dietrich3, A. Freyberger4, A. Jungbauer5, O. Mekenyan6, H. Nau7, B. Van der Burg8, H. Witters9 and A. Mantovani10

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Of the three in vitro embryotoxicity tests validated to classify the embryotoxic potential of unknown chemicals, only the Whole Embryo Culture (WEC) is able to distinguish between specific dysmorphogenesis of organ primordia and general embryotoxic effects.

The embryotoxic effects of the organic solvent N-methylpyrrolidone (NMP), and its metabolites 5-hydroxy-methylpyrrolidone (5H-NMP), N-methyl-succinimide (MSI) and 2 hydroxy-methyl-succinimide (2H-MSI) were assessed. Furthermore, underlying mechanisms for occurring dysmorphogenesis in the head region of the cultured embryos were investigated using whole-immuno-staining (WIS) analysis. 9,5-day old embryos were exposed to the test compounds at increasing concentrations. WIS was performed using antibodies specific for cellular-retinoic acid binding protein I (CRABP-I) indicating neural crest cells (NCC) and specific for 2H3-neurofilament indicating Central Nerves (CN). Specific dysmorphogenes in neurulation and abnormal development of the second visceral arches were induced by NMP and 5H-NMP. WIS revealed disturbed development of CN causing of observed dysmorphogenesis. NMP induced a significantly changed progression of the CN, but did not interfere with NCC. In the order of decreasing embryotoxicity, the ranking of the test substances was as follows: NMP>5H-NMP>2H-MSI>MSI. NMP and 5H-NMP were classified as weakly embryotoxic; MSI and 2H-MSI as non-embryotoxic.

This study suggests that embryotoxicity previously observed after NMP administration to experimental animals is predominantly caused by the parent compound, NMP.
The Key Area Reproductive Toxicity is implemented into an action of the European Centre for the Validation of Alternative Methods (ECVAM), addressing the validation of alternatives for the European chemicals and cosmetics policies. Activities in establishing an ECVAM task force (chair Prof. H. Spielmann) guiding actions in this key area are ongoing. The current activities, targeting embryotoxicity testing based on embryonic stem (ES) cells, neurodevelopmental toxicity and endocrine disruptors are closely linked to the Integrated Project ReProTect, in which ECV AM is responsible for scientific input and the daily management. ReProTect aims for the development and implementation of a tiered testing strategy based on alternative tests for reproductive toxicity. In order to advance this approach, workshops on the test substances selection, metabolic activation, implantation and identification of applicable sensor technologies have been held. Ongoing studies on ES cells embrace further exploitation of the Embryonic Stem Cell Test (EST) and feasibility studies on the transferability to human ES cells. Furthermore, approaches to assess developmental neurotoxicity using different sources of stem cells, namely embryonic, fetal and umbilical cord stem cells are compared. Prevalidation exercises of in vitro methods for detection of compounds with (anti)-estrogenic and (anti)-androgenic activity are currently ongoing in collaboration with US EPA and Japan/CERI. In summary, the development, optimisation and integration of in vitro models into suitable testing strategies is aimed for, not only to decrease animal test numbers in reproductive toxicity testing, but also to gain more detailed information on the toxicological mechanism in different target tissues.

**Poster**

**ECVAM Key Area Reproductive Toxicity:**

**Summary of ongoing activities**

*Lars Hareng, Cristian Pellizzer, Sarah Adler, Tina Stumann, Patrizia Pazos, Miriam Jacobs and Jan de Lange*

ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra, Italy

The Key Area Reproductive Toxicity is implemented into an action of the European Centre for the Validation of Alternative Methods (ECVAM), addressing the validation of alternatives for the European chemicals and cosmetics policies. Activities in establishing an ECVAM task force (chair Prof. H. Spielmann) guiding actions in this key area are ongoing. The current activities, targeting embryotoxicity testing based on embryonic stem (ES) cells, neurodevelopmental toxicity and endocrine disruptors are closely linked to the Integrated Project ReProTect, in which ECVAM is responsible for scientific input and the daily management. ReProTect aims for the development and implementation of a tiered testing strategy based on alternative tests for reproductive toxicity. In order to advance this approach, workshops on the test substances selection, metabolic activation, implantation and identification of applicable sensor technologies have been held. Ongoing studies on ES cells embrace further exploitation of the Embryonic Stem Cell Test (EST) and feasibility studies on the transferability to human ES cells. Furthermore, approaches to assess developmental neurotoxicity using different sources of stem cells, namely embryonic, fetal and umbilical cord stem cells are compared. Prevalidation exercises of in vitro methods for detection of compounds with (anti)-estrogenic and (anti)-androgenic activity are currently ongoing in collaboration with US EPA and Japan/CERI. In summary, the development, optimisation and integration of in vitro models into suitable testing strategies is aimed for, not only to decrease animal test numbers in reproductive toxicity testing, but also to gain more detailed information on the toxicological mechanism in different target tissues.

**Lecture**

**ReProTect – WP2 implantation**

*Lisbeth E. Knudsen*

Institute of Public Health, University of Copenhagen, Denmark

Work Package 2 (WP2) covers the time-window of female reproduction from implantation and maturation of the conceptus till birth, focusing on the process of implantation, including the preparation of the uterine environment, and on placental growth and function.

The main corresponding outcomes from in vivo animal studies in this time window, as investigated in 1 and 2 generation studies (described in OECD guidelines), are:
- pre- and post-implantation loss which are measured as low number of pups
- early and late resorptions
- growth retardation

All these negative outcomes found in animal studies are known from human pregnancies as well, occurring “spontaneously” and/or being induced by chemicals such as pharmaceuticals and recreational drugs. These are endpoints where very little information on mechanisms of action is available in the literature, be it in women or in experimental animals.

The human endometrium, the implantation process and placenta formation and function differ significantly from those of rodents, and only some monkey species are fully comparable with humans. To obtain valuable information from in vitro test systems, the use of human tissue is thus if not mandatory, at least favourable. This approach is unique among ReProTect sub-projects and can be considered a major input and at the same time a challenge.

Work Package II is co-ordinated by professor Lennart Dencker, University of Uppsala – this work package will start after 24 months; new partners will be recruited after the 18 months.
W.P. III.1. Early prenatal development: All test guidelines for regulatory embryotoxicity testing are based on experiments in pregnant animals. Recently, however, several in vitro embryotoxicity tests have successfully been validated in an ECVAM validation study. One of them was the Embryonic Stem Cell Test (EST), in which a mouse ES cell line is used, since ES cells are pluripotent and can differentiate in culture into cells of most organs. In the EST the effects of test chemicals on differentiation into beating myocard cell is tested. The EST has the advantage that no embryos or embryonic tissues have to be obtained from pregnant animals. In the ReProtTect project the database of chemicals tested in the EST will be extended and development of ES cell into other major target tissues will be standardised, e.g. nerve cells, cartilage and bone cells. In addition, the potential of human ES cells for embryotoxicity testing will be exploited, since this may allow to avoid problems arising from species specificity.

W.P. III.2. Late prenatal development: Rodent post-implantation Whole Embryo Culture (WEC) is an ex vivo test focusing on organ formation during embryogenesis. In the ECVAM validation study the WEC provided reproducible results in four laboratories. However, since metabolic activation of xenobiotics is important for proper determination of toxic effects of chemicals in culture systems, the project will focus on introducing a metabolising system as an adjunct to WEC. Therefore, an existing in vitro metabolising system will be introduced as a pre-incubation to the established WEC.
Recent years have seen a growing emphasis towards the use of computational methods, such as Quantitative Structure-Activity Relationships (QSARs), to predict toxicity and fate. Interest has been highlighted by their possible use in product development and regulatory applications, not least the Cosmetics Directive and REACH within Europe. The time for rhetoric is at an end, and there is now a need towards implementing these techniques. This presentation will discuss the possibilities, practicalities and limitations of the use of in silico technologies, specifically in a regulatory setting. In particular, the vision for the use of in silico techniques goes beyond traditional QSARs and expert systems. There is the potential for a shift in the toxicological paradigm towards intelligent and integrated testing strategies that incorporate decision support systems. These could bring together existing data, read-across as well as predicted values. To assist the vision becoming reality, the practicalities of implementation into a regulatory setting must be borne in mind. These will include whether we accept valid, as opposed to validated, models, and how integrated strategies may be assessed and used.
In regulatory toxicology, the experimental assessment of reproductive toxicity is most costly, complex and time-consuming and requires the highest number of test animals. Grouping of chemicals into categories is one of the approaches that can be used to reduce the number of animal tests in safety and risk assessments of chemicals. This is expected to play an important role also in the future new EU chemical policy called REACH. The application of the category approach for reproductive toxicity endpoints was investigated, using phthalates as an example. A group of 10 ortho phthalate esters was selected to categorise the phthalates that produce severe reproductive effects in experimental animals and those that do not. The differences in physicochemical properties, absorption rates and metabolism between the phthalates investigated could not explain the difference in their reproductive toxicity. It appeared that phthalates with side chain length from C4 to C6 can produce similar reproductive effects in experimental animals. The anti-androgenic effects observed in post-organogenesis in vivo studies seem to be the most crucial. From this investigation it is expected that phthalates included in the tight boundaries of this category would all show these anti-androgenic effects. Further testing might not be needed for phthalates within these boundaries. For phthalates outside the boundaries the reproductive potential remains unclear.

(Q)SARs will need to be used for assessing chemicals to limit animal testing in the framework of REACH and cosmetic directive. Regulatory use of these methods will increase when the tools are officially validated. We present the validation of the existing (Q)SAR rule base of the BfR (former BgVV), based on new chemicals for predicting the skin irritation and corrosion potential of chemicals. This rule base predicts non-irritation and non-corrosion by means of physical-chemical limit values and special classes of empirical formulas, such as CHal and CN. Within this project we will re-evaluate the rules, taking into account the OECD principles on (Q)SAR validation. We will also externally validate the rule base using 200 new chemicals that were not used for developing the model. Since these rules address the absence of irritation/corrosion, the number of false negatives, if any, will be reported. The number of chemicals of the validation set that predicts the absence of corrosion/irritation will be shown. This number also presents the number of tests that can be omitted in case this rule base is applied. The question as to whether this external training set of 200 chemicals fully covers the applicability domain of the rule base will be presented. This work is directly related to the presentation on the SICRETool that is a tiered approach assessing the skin irritation/corrosion potential without in vivo testing of Walker et al. as it validates the first step in this tiered approach.
Lecture

**Applicability domain of a QSAR assessed in parameter and structural space**

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Applicability domain (AD) of a QSAR has been so far estimated on the basis of model descriptors’ space coverage by the training set. Different numerical approaches to estimate this subspace yield different ADs. Particular choice depends on training set data distribution and dimensionality of the model (i.e. number of descriptors). In general, interpolative predictive accuracy within descriptor space is on average greater than extrapolative predictive accuracy. That however, is only true on average, i.e. there are many individual compounds with small error outside of the descriptors space coverage, as well as individual compounds with large error inside the domain. By identification of descriptor coverage, we make only a partial step towards defining model’s AD. There is always a possibility that the model is missing a descriptor needed to correctly predict a queried chemical’s activity. Then, despite the chemical being in the parameter domain, the chemical’s activity is predicted with error. To address this problem and refine AD estimation we propose to add a global structural similarity test to ensure that the structural features in a new test compound are covered in the original training set of chemicals. These two conditions: 1) being in the model’s parameter space coverage and 2) structural similarity to the training set are complementary. Therefore, in order to describe the domain robustly, the full training set comprising both structures and descriptor set is required. We will provide case study of mutagenicity QSAR by Debnath et al. (1998) AD estimation as example of the proposed approach.

Poster

**Introduction to the Artificial Neural Networks (ANNs) and their applications in QSAR studies**

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QSAR studies rely upon statistics and other techniques to derive mathematical models which relate the biological activity of a series of compounds to one or more properties of the molecules. These properties, or descriptors, may be derived from numerous sources including refractive index, octanol/water partition coefficient or spectral data. Many methods were used for mathematical modelling in QSAR studies with few or many limitations and assumptions e.g. regression analysis in QSAR model building with assuming linear relationship between the biological activity and one or more descriptors. Artificial Neural Network modelling is one of such affords originated from a field called Artificial Intelligence (AI). Artificial Neural Networks (ANNs) are the mathematical algorithms generated by computer that approach the functionality of small neural clusters in a very fundamental manner. For their application in QSAR studies, the individual network is built and trained with the source data and actual biological activity. The trained network is useful for predicting the biological activity of presented new chemical compounds. In this paper, the author is going to introduce the Artificial Neural Network technology, the basic concepts and working of Artificial Neural Networks and their possible applications in QSAR studies.
Consensus classification of chemicals according to the mechanism of toxic action to fish

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Quantitative Structure-Activity Relationships (QSARs) rely on the paradigm that chemicals belonging to the same or similar chemical classes behave in a similar manner. In the field of aquatic toxicology, it is widely agreed that the QSARs are valid for prediction within the same applicability domain, i.e. for the same mechanism of toxic action (MOA). The aim of this study was to perform consensus classification according to MOA of 177 chemicals taken from the OECD Screening Information Data Set (SIDS) for high production volume chemicals. For this purpose four classification schemes were compared. The first scheme was applied in-house and used to classify chemicals into seventeen MOA. The second one was done by an expert and included a similar number of mechanisms. The third one was performed according to the rules implemented in ASTER (Assessment Tools for the Evaluation of Risk, U.S. EPA – Duluth MN). A consensus classification based on the majority principle was achieved comprising nine MOA. The consensus MOA of the 177 chemicals were then compared with the classifications obtained by applying the scheme of Verhaar et al., 1992, Chemosphere 25, 471-491. As a result, 75 chemicals were classified as non-polar narcotics (NPN) and 12 as polar narcotics (PN). Their acute toxicity to fish can be predicted confidently by the NPN and PN models, including those recommended by the Technical Guidance Document of the European Commission. The remaining 90 chemicals were classified as reactive and for prediction of their toxicity we suggest the use of MOA-specific QSAR models.

The use of similarity measures in defining the applicability domain of skin sensitisation SARs

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In the (Q)SAR field, the applicability domain (AD) is widely understood to express the scope and limitations of a model, i.e. the range of chemical structures for which the model is considered to be applicable. For QSAR models, the parameter space is typically represented by defined ranges of physico-chemical descriptors. For SAR models in the form of structural alerts, the parameter space is typically represented by the structural feature that defines the presence of a hazard.

One potential approach to prevent the inappropriate application of a (Q)SAR model involves the use of similarity measures to compare a new query chemical with those present in the training set. Such a similarity measure should ideally reflect the mechanistic basis for the (Q)SAR, although the use of structural analogy alone may be appropriate in situations where the mechanism is unclear.

This work explores the use of similarity measures for a set of structural rules and investigates their utility in the validation of (Q)SARs. Examples are based on structural rules for skin sensitisation.
Lecture

SAR meets LLNA – Structure Activity Relationship triggers the Local Lymph Node Assay for testing the skin sensitisation potential

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One of the fundamental toxicological issues for chemical substances is their skin sensitisation potential. Therefore, regulators require proper skin sensitisation tests both with guinea pigs or the Local Lymph Node Assay (LLNA) with mice for hazard identification, and proper risk assessment when chemicals are intended for dermal contact, like cosmetics.

Driven by the interest to follow the 3R principles: refinement, reduction and replacement of animal tests, there is an urgent need to optimise currently accepted test methods. Chemical specific parameters have been calculated by a newly developed computer program and result in a Structure Activity Relationship (SAR), predictive for the estimation of LLNA test results. Such theoretic estimation of sensitisation potential is useful to focus the animal tests on the most promising chemicals.

Based on the hypothesis that the skin sensitisation potential is related to specific chemical structures, the SAR has been calculated and in parallel, officially required LLNA sensitisation tests have been conducted. Out of about 100 test substances, chemical groups were defined according to their complex physico-chemical parameters in respect to these test results. Such SAR information was found to be an added value for the screening of new chemical substances as well as for optimising individual LLNA test protocols.

The results demonstrate that SAR data are scientifically helpful to understand the interaction of chemicals with the immune system and prospectively helps to reduce the need of animal tests.

Lecture

Roles for QSAR in risk assessment

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Early pioneers in QSAR development believed in the premise that laboratory experiments should not be performed without a firm expectation of the results before going into the laboratory. The value of QSARs in risk assessment will be to provide us with those expectations for a wide variety of exposure and hazard assessment endpoints before the decision to require specific testing is made. Most of the current QSAR models are limited only by the lack of designed databases; however, as they evolve, QSAR models for most endpoints will undoubtedly be used to provide us with test expectations for thousands of untested chemicals. In so doing, QSAR will complement the 3Rs with a powerful new tool to minimise animal testing which is not likely to influence regulatory decisions. If it is true that 95% industrial chemicals have lower probability to be classified as an EDC than n-butyl aniline, avoiding testing on those chemicals can be achieved by QSAR screening. With the recent development of computer simulators of metabolic activation (e.g. the virtual liver), improved QSAR models for skin sensitisation, respiratory irritation and genotoxicity will follow quickly. Finally, the integration of QSAR models with in vitro methods holds great promise in the prudent use and interpretation of our testing and assessment resources.
Lecture

The assessment of the skin irritation/corrosion potential without in vivo testing

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This presentation shows the possibility of assessing the skin irritation/corrosion endpoint without or limited further animal testing using a tiered approach called SICRET (Skin Irritation Corrosion Rules Estimation Tool). The proposed mechanism behind irritation is that an organic chemical first needs to penetrate the skin before the reactivity of the chemical can cause cytotoxicity leading to irritation or corrosion. This mechanism is used as a starting point in the tiered approach. As a first step physicochemical limit values were derived from a database of circa 1300 chemicals. For chemicals outside these limit values no irritation/corrosion is expected and therefore need not be classified. The validation of these physicochemical rules will be presented by Hulzebos et al. Chemicals that fall within these limit values follow the second path in the tiered approach. These chemicals need to be checked for irritation or corrosion alerts and if present they can be classified accordingly. For chemicals with dermal absorption potential and no structural alerts prevalidated in vitro testing is proposed. If also this result shows no irritation/corrosion we propose to delete the in vivo test for confirmation requested in the OECD guideline. Though this endpoint and this strategy might not score high on the number of animal saving it scores on animal welfare. In summary, SICRET is a “tiered approach” that uses physicochemical property limits, structural alerts and in vitro tests to classify chemicals that cause skin irritation or skin corrosion without further animal testing.
During the last years data mining techniques have gained much popularity for the prediction of toxic activities. This presentation starts with a review of this approach, a summary of the present state of the art in this area and a brief discussion of application areas and limitations.

The second part will describe a specific realisation of these ideas and techniques in the lazar prediction system and its application for the prediction of carcinogenicity at various levels of detail. In crossvalidation experiments lazar is capable to predict rodent carcinogenicity for the Carcinogenic Potency Database (1376i compounds) with more than 70% accuracy. This accuracy is very competitive for an endpoint, that is very hard to predict with traditional (Q)SAR techniques.

Lazar is capable of discriminating reliably between trustworthy and untrustworthy predictions. An inspection of misclassified structures reveals, that the majority of misclassified instances falls indeed beyond the prediction scope of the training data. As lazar provides the rationales for predictions in an understandable and traceable manner it can be applied for 1. The prediction of untested structures (replacement of animal experiments for predictions with high confidence), 2. The identification of information deficits and the priority setting for further testing (refinement of animal experiments), and 3. The identification of hypothesis about toxicological mechanisms.
Lecture

Quantitative Structure-Permeability Relationships (QSPeRs) in reproductive toxicology: Crossing the placental and blood-testis barriers

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The replacement of animal testing for endpoints such as reproductive toxicity is a long-term goal. It is probable that integrated testing strategies, combining together in silico and in vitro approaches, are likely to be the most productive. This study describes the possibilities of using simple (quantitative) Structure-Permeability Relationships ((Q)SPeRs) to predict whether a molecule may cross the placental membrane, or the blood-testis barrier. The concept is straightforward, if a molecule is not able to cross one of these barriers, then it will not be a reproductive toxicant. Such models could be placed at the start of any integrated testing strategy. To develop these models literature data were collected for the transfer of molecules across the membranes. Whilst a reasonable number of data are available for the modelling of the ability of a molecule to cross the placenta, relatively few are available for the penetration of the blood-testis barrier. This indicates a possible need for more work in this area. Modelling of the permeability data indicates that significant (quantitative) Structure-Activity relationships can be developed for the ability of molecules to cross these biological barriers.

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Poster

Prediction of human skin permeability of chemicals in various vehicles using artificial neural network

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This study was carried out to develop a novel method for predicting the human skin permeability coefficient (logKp) of chemicals, where vehicle effects were considered. The data set consisting of 359 measured logKp for 151 chemicals dissolved in various vehicles, was analysed. Molecular weight (MW) and log (octanol-water partition coefficient) (logP) of chemicals, and logP of vehicles were calculated by Pallas (CompuDrug International Inc., South San Francisco, CA) as molecular descriptors for prediction. The relation between these descriptors and logKp was examined using feed-forward back-propagation neural network. The neural network model with a configuration of 3-5-1 for input, hidden and output layers was much superior to the multiple linear regression model in terms of root mean square (RMS) errors (0.675 vs 0.887). A leave-some-out cross-validation demonstrated that the neural network model predicted Kp with a reasonable accuracy (predicted RMS error of 0.723).

In addition, we also developed a novel method for predicting the human skin apparent diffusion coefficient (D) of chemicals. The data set consisting of 107 measured logD for 61 chemicals was analysed. MW and logP of chemicals are used as descriptors to predict logD of chemicals. The RMS error of neural network model with a configuration of 2-5-1 was 0.553 and a leave-some-out cross-validation revealed that the neural network model predicted D with a reasonable accuracy (predicted RMS error of 0.606).
Lecture

**In vitro prediction of human dermal and oral absorption for Physiologically-Based Pharmacokinetic (PBPK) modelling**

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Traditional toxicokinetic (TK) studies provide data on the absorption, distribution, metabolism, and excretion (ADME) of a chemical to support interpretation of repeat dose toxicity tests i.e. relevance of adverse effects observed at high doses to human health. The introduction of the 7th Amendment to the EU Cosmetics Directive (76/768/EEC) will result in the ban of repeat dose toxicity and TK studies of chemicals used as cosmetic ingredients in animals by 2013. Therefore, there is a need to develop *in vitro* alternatives to these traditional *in vivo* studies.

PBPK models enable the study of chemical concentration time profiles in individual organs, tissues and plasma and can form the basis of human health risk assessment. These models are populated by *in vitro* data, including those derived from simple absorption models. We have investigated the Parallel Artificial Membrane Permeation Assay (PAMPA), Immobilised Artificial Membrane (IAM) chromatography and Caco-2 cell lines, to provide an early indication of whether these systems can adequately model oral and dermal absorption of chemicals. The hypothesis is that the data generated from these models can then be integrated into PBPK models as an alternative to data derived from TK studies in laboratory animals. In the future, the linkage of absorption simulation and PBPK models, coupled together with Pharmacodynamic (PD) modelling, will bring us closer to a full simulation of chemical disposition and effect, one that ideally could be based on only a few properties readily measured *in vitro* and/or computed.

Lecture

**Integration of PBPK and reaction network modelling: Predictive xenobiotic metabolomics**

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The recent emphasis on the application of “systems biology” to biomedical research invariably traces its origin to “cybernetics”, as advanced by Norbert Wiener in the mid 20th century. In those early days, the integration of “computing machines” and biology was advocated by a handful of visionaries. Our research group, in the past 15 years, has attempted such a systems biology approach towards the advancement of chemical mixture toxicology. Specifically, we aim to integrate computational modelling with *in vitro* and *in vivo* experimentation to address the question “How does one deal with the potentially astronomical number of combination of chemicals and other possible stressors in the context of cumulative risk assessment?” Our answer is to first focus on the fundamental biological and toxicological processes occurring in the normal system. The idea is that once we have sufficient understanding of normal biological processes, all stimuli and insults from external stressors can be treated as perturbations to these processes. The next step is to capture the essence of these processes into modelling frameworks by integrating recent advances in computational technology and modern biology. In the case of complex chemical mixtures and their interactions, the computer-assisted approach of Biochemical Reaction Network Modelling offers a ray of hope. The possible linkage between this novel computational methodology and Physiologically-Based Pharmacokinetic (PBPK) modelling could result in a multi-scale computer simulation platform capable of predicting complex pathway interactions and metabolite concentrations at the molecular level up to tissue and organ concentrations and exposures at the organism level.
Living organisms from single cells to people can be thought of as “biological machines” – feedback control systems following genetically-determined developmental programs and in adulthood focusing on homeostasis and reproduction. Systems engineering principles that define the control circuits in man-made machines are also applicable to living systems. In fact, striking parallels exist between control circuits in complex machines and in biological cells and tissues (Carlson and Doyle, *PNAS* 99, Suppl. 1, 2538-2545, 2002). Regulatory networks exist at all levels of biological organisation – molecular, cellular, tissue and organism – and a systems engineering approach to characterising their structure and function appears to be possible. We can ask if and when computational models will be ready to replace laboratory animals in toxicological research and testing. First, however, we should recall a cardinal rule of computer programming – garbage in – garbage out. In other words, a robust, predictive computational model of a biological system must be based on a sound understanding of that system. The rate-limiting step in the development of these models is the rate of our progress in understanding the relevant biology. Although a revolution is underway in the study of basic biology it will be some time before we can draw inclusive circuit diagrams of living cells and tissues. Today’s computational models are thus incomplete and are not suitable replacements for laboratory animals. Computational models do, however, have important roles to play as adjuncts to classical toxicological methods. Three dimensional modelling of protein structure, for example, can be used to screen chemical structures for binding behaviours potentially associated with toxic effects. Physiologically-based pharmacokinetic models help to ensure efficient experimental design and thereby refine animal use. The ongoing, rapid development of new biological understanding and the explosive growth of computer hardware and software technologies guarantee that the role of computational modelling in toxicology will expand continually. While these developments will not, in the foreseeable future, eliminate the need for laboratory animals, they will lead to significant refinement and possibly to reduction of animal use.

Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy.
The Tissue Metabolism Software (TIMES) was used to facilitate the interface of skin sensitisation model which incorporates skin metabolism and consider the potential of parent chemicals and/or their activated metabolites to react with skin proteins. The training set comprised 634 diverse chemicals classified as significant, weak and non-sensitisers. Since skin sensitisation potential depends upon the ability of chemicals to react with skin proteins either directly or after appropriate metabolism; a metabolic simulator was constructed to mimic the enzyme activation of chemicals in the skin. This simulator contains 203 hierarchically ordered spontaneous and enzyme controlled reactions. The covalent interactions of chemicals and their metabolites with skin proteins were described by 83 reactions falling within 39 alerting groups. For some of these groups specific (Q)SARs were utilised to determine stereo-electronic characteristics that might enhance or inhibit activity. The present skin sensitisation model was able to predict correctly 80% of the significant sensitisers, 34% of the weak sensitisers and 72% of the non-sensitisers. A set of 96 chemicals tested for skin sensitisation and not used in the training set were used for external validation of the model. The model predicts the external data fairly well if a model domain was determined based on the concept of the mutual influence amongst first or second neighbour atoms in a molecule. In this case, the correctness of predictions was either 71% or 87% depending on how strict the domain was defined. The correctness of predictions was reduced to 52% if the model domain was ignored.

Lecture

TIMES skin sensitisation model: Development and validation

Sabcho Dimitrov1, Lawrence Low2, Grace Patlewicz3, Petra Kern4, Gergana Dimitrova1, Mike Comber5, Richard Phillips2, Jay Niemela6, Paul Bailey2 and Ovanes Mekenyan1

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The process of validation is an integral part in the establishment of new toxicological methods. A crucial part of this process is based on the application of adequate biostatistical methods.

Especially when new technologies shall be applied within existing test systems, qualified procedures for comparisons of different endpoints and their results are essential.

In this study the results of a joint project by ZEBET and German pharmaceutical companies to improve the Embryonic Stem Cell Test (EST) were taken as example for the biostatistical evaluation of new endpoints.

The validated endpoint of the EST consists of the microscopic analysis of cardiomyocyte differentiation after application of reference substances. Additionally, molecular endpoints assessing gene expression by flow cytometry (FACS) and real-time-PCR (RT-PCR) were selected. Ten reference substances with different embryotoxic potentials (non, weakly and strongly) were tested by microscopic analysis, FACS and RT-PCR.

Differences between substance effects and test methods for ZEBET (Microscope/FACS) and for Schering (Microscope/RT-PCR) were assessed with univariate analysis of variance (ANOVA). The inter-laboratory reproducibility was determined using results obtained with the validated endpoint. The already validated biostatistical prediction model was applied to the new endpoints and the predictive power assessed.

It could be shown that the occurring variance in the test results is mostly caused by reference substances and only to a minor part depends on the applied method or the conducting lab.

In conclusion, new endpoints may be implemented in a test system if they do not differ significantly from the validated ones and the results lead to the same predictive outcome.
Poster

Strategy for (Q)SAR evaluation of chemical genotoxicity

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There are approximately 20,000 existing chemicals that should be evaluated urgently for human health effect but only 10% of them have been, more or less, evaluated. It is, however, not realistic to perform toxicological tests on all chemicals. In 2003, the law concerning the examination and regulation of manufacture/import of new/existing chemical substances in Japan had been amended, and became effective last year. The (Quantitative) Structure Activity Relationships (Q)SAR approach has also been recommended for consideration as an aid in risk evaluation. We have validated commercially available (Q)SAR systems (DEREK, MultiCase, and AdmeWorks) employed widely in overseas regulatory agencies, by using data on more than 200 chemicals registered for existing chemicals in Japan. These chemicals had been tested under GLP compliance, thus the quality of test results could be considered sufficient for the learning dataset. We evaluated the (Q)SAR systems individually using existing chemicals with genotoxicity data and using also the database published by Kirkland et al.. An in silico evaluation flow was constructed, combining these systems after filtering with molecular weight of the chemical. We obtained satisfactory outcomes of evaluation of Ames assay results applied to the proposed flow.

Poster

The combined use of (Q)SARs and in vitro testing methods for creating intelligent testing strategies for local effects – potential and current limitations

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In the EU, for both animal welfare and economical considerations, the future policy for the risk assessment of chemicals (REACH proposal) relies on the use of both (Q)SARs and in vitro testing methods. Consequently, so-called intelligent testing strategies that are based completely on non-animal testing and/or in silico prediction methods have lately received increasing attention. In the past 15 years, activities at the BfR in this field have focused on both the validation of in vitro methods at ZEBET and the development of valid (Q)SARs for the prediction of the presence or absence of substance-related adverse effects.

The work of Ingrid Gerner and colleagues at the BfR to predict/exclude local effects such as skin/eye irritation and corrosion or skin sensitisation has led to a set of physico-chemical exclusion rules and structural alerts that have been submitted to the European Chemicals Bureau for external validation in early 2005.

The potential and possible limitations of these rules and alerts in combination with well-established in vitro testing methods to be used in the frame of animal-free testing strategies for local effects are discussed. Both components could complement each other to close data gaps and extend the applicability domain of such a testing strategy. Future work needs are addressed.
Poster

Optimisation of pyrogen testing in parenterals according to different pharmacopoeias by probabilistic modelling

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The rabbit test to detect pyrogenic contamination in parenterals is crucial to ensure patient safety. The pharmacopoeial tests in Europe, the US and Japan are based on the fever reaction of rabbits, but differ in their experimental design and in their algorithms to assess contamination. Employing an international reference endotoxin, fever can be induced in rabbits. Data from 171 rabbits built the base for probabilistic modelling of the fever reaction and for the comparison of the pharmacopoeial tests. The rabbit fever reaction could be modelled as a function of the amount of injected endotoxin (per kg body weight) by linear regression. Combining the pharmacopoeial algorithms of the rabbit pyrogen test with the developed model allowed analysis of differences regarding test results and animal consumption. This showed that the assessment of pyrogenic contamination strongly depends on the respective pyrogen test stipulated by regulations. Additionally, the approach was used to develop a new experimental design. Two specific versions of this design resulted in a reduction of the number of animals used by about 30% while the safety of the test was maintained. A need for harmonisation is evident, allowing optimisation of the experimental design, which promotes animal welfare.

Lecture

Prevalence and test interdependence: Pivotal parameters in the design and validation of testing strategies

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Testing strategies in toxicological hazard assessment are usually defined by the sequential use of existing knowledge, chemical information, in silico, in vitro and in vivo approaches. As stand-alone alternatives, replacements to animal tests seem to be achievable for only a few toxicological endpoints. Thus, a combination of in vitro and other tests applied in a strategic manner is needed to replace or at least optimally reduce in vivo testing. Besides animal welfare, such a strategic approach needs to accommodate safety and economical aspects. These three factors can be optimally addressed only when the prevalence of the toxicological effect of concern and test interdependence are analysed and incorporated. As pilot cases, the prevalence according to the current in vivo test for skin irritation (<10%) and eye irritation (15-20%) for the applicability domain of new chemicals were assessed by employing the European New Chemicals Database. Predictive values of alternative tests, calculated with such prevalence estimations, enable an optimised test assessment and strategy design, e.g. in low prevalence situations they demand a strategy first focusing on the correct identification of non-toxic substances. To highlight this, we modelled two strategies proposed for eye irritation with three steps each based on assumptions of tests’ predictive capacities and test interdependence. Consequences for test development and the validation process are discussed. We conclude that information on prevalence and test interdependence is essential for the design of testing strategies in which the objectives of safety, animal welfare and costs have to be balanced.
The ECVAM Key Area “Biostatistics and computational toxicology”, established in 2005, was set-up in order to address the growing analytical demands of rapidly developing validation studies and concepts. Anticipating an increasing number of validation exercises, this key area shall support their design, conduct and analysis. Emerging fields in validation such as retrospective approaches or testing strategies bear new statistical challenges. Furthermore, this key area shall serve as a contact point for validation efforts dealing with computational approaches, e.g. (Q)SARs or expert systems. One of its first activities is to build up a network of experts especially addressing the emerging challenges, e.g. retrospective data analysis, evidence-based approaches to toxicological problems and decision making aspects, foreseeing a first meeting at the end of this year. However, two recent publications (Hoffmann et al., 2005, *Regul. Toxicol. and Pharmacol.* 41; Hoffmann and Hartung 2005, *Tox. Sci.* 85) constituted first achievements demonstrating ways to introduce evidence-based approaches into validation. A feasibility study on more efficient validation study designs and a case study highlighting statistical aspects in the design of testing strategies are just finalised. Regarding actual studies, the major achievement was the integral statistical supervision of validation projects on *in vitro* pyrogen tests (Hoffmann et al., 2005, *J. Immunol. Methods* 298). These were accompanied by a thorough analysis of the corresponding animal experiment (Hoffmann et al., 2005, *J. Endotoxin. Res.* 11). Ongoing validation activities involving this key area are *inter alia* studies on skin and eye irritation, cell transformation assays and acute systemic toxicity.

To balance limited possibilities to generate experimental data, safety assessments will increasingly rely on data from the non-test methods. *In silico* based information may supplement available experimental test data making it possible to reach a conclusion with more certainty on an endpoint of concern, or it can be used to replace experimental test where there is no test data available. The non-test methods include SAR and QSAR. SAR can be applied for read-across/analogue/chemical category identification while QSAR for a quantitative endpoint prediction. Formal derivation of SAR is very similar to QSAR development. (Q)SARs (QSARs and SARs) can be developed for both *in vivo* and for *in vitro* endpoints. Validated (Q)SAR models of *in vivo* data may potentially lead to replacement. Feasibility to develop reliable (Q)SARs depends on an endpoint. For example NOEL for systemic toxicity is not a well defined endpoint from modelling point of view as it represents already interpreted data coming from a suite of endpoints and inherently "noisy". Further some of the *in vivo* endpoints such as eye irritation elicit substantial intra chemical variability due protocols used. Quality of QSAR models of such “noisy” data is limited. QSAR for *in vitro* endpoints can be built to further reduce costs and time. These models can likely achieve higher predictivity compared to QSARs for *in vivo* endpoints because they will be based on a higher quality data, will model simpler (in terms of biology) endpoints, and there will be a better mechanistic knowledge about the endpoint.
Lhasa pioneered the sharing of knowledge about mechanism-based structure-activity relationships by computer, and many commercial and regulatory organisations have contributed to the knowledge base. Information can be shared in sufficient detail both to make predictions about potential toxicity and metabolism, and to support and justify the predictions to a user, without disclosing commercially sensitive material. More recently, projects run by the International Life Sciences Institute in Washington, Lhasa, and a consortium in the USA, have explored ways of encouraging the sharing of toxicological data with some success. This talk will discuss how issues that have constrained data and knowledge sharing are being addressed. The indications are that data and knowledge sharing will become established as ways to make research more productive, to save cost, and to reduce the need for animal experiments.

**Poster**

**Realistic approaches to sharing data and knowledge**

*Philip Judson*

Lhasa Limited, Leeds, UK

Lhasa pioneered the sharing of knowledge about mechanism-based structure-activity relationships by computer, and many commercial and regulatory organisations have contributed to the knowledge base. Information can be shared in sufficient detail both to make predictions about potential toxicity and metabolism, and to support and justify the predictions to a user, without disclosing commercially sensitive material. More recently, projects run by the International Life Sciences Institute in Washington, Lhasa, and a consortium in the USA, have explored ways of encouraging the sharing of toxicological data with some success. This talk will discuss how issues that have constrained data and knowledge sharing are being addressed. The indications are that data and knowledge sharing will become established as ways to make research more productive, to save cost, and to reduce the need for animal experiments.

**Poster**

**Development of topical formulations with corticosteroids utilising physicochemical and in vitro methods**

*Monika Kaca*, *Udo Bock*, *Rolf Daniels*, *Claus-Michael Lehr* and *Eleonore Haltner*

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**Introduction:** The objective of this work is to define a practical sequence scheme for a fast and reasonable development of semisolid formulations without testing on animals. For this purpose four active compounds were chosen from the corticosteroids group: Betamethasone valerate, clobetasol propionate, hydrocortisone and mometasone furoate. Two OECD-markers for *in vitro* percutaneous absorption (OECD 428): caffeine and testosterone were taken additionally as controls. The first step summarised information on physico-chemical properties of the substance. Physico-chemical profiling of the model drug Hydrocortisone are here presented.

**Experimental methods:** The solubility was estimated by nephelometric analysis. The saturation solubility was measured by the shake flask method (32°C, KRB buffer). The partition coefficient was determined in experimental systems by measuring octanol/water partitioning also by the shake flask method. The Immobilised Membrane Partition Coefficient (KIAM) and protein binding were determined by HPLC.

**Results:** Table 1 Summary of parameters determined for Hydrocortisone: Molecular weight 362.47 g/mole; CAS Number 50-23-7; Molecular formula C21H30O5; UV maximum 242 nm; pKa none; Log P 1.56; Protein binding 49%; Solubility (nephelometric) >202.46 µg/ml; Saturation solubility 333.00 µg/ml; KIAM 7.56.

**Discussion:** Hydrocortisone is a very slightly soluble compound. The data summarised in Tab. 1 indicate that Hydrocortisone is highly permeable. This model drug is a moderate lipophilic substance with approximately 36 times higher affinity to the lipophilic octanol phase than to the water phase. The KIAM value correlates well with log P. The *in vitro* drug release profile of the test drugs from semisolid formulations has to be determined in outgoing studies.
Poster

3D-Quantitative Structure-Activity Relationships (3D-QSARs) to predict binding to the human oestrogen receptors $\alpha$ and $\beta$: Use in risk assessment

Assia Kovatcheva$^1$, Emilio Benfenati$^2$, Alessandra Roncaglioni$^2$ and Mark Cronin$^*$$^1$

$^1$Liverpool John Moores University, School of Pharmacy and Chemistry, Liverpool, UK; $^2$Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy

Quantitative Structure-Activity Relationships (QSARs) are increasingly being used to predict the effects of a diverse series of toxic endpoints. As part of these efforts there has been considerable interest in predicting whether a compound will cause endocrine disruption. In particular there has been much success in using tiered computational approaches for the prediction of endocrine disruption. These start with simple and easily applicable structural rules and go through to more complex receptor binding studies. The present study has investigated the use of a 3D QSAR approach, Comparative Molecular Field Analysis (CoMFA), to model the relative binding affinity of 99 compounds to the human oestrogen receptors $\alpha$ and $\beta$ (hER$\alpha$, hER$\beta$). The binding data were obtained from the literature (Malamas M. S. et al., 2004, J. Med. Chem. 47, 5021-5040). The study indicates that for compounds known to bind to hER, relative binding affinity (RBA) can be predicted accurately. Such predictions could form part of an integrated strategy to assess whether a compound has the potential to be a significant endocrine disruptor.

Funding from the EU 5th Framework EASYRING project (QLK4-2002-02286) is gratefully acknowledged.

Poster

DEREK for Windows: A computer system for sharing toxicological knowledge

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Computer systems have an important role to play in reducing the use of animals in toxicity testing. This includes providing mechanisms of dissemination whereby maximum use can be derived from the results of tests which are necessarily carried out to avoid repetition of the same or similar experiments. In some cases, however, it may not be possible to share either the exact nature of the chemical tested or the results of the test because of commercial sensitivity. In such instances, it is often nevertheless possible to share the general conclusions of the study. Knowledge of this type can be stored and utilised in a toxicological expert system such as DEREK for Windows.

DEREK for Windows is a knowledge-based expert system designed to predict the toxicity of a chemical from its structure. The knowledge base is composed of alerts, example compounds and rules which each contribute to the predictions. The alerts define chemical environments which are associated with a particular toxicological endpoint such as genotoxicity, carcinogenicity or skin sensitisation. Each alert is based on toxicity data for specific compounds together with other relevant information, including mechanistic understanding where this is available.

Examples will be presented to illustrate how proprietary toxicity data contributed by DEREK for Windows users have been used in conjunction with published evidence to derive and refine alerts without compromising confidentiality.
Poster

**A pre-validation of in vitro photo
genotoxicity tests and the effort to find a useful statistical prediction model**

Elisabeth Schmidt1, P. Aeby2, S. Brendler-Schwaab3, B. Epe4, R. Frötschl5, E. Gocke6, C. Hertel4, P. Kasper5, S. Kirchner6, M. Liebsch6, K. Meurer7 and U. Plappert-Helbig8

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Two *in vitro* tests – the Photo Micronucleus Test (PMNT) and the Photo Comet Assay (PCA) – were evaluated to proof their ability to identify the photogenotoxicological potential of chemicals. Thirteen photoactive substances were tested under blind conditions in three to five laboratories in the years 2002 to 2004 in a ring trial coordinated by the Federal Institute for Drugs and Medical Devices (BfArM), Bonn, in two independent runs.

Statistical methods used in this validation study will be discussed: (1) The definition of useful toxicological endpoints and the handling of the data as a first and very important step in the process of the statistical analysis. (2) The repeatability and reproducibility must be given for the test to be of practical use and has to be assessed.

For the interpretation of the results, “individual” prediction models were developed by all of the participating laboratories: (3) Comparison of the experimental findings with the classification of photogenotoxicity shows the sensitivity and specificity of the tests. A 2x2-contingency table proved to be most useful for this purpose. (4) Despite the small number of laboratories, the limited number of replicates, and chemicals used, an attempt is made to develop a general prediction model.

Lecture

**The role of electrophilic reactivity in Quantitative Structure-Activity Relationships (QSARs) for toxicity**

Terry Schultz1, Moges Woldemeskel1 and Mark Cronin*2

1 The University of Tennessee, College of Veterinary Medicine, Department of Comparative Medicine, Knoxville, USA; 2 School of Pharmacy and Chemistry, Liverpool John Moores University, Liverpool, UK

There is great interest in the use of Quantitative Structure-Activity Relationships (QSARs) to predict toxicity. QSARs and *in silico* approaches have traditionally been applied most successfully in toxicology to predict endpoints, such as narcosis, where potency can be related to passive, steady-state, effects such as accumulation at the site of action. However, many toxicities (e.g. mutagenicity, sensitisation and numerous others) relevant to risk assessment are not elicited in this manner and are brought about by the covalent interaction of the xenobiotic with a biological macromolecule. Many of these mechanisms are electrophilic in nature, and are considered to constitute reactive toxicity. Predictions for such compounds and endpoints have been rather poor. There are a number of reasons for this, most notably the failure of QSAR descriptors to parameterise electrophilicity adequately. To address this problem this study has investigated chemical reactivity as quantified experimentally by reaction with the model nucleophile glutathione (GSH). The compounds chosen to study were olefins conjugated to a carbonyl group. There are inherently electrophilic and convey the potential to act by Michael-type nucleophilic addition. The measured reactivity parameter (ReacGSH) endpoint was then related to acute toxicity assessed in the 40 hour *Tetrahymena pyriformis* population growth impairment assay. A high quality linear relationship was observed between toxicity and reactivity for compounds most of which act via Michael addition. This approach is successful and may have possible applications to other toxicity endpoints.
Lecture

Comparative analysis of gene networks at multiple doses and time points in livers of rats exposed to acetaminophen

Hiroyoshi Toyoshiba¹, Hideko Sone¹, Fred Parham², Richard D. Irwin², Gary A. Boorman² and Christopher J. Portier²

¹ National Institute for Environmental Studies, Tsukuba, Japan;
² National Institute of Environmental Health Sciences, RTP, USA

Gene interaction network analysis using microarray data sets has been developed to quantify systematic changes of gene expression after chemical exposure. In this study, methods based on Bayesian networks for identifying and quantifying linkages between genes was applied to detect differences in gene expression interaction networks between multiple doses and time points. Seventeen (17) genes were selected from the gene expression profiles of microarrays from livers of rats orally exposed to 50, 150 and 1500mg/kg acetaminophen (APAP) at 6, 24 and 48 hours after their exposure. The selected genes are related to three biological categories that are associated with response to acetaminophen: apoptosis, oxidative stress and acetaminophen-influenced genes. Gene interaction networks between all 17 genes were identified for the nine dose-time observation points by the TAO-Gen (Theoretical Algorithm for identifying Optimal Gene interaction networks) algorithm. Using k-means clustering analysis, the estimated nine networks could be clustered into two clusters, the first consisting of the low and middle dose groups, and the second consisting of the high dose. The analysis suggests that the networks could be segregated by doses but within doses, was consistent over time of observation. The networks formed by these two clusters were quantified to calculate the probability distribution for the strength of the linkage between any two genes in the networks at different times, suggesting that there are different molecular mechanisms between lower doses and high dose or different time of APAP. The approaches shown here could provide predictive information to understand high-versus low-dose mechanisms of toxicity.

Poster

Developing a QSAR framework to predict the toxicity of electrophiles: Findings of an expert workshop

Gilman Veith¹, Mark Cronin² and Terry Schultz³

¹ International QSAR Foundation to Reduce Animal Testing, Two Harbors, USA; ² Liverpool John Moores University, School of Pharmacy and Chemistry, Liverpool, UK; ³ The University of Tennessee, College of Veterinary Medicine, Knoxville, USA

The potency of chemicals which has reversible interactions with macromolecules is often much lower than that of similar chemicals which can bind irreversibly to proteins and DNA. Direct acting electrophiles and pro-electrophiles may react covalently with cellular nucleophiles. Depending on the nucleophile involved, reactive toxicity can be expressed by a variety of endpoints from cytotoxicity to sensitisation and genotoxicity. There are many mechanisms by which electrophiles can interact with nucleophiles, and the molecular descriptors required to model these reactions are complex. The First Annual Knoxville Reactivity Workshop was held in Knoxville (May 2005) to initiate the development of a modelling framework for reactive toxicity. Beginning with soft electrophiles, a new assay using glutathione (GSH) as a model nucleophile was evaluated for its ability to rank chemicals as skin sensitisers, aquatic toxicants and respiratory irritants. Reactivity data from the GSH new assay were found to be related linearly to Tetrahymena pyriformis toxicity. These data suggest a direct relationship between toxicity and reactivity for esters and amides predisposed to undergo a SN2 displacement reaction with soft nucleophiles. These results further suggest that the GSH assay will be of assistance in determining the chemical domain of reactivity with the thiol nucleophile. In addition, QSAR methods to predict GSH reactivity, using structural rules for the different reaction mechanisms, were evaluated. Finally, research implementation plans were made for the development of other model nucleophiles that can serve as a surrogate for the many important molecular initiating events in the toxicity pathways of reactive chemicals.
Stem cells are of increasing scientific interest because of their potential use in a number of biomedical and research applications. Stem cells are inherently self-renewing and as a consequence can be cultured in an undifferentiated state or programmed to give rise to more specialised cells of representative of cell types found in specific human tissues or organs. These properties allow stem cell approaches to be used to investigate an array of diseases, and identify novel therapeutic targets for drug discovery programs. Equally, stem cell models and their differentiated progeny promise to significantly improve in vitro toxicity assessment. Indeed, an embryonic stem cell test for developmental toxicity has already been endorsed by ECVAM Scientific Advisory Committee. Other systems which rely on programmed stem cell culture to generate complex 3D tissue equivalents for the assessment of properties such as barrier function are also on the horizon. This presentation provides an overview of the use of stem cell technology to generate in vitro models of toxicity. Specific emphasis is placed the use of stem cell models as alternatives to testing in animals with regards their use in models of barrier function, hepatotoxicity and genotoxicity and in reproductive toxicology screens.
**Poster**

**Establishing predictive molecular markers of differentiation as toxicological endpoints in the Embryonic Stem Cell Test (EST)**

Roland Buesen¹, Johanna Kaltenhäuser², Elke Genschow¹, Anke Visan¹, Birgitta Slawik¹, Katharina Schlechter¹, Klaus Becker², Horst Spielmann¹ and Andrea Seiler¹

¹Federal Institute for Risk Assessment (BfR), National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany; ²Schering AG, Berlin, Germany

In the field of reproductive toxicity mandatory test guidelines require in vivo experiments for the detection of the embryotoxic potential of chemicals and drugs. A promising alternative method for these purposes has been provided with the Embryonic Stem Cell Test (EST). This assay is based on the capacity of murine embryonic stem cells (ES cells) to differentiate in vitro into a variety of cell types. The EST is able to assess the embryotoxic potential of chemicals by the evaluation of inhibitory effects on differentiation of contracting myocardial cells which can be detected by microscopical analysis. Using a biostatistical prediction model (PM) the assay passed an international validation study and was able to predict the embryotoxic potential of test chemicals with an accuracy of 78%.

A joint project was carried out by ZEBET and German pharmaceutical companies to improve the EST by establishing molecular endpoints of differentiation. Cardiac-specific gene expression has been studied at protein and RNA levels by flow cytometry and real-time-PCR under the influence of 10 chemicals with different embryotoxic potentials. The results obtained using cardiac-specific molecular endpoints were comparable to the validated microscopic analysis of beating areas and led to the same predictive outcome. The data clearly demonstrated that the selected molecular markers provide objective endpoints of early embryonic differentiation and are able to predict developmental toxicity in vivo from in vitro data for reference compounds.

In conclusion, a modified EST holds promise to be a new predictive screening system for hazard assessment with regard to developmental toxicity.

The first two authors contribute equally.

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**Lecture**

**Human Umbilical Cord Blood Neural Stem Cell Line (HUCB-NSC) – implementation for studying developmental neurotoxicity**

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¹Medical Research Institute, Warsaw, Poland; ²ECVAM, Institute for Health and Consumer Protection, Joint Research Center, Ispra, Italy

Previously we have established non-immortalised, stable HUCB-NSC line with potential to differentiate into neuron, astrocyte and oligodendrocyte-like cells. In this report we are introducing HUCB-NSC as a model system for neurotoxicity testing. Standardised conditions for the growth and differentiation of HUCB-NSC line were established in multi-well plate format culture. The influence of selected growth factors and neuromorphogenes on cells differentiation and growth rate/survival has been tested in parallel.

Cells were incubated in the presence of twelve different combinations of growth factors and neuromorphogenes for 2 or 3 weeks at different plating densities. Their growth rate and survival was estimated by the MTT test and Live/Dead, Viability/Cytotoxicity Kit assays, as well as their differentiating potential by the quantified immunocytochemical expression of neural specific markers.

Under the standardised conditions the most potent for differentiation were: 1) CNTF and dBcAMP for neuron-like cells (~80% β-Tubulin III cells), 2) combination of PDGF-BB and RA for astrocyte-like cells (~60% S100β² cells) and 3) PDGF-AA followed by T3 for oligodendrocyte-like cells (~10% of Gal-C² cells). In addition, CNTF promoted, whereas dBcAMP and RA significantly depleted the HUCB-NSC number.

The availability of the HUCB-NSC line in culture promises a detailed examination of the compounds that influence the dynamics of neural somatic stem cells expansion and differentiation and opens possibilities for neurotoxicity testing.

**Poster**

**Expansion of the Embryonic Stem Cell Test: Differentiation into neural cells**

Katrin Hayess, Roland Buesen, Katharina Schlechter, Birgitta Slawik, Horst Spielmann and Andrea Seiler  
Federal Institute for Risk Assessment (BfR), National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

The Embryonic Stem Cell Test (EST) is a validated *in vitro* assay that has been established to classify compounds with respect to their embryotoxic potential. The current experimental procedure involves differentiation of murine embryonic stem cells (D3) into contracting cardiomyocytes. However, potentially embryotoxic drugs may effect primarily other tissues than myocard. Consequently, this consideration prompted us to expand the EST to other major target tissues.

Here, we present a protocol for differentiation of murine embryonic stem cells into neurons designed with special regard to the testing of chemicals. This modified protocol is based on a monolayer differentiation procedure and offers the advantage of a reproducible development of neural cells in a comparatively short time. The differentiation of D3 cells into neural cells was characterised by analysis of neuron-specific marker gene expression using flow cytometry. In addition, the developing neurons were examined by immunofluorescence staining using neuron-specific antibodies. As a result, we were able to define neuron-specific molecular endpoints for the detection of chemical effects on embryonic development.

The expansion of the EST to more than one target tissue will considerably improve the accuracy of this predictive screen by preventing false negative results.

**Poster**

**In vitro approaches to developmental toxicity**

Helena Hurst, Richard Clothier and Margaret Pratten  
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This study compared the effects of potential teratogens/embryotoxins *in vitro* on chick heart micromass (MM) model and the differentiation of cardiomyocytes with the D3 stem cell systems. White Leghorn 5 days old embryo hearts were dissociated to produce a cardiomyocyte suspension in Dulbecco’s Modified Eagle’s Media (DMEM). D3 murine embryonic stem cells (ESC) were induced to form Embryoid Bodies (EB’s) using the hanging drop technique upon removal of LIF. Cultures were incubated at 37°C in CO₂ (5% v/v in air) and observations made every 24 hours over 5 days. Culture viability was assessed using the resazurin reduction and total protein via the kenacid blue assays.

All-trans-retinoic acid (tRA) and sodium valproate (VPA) were used as controls. tRA significantly (P<0.05) reduces cell activity and beating whilst not affect total cell number. There is no cytotoxicity in the MM cardiomyocyte cultures when exposed to sodium valproate (VPA) up to 500 µM whilst all VPA concentrations (<500 µM) reduced contractile activity. The D3 studies with tRA and VPA were comparable with the MM results.

Blind studies have now been performed testing embryotoxic potential using structurally related compounds, one a known teratogen and the other non-teratogen (analogue). All studies were compared with D3 ESCs. This would provide an alternative battery of system/organisms to the use of rat micromass, for embryotoxicity testing *in vitro* and for mechanistic studies of embryonic development perturbation. This work was funded by a grant from the FRAME research council.
In vitro embryotoxicity testing of some components of dental biomaterials by the Embryonic Stem Cell Test

Koichi Imai¹, Horst Spielmann², Tohru Hayakawa³ and Masaaki Nakamura⁴

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The effects of some components of dental biomaterials on the differentiation of embryonic stem cells of the mouse cell line D3 (ES-D3 cells) were examined using the Embryonic Stem Cell Test (EST), which consists of three parameters: the differentiation rate into contracting myocard, 50% cell viability of ES-D3 cells and of Balb/c 3T3 cells, and clone A31 (3T3 cells), which were finally calculated by a designated formula. Of the 24 monomers tested, Bis-GMA, UDMA, Bis-MPEPP, TEGDMA, Bis-GMA(6F), 6-HHMA, BPE-1300 and MTYA were classified as weak embryotoxic, and 2.0-EPDMA, 3.0-EPDMA, 4.0-EPDMA, 1.6-ADMA, 1.8-ADMA, 1.10-ADMA, MEPC, Phosmer M, BSNa, EDMABA, GAM, GMA, GMR, NPG, PTSNa and QTX as non embryotoxic. In the case of metal powders extracted in culture media, it was found that Ag was classified as weak embryotoxic, while Co, Cr, Ni and Pd were classified as non embryotoxic. On the other hand, among the standard chemicals used for atomic absorption spectrophotometry, it was found that Cr and Hg ions were classified as strong embryotoxic, and In, Sn, Sb and V ions were classified as weak embryotoxic, while Ag, Co, Cu, Ni, Pd and Zn ions were classified as non embryotoxic. Five plasticisers, dibutyl phthalate, n-butyl benzylphthalate, n-butyl phthalyl, n-butyl glycolate, di-2-ethylhexyl phthalate, di-2-ethylhexyladipate were classified as weak embryotoxic.

It is considered that further intensive study on the embryotoxicity of dental biomaterials is needed, in addition to conventional biological aspects, from the perspective of biological safety.

Proposal for modification of the Embryonic Stem Cell Test to expand its applicability

Koichi Imai¹, Horst Spielmann² and Masaaki Nakamura⁴

¹ Osaka Dental University, Department of Biomaterials, Osaka, Japan; ² BfR, ZEBET, Berlin, Germany

Developed by Spielmann et al. in 1997 and already validated in EU countries, the Embryonic Stem Cell Test (EST) protocol is a screening test method that easily and promptly analyses differentiation toxicity of chemical substances in vitro. It is not applicable to insolubles or very slight solubles, but to solubles. Modification is necessary to test dental biomaterials that have a variety of compositions and are either soluble or insoluble, or have a variety of usage. In the present study, we tried to modify the protocol for expanding EST applicability, by exchanging the treating vehicle from a liquid medium to a type I collagen gel matrix. Accordingly, we embedded the test samples in type I collagen gel matrix based on a three-dimensional cytotoxicity method. That is, a method was employed to estimate the in vitro embryotoxicity from the time of placement of the test substances to the differentiation of ES cells. We examined the influence of three kinds of dental restorative materials on the ES cells. The differentiation rate was the highest for glassionomer cement, followed by two kinds of light-cured composite resins, and was nil for dental amalgam. It seems possible to perform in vitro embryotoxicity testing of dental biomaterials using this modification. Although this modified protocol has not been subjected to a validation process, it can be positively assessed to some extent by an in vitro examination of provisional embryotoxicity levels of dental biomaterials with complex compositions.
Lecture

A perspective on embryonic and adult stem cells for in vitro and in vivo testing

Aernout Luttun
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Like recombinant DNA in the 1980’s and transgenic animals in the 1990’s, stem cell technology is well on its way to become a central research tool for this and the following decades. Stem cells are generally derived from two main sources: embryos or adults. They can be further subcategorised according to their differentiation potential. While embryonic stem cells are considered pluripotent given their ability to give rise to all somatic and germ line cell types, the differentiation repertoire of adult stem cells is more limited. On the other hand, unlike stem cells from adult sources, the use of embryonic stem cells has met with significant ethical concerns. Recently, adult stem cells were derived, termed “Multipotent Adult Progenitor Cells” (MAPCs) that have many features of embryonic stem cells, including the ability to differentiate into many cell types representing all three germ layers of the embryo. This broad differentiation potential along with the availability without ethical restrictions offers many possible applications. Here we give an overview on how stem cells in general, and MAPCs in particular, can be exploited in addressing diverse questions at different levels ranging from in vitro testing to in vivo therapy.

Lecture

Differentiation of hepatocyte-like cells from human embryonic stem cells and adult liver progenitors

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University Montpellier, Inserm U632, Montpellier, France

Primary cultures of differentiated human hepatocytes represent a powerful tool for basic and applied research, in particular for drug metabolism and hepatotoxicity testing, as well as for biotherapy of liver diseases. Hepatocytes are currently isolated from livers not used for transplantation or from lobectomies resected for medical purpose. However, these sources of tissue present various inconveniences including scarcity, limited availability and technical difficulties for cell isolation. Obtained of differentiated human hepatocytes from stem cells is therefore indispensable and represents an exciting challenge. Several potential sources of stem cells have been considered in this respect, including Embryonic Stem Cells (ESC) and intrahepatic progenitors. Several groups have reported that, under appropriate culture conditions and stimulation by cytokines, growth factors or chemical reagents, ESC differentiate to hepatocyte-like cells expressing markers such as albumin and other plasma proteins, CK8/18, production of glycogen, expression and xenobiotic-mediated induction of cytochrome P450 genes. Similarly, intrahepatic progenitor cells have been shown to be able to generate hepatocyte-like cells expressing the above-mentioned markers. Another exciting and promising advantage of stem cells is the possibility to modify their genome by different approaches including gene transfer and siRNA-mediated gene down-regulation. The technical means allowing such modifications including homologous recombination and lentivirus vector-mediated transfection are now available. Evaluating the impact of up- or down-expression of candidate genes on the process of stem cell differentiation and/or on the phenotype of generated hepatocytes opens the way to basic and applied investigations on the process of human liver ontogenesis and liver diseases.
The development of suitable test systems is important in order to protect foetuses and neonates against the toxic action of chemicals. Our goal is to establish a human in vitro model to test chemicals for their neurotoxic effects.

We have established the culture and propagation of Normal Human Neural Progenitor (NHNP) cells, which differentiate into different neuronal subtypes, astrocytes and oligodendrocytes. To validate our cell model for testing developmental neurotoxicity, NHNP cells were exposed to different chemicals exhibiting neurotoxic effects. Cell differentiation, viability, apoptosis, migration and MAP kinase signaling were used as biological endpoints for testing the influence of these chemicals on these cells.

Exposure of undifferentiated NHNP cells to low concentration of HgCl2 for one week lead to a significant decrease of differentiated neurons relative to astrocytes. Exposure of differentiating cells to HgCl2 resulted in reduced cell migration. The cell viability was not affected.

Ethanol (EtOH) was reported to affect cellular signal transduction in the rodent brain. Exposure of undifferentiated NHNP cells to 200 mM EtOH for 30 and 60 minutes lead to a significant inhibition of ERK 1/2 phosphorylation. The cell viability was also not affected by EtOH treatment.

In summary, our preliminary data show that exposure of NHNP cells to neurotoxic compounds leads to alterations of endpoints, which were reported in in vivo studies.

**Lecture**

**Normal human neural progenitor cells: An in vitro model for testing developmental neurotoxicity**

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The HALO (Hemotoxicity Assays via Luminescence Output) Platform provides a standardised, validated in vitro proliferation test system with high-throughput and predictive capability for drug screening and all later stages of drug development. The newly developed HALO 7-population predictive paradigm can test compounds on 7 lympho-hematopoietic populations (2 stem cell, HPP-SP, CFC-GEMM; 3 progenitor, (BFU-E, GM-CFC, Mk-CFC); 2 lymphopoietic, T-CFC, B-CFC) derived from human, primate, dog, rat or mouse bone marrow target cells simultaneously. The response by these early cell populations, especially the stem cells, allows prediction of compound effects in all peripheral lineages. Since HALO has been validated against the Registry of Cytotoxicity Prediction Model, the IC50, IC75 and IC90 values obtained from screening compounds on animal bone marrow cells can be used to calculate the estimated in vivo doses for pre-clinical studies. When primary human bone marrow target cells are used, the initial or clinically-relevant doses for human trials can also be predicted. If used during ADME/Tox compound screening, the HALO Platform provides a powerful decision-making tool that is an alternative surrogate assay for animal testing that can save time and money during drug development.

**Poster**

**Global in vitro predictive stem cell hemotoxicology for early drug screening and estimating pre-clinical and clinical trial dosing**

Ivan N. Rich and Karen Hall
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The Embryonic Stem Cell Test (EST) is a well-established and standardised in vitro method to predict the embryotoxic potential of chemicals and is based on the capacity of murine embryonic stem (ES) cells to differentiate under specific culture conditions into beating cardiomyocytes. An efficient differentiation requires the use of cell culture medium supplemented with 15% foetal calf serum (FCS). Unfortunately, FCS has a great lot to lot variability with regard to its composition. Therefore selected batches of FCS have to be pre-tested if they support differentiation and proliferation of ES cells before the EST can be performed. In order to optimise the culture conditions in the EST our aim was to establish serum-free culture conditions. Chemically defined serum-free culture conditions would provide several advantages: (1) improved protocol transfer to other laboratories (2) improved reproducibility of the differentiation assay, (3) no interference of undefined serum component with the test substance (reproducible bioavailability) and (4) application of the EST in high/medium-throughput screening systems.

Chemically-defined serum-free media supplemented with several growth and differentiation factors known to be important for cardiogenesis in vivo have been tested. Our results demonstrate that under these conditions cardiac embryonic stem cell differentiation can be achieved in vitro in a highly standardised and reproducible manner. In addition, we were able to demonstrate the applicability of the serum-free EST to test chemicals with different embryotoxic potentials.

**Poster**

**The use of serum-free culture conditions in the Embryonic Stem Cell Test: Defined culture conditions for cardiac stem cell differentiation**

*Katharina Schlechter¹, Roland Buesen², Birgitta Slawik², Horst Spielmann² and Andrea Seiler²*

¹ University of Applied Sciences, Giessen, Germany; ² Federal Institute for Risk Assessment (BfR), National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

The Embryonic Stem Cell Test (EST) is a well established and standardised in vitro method to predict the embryotoxic potential of chemicals and is based on the capacity of murine embryonic stem (ES) cells to differentiate under specific culture conditions into beating cardiomyocytes. An efficient differentiation requires the use of cell culture medium supplemented with 15% foetal calf serum (FCS). Unfortunately, FCS has a great lot to lot variability with regard to its composition. Therefore selected batches of FCS have to be pre-tested if they support differentiation and proliferation of ES cells before the EST can be performed. In order to optimise the culture conditions in the EST our aim was to establish serum-free culture conditions. Chemically defined serum-free culture conditions would provide several advantages: (1) improved protocol transfer to other laboratories (2) improved reproducibility of the differentiation assay, (3) no interference of undefined serum component with the test substance (reproducible bioavailability) and (4) application of the EST in high/medium-throughput screening systems.

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**Lecture**

**Current status of the Embryonic Stem Cell Test: The use of recent advances in the field of stem cell technology and gene expression analysis**

*Andrea Seiler, Roland Buesen, Katrin Hayess, Elke Genschow, Anke Visan, Birgitta Slawik, Katharina Schlechter and Horst Spielmann*

Federal Institute for Risk Assessment (BfR), National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET), Berlin, Germany

All guidelines that are currently used for regulatory developmental toxicity testing of chemical and drugs are based upon animal experiments. The most promising alternative is based on embryonic stem (ES) cells of the mouse. The ability to differentiate into numerous cell types has made ES cells a popular system to study gene function and developmental processes during differentiation in vitro. The Embryonic Stem Cell Test (EST) makes use of this capacity and is detecting developmental toxicants during stem cell differentiation into cardiomyocytes.

In the present study our investigations aimed at the further development of the validated EST protocol. Here we present improvements that focused primarily on (i) the quantitative assessment of drug effects at the cellular level, using a novel approach in which the expression of tissue-specific marker proteins under influence of the test chemical is quantified by intracellular flow cytometry in ES cells, (ii) the development of protocols for ES cell differentiation into various cell types other than cardiomyocytes and (iii) on the standardisation and optimisation of ES cell culture and differentiation conditions in chemically defined serum-free medium.

An important strength of the serum-free, molecular approach is that in this way the ability of the test to monitor the cellular response to toxins could be expanded to proteins of many signal transduction pathways in a highly standardised form.
The embryonic stem cell test was implemented at J&JPRD in order to help early drug research in the selection process of non-embryotoxic pharmaceutical compounds. In order to gain a better yield of strongly contracting embryoid bodies, a few optimisations to the validated ECV AM protocol were executed. In a former study we already demonstrated that by using the optimised protocol and the prediction model of the validation study, the classification of 6 reference compounds reflected the in vivo classification.

The aim of this study was to test more compounds in the optimised EST in order to know better its prediction potential. In a first phase, 14 reference compounds used in the ECV AM pre-validation and validation study were tested. The results showed that the prediction by using the optimised protocol was comparable with the validation study. The compounds that caused misclassifications in the validation study also caused misclassifications with the optimised method.

In a second phase two analogous J&J compounds were tested of which the first one was teratogenic and the second one was not teratogenic in vivo. The results showed that the first compound caused an inhibition of differentiation at lower concentrations compared to the inhibition of cell growth, while the second compound caused inhibition of differentiation in the same dose range as cytotoxicity occurs. However the prediction model classified both compounds as moderate embryotoxic compounds.

More in house compounds with known in vivo data will be tested in order to optimise the prediction model.

Poster

Further evaluation of the optimised in vitro Embryonic Stem Cell Test (EST) by testing reference compounds and J&J compounds

Ann De Smedt, Margino Steemans, Ann Lampo and Philippe Vanparys
J&JPRD, Genetic and In Vitro Toxicology, Beerse, Belgium

Lecture

Mimicking liver development by sequential exposure to hepatic cytokines: The key to differentiate rat bone marrow stem cells into hepatocyte-like cells

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Background/Aims: Differentiation of adult Bone Marrow Stem Cells (BMSC) into hepatocyte-like cells is commonly carried out by continuous exposure of the cells to a mixture of cytokines. In the present study, it was investigated whether the differentiation efficacy can be importantly enhanced by sequential exposure of the cells to liver-specific (LSP) cytokines [fibroblast growth factor-4 (FGF-4), hepatocyte growth factor (HGF), insulin-transferrin-sodium-selenite (ITS) and dexamethasone (Dex)] comparable to the secretion pattern occurring during in vivo embryonic liver development.

Methods: The differentiation process was characterised by means of quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis and immunofluorescence. ALB secretion was analysed using ELISA.

Results: Upon sequential exposure to LSP cytokines, BMSC-derived hepatocyte-like cells undergo different stages of hepatocyte differentiation, as seen during liver embryogenesis. Indeed, expression of the early hepatocyte markers alpha-foetoprotein (AFP) and hepatocyte nuclear factor (HNF)3β decreased as differentiation progressed, whereas levels of the late LSP markers albumin (ALB), cytokeratin (CK)18 and HNF1α were gradually upregulated, suggesting hepatocyte commitment and maturation. In contrast, simultaneous treatment with a mixture of all cytokines did not significantly alter the expression pattern of the LSP markers. Moreover, only upon sequential exposure to LSP cytokines, cells expressed phase I cytochrome P450 (CYP) proteins and showed significantly increased ALB secretions, pointing to a functional hepatic status of these cells.

Conclusion: Sequential induction of the differentiation process, analogous to in vivo liver embryogenesis, is crucial for in vitro differentiation of BMSC into mature hepatocyte-like cells.
Lecture

Human embryonic stem cells – The source of normal human cells for in vitro assays

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¹Cellartis AB, Göteborg, Sweden; ²RCEM1, Gothenburg University, Göteborg, Sweden

Background: The most commonly used models for toxicological studies today are animals or animal cells from rats, mice and other species followed by human cell lines. Animal experiments cause suffering while transferability of results from animal experiments and animal cells to humans is often low. Human cell lines are available, but usually have a low degree of functional differentiation. Human primary material is problematic with respect to quality and availability. Therefore there is a demand for available, normal and functional human cells for in vitro assays.

Procedure: Human Embryonic Stem Cells (hESC) offer unique possibilities and provide new opportunities to generate a virtually limitless supply of normal human cells for assays. Cellartis has established 30 hESC lines and generated extensive knowledge on methods for the culture, handling and characterisation as well as the differentiation of these cells. We are now directing our efforts towards the development of assays based on hESC and their differentiated derivates.

First generation assays based on undifferentiated hESC and derivates can be utilised to evaluate early human development in vitro and to detect embryotoxic and teratogenic effects in a human-relevant system. Once functionally differentiated somatic cells such as cardiomyocytes and hepatocytes can be created from undifferentiated hESC, these cell types can form the basis of an even more comprehensive generation of hESC based assays.

Poster

On the replacement of in vivo tests on germ cell mutagenicity and fertility impairment

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In contrast to drugs, industrial chemicals are not intended to be uptaken directly by humans. They mainly serve as intermediates for preparations and products. In case of proper use exposure to the general public seems, therefore, to be negligible. However, uncontrolled exposure may happen e.g. by accidents. Often little is known about the toxicological profile of a chemical substance. Consequently, a risk on human health can not be estimated. Realising this fatal situation, the EU enacted a Directive for the notification of new and existing chemicals. Impairment of fertility is one of the most important endpoints to be determined using in vitro generation studies, which are extremely time-, money-, and animal-consuming.

Besides the prediction of a carcinogenic risk, one of the most important issues of mutagenicity data is the detection of adverse effects on gametes and thus on fertility as well as on early embryonal development in mammals. For this purpose tests on mammalian germ cells need to be conducted. In practice, however, germ cell tests are rarely used because of the costs and the number of animals required. Therefore, we tried to develop a sensitive and predictive in vitro test system, which could serve as a model for mammalian germ cells: Murine female and male embryonic germ cell lines had been established in our laboratory and their sensitivity upon mutagen/non-mutagen treatment had been tested in comparison to adult cell lines. Applying linear discriminant analysis, all test chemicals used could be classified correctly!
Poster

Can the embryonic stem cell test be used for the early selection of pharmaceutical compounds?

Sonia Whitlow, Heinrich Buergin and Nicole Clemann
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Teratogenicity is one of the potentially critical side effects of new compounds. For chemicals used as drugs, embryotoxicity studies in two species have to be conducted to cover embryofetal development (ICH guidelines). These \textit{in vivo} protocols are time-consuming, expensive and use a large number of animals. In developmental toxicology, many \textit{in vitro} tests have been developed using a wide spectrum of cell and tissue cultures.

The scope of this “internal validation” was to implement the Embryonic Stem Cell Test (EST) validated by the ECVAM in 1999 as a tool to detect the embryotoxic potential of new pharmaceutical compounds early in the drug development process.

The “internal validation” with six well known compounds and 10 Roche compounds gave an overall accuracy for the embryonic stem cell test of 81%. The overall predictivity for this limited set of test compounds was very good (85% for non teratogenic compounds and 83% for strong teratogenic compounds). The relatively poor prediction (66%) for weakly teratogenic compounds was because only three weakly teratogenic compounds could be tested. Further testing of pharmaceutical compounds, ideally strong and weak teratogenic compounds, to improve the prediction is still necessary.
Session 7.2
Innovative approaches for alternative methods development

Poster
Study on cytotoxicity assay and fluorescence probe on in vitro sensitisation assay using h-CLAT (human Cell Line)
Takao Ashikaga, Sakiko Sono, Kasturako Yoneyama and Hiroshi Itagaki
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In this study, several kinds of cytotoxicity assays and fluorescence probes were examined in the h-CLAT. In the first protocol, MTT assay was performed to determine the IC50 as a basis for dose setting, and then cell viability was measured by PI assay when CD86/CD54 expression was evaluated. Now we use PI assay for both dose setting and actual test. Four kinds of cytotoxicity assays (MTT, PI, FDA and 7-AAD) were compared using four chemicals (three allergens, DNCB, Ni, pPD, and one non-allergen, SLS). In the cases of Ni and pPD, the presence of the chemicals in the culture medium influenced the results of MTT assay. pPD influenced the results of FDA assay due to its intrinsic fluorescence. On the other hand, the IC50s of the four chemicals were almost unaffected in the cases of both PI and 7-AAD assay. Therefore, these two assays were thought to be suitable for this test. FITC-labeled antibodies are used in the h-CLAT and showed high prediction. PE, another kind of commercially available fluorescence probe, was evaluated using DNCB, SLS and Tween 80 (a non-allergen). Previously these three chemicals were discriminated actually by FITC-labeled antibody. In the case of DNCB, the augmentation of CD86 stained with PE-labeled antibody was higher than that obtained with FITC. In the case of SLS, no augmentation was observed when using either FITC or PE-labeled antibodies. However, for Tween80, augmentation of CD86 was observed only when PE-labeled antibody was used. Further study will need on the usefulness of PE.
Poster

Applying the Three Rs to mouse mutagenesis studies

Nirmala Bhogal
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Mouse mutagenesis studies are either phenotype-driven or genotype-driven. The former are reputedly more relevant with regards genomic annotation and methods have traditionally involved inducing mutations using chemicals in live animals. Such methods rely heavily on germline transmission to any offspring. Generally, mutation rates are low and germline transmission poor with many of the offspring not surviving to reproductive age or being sacrificed or subjected to invasive genotyping. As chemical mutations occur randomly, it is not uncommon for many mice to have been used without a single new strain being found. Transgenic methods, although gene-specific, are also very inefficient. Recent developments in the field of embryonic stem cell manipulation have resulted in higher mutation and transmission rates and the use less invasive genotyping protocols. RNA interference and gene induction methods allow genomic annotation based on transient rather than permanent genetic defects. However, the application of such techniques relies heavily on understanding the interplay between genes and their expression products. Furthermore, without appropriate information management, it is difficult to assess the relevance of mouse mutagenesis studies to human health. The sequencing of the human genome and the discovery of DNA variations, including single nucleotide polymorphisms, may facilitate discovery of biomarkers of toxicity and disease susceptibilities from genotyping and phenotyping studies with human volunteers and from genomics-based research. The significance of biotechnology, bioinformatics and human population research to the Three Rs and the development alternatives to mouse mutagenesis studies will be reviewed.

Poster

Changes induced by heat shock in in vitro culture of gastrulating mammalian embryo- application of FTIR and RAMAN spectroscopy

Floriana Bulic-Jakus¹, Ana Katusic¹, Maja Balarin², Maja Vlahovic¹, Ozren Gamulin², Gordana Juric-Lekic³ and Jasminka Brnjac-Kraljevic²
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An original organ culture model of the rat gastrulating embryo-proper was used to investigate teratogenic effects of a severe heat shock. Additionally, FTIR and RAMAN spectroscopy were applied to detect possible changes in composition of the culture medium.

Embryonic parts of 9.5-days-old rat embryos were cultivated for two weeks at the air-liquid interface in serum-supplemented Eagle’s MEM (50%). Heat shock (43°C) was applied for 24 hours and embryos were transferred to 37°C. Controls have spent the 14-day culture period at 37°C. Culture medium was changed every other day. Culture medium as well as culture-conditioned medium was frozen at -20°C. Two diameters of explants were measured several times by an ocular micrometer and compared by t-test. FTIR and FT-Raman spectroscopy were done on defrosted and dyalised culture media. The infrared spectra in transmission mode were recorded using Perkin Elmer GX spectrometer and Raman spectra with Raman module of same spectrometer. The changes in spectra of heat shocked and control group were followed in region of 1800 cm⁻¹ to 600 cm⁻¹ which is the region of active vibrations of biological macromolecules.

The diameters of heat shocked embryos were always significantly smaller than in controls (p< 0.01) which showed that the growth of embryos was impaired by severe heat shock. FTIR and RAMAN spectroscopy showed comparable results. It seems that either of these complementary spectroscopical methods may serve as a suitable method for a quick assessment of the impact of extraneous factors on a complex biological system.
Lecture

A novel strategy applied on hepatocytes allowing to mimic in vitro the human metabolic idiosyncrasy

Jose Castell\textsuperscript{1}, Augustin Lahoz\textsuperscript{2}, Daniel Henández\textsuperscript{1}, Maria J. Gómez-Lechón\textsuperscript{1} and Ramiro Jover\textsuperscript{1}

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Drug metabolism is a key determinant of drug pharmacokinetics variability in human beings. Well-recognised causes of such interindividual differences are phenotypic as well as genotypic differences in the expression of the enzymes involved in drug metabolism. Indeed, a major characteristic of CYP enzymes is the large range of interindividual variability in the expression of enzyme protein. Variable CYP activities are important sources of variability in the response (effect vs. side effects) to many drugs.

Genotypic variability is easy to identify by means of polymerase chain reaction-based or DNA chip-based methods, whereas phenotypic variability requires direct measurement of enzyme activities in liver, or, indirectly, measurement of the rate of metabolism of a given compound in vivo. There is a great deal of phenotypic variability in human beings, only a minor part being attributable to gene polymorphisms. Enzyme activity measurements in a series of human livers, as well as in vivo studies with human volunteers, show that phenotypic variability is, by far, much greater than genotypic variability. The sources of such variability are many, including diet, age, disease and exposure to a variety of environmental factors, including smoking.

Reproducing in vitro the variability and metabolic idiosyncrasy of human beings has been hampered by the considerable difficulty in governing simultaneously the expression of several genes in hepatocytes by conventional molecular biology tools. By the use of suitable viral expression vectors encoding all major drug metabolising enzymes, we have succeeded in generating cells that can virtually reproduce any human phenotype providing a valuable tool to investigate the role of idiosyncrasy in drug metabolism and toxicity in vitro.

Lecture

Development of a novel diagnostic ELISA for human insulin using serum-free cell culture

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Background: Concerns for animal welfare led us to develop a custom ELISA, for the measurement of human insulin, that replaced both the use of the ascites method and the use of fetal calf serum, while providing sufficient precision and reliability for clinical and research applications.

Methods: Insulin monoclonal antibodies were grown in vitro (derived from hybridoma cultures) in RPMI 1640/DMEM (1:1 v/v) supplemented to 4 mmol/l L-glutamine, 4% MaxiMab Mark II Supplement, and 2% Complex Lipid Solution. After weaning of the cells, fetal calf serum was not used for antibody production. A two-step ELISA was developed using recombinant human insulin (standard), charcoal-treated human serum (matrix), 50 mmol/l PBS containing human serum (assay buffer), and HRP-TMB detection system.

Results: The assay characteristics include sensitivity of 1.56 uU/ml, dynamic range of 1.56 to 200 uU/mL, no cross-reactivity with human C-peptide or pro-insulin, intra- and inter-assay CVs of <10\%, recovery of exogenously added insulin to plasma samples of 102.2-105.7\%, and linearity of dilution (1/2, 1/4, and 1/8) of insulin spiked plasma samples as 93-110\% of undiluted plasma samples. Circulating insulin levels in ten healthy volunteers were measured using both conventional ELISA methods and our new ELISA with absolute values similar between the two assays.

Conclusions: A highly specific and sensitive insulin ELISA was developed without using the ascites method or fetal calf serum for monoclonal antibody production. These methods could serve as a guide for reducing animal use for antibodies produced for other types of immunoassays and diagnostic tests.
Poster

In vitro model to compare surgical meshes in cell cultures

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Purpose: Regarding the recently demonstrated different effects of surgical polypropylene meshes on different cell lines, we established an in vitro model to compare surgical meshes without animal use.

With this model we examined different meshes with resorbable and non resorbable filaments. Some meshes were examined after resterilisation by autoclaving at 121°C for 20 minutes.

Methods and Materials: A variety of surgical meshes were tested in our model. Parts of the meshes (1 cm²) were sterile incubated with human cells for 72 hours. Apoptotic and proliferation index were measured. Scanning electron microscopy was carried out on the mesh-samples.

Results: With this in vitro model we could demonstrate some differences between the examined meshes. We could measure differences in the apoptotic index and proliferation index as well. In some meshes the apoptotic index was significantly increased while another one did not alter this index.

Scanning electron microscopy demonstrated alteration of the filament structure in all meshes after incubation with cell cultures.

Discussion and conclusion: Surgical implants are normally tested using animals prior to introduction into clinical use. With our model we demonstrate differences in the effect of the meshes on human cell lines. The apoptotic effect was different between the meshes, in all meshes the incubation with cell cultures lead to alteration in scanning electron microscopy pictures.

In our opinion this in vitro model can serve as a pre-clinic model to compare surgical meshes in view of biocompatibility and can lead to a reduced use of animals.

Poster

Assessment of infectivity of cryptosporidium oocysts by Fluorescent In Situ Hybridisation (FISH) as an alternative method to mouse bioassay

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Introduction: Cryptosporidium is a waterborne pathogen which significantly contributes to mortality of immunosuppressed people. Over 75% of AIDS patients die due to cryptosporidiosis as there is no treatment for this disease. Diarrheal disease is initiated by a microscopic size transmissive stage (i.e., 5 Fm), the oocyst.

Methods: Infectivity of Cryptosporidium is routinely assessed by mouse bioassay which uses large numbers of neonatal mice for challenge with Cryptosporidium; it is followed by animal sacrifice, necropsy, and histological analysis. Mouse bioassays have several serious deficiencies in addition to having to use large numbers of animals. Therefore, we propose a new molecular method for assessment Cryptosporidium infectivity, i.e., Fluorescent In Situ Hybridisation (FISH). This method will eliminate the need for the mouse bioassay by the U.S. and world-wide laboratories concerned with Cryptosporidium and cryptosporidiosis.

Results and Discussion: Our preliminary data indicates that the FISH method is superior to the mouse bioassay because it is several-fold more sensitive and specific, provides visual color-coded information on pathogen morphology, has extraordinarily high detection threshold, i.e., single pathogen vs 103-106 pathogens in mouse bioassay, and is easy to use and cost-and-labor effective, i.e., $5 vs $40 and 1.5 hr vs 10 days, respectively.
Neuronal network cultures respond to transmitters, their blockers, and other neurotoxic compounds in a substance-specific manner. Networks grown on 64-microelectrode neurochips remain spontaneously active and stable for many months providing a suitable test platform. This hybrid system of cells and microelectrodes forms a sensing device based on quantitative analyses of the complex signal patterns of living neuronal networks. High-content screening is a substantial improvement for detecting undesired effects of test compounds on neuronal activity at an early phase of drug development.

With several examples of neurotoxic, sedative and narcotic substances we underline the suitability of this test system. Experiments with the neurotoxic antifungal and antifouling compound trimethyltin chloride show that spinal cord and auditory cortex cultures exhibit characteristic and dose-dependent changes of their electrical firing patterns (Gramowski 2000, NeuroToxicology 21). Data derived from dose-response curves for the anaesthetic ketamine confirm its strong receptor-specific effects on the electrical activity. Employing refined pattern recognition analyses we demonstrate that it will be possible to ascribe the network impairment to different receptors and ion channels. This is shown for strychnine, bicuculline and picrotoxin which bind to the glycine and GABA-A receptors respectively.

These sensitive and quantitative (Gramowski 2004, Eur. J. Neurosci. 19) responses have triggered strong interest in using such platforms as broadband biosensors for screening for adverse side effects of various classes of compounds during early drug development and at the same time as a means for reducing in vivo animal tests.

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Lecture

Cell array-based RNA interference as an alternative to the genetically modified animal experimentation

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Loss-of-function studies using knock-out mice and gain-of-function studies using transgenic mice are a well-established approach to study gene function in in vivo situation. Besides undisputed contribution in the field of functional genomics these techniques intrinsically require a large number of animals for the production of the gene-manipulated animals as well as for the testing of gene function in the various physiologic cell populations. This requirement raises ethical issues concerning experimentation on animals, but it is also expensive due to the high cost of animal maintenance. Therefore, there is a clear need for development and optimisation of in vitro technologies, which could contribute to reduction, if not replacement, of animals in the existing knockout and transgenic mouse technology.

In the present study a development of a high-throughput in vitro experimental approach, the transfected cell array (TCA), in primary mammalian cells as an alternative to generation of genetically manipulated animals for loss-of-function as well as for gain-of-function studies will be presented. For that purpose we applied the RNA interference (RNAi) technology, which allows a specific inactivation of target genes. Thus, genes functions in various cell types could be investigated. The array-based method allows for high throughput functional analysis of hundreds of genes with the minimal cell number requirements. Moreover, usage of primary cells implies that experimental results can be directly transferred into in vivo situation in animal models and man.
Poster

Toxicity of nanoscale cationic polymers in vitro and in vivo

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The toxicity issues concerning nano-scale compounds are constantly gaining more attention. Lack of inherent toxicity is of strategic importance if nanomaterials are planned for medicine. Nanoparticles (e.g. dendrimeric macromolecules, cationic polymers, functionalised fullerenes) are widely studied for their use in targeted drug delivery, particularly drugs based on proteins, DNA and RNA. We have analysed the toxicity of two cationic polymers: poly(amidoamine) PAMAM G5 dendrimer (Mwt ca 25,000) and branched polyethyleneimine (PEI, Mwt ca 25,000) to biological systems of different complexity (a test battery). Different acute toxicity endpoints: the 30-minute inhibition of light output of photobacteria Vibrio fischeri, 24 h impairment of growth of protozoa Tetrahymena thermophila, 24 h viability of human cell line K562 (trypan blue exclusion) and mortality of mice (i.p.) were evaluated. In all tests dendrimer was 2 10-fold less toxic than PEI. For the in vitro test the average acute toxicity of PEI and dendrimer were 18 and 180 mg/l, respectively. The acute toxicity of PEI to mice was 74 and that of dendrimer 150 mg/kg. The 24 h IC₅₀ for K562 cells and 30 min EC₅₀ for photobacteria were practically similar: 25 mg/l for PEI and about 270 mg/l for dendrimer, showing the potential of rapid ecotoxicological tests in toxicity screening of nanoparticles. In addition, the study of the adverse effects on different biological organisational levels helps to discover the mechanisms of toxicity of these emerging chemicals and to predict their hazard to ecosystems as the production of some nanomaterials is already in high production volume scale.

Poster

Quantitative evaluation of activity of osteoclasts derived from peripheral blood precursors

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Osteoporosis is nowadays the medical as well as sociological problem because of the pronounced progress of the loss of skeletal tissues found in high percentage of the population of postmenopausal women in Europe. The animal tests for experimental osteoporosis are cruel and unacceptable. Two populations of cells – osteoblasts and osteoclasts – are responsible for the balance and regulation of bone rebuilding process. The precursors of both kinds of these cells are found in bone marrow. Peripheral blood is used as the source of preosteoclasts differentiated in vitro.

The proposed methodology is the modified technique called “PITS”. This technique is based on direct action of osteoclasts on thin bone plates in vitro. Osteoclasts seeded on the bone plate adhere to it, and form underneath a depression called “pit” thanks to the activity of their proton pump which leads to lowering of local pH. The original technique based on counting the pits is not accurate and reliable. Therefore we decided, to measure the activity of osteoclasts by biochemical technique of estimation of N- or C-telopeptides released by digestion of bone matrix by MMPs (matrix-metalloproteinases) secreted by osteoclasts. The increase of concentration of telopeptides is supposed to be specific marker of activity of osteoclasts. The whole process is observed in the tissue culture wells in which bone fragments and osteoclasts are incubated in proper medium.
Lecture

Assessing the toxicity of smoke derived from polymer combustion using human lung cells (A549)

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Many synthetic polymers have been introduced in building materials and mass transport, which have resulted in the generation of more toxic and hazardous combustion products. An alternative method using in vitro techniques has been developed to assess the toxicity of smoke derived from polymer combustion on human lung cells (A549). The lung cells were grown on a porous membrane and exposed to the combustion toxicants at the air/liquid interface with a dynamic exposure method using the Harvard Navicyte Chamber. A laboratory small-scale fire model using a vertical tube furnace was designed for the generation of combustion products. A range of building and mass transport materials, including PMMA (Polymethyl methacrylate), Polyethylene, Polycarbonate, Polypropylene, and PVC (Polyvinyl chloride) were investigated. Three in vitro methods were studied including: MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), NRU (Neutral Red Uptake) and ATP (Adenosine Triphosphate) assays. The thermal degradation products were analysed using ATD-GCMS (Automatic Thermal Desorption – Gas chromatography Mass Spectrometry) method, coupled with a direct reading measurement using a CO/CO² sensor. IC₅₀ (50% inhibitory concentration) values were generated using this method, these included: PVC = 1.5-2.3 mg/L air; Polyethylene = 2.6-4.5 mg/L air; Polypropylene = 4.1-5.1 mg/L air; PMMA = 4.4-6.4 mg/L air; and Polycarbonate = 7.9-13.3 mg/L air. The toxicity rank of the polymers as determined using the three assays from most toxic to least toxic were: PVC>Polyethylene>Polypropylene>PMMA>Polycarbonate. The technique developed here has the potential to be an alternative method to the current fire smoke toxicity standard.

Lecture

RNA interference: A novel alternative approach in nephrotoxicity studies

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One of the main goals in functional genomics has been the development of tools that allow easy manipulation of gene expression levels, that would be suitable for high through-put screening. RNAi has emerged as one of the preferred approaches to achieve this goal. It is an important biological mechanism in the regulation of gene expression in animals and plants.

Functional investigation of the complex regulation of molecular switches and their effectors are key to understanding nephrotoxicity. Nephrotoxicity is caused by several drugs such as immunosuppressive agents and is associated with the development of renal fibrosis. We have established several in vitro models of nephrotoxicity and have identified several key genes, using both micro-array and differential gene expression (SSH) technology, which we believe are involved in this process. We have used RNAi to analyse the role of these genes in the development of nephrotoxicity. A number of interesting differentially expressed genes involved in signal transduction, cell cycle regulation and cytoskeletal dynamics were identified and are being examined further as potential therapeutic targets.

In conclusion we have demonstrated that silencing of key genes with RNAi has helped to elucidate their role in the development of renal fibrosis and nephrotoxicity. Further examination of these differentially regulated genes may lead to the identification of novel therapeutic targets and potential adjunct therapies. Finally we believe that the potential role of RNAi as an alternative to animal models is just beginning to be realised.
Cell cultures are well established in active substance testing. In an open perfusion system cells are fed continuously by nutrient medium and metabolic products are removed continuously. Because glucose is the main energy source, glucose consumption of cell culture can be estimated by an amperometric enzyme biosensor continuously and non-invasively. Kinetics of this metabolic parameter is used to characterise activity profiles of substances by an in vitro test system, under conditions closely approximated to the in vivo situation.

24 hours after the transfer of human amniotic epithelial cells (FL cells) into the perfusion system, a metabolic equilibrium is reached characterised by stable glucose consumption. It was significantly reduced during cell exposition to 30 mg/l hydrogen peroxide, occurring physiologically in the micro-environment of macrophages. Similar effects have been induced by low heavy metal concentrations being significant as water pollutants (e.g. 390 µg/l copper), which opens the possibility for early prediction of disturbances of ecosystems [1, 2]. In search of new active substances, both kinetics of polio virus infection and protecting effects of natural antiviral substances were monitored [3]. Recently, stimulating activities of natural products on cell metabolism were demonstrated. Actually, the biosensor-controlled perfusion cell culture is on an experimental stage of test development, but has a great potential to be further evolved into a validated laboratory system to supplement and/or substitute animal tests.


Poster

**Biosensor-controlled perfusion cell culture: An innovative biomonitoring system potentially useful to supplement or partially substitute animal studies**

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Poster

**Comparison of tissue barriers – evaluation of the permeability in vitro**

*Heidi Nevala, Tarja Toimela and Hanna Tähti*

University of Tampere, Medical school, Tampere, Finland

When developing new drugs it is important to investigate their passage to different tissues. Specific barriers regulate this permeability by forming tight intercellular junctions. The brain is protected by a blood-brain barrier (BBB) which is composed of brain microvessel endothelial cells. Similarly, the retina has a blood-retinal barrier that resembles the BBB. Due to a tight epithelial cell structure, the absorption of drugs through the small intestine is restricted as well.

The aim of this study was to compare the barrier integrity of primary porcine microvessel endothelial cells (PMEC), human retinal pigment epithelial cell line (ARPE-19), and human colonic adenocarcinoma cell line (Caco-2). The cells growing on filter inserts, and the integrity of the barrier was evaluated with the measurement of trans-epithelial and trans-endothelial electric resistance (TEER). The cells growing on chamber slides were immunostained for a tight junction protein, occludin. The TEER of ARPE-19 cells appeared to be low when compared to PMEC and Caco-2 cells. Immunocytochemistry revealed the existence of occludin in all cell types studied.

In conclusion, PMEC and Caco-2 cells seem suitable for in vitro permeability studies, whereas ARPE-19 yielded less optimal TEER values. The characterisation of transport proteins and the inducibility of tight junctions in these cells is under investigation.

Acknowledgements: EDIT Project, TEKES (The National Technology Agency of Finland).
Poster

Validation of various blood flow-meters in an artificial circuit as a 3R concept

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Introduction: Intra-operative flow measurement during bypass operation is helpful to exclude technical failures. Blood flow-meters validation is mandatory to confirm reliability of measured results. Preclinical validation studies may be performed in the animal models and artificial circuits. The aim of this study is to evaluate the feasibility and the impact of artificial circuit tests before animal trials as part of the 3R concept.

Methods: The artificial circuit was constructed with PVC tubing sets, one roller pump and two reservoirs. Pulsatile flow was obtained with short length, small diameter tubes. Flow conditions were continuously controlled with invasive pressure and flow signals. A 6 cm long swine carotid artery segment was inserted in the circuit, which was filled with pig blood and immersed in a 37°C water bath. Swine blood and arteries were collected from euthanised animals from other studies. The following devices were tested: Quantix OR®, CardioMed® and Medi-Stim®. Time collected true flow amount was used as reference during each measurement. The following tests were performed with the artificial circuit: 1. Correlation and agreement analysis, 2. Device reproducibility and measurement stability, 3. User accuracy (intra- and inter-observer variability).

Results: All devices showed good results in the reproducibility tests, the correlation coefficients between flow-meters and time collected true flow being over 0.98 (p=0.01).

Discussion: The testing of blood flow-meters in the artificial circuit was reproducible. The use of artificial circuits is a useful previous step for animal studies facilitating device and probe design improvement, thus decreasing the number of animal trials.

Poster

Aquatic hazard assessment of petroleum products using biomimetic solid phase microextraction

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Petroleum products often consist of multi-component hydrocarbons with variable composition. Despite this complexity, the constituent hydrocarbons typically act via narcosis in aquatic toxicity tests. Consequently, adverse effects are expected once the total molar sum of the individual hydrocarbons in organism lipid (i.e. Tissue Body Residue) exceeds a critical threshold (i.e. Critical Body Residue) corresponding to narcosis. Recent research has shown that solid phase microextraction (SPME) fibers provide a surrogate for organism lipid thereby providing a convenient analytical tool for estimating the TBR and predicting narcotic effects of complex mixtures. To investigate the applicability of this analytical tool for predicting the aquatic toxicity of petroleum substances, acute toxicity tests for an invertebrate (Daphnia magna), fish (Onchorynchus mykiss) and algae (Pseudokirchneriella subcapitata) were performed in parallel with SPME measurements for a model petroleum product (No. 2 Fuel Oil). Total molar hydrocarbon concentrations on polydimethylsiloxane SPME fibers were quantified using gas chromatography and flame ionization detection. From the observed fiber concentration-effect relationship, Critical Fiber Residues (CFR) corresponding to 50% response in daphnia, algae and trout were determined. Fiber measurements performed for 38 petroleum substances including crude oils, fuels, hydrocarbon solvents and petrochemicals were then used to determine if CFRs derived from No. 2 Fuel Oil could be used to predict observed effects. Results indicate SPME biomimetic extraction correctly predicted the hazard classification for most of the products investigated but overstated observed aquatic toxicity in a few cases. Consequently, this technique provides a simple, conservative and cost-effective alternative test to support environmental classification of petroleum substances that avoids animal use.
The basic *in vitro* genotoxicity tests like the Ames test, mouse lymphoma test, chromosomal aberration or micronucleus test, exhibit major limitations such as the lack of human-like metabolic capacity, toxicokinetics, use of cell lines that are not relevant to the target organs and oversensitivity compared to *in vivo* situations. A recent analysis of over 700 chemicals tested in the current *in vitro* genotoxicity tests demonstrated that, whilst they are efficient at detecting rodent carcinogens, 75%-95% of rodent non-carcinogens also induce false positive results in one or more of these assays (Kirkland et al., *Mut. Res.*, in press) which leads to most chemicals requiring *in vivo* animal tests.

Acknowledging these limitations of the present *in vitro* assays, a task force initiated by the European Commission and led by ECVAM recommended the introduction of an additional *in vitro* step using skin models (Maurici et al., *ATLA*, in press). Taking on this recommendation, the COLIPA Task Force Genotoxicity has developed a concept for dermally exposed substances that should form part of a strategy for replacement of animal experiments. A preliminary work plan based on this concept will be presented. This aims at clarifying positive results from *in vitro* genotoxicity tests on the basis of *in vitro* experiments that adequately cover skin metabolism, skin penetration and genotoxicity. Ideally, the development and future validation of these methods will not only lead to replacement of animal experiments but also to the generation of results with higher significance for the dermal route of exposure.

**Poster**

**The COLIPA strategy for the development of *in vitro* alternatives: Genotoxicity**

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The basic *in vitro* genotoxicity tests like the Ames test, mouse lymphoma test, chromosomal aberration or micronucleus test, exhibit major limitations such as the lack of human-like metabolic capacity, toxicokinetics, use of cell lines that are not relevant to the target organs and oversensitivity compared to *in vivo* situations. A recent analysis of over 700 chemicals tested in the current *in vitro* genotoxicity tests demonstrated that, whilst they are efficient at detecting rodent carcinogens, 75%-95% of rodent non-carcinogens also induce false positive results in one or more of these assays (Kirkland et al., *Mut. Res.*, in press) which leads to most chemicals requiring *in vivo* animal tests. Acknowledging these limitations of the present *in vitro* assays, a task force initiated by the European Commission and led by ECVAM recommended the introduction of an additional *in vitro* step using skin models (Maurici et al., *ATLA*, in press). Taking on this recommendation, the COLIPA Task Force Genotoxicity has developed a concept for dermally exposed substances that should form part of a strategy for replacement of animal experiments. A preliminary work plan based on this concept will be presented. This aims at clarifying positive results from *in vitro* genotoxicity tests on the basis of *in vitro* experiments that adequately cover skin metabolism, skin penetration and genotoxicity. Ideally, the development and future validation of these methods will not only lead to replacement of animal experiments but also to the generation of results with higher significance for the dermal route of exposure.

**Poster**

**Evaluation of U937 cell line for the identification of contact allergens**

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The development of *in vitro* testing allows to reduce animal testing and to better analyse the complex mechanisms underling dermal sensitisation. Having a central role in the initiation of allergic contact hypersensitivity, dendritic cells may represent an alternative to animal tests. Recently, our group and others reported promising results obtained with human peripheral blood monocytes derived dendritic-like cells (PBMDCs). However, PBMDCs major drawbacks are the complex and expensive method for obtaining them and donor-to-donor variability. To solve these problems, the U937 cell line was evaluated as a source of dendritic-like cells. Thus, we have developed an *in vitro* test for the identification of contact allergens based on the activation of dendritic-like cells. Cultured in presence of low concentration of interleukin-4 (IL-4), U937 cells were seeded in 12-well plates and exposed to test items for 24 hours, 48 hours and 72 hours. Cells were then analysed by flow cytometric measurement of the co-stimulatory molecule CD86 and by quantitative real time Reverse Transcriptase-Polymerase Chain Reaction analysis of IL-1β and IL-8 gene expressions. Standard sensitisers, standard irritants and oxidative hair dye precursors were tested at non-toxic to sub-toxic concentrations. For each test item, specific modulation of the chosen markers (CD86, IL-1β and IL-8) was observed, indicating that the described *in vitro* assay may be able to discriminate contact allergens from irritants. Five oxidative hair dye precursors were identified as potential sensitisers, confirming the results obtained with the murine local lymph node assay. A classification scheme based on the *in vitro* results is proposed.
**Poster**

**Primate and mouse Precision Cut Lung Slices (PCLS) as alternative for in vivo respiratory toxicity testing**

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Precision cut lung slices (PCLS) offer the distinctive opportunity to gain insight into lung functions under cell culture conditions. PCLS possess all the advantages of an in vitro technique but still maintain many functions of the intact organ. The objective of our work is the development of measurements of different proteomic and genomic endpoints for PCLS for rapid in vitro assessment and prediction of the respiratory sensitising/toxicological potencies of (bio)pharmaka and air pollutants.

Therefore, lungs of different species are taken to be filled with a medium-agarose solution and cut with a microtome (Krumdieck tissue slicer). After preparation of PCLS with a thickness of 250 µm slices are washed and cultivated for few days at 37°C. Vitality of the lung slices is remained for over 48 hours as controlled by LDH measurements and propidium iodide staining.

Here, we report the effects of direct exposure of primate or mouse lung slices to increasing concentrations of immunostimulants e.g. lipopolysaccharides (LPS), MALP-2 and TNFα. We show the quantification of cytokine generation assayed by flow cytometry on beads (IL1alpha, IL2, IL4, IL5, IL6, IL10, IL17, TNFα, GM-CSF, IFNγ) and ELISA (IL6, IL8, IL12, TNFα). Further studies using allergens like trimellitic anhydride (TMA), dinitrochlorobenzene (DNCB), amylase or detergent proteases and immune suppressive dexamethasone are planned for the near future. Beside this, gene microarray experiments of differentially expressed genes in the monkey model will be performed.

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**Poster**

**Improving drug product development and outcome with validated target organ specific human cell based three dimensional in vitro models**

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A recent US Food and Drug Administration report “Innovation Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products” (March 2004) concludes that “traditional tools used to assess product safety have changed little and have not benefited from gains in scientific knowledge.” Advances in harvesting normal human cells, optimisation of proliferation and differentiation media, and the application of the principles of GMP manufacturing have allowed MatTek to produce various target organ specific in vitro models which obviate or dramatically reduce the need for animals for a number of test methods. These models are based on normal human cells and thus avoid problems associated with species extrapolation from animal systems, an important consideration given the increasing reliance on genomic and proteomic studies. Careful control of the culture system allows production of highly reproducible tissues which exhibit dramatically improved reproducibility versus animals or explants tissues. The expression of highly differentiated function within these tissues makes them suitable for a number of investigations involving tissue function, gene expression, permeability, and other differentiated phenomena for which simpler, monolayer cell systems are not well suited. In addition, in a number of instances, the in vitro tissue models are preferable to whole organism testing since the isolation of specific phenomena are possible allowing easier, more accurate, and reproducible test results. Finally second generation tissues that incorporate multiple cell types allow researchers to determine the contribution of the various cell types to the phenomena under study.
Introduction: Biologic heart valves substitutes are currently tested in large animals. The objective of this study was to design a bioreactor to simulate mammalian heart rate, blood pressure and flow in order to reduce animal experiments.

Methods: The entire hydrodynamic pulse replicator is assembled of medical graded materials to be easily and repeatedly autoclaved. The system consists of a cylindrical culture reservoir made out of borosilicate glass. A polycarbonate disc to house the heart valves is attached to a steel tripod. This tripod passes a steel lid and is connected to a pressure piston with a return spring. The steel lid covers the reservoir airtight allowing a prepressurisation with compressed air-carbon-dioxide-mix to maintain a physiologic oxygen and carbon dioxide content. The housing disc with the heart valves is pushed down in the culture reservoir using an attached pressure line and moves backwards using the return spring. The application and release of pressure is microprocessor controlled. There are no heat-radiating components. The entire bioreactor can be placed in a conventional incubator.

Results: The microprocessor controlled movement of the housing disc with the heart valves results in a cardiac-like cycle by means of complete opening and competent closing of the leaflets. The pulse rate can be adjusted up to 2 Hertz. Pressure recordings revealed a dicrotic pressure profile resembling a physiologic pressure environment.

Conclusion: The simple, basically maintenance free, bioreactor design allows easy and reproducible simulation of a physiologic heart cycle. This is the first step to conduct feasibility studies of new biologic heart valves ex vivo, reducing currently required large animal experiments.

Poster

A novel bioreactor for ex vivo testing of biologic heart valves

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Poster

Evaluation of neuroactive compounds with neuronal networks in vitro on microelectrode neuro-sensor chips

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Nerve cells growing directly on microelectrode neurochips organise into networks and communicate via chemical and electrical signals like in vivo. They generate typical signal patterns of electrical activity which can be recorded and analysed. The patterns are stable, reproducible, receptor- and tissue specific. Neuroactive compounds modify network activity in a substance-specific manner. By refinement of data analysis methods and machine learning approaches the vast amount of complex data can be assigned to substance specific profiles.

We compared several anticonvulsants and anaesthetics with varying modes of action with respect to their typical changes of electrical network activity patterns in cultures of murine frontal cortex. Simultaneous extra-cellular multielectrode recordings reveal a concentration-dependent decrease of activity for all test compounds. Besides a general activity decrease (compound-specific effective concentration values), different compounds show distinguishable special effects on pattern synchronicity, oscillatory behaviour and intra-burst structure as well as reversibility.

Neuronal networks on microelectrode arrays have developed into a valuable technology that yields highly detailed insights into mechanisms of action and side effects of neuroactive drugs and prevents animal trials at an early stage of drug development.

Supported by Landesforschungsschwerpunkt Mecklenburg-Vorpommern Biosystemtechnik and European Community (ERDF).
Introduction: This project has been propounded by ATRA and I-CARE with the purpose of establishing in Switzerland one or more human tissue banks for the sole purpose of bio-medical research. These human tissue banks will work to source, treat, preserve and supply human tissue material for research purposes.

Methods: Along with the establishment of human tissue banks the project will also emphasise the need to set up regulations to protect surgeons and researchers, in making clear that the use of human tissue for research purposes must never come in conflict with organ donation for transplantation and in creating a centre that can co-ordinate and catalogue all tissue suppliers and users.

Results: With this one project, it would be possible 1. To reduce the use of animals for experimentation in Switzerland by 15-20%, thereby saving up to 100,000 animals annually 2. To improve the quality of biomedical research 3. To improve the collaboration between specialists/surgeons and researchers who work in the laboratory.

Discussion: A large number of experiments in biomedical research are carried out on tissues of animal origin. More-meaningful results can be obtained by using human material in pre-clinical studies. It has been ascertained that the demand in Europe for human tissue by researchers is far greater than the supply. Surplus surgical tissue is considered a sanitary waste, but could be sourced and used as per national regulations. A human tissue bank would actually turn what is now considered waste into a resource for biomedical research.

Poster

Establishment of human tissue banks for bio-medical research in Switzerland

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Poster

Daphnia magna as alternative bio-object in ecotoxicology

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We have been developing non-traditional methods of the identification of pollutants, using various hydrobionts as biological objects and the study of the mechanism of toxic action of xenobiotics. The experiments were carried out with using of Daphnia magna. The toxicity of xenobiotics was determined by the value of LC₅₀, a concentration of the compounds causing death to 50% of hydrobionts during incubation with toxicants for 24 hours. The toxicity of organophosphates (Dipterex, DFP, DDVP, Paraoxon, Malathion, Malaoxon), heavy metals ions (Hg, Pb, Cu, Co, Cd, Cr, As, Al), organochlorines (Aldrin, Dieldrin, Endrin, Acroclor, DDT, Lindane, PCBs etc.), cyanides (sodium cyanide) and pyrethroids (Cypermethrin, Fenvalerate, Deltamethrin, Permethrin, Allethrin, Resmethrin, Phenothrin, Kadmethrin, Cyphenothrin) was determined. The effects of a number of antagonists on the toxicity of xenobiotics were studied. At the first time we discovered that in experiments to Daphnia magna some muscarinic cholinoreceptor blockers (atropine, amyizil etc.) reduced the toxic effect of organophosphates. In the case of heavy metals the chelating agents (EDTA, Dithioethlycarbamate, Unithiolum, Sodium thiosulphuricum, L-Aspartic acid) were effective, for certain organochlorine poisonings – anticonvulsive drugs (diazepam, phenobarbital), for cyanide poisoning – sodium nitrite and anticyane. In the case of pyrethroid’s poisonings the antagonist of glutamate receptor (ketamine) and agonists of GABA-receptor (phenazepam, ethanol) reduced the toxicity of xenobiotics. As far as these antidotes have a specific treatment action only against definite classes of pollutants, we have elaborated the sensitive express-methods of bio-identification of pollutants with the usage of alternative bio-object.
**Poster**

**Precision-cut fibrotic liver slices as a new in vitro model to study fibrosis and to test anti-fibrotic drugs**

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Introduction: Liver fibrosis is one of the leading causes of death worldwide. Studies on anti-fibrotic drugs rely mostly on *in vivo* models involving substantial animal discomfort. Current *in vitro* models cannot mimic the complex *in vivo* milieu. Therefore, we evaluated fibrotic liver slices as a new, physiologic *in vitro* model to study fibrosis and to test anti-fibrotic compounds.

Methods: Precision-cut liver slices (8 mm diameter, 250 µm thickness) were prepared from livers of rats three weeks after bile-duct ligation (BDL) and incubated for 0-48 hours with 0-2 mM pentoxifylline. Expression of fibrosis-markers was studied using real-time PCR, Western blot, and histochemistry. Viability was assessed by measuring ATP content.

Results: BDL-livers showed clear signs of fibrosis, like increased collagen content and α-smooth-muscle actin (αSMA) expression. Fibrotic liver slices remained viable during 48 hours of incubation with significant increase of pro-collagen-1a1 mRNA expression and collagen protein content (5.7±0.7 and 1.5±0.07 fold compared to non-incubated slices), indicating progression of fibrosis. Addition of the anti-fibrotic drug pentoxifylline inhibited pro-collagen-1a1 mRNA, αSMA mRNA and αSMA protein expression significantly after 24 hours of incubation (0.26±0.04, 0.22±0.13 and 0.82±0.02 fold compared to control incubation) without influencing slice viability.

Conclusion: Fibrotic liver slices are a promising tool to study fibrosis *in vitro* in a physiological, multicellular context and to test anti-fibrotic drugs. Importantly, this method may provide the opportunity to study these processes not only in animal, but also in fibrotic human liver tissue and will contribute substantially to the reduction, refinement, and potential replacement of animal experiments.

**Poster**

**Differential susceptibility of lung cell lines to cytotoxic effects of respiratory irritants and sensitisers**

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Occupational exposure to chemicals is one of the main causes of respiratory allergy and asthma in Western countries. Identification of chemicals that trigger asthma is difficult due to the lack of a validated *in vitro* test method. In our approach to create an *in vitro* assay for respiratory toxicity, the choice of a relevant cell model is important. Different human cell models modelling for different structures of the upper and lower airways may demonstrate variable susceptibility to cytotoxicity caused by model respiratory sensitisers and irritants. The objective of the current work was to compare the susceptibility of selected cell lines and examine whether this susceptibility correlates with the *in vivo* localisation of the represented lung structure and its accessibility to inhaled chemicals.

The human bronchial epithelial cell line Beas-2B, the alveolar epithelial cell line A549 and macrophages derived from the myelomonocytic cell line THP-1 were exposed to the respiratory sensitising chemical ammonium hexachloroplatinate IV and the irritant tributyltin. After 24 hours cytotoxicity was measured by a neutral red uptake assay.

The cytotoxic effects of the chemical ammonium hexachloroplatinate IV according to EC50 values could be ranked as follows: Beas-2B (most sensitive) < THP-1 < A549 (least sensitive). Similar observations were made after exposure to the respiratory irritant tributyltin.

Our observations suggest that human cell lines modelling for different lung structures show a differential susceptibility to respiratory sensitising and irritating chemicals which correlates with their ability to physically contact inhaled chemicals *in vivo*. 
Neuronal tissue and a suitable recording/stimulating electronic system form a functional bio-electronic hybrid system. This system provides a novel in vitro platform for pharmaceutical drug development, for high-content drug screening, and for safety pharmacology.

We culture electrically active neuronal networks from embryonic mouse spinal cord or brain directly on glass/ITO- or silicon-based multi-electrode arrays with stable cell-electrode coupling for several months. This allows the monitoring of the onset of electrical activity, of bursting activity stabilisation and of the development of histiotypic native or drug-modified electrical activity patterns. The glass neurochip sensor system was extensively used over the last years to monitor states of toxic or metabolic impairment of neurons accompanied by characteristic electrical activity changes. Results will be reported from studies on the effects on the electrical activity of neurotoxins, ammonia, neurosteroids, benzodiazepines, anaesthetics and anticonvulsive drugs as well as studies on detecting neuronal side-effects of compounds.

In addition, a new standard CMOS technology-based silicon chip with unique features has recently been introduced. Besides the recording electrodes for action potentials, temperature diodes and ion sensitive field effect transistors (ISFET) were integrated to measure temperature and pH changes and oxygen concentration of the cultures at the silicon chip.

This NeuroSensorix® approach reduces the number of animal experiments, refines the quality of data analysis and will replace animal tests.

Supported by the State of Mecklenburg-Vorpommern and European Community (ERDF).

Poster

Influence of hypothyroidism induced by thiamazole on the toxic interaction between propranolol and disopyramide in chick embryos

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In order to develop alternative methods, we have studied the biological effects of drugs on the cardiovascular system of chick embryos using physiological techniques. The present study evaluated the effect of the hypothyroidism induced by thiamazole on the toxic interaction between propranolol and disopyramide in chick embryos.

Fertilised eggs of White Leghorns were incubated and investigated. 1.2 mg/0.2 ml/egg of thiamazole was injected into the albumen of fertilised eggs of incubation. The control group was given 0.2 ml/egg of physiological saline in the same manner. Propranolol at 0.1 mg/egg and disopyramide at 0.3 mg/egg were injected into the air sac of fertilised eggs of incubation. Electrocardiograms (ECGs) were recorded 0 to 60 min after the injection.

Results: After the injection of propranolol and disopyramide into the thiamazole treated eggs, the heart rate was significantly decreased compared with the thiamazole untreated eggs. In addition, this toxic interaction between propranolol and disopyramide was more severe at the chick embryos with hypothyroidism induced by thiamazole.

Discussion: An experimental animal model with heart disease originated from abnormalities of the thyroid gland in chick embryos has been produced by the treatment with thiamazole. With the recent concern for animal rights, experimental studies using mammals have been limited. In the present study, the influence of the hypothyroidism on the toxic interaction between propranolol and disopyramide was demonstrated in chick embryos. In conclusion, the chick embryonic model of hypothyroidism produced by thiamazole may be useful for investigating the pharmacological and toxicological effects of cardiovascular drugs.
Session 7.3
The contribution of the OMICS technology to the 3Rs

Lecture
Characterisation of in vitro cultures of primary cells by expression profiling

Hans Jürgen Ahr, Heidrun Ellinger-Ziegelbauer, Grazyna Wasinska-Kempka and Christina Weiland
Bayer HealthCare AG, Molecular and Special Toxicology, Wuppertal, Germany

The toxicological evaluation of drugs and chemicals still relies almost exclusively on studies in animals. However, both ethical and economic reasons strongly advocate to find alternative animal free methods, which may at least in part replace these in vivo studies. In vitro cell culture systems of different organs and species including humans are widely considered as the method of choice in this respect. It is however, necessary to clearly understand the advantages, but also the limitations of such in vitro systems before they can be used for specific purposes. The recently developed gene expression techniques using microarrays now offer an unprecedented opportunity for the characterisation of these cell systems. This technology allows the simultaneous analysis of mRNA expression levels of thousands of genes, thus enabling a broad insight in the functional state of cells in culture as compared to their in vivo counterpart. Examples will be given of the expression patterns of cells of different origin (liver, kidney) in culture in comparison to the in vivo situation. The functional limitations of cell culture systems will be exemplified under the influence of model toxins. The findings will then be discussed with respect to the reliability of in vitro systems for toxicological testing of compounds.
Lecture
Detection of pain and stress by monitoring gene expression

Paolo Cinelli, Margarete Arras and Kurt Bürki
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One of the main concerns with regard to animal experimentation is that, if animals must be used for experimental purposes, pain and distress should be abolished or reduced to an absolute minimum. Considerable progress has been made concerning the application of the principles of reduction and replacement, however to implement refinement it is necessary to improve our ability to objectively recognise signs of pain and distress.

Pain can be thought of as having both sensory (discriminative) and affective (the unpleasantness) dimensions, and is usually classified as acute or chronic depending on its duration and as neuropathic when it derives from direct damage to the nervous system. Molecular dissection has begun to reveal distinct functions for these separate pathways and their contribution to the final behavioural outcome. Specific patterns of phenotypic change characterise different chronic pain conditions and it is these distinct molecular signatures that need to be considered if effective pain detection and control have to be achieved.

DNA microarrays are among the most powerful and versatile tools for genomics and genetics research. The main goal of our project is the identification of a set of genes that change their expression levels during pain and stress conditions in the mouse. With a pool of selected genes we designed a low density microarray and monitored changes in gene expression levels in mice under different pain/distress conditions. Our results indicate that the genes present on the microarray are useful for an objective detection of different pain levels in the mouse.

Lecture
Gene expression as the basis for alternatives methods: Estrogens as an example

George Daston, Jorge Naciff, Gary Overmann, Leslie Foertsch and Zubin Khambatta
Procter and Gamble, Miami Valley Labs, Cincinnati, USA

Microarrays have made it possible to evaluate gene expression on a scale that had not been technically feasible before. Changes in gene expression are sensitive indicators of biological response and specific patterns of changes can identify toxicological mechanism. Evaluation of gene expression can be added to traditional toxicity tests as a refinement, to gain more and better information, can reduce the number of animals used in testing, or as the foundation for in vitro replacement. Each of these is illustrated using endocrine disrupter (ED) screening as an example. Estrogens of varying potency produce a characteristic profile of gene expression in reproductive tissues of rat fetuses. The dosing protocol used is comparable to the OECD 414 assay. Evaluation of gene expression can be added as a refinement that makes the assay a screen for ED as well as a developmental toxicity. It is also possible to evaluate gene expression in a limited number of animals as a substitute for the mammalian portion of the USEPA's ED screening battery, resulting in a marked reduction in animals. Finally, gene expression provides a more specific readout of hormonal activity in cell-based assays. We have evaluated gene expression in MCF-7 cells (breast cancer-derived) after estrogen exposure, and are comparing these results to human uterine cells. The causal relationship between gene expression and higher order effects, along with its conservation across species, makes it an excellent basis for a 3R approach to alternatives development.
The first step of drug discovery is “target identification” in which the disease state is studied to identify a molecular target for modulation through chemical intervention (i.e., a drug). Until recently, target identification relied heavily on finding targets by studying animal models of human disease (natural or engineered). However, animal-based targets are only successful to the extent that the relevant biology is replicated in humans – a hit-or-miss proposition due to differences between species and between the animal and human diseases. However, a new paradigm is emerging as genomics, proteomics, and other “omics” technologies are facilitating the molecular level study of humans. Targets are increasingly found by studying human tissue (e.g., normal vs. diseased or early vs. late stage) for differential gene or protein expression to identify genes that are involved in the disease process. Cellular pathways associated with the human disease can be mapped using such techniques, leading to the identification of high-quality human-relevant disease-specific targets. Another application of “omics” technologies comes later during drug development, when these technologies can be used to identify early biomarkers of drug efficacy or toxicity. Early stage human drug testing or “experimental medicine” utilising such biomarkers is increasingly popular and is replacing some animal preclinical testing. The identification of “omics” biomarkers is also being done within animal experimentation. While this enables more humane endpoints, the fact remains that attempting to extrapolate from animal-based human drug research is inefficient compared to studying humans directly, and resources should shift more quickly towards human-based “omics” approaches.

"Omics" technologies enabling the 3Rs in drug discovery and development: Treating human disease by studying humans

Sadhana Dhruvakumar
People for the Ethical Treatment of Animals (PETA), Research and Investigations, Norfolk, VA, USA

The use of toxicogenomics in risk assessment: Perspectives and challenges

Ursula Gundert-Remy, Hans-Bernhard Richter-Reichhelm and Axel Oberemm
Federal Institute for Risk Assessment, Safety of Substances and Mixtures, Berlin, Germany

Technologies have been established that generate complex information on molecular changes in animal and man following exposure to chemicals. Having a greater understanding of this information alongside, empirical toxicological reference data provides for the continued evolution in our ability to understand toxicological mechanisms of action, thereby providing a more scientific basis for species extrapolation. Performing toxicogenomic analyses in traditional short-term animal studies will improve the quality of information available from such studies, leading to reduction of possible follow-up studies. In addition, toxicogenomics will aid the identification, development and validation of improved in vitro alternatives. On the other hand, toxicogenomics also provides specific opportunities for improvements at different stages of the risk assessment process such as the development of new predictive models for identifying human health hazards and more significant molecular biomarkers of exposure. Molecular profiling can be used for screening of existing chemicals and to quickly identify potentially hazardous substances and to categorise chemicals and mixtures of chemicals into different mode of action groups. As there is indication that molecular signals differ at dose levels, it is hoped that toxicogenomic information can also contribute to the understanding and interpretation of effects seen with low dose exposure. Gene polymorphisms are known to play a role in the different intra-species susceptibilities to chemicals, thus explaining for the observed differences in effects. Growing knowledge of genomic variability will enable a greater insight into the factors behind the observed variability in susceptibility to chemical exposure that can be seen in human populations.
Lecture

**In vitro models and quantitative differential proteomics technologies for molecular signatures of neurotoxicity and neuroprotection**

Martina Klemm and André Schrattenholz
ProteoSys AG, Mainz, Germany

New challenges for hazard and risk assessment in pharmaceutical and chemical industries and problematic animal testing require the development of a human *in vitro* models for the molecular characterisation of drug effects. The outstanding potential of human Embryonic Stem Cell (hESC)-based *in vitro* models is put in context with results from corresponding murine ESC-screening system. In combination with quantitative differential proteomic display techniques, biomarkers for neurotoxicity are developed. Results are superior to those of conventional array technologies (nucleic acids), because the proteomic analysis covers posttranslational modifications.

The main task of a comprehensive analysis of proteins ("proteomics") is the establishment of a reliable methodology for complexity reduction. Here we present data from experiments with embryonic stem cells during conditions of neuronal stress and rescue, demonstrating the feasibility of quantitative pattern control of complex samples.

Taken together proteomic strategies presented here are able to reliably and quickly detect and identify key molecular events in mode of action or toxicological studies, providing information which is not accessible by standard array technologies, which currently only display the amino acid backbone information of proteins (cDNA or recombinant protein) without detecting posttranslational modifications and thus missing important functional details. Given the enormously complex and dynamic nature of these modifications, and the redundant and pleiotropic organisation of almost all major signal transduction pathways, we envisage the emerging importance of protein signatures of functionally related sets of posttranslational protein isoforms, rather than single targets or surrogate biomarkers.

Poster

**Toxicoproteomics – Identification and pre-validation of potential early biomarkers in chemically induced hepatocarcinogenesis**

Michaela Kroeger1, Kerstin Fella1, Matthias Glueckmann2, Volker Kruft2, Juergen Hellman1 and Peter-Juergen Kramer1

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A common animal model of chemical hepatocarcinogenesis was used to identify early protein biomarkers as an alternative to the classical toxicological endpoints. N-nitrosomorpholine was administered to male Wistar rats for 7 weeks followed by an exposure-free period of up to 25 weeks. Five animals per group were sacrificed at different time points during and after exposure.

Proteome analysis is mainly based on the separation of proteins by 2D-electrophoresis (2DE) as a first step prior to characterisation by mass spectrometry (MS). By using 2DE, many differentially expressed proteins could be detected after 3 weeks as well as 25 weeks of NNM treatment in rat liver tissue. Subsequent MS analysis has been able to identify most of these proteins. The intersection of differentially expressed molecules in both time-points revealed that many endpoint related proteins of week 25 are already detectable in week 3. For verification of these potential early biomarkers identified by 2DE/MS, we have utilised an alternative MS-based protein quantitation method, the iTRAQ™ reagent technology. Many of the proteins deregulated in week 3 could be confirmed by this method. These results show that detection of early protein biomarkers is possible with proteomic approaches. Whether these new biomarkers can support predictive toxicology in order to improve and shorten regulatory carcinogenicity studies is under further investigation.
Lecture

Gene expression analysis of responses to xenobiotics of primary rat hepatocytes in double-layered co-culture systems with small intestinal cells

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Introduction: Usual cytotoxicity tests do not include important metabolic processes such as absorption or biotransformation. To overcome this problem, we proposed physiologically-based simple double-layered co-culture system using cell lines representing the small intestine and liver tissue, where we observed enhanced metabolic functions of the liver by the possible interactions between the two cell lines. In this study, to verify physiological relevance and the mechanisms responsible for this metabolic enhancement, we investigated DNA microarray analysis of primary rat hepatocytes co-cultured with rat small intestinal cells, IEC-6.

Methods: We co-cultured IEC-6 and primary adult rat hepatocytes using a highly-O₂-permeable material, polydimethylsiloxane (PDMS), for the hepatocytes culture. Next, we analysed the poly-cyclic aromatic hydrocarbons (PAHs)-triggered alterations of gene expressions in the hepatocytes using DNA microarray and compared them with those of in vivo oral administration.

Results and discussion: The improved double-layered co-culture system using PDMS successfully maintained the functions of hepatocytes beneath the IEC-6 membranes, whereas conventional double-layered co-cultivation failed to sustain the hepatocytes viability by the lack of O₂ supply. DNA microarray analysis demonstrated IEC-6 has many different influences on hepatocytes activities, such as cell attachment, fat or fatty acid metabolism, or hormone responses. In addition, we observed better in vivo-mimicking gene expression profiles in co-cultured hepatocytes indirectly attacked by PAHs through the IEC-6 cell layers than those in pure culture hepatocytes directly attacked by PAHs without IEC-6 cells. These results show the potential of the co-culture system in in vitro estimation of in vivo human responses when xenobiotics are orally administered.

Poster

Identification of potential markers for an in vitro test system of renal carcinogenesis

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The explanation of the toxicological potential of a substance currently requires large numbers of animals. One promising approach for an in vitro alternative is the application of microarrays to elucidate sets of early toxic insult markers with high correlation to in vivo systems. The aim of our study is the development of an in vitro pre-screening system, using primary and continuous renal cell cultures to characterise the carcinogenic potential of substances in the kidney. In a first phase of this study, male Eker rats were orally treated with the renal carcinogen aristolochic acid (AA). After 1, 3, 7, and 14 days of exposure, three animals from each group were sacrificed, RNA from the kidney cortex was isolated and gene expression profiles were analysed on Affymetrix RAE230A chips. Characteristically deregulated genes were extracted and functionally annotated using statistical and clustering tools. DNA-damage repair genes including p21 and MGMT were up-regulated in all exposure groups. Real-time PCR, allowed the verification of AA-dependent deregulation of both markers in NRK-52E and primary kidney cells from F344 rats. Since DNA damage and deficiencies in the corresponding repair are integral in the process of carcinogenesis, both genes represent promising in vitro markers and should be validated in further cell culture systems from different species.
Poster

A novel TOSHI scaffold useful for inducing cell behaviour and its application to the cellomics study

Toshiaki Takezawa and Tomoyo Takeuchi
National Institute of Agrobiological Sciences, Laboratory of Animal Cell Biology, Tsukuba, Japan

We established a novel concept for the cellomics study to systematically analyse cell behaviour and phenotypes by culturing various cells on the TOSHI (tissue/organ section for histopathology) scaffold reflecting tissue conditions in vivo.

We noticed that thin tissue sections commonly prepared for histopathology retained the original microarchitecture and composition of the tissue, developed a breakthrough technology of culturing animal cells on a TOSHI scaffold, and demonstrated the application of TOSHI scaffolds made of a bovine placenta in tissue reconstruction and a serum-free culture. The labyrinth region of the scaffold induced unique cell behaviours to form multicellular spheroids of BeWo cells (human choriocarcinoma cell line), a capillary network-like structure for CPAE cells (bovine pulmonary artery endothelia), and a neuronal network-like structure for PC-12 cells (rat pheochromocytoma cell line). The scaffold provided a microenvironment to maintain the viability of PC-12 cells in a serum-free condition. Also, we succeeded in preparing a multicellular mass of NHDFs (normal human dermal fibroblasts) involving acellularised section-derived components.

TOSHI scaffolds conserve many of the biochemical factors that serve as signalling cues for inducing cell behaviour and phenotypes, and those factors are easily detected by ordinary techniques such as immuno-histochemistry or in situ hybridisation. Also, TOSHI scaffolds can be prepared not only from all animal tissues/organs of any age regardless of pathology, but also from the entire bodies of small animals. Taking these advantages, the analysis of interactions between different cell types and various TOSHI scaffolds will play an important role for a novel approach to study cellomics.

Poster

3R potentials of “-omics” technologies

Marjolein van Boxel, Marian Moelands and Coenraad Hendriksen
Netherlands Centre Alternatives to Animal Use, Faculty of Veterinary Sciences, Utrecht University, Utrecht, the Netherlands

In a relatively short time-span, biomolecular engineering has evolved into a scientific discipline with substantial promise to revolutionise biomedical research. High-throughput genomics, proteomics and metabolomics technologies enable simultaneous monitoring of the expression of large numbers of individual genes and proteins, resulting in a more profound mechanistic insight in (patho-) physiological processes. Great advances can be made in a wide variety of research areas, such as for drug discovery, vaccine development and safety assessment of chemicals. Little was known about the consequences of these new methodologies for laboratory animal use, although some technologies will probably initiate scientific questions that require (additional) animal experimentation. Apparently, though, “-omics” methodologies may also have potentials for the replacement and, notably, the reduction and refinement of animal experimentation. Increased predictability of tests enables the application of earlier, more humane endpoints. Animal testing may be shifted to later stages in the drug development process and low, subclinical doses of chemicals/drugs can be studied. More and scientifically more relevant data may be attained from a smaller number of animals, due to a better general set-up and a science-based selection of the most appropriate animal model. In cooperation with the Netherlands Genomics Initiative (NGI), the Netherlands Centre Alternatives to animal use organised the first international conference on the potentials of genomics technology as a 3Rs tool, “Genomics & Alternatives to animal use 2004”. A presentation will be given of the most important results of this conference, which was held in June 2004 in Maastricht.
Session 7.4
Non-invasive techniques for monitoring and imaging (Doerenkamp-Zbinden-session)

Lecture
Magnetic Resonance Imaging (MRI) of the lung as a tool for the non-invasive evaluation of drugs in rat models of airways diseases

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The inflammatory status of the lungs in rat models of airways diseases is traditionally inferred from Bronchoalveolar Lavage Fluid (BALF) analysis and/or histology. For lung function analysis rats are usually tracheotomised and artificially ventilated. To suppress spontaneous respiration animals receive a muscle relaxant. From measurements of airflow and transpulmonary pressure, airway resistance is calculated after each respiratory cycle. Clearly, the invasive character of these procedures precludes repeated assessments in the same animal.

We have developed MRI approaches in which acquisitions are performed on spontaneously breathing, anaesthetised animals. Neither artificial ventilation nor tracheotomy is necessary. To suppress spontaneous respiration animals receive a muscle relaxant. From measurements of airflow and transpulmonary pressure, airway resistance is calculated after each respiratory cycle. Clearly, the invasive character of these procedures precludes repeated assessments in the same animal.

Two illustrative examples are allergen (ovalbumin, OVA) and lipopolysaccharide (LPS) inducing distinct inflammatory responses in rats. For OVA, MRI signals correlate with eosinophilia and increased protein content in BALF. Following LPS, MRI signals reflect secreted mucus as revealed in BALF. Histology confirms these results.

The effects of airways remodelling and hyporesponsiveness induced by respectively OVA or LPS can also be monitored by detecting modulations of the parenchymal signal caused by changes in oxygenation levels. This opens the avenue for non-invasive assessments of lung function.

Overall, these approaches provide the basis for non-invasive testing of anti-inflammatory compounds for respiratory diseases.

Grant from the 3R Foundation, Muensingen, Switzerland (Project 82/02)
Poster

**Analysis by FIB imaging technique of Caco-2 cell lines cultured on a new tridimensional substrate**

Isabella De Angelis¹, Francesca Caloni², Gianfranco Friggè², Simona Stacchezzini², Annalaura Stammati¹ and Marziale Milani³

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Epithelial cells cultured in a tridimensional (3D) environment could improve their growth and differentiation, offering an *in vitro* model closer to *in vivo* situation. Human intestinal Caco-2 cells, able to spontaneously differentiate in long-term culture to small enterocytes and extensively used as model of intestinal barrier, are particularly suitable and intriguing for this advanced culture technique.

Biopolymers have been demonstrated to be an efficient support for epithelial cells; in particular alginate, an anionic mucus-adhesive polymer produced from different species of brown algae, has several unique properties of entrapment and/or delivery of the cells.

In this study, we have investigated growth capability and morphology of parental Caco-2 cells and Caco-2/TC7 clone, on a new 3D alginate concave encapsulation model. In this system, both cell lines actively grow, increasing their number (till to 500%) for about 15 days and remaining viable until the 21 day of culture, as in monolayer condition.

Our 3D culture model has been analysed by Focused Ion Beam-Scanning Electron Microscope (FIB/SEM), an innovative imaging and manipulation technique for biological samples; it allows selective sectioning and imaging at nanoscale, collecting secondary particles generated by primary ions or electrons. The technique proved viable for investigation of the boundary between cells and host matrix.

Even if further functional studies must be conducted, present results show that alginate matrix is able to support cellular growth and morphological features of Caco-2 and Caco-2/TC7 cells, opening new perspectives for a more physiological organisation in culture of these cells.

Poster

**Biophotonic imaging and its uses for monitoring and tracking disease processes in live animals**

Kevin Francis
Xenogen Corporation, Alameda, USA

Xenogen Corporation is a leader in the field of biophotonic imaging and has developed a technology which allows biological processes, including gene expression that is both temporal and spatially defined (i.e. occurring in defined tissues and organs), to be monitored in live animals in real-time. Genes encoding luciferase and fluorescent proteins are engineered into cells (e.g. cancer cell lines and infectious disease agents) and animals (transgenic mice and rats) to enable them to produce light that can be visualised through the tissues of a live animal using specialised imaging equipment and software designed and built by the company. To date, Xenogen’s technology has been used predominantly to facilitate research in areas such as oncology, infectious disease, inflammation, neurology and toxicology. This non-invasive imaging technique allows significantly fewer animals to be used due to the generation of superior data and better biostatistics. An overview of this technology will be presented along with specific examples in each of the above disease areas.
Lecture

Measuring nociception by fMRI in anaesthetised animals

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¹ Institute for Pharmacology and Toxicology, Non-invasive imaging, Erlangen, Germany;
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There is a demand for novel analgesics to provide relief for different types of pain. The development of new analgesics depends on traditional pain tests in animals. They measure behavioural reactions of awake animals suffering from pain. A solution could come from measuring responses to painful stimuli in the anaesthetised animal non-invasively by functional Magnetic Resonance Imaging (fMRI). This method provides highly resolved objective functional information on the processing of nociceptive stimuli throughout the whole brain as has been demonstrated in human pain studies. Consequently, this method could also improve the objective measurement of modulatory effects of analgesics.

We established such a fMRI testing system in anaesthetised rats using a mild noxious heat stimulation applied to the rat hindpaw. Because the testing is applied to animals under anaesthesia and the stimulation is mild (temperature: 34-45°C, suitable for humans too) we are minimising the stress for the animal. Moreover, we obtain reliable objective information of different pain competent structures along the pain pathway throughout the whole brain. Having established an optimised analytical framework we could define the degree of pain suppression of different conventional analgesics. Moreover, such a model can be applied for investigating (chronic) pain processes. This would open a new avenue for research on pain chronication and it may contribute to the evaluation of novel analgesics intended to inhibit or even reverse chronic pain.

Poster

Non-invasive imaging of pulmonary tumours in mice using Flat-Panel Detector-based Volumetric Computed Tomography

Sarah Kimmina¹, J. Missbach², J. Rieker³, S. Obenauger³, F. Alves², E. Grabbe³ and K. Nebendahl¹

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There is a strong need for non-invasive methods to monitor tumour growth and progression over time. This study was aimed at examining the usefulness of Flat-Panel Detector-based cone beam Volume-Computed Tomography (FPD-VCT) as a non-invasive technique to monitor tumour growth and metastatic spread at defined time intervals.

1x10⁶ A549 cells (human lung adenocarcinoma cell line) were injected through the intercostal muscular tissue directly into the left lower lobe of the lung of adult SCID mice (n=8). Two weeks after tumour cell inoculation, scans of anaesthetised mice were performed using FPD-VCT (General Electrics Prototype) and were repeated at distinct time intervals.

The results obtained by FPD-VCT were subsequently related to histological analyses. Pulmonary tumour nodules of about 500 µm in diameter could reproducibly be detected by FPD-VCT. Monitoring of tumour growth pattern, morphological characteristics, perfusion rate and tumour extension by FPD-VCT allows a continuous view inside the animal and assessment of the course of the disease. Furthermore this imaging technology might be a powerful tool to reduce animal pain and distress, by assessing the tumour load of the animals. It will also allow for a significant reduction of the number of animals needed to sacrifice for experimental purposes, because imaging can be repeated as often as necessary.

Thus, FPD-VCT analysis has the potential to establish a novel standard for real-time non-invasive tumour assessment over time in animal models.
A multi-parametric live cell imaging approach with state-of-the-art high resolution video-enhanced, confocal, DIC, POL and digital fluorescence microscopy is used as test battery for the monitoring of physiological reactions to the addition of test compounds. Application to neuronal network cultures allowed a distinction of the different glial and neuronal cell types, their axonal and dendritic processes and a direct comparison of the pharmacological impairment of electrical activity with Ca2+ level oscillations. Further studies to compare intracellular activity such as organelle movement, cytoskeleton restructur-}

ting, or Ca2+ signals with electrical activity in a physiologi-

cally active neuronal cell ensemble are in progress. The system allows highly complex studies of cellular behaviour which give new insight into the molecular mechanisms of drug action. Since multiple endpoints can be determined, this test battery will be especially useful in studies where toxic or adverse side effects of test compounds need to be excluded, because its multi-parametric feature reduces the extent of false negative results considerably.

Supported by the Ministry of Education and Research of the State of Mecklenburg-Vorpommern.

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Poster

**Multi-parametric digital imaging test battery for optical monitoring of the physiology of living cells in vitro**

*Willfried Kröger¹, Simone Stüwe², Alexandra Gramowski¹, Sergei A. Kuznetsov¹ and Dieter G. Weiss*¹

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A multi-parametric live cell imaging approach with state-of-the-art high resolution video-enhanced, confocal, DIC, POL and digital fluorescence microscopy is used as test battery for the monitoring of physiological reactions to the addition of test compounds. Application to neuronal network cultures allowed a distinction of the different glial and neuronal cell types, their axonal and dendritic processes and a direct comparison of the pharmacological impairment of electrical activity with Ca2+ level oscillations. Further studies to compare intracellular activity such as organelle movement, cytoskeleton restructur-

ing, or Ca2+ signals with electrical activity in a physiologically active neuronal cell ensemble are in progress. The system allows highly complex studies of cellular behaviour which give new insight into the molecular mechanisms of drug action. Since multiple endpoints can be determined, this test battery will be especially useful in studies where toxic or adverse side effects of test compounds need to be excluded, because its multi-parametric feature reduces the extent of false negative results considerably.

Supported by the Ministry of Education and Research of the State of Mecklenburg-Vorpommern.

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Poster

**Imaging induced neurodegeneration in living fluorescent cells using organotypic hippocampal slice cultures derived from transgenic fluorescent mice**

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Transgenic mice expressing fluorescent proteins in neurons and glia provide new tools for on-line visualisation of dynamic degenerative and regenerative structural changes. In initial trials we have established hippocampal slice cultures derived from 7 day old transgenic fluorescent mice of several strains. With thy1 as promoter, one strain expresses EYFP (Enhanced Yellow Fluorescent Protein) in subpopulations of pyramidal cells and processes; a second expresses CFP (Cyan Fluorescent Protein) in the same subpopulations, as well as in a subpopulation of den-

tate granule cells and their mossy fibres. A third strain expresses DsRed in all astrocytes under the GFAP promoter. Combining the last two strains has provided double transgenic pups with red glial cells and cyan neurons expressed in hippocampal slice cultures. Most recently we have characterised hippocampal slice cultures from PLP-GFP (Green Fluorescent Protein) mice expressing fluorescence in oligodendrocytes. Here we report the presence and developmental expression of the transgene pro-

teins allowing visualisation of neuronal and glial cell bodies and processes in hippocampal slice cultures, as well as the disappear-

tance of fluorescence in relation to experimental excitotoxic and mechanical lesions. Fluorescence was recorded before and then followed after the lesion induction by time-lapse fluores-

cence microscopy. The method is useful for screening of com-

pounds for neurotoxicity as well as glial toxicity. Examples, including time-lapse videos, will be presented at the meeting.

Transgenic mice were kindly provided by Prof. Aagaard Jensen, MBC and Dr. Zalc, Inserm and obtained from Jackson Laboratories. The study was supported by the Danish MRC and the FP5 EUgrant (QLK3-CT-2001-00407).
Lecture

New approaches in non-invasive molecular imaging: Combining PET and MRI

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Positron Emission Tomography (PET) provides quantitative information about functional processes such as glucose metabolism, receptor-ligand binding, and even gene expression. Its high sensitivity in the picomolar range and the large variety of radiolabelled tracers and markers makes PET a powerful tool for functional in vivo molecular imaging. Since PET is absolutely non-invasive, the number of used animals for longitudinal follow-up studies can be drastically reduced and the revealed data become more reliable since the same animal is used over the entire period. However, PET studies show only very little anatomical information and especially for new tracers and biomarkers with an unknown biodistribution, it is often hard to determine their uptake localisation in vivo. Many efforts are made to combine high resolution PET with Computed X-Ray Tomography (CT), an imaging modality providing advanced information about the anatomy. However, CT has low soft tissue contrast and uses relatively high doses of ionising radiation, which might have biological effects in the animal models being studied. As an alternative, Magnetic Resonance Tomography (MRT) can provide high spatial resolution and excellent soft tissue contrast for morphological imaging, but suffers from poor signal strength leading to low sensitivity for functional imaging. Thus, it would be ideal to combine PET and MRI in one device and derive functional and anatomical information at the same time. Our groups focus on the development of a combined PET-MRI scanner for small animals based on novel avalanche photodiodes. First studies proved the feasibility of such a combined system.

Lecture

In vivo imaging in drug discovery and development

Detlef Stiller
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Drug discovery and development is a long-lasting process (on average 15 years) involving many different disciplines and producing a lot of costs up to 1.2 billion US$/compound until launched to the market. Recently the FDA stated solicitorously an ongoing tendency of increasing costs for drug development which might lead to an unbearable burden for the health care system. Therefore the FDA started several initiatives to involve more strongly new promising technologies to accelerate this process to reduce costs and to increase success rates for compounds.

In vivo imaging, especially Positron Emission Tomography (PET) and Magnetic Resonance Imaging (MRI) became important tools to improve that process. With imaging, we can perform longitudinal studies on single animals without the sampling error inherent to biopsy; however, the most important attribute of imaging is the provision of structural and functional information under physiologic conditions, mimicking the situation observed in the clinic. Disease is a biological process caused by changes at the molecular level, therefore molecular imaging, mostly using PET together with tracer amounts of radio-actively labelled compounds, can hasten drug development at the target identification and validation stages, in the synthesis and optimisation of drug candidates, and in pre-phase I to phase II clinical trials, i.e., at almost any point in the process. Furthermore the translational aspect of both technologies to look at the same readout in animal as in man helps to improve the predictability of animal models, to reduce the number of animals needed and to decrease development time for drugs.
Poster

Online monitoring of physiological parameters of cell cultures

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Physiology parameters such as oxygen consumption and acidification showed to be important parameters in cell metabolism. Parameter changes in dependence of test compounds or supplements are meaningful indicators for metabolic interferences like receptor activation, initiation of signal transduction pathways, irritation/corrosion, inhibition or activation of metabolic pathways and apoptosis.

Therefore, we developed our “BIONAS®2500 analysing system” suitable for online detection of long term effects on physiological parameters on living cells. Our system is able to monitor oxygen consumption, acidification and adhesion of living cell cultures. It is a useful tool for analysing cell reactions in dependent of test substances. The detection can be performed from a few hours to several days. Regeneration/recovery effects can also be monitored after displacement of the substance or supplement.

Another point of interest is the measurement of changes in cell adhesion and confluence. As above mentioned, it can also be measured by the “BIONAS®2500 analysing system”. Especially for adhesion detection we developed a new Multiwell analysing system “BIONAS®9600 AdCon Reader”. It allows monitoring of changes in cell morphology and cell shape, cell proliferation, cell adhesion and spreading and also cell death (apoptosis/necrosis). Furthermore receptor activation, signal transduction, irritation/corrosion and inhibition/activation of metabolic pathways can be detected. It can be also very useful for cell differentiation analysis and other applications.

Poster

The optical probe technique for the analysis of drug resistance profiles in multicellular tumour spheroids

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The development of multidrug resistance (MDR) is a major impediment for the success of chemotherapeutic cancer treatment. MDR mainly occurs due to increased expression of MDR transporters belonging to the ABC-family of transporters, e.g. P-Glycoprotein (Pgp), Multidrug Resistance-associated Protein-1 (MRP-1), and Breast Cancer Resistance Protein (BCRP). To circumvent MDR an increasing number of MDR-reversing agents have been developed and are tested either in two-dimensional cell cultures or in animal experiments of cancer. In a novel approach to replace animal experiments the optical probe was developed. This new technique uses the optical sectioning properties of confocal laser scanning microscopy to record drug penetration profiles in the depth of three-dimensional tumour tissues, i.e. either multicellular tumour spheroids or tumour fragment spheroids. Following staining with either fluorescent anti-cancer agents or fluorescent test substances drug penetration and diffusion kinetics of these probes can be monitored semi-automatically over time in living tissues. This allows routine testing of MDR reversing agents which increase the diffusion of anti-cancer agents in treated tissues, and is applicable for large throughput screening.
The limitations of conventional static cell culture systems prevent a continuous exposure of cells to toxin. In order to investigate the toxic effects of continuous exposure of the nephrotoxin cadmium chloride (CdCl₂), we utilised a perfusion cell culture apparatus (EpiFlow®).

The porcine proximal tubule cell line LLC-PK₁ and the human proximal tubule cell line HK-2 were exposed to CdCl₂ for up to 72 h as a single static dose or under continuous perfusion. Lactate dehydrogenase (LDH), adenylate kinase (AK), glucose consumption and lactate production were assayed in the supernatants. Cell viability was assayed by resazurin reduction.

Perfusion experiments with control, NOEL (1 µM for the HK-2, 3 µM for the LLC-PK₁) and EC₅₀ (5 µM and 15 µM, respectively), as detected after 72 h under static conditions, were performed for 4 days.

Under perfusion conditions, the 5 µM and 15 µM concentrations increased enzyme release strongly; a marked peak was observed at 12 h for the LLC-PK₁ and at 25 h for the HK-2 cells. The 15 µM concentration resulted in 100% lethality to the LLC-PK₁ and the 5 µM concentration resulted in 70% lethality to the HK-2 cells.

The results indicate a greater susceptibility of renal epithelial cell lines to CdCl₂ under continuous exposure in perfusion culture than compared to a single dose in static culture.

In conclusion, the EpiFlow system is well suited for long-term toxicity testing under continuous perfusion as well as for testing of acute toxicity.
We examine the alternative method in vitro procedure of the developmental toxicology test of chemicals. Although the effects of bisphenol A have been extensively investigated, there is little information concerning the effects of tetrabromobisphenol A (TBrBAP). TBrBPA, which is widely used as a flame retardant in the building industry in USA. We have reported that TBrBPA had similar embryological toxicity on cultured rat embryos to that of bisphenol A at the concentration (1 ppm). At higher concentration bisphenol A had a severe toxicity on rat embryo in culture. In the present experiments we examined the embryological toxicity of TBrBPA at a lower concentration. Rat embryos on day 11.5 of gestation were removed from the uterus and cultured as whole embryo for 48 hours in normal medium. Two hours after incubation in normal medium, TBrBPA or its solvent DMSO, was added to the medium. The rat embryos were further cultured for 46 hours.

In embryos cultured in the medium containing TBrBPA at the concentration of 1ppm a clear decrease in heart rate and blood circulation in yolk sac vessels were observed at 24 and 48 hours in culture. In rat embryos cultured in medium containing vehicle only cacogenesis was observed at the corresponding times. No clear abnormality was observed in cultured embryos at the concentration below 1 ppm of TBrBPA. It was found that TBrBPA had the toxicity on the circulation of rat embryos at concentration up to 1 ppm.

**Poster**

**The effect of tetrabromobisphenol A at a low concentration on rat embryos in culture**

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Most human hepatoma cell lines are deficient in the major CYP-related enzyme activities, making them unsuitable for xenobiotic metabolism and toxicity studies. The aim of this work was to analyse the xenobiotic metabolism capacity of HepaRG cells derived from a human hepatocellular carcinoma, that exhibit a highly differentiated pattern after 2-3 weeks at confluence in the presence of DMSO (Gripon et al., PNAS, 2002, 99, 15655). mRNAs encoding various nuclear factors (AhR, PXR, CAR, PPARα), CYPs (1A2, 2C9, 2D6, 3A4) and phase 2 enzymes (GSTA1, A4, M1 and GT1A1) were measured by qRT-PCR in HepaRG cells at their optimum level of differentiation and were found to be expressed for most genes, at levels close to those estimated in primary human hepatocyte cultures and equal or much higher than those in HepG2 cells. Similarly, values of basal CYP activities and their response to prototypical inducers as well as metabolic profiles of the compounds studied were comparable to those obtained with cultured human hepatocytes. Moreover, the hepatotoxicants tested were more cytotoxic to HepaRG than to HepG2 cells. In conclusion HepaRG cells represent the first human hepatoma cell line expressing the major CYPs involved in xenobiotic metabolism and appear to be a unique tool for investigating xenobiotic metabolism and toxicity in human liver.

**Lecture**

**Human hepatoma HepaRG cells: a reliable surrogate to primary human hepatocytes for xenobiotic metabolism and toxicity studies**

*Caroline Aninat¹, Amélie Piton¹, Denise Glaise², Tifenn Le Charpentier², Sophie Langouet¹, Fabrice Morel¹, Christiane Guguen-Guillouzo² and André Guillouzo¹*

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The evaluation of the mutagenic activity of mainstream cigarette smoke is mostly based on studies with condensates or extracts in the standard Ames assay. Due to the methodological difficulties of testing air contaminants in their natural gaseous or aerosolised state, there are no accepted concepts and techniques for effective exposure of bacteria under such conditions. Therefore, we established a novel approach using an exposure device based on the cell exposure system CULTEX®, which was connected to a smoking machine (smoking robot VC10). This allows us the investigation of chemically and physically unchanged mainstream cigarette smoke by exposing bacteria of Salmonella typhimurium strains directly to diluted mainstream smoke of the research cigarette K2R4F. In preliminary experiments the treatment of strain TA98 to whole smoke resulted in the induction of revertants dependent on dilution and the number of cigarettes smoked, whereas the gas phase induced no mutagenic signal. In comparison to studies with condensates by using the plate incorporation assay, the exposure of the bacteria directly to native cigarette mainstream smoke seems to enhance the susceptibility of the bacteria to mutation. The introduction of our exposure device in the field of inhalation genotoxicology offers new possibilities in the evaluation of genotoxicity in the Ames assay by taking in consideration not only hydrophilic but also hydrophobic substances in the particulate and gas phase.

**Poster**

**The use of the modified CULTEX® system for the direct exposure of bacteria to mainstream cigarette smoke**

*Michaela Aufderheide and Ulrich Mohr*
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The evaluation of the mutagenic activity of mainstream cigarette smoke is mostly based on studies with condensates or extracts in the standard Ames assay. Due to the methodological difficulties of testing air contaminants in their natural gaseous or aerosolised state, there are no accepted concepts and techniques for effective exposure of bacteria under such conditions. Therefore, we established a novel approach using an exposure device based on the cell exposure system CULTEX®, which was connected to a smoking machine (smoking robot VC10). This allows us the investigation of chemically and physically unchanged mainstream cigarette smoke by exposing bacteria of Salmonella typhimurium strains directly to diluted mainstream smoke of the research cigarette K2R4F. In preliminary experiments the treatment of strain TA98 to whole smoke resulted in the induction of revertants dependent on dilution and the number of cigarettes smoked, whereas the gas phase induced no mutagenic signal. In comparison to studies with condensates by using the plate incorporation assay, the exposure of the bacteria directly to native cigarette mainstream smoke seems to enhance the susceptibility of the bacteria to mutation. The introduction of our exposure device in the field of inhalation genotoxicology offers new possibilities in the evaluation of genotoxicity in the Ames assay by taking in consideration not only hydrophilic but also hydrophobic substances in the particulate and gas phase.

**Poster**

**Harvest, proliferation, and functional testing of human dendritic cells**

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Difficulty in harvesting large numbers of cells, short survival time, and rapid phenotypic changes in culture have prevented the widespread use of human dendritic cells (DC) for many fundamental studies applicable to the initial stages of pharmaceutical discovery and development. To develop a commercial source of DC, precursor cells were harvested from umbilical cord blood samples, proliferated, and induced to differentiate into DC with a newly developed medium. Use of the novel culture medium allowed increases which averaged >400 fold in DC number. FACS analysis showed that the DC expressed CD1a, HLA-DR, CD11c, CD40, CD80, CD83, and CD86 for up to 35 days in culture; Birbeck granules were also observed over this culture period by TEM. Upon stimulation, the DC showed gene and protein responsiveness in terms of IL-12, MIP-1α, MIP-3α, IL-6, and TNF-α expression and DC were able to stimulate allogeneic TC. In addition, DC exposed to allergens enhanced both primary and secondary TC responses and the DC were infectible with HIV-1. Thus, improved culture conditions have allowed the harvest and expansion of functional DC. MatTek’s new DC product (DC-100) will be useful in: 1) allergenicity, 2) viral infection, 3) antigen presentation, 4) immuno-therapeutic, and numerous other studies related to the development of prophylactic and therapeutic pharmaceuticals.
**Poster**

**Preliminary characterisation of an *in vitro* model of blood brain barrier useful for toxicological studies**

*Maria Balduzzi¹, Silvia Paradisi², Andrea Matteucci², Emanuela Testai³ and Isabella De Angelis*³

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The blood-brain barrier (BBB) plays a pivotal role in maintenance of brain homeostasis and is crucial for neuronal functions. *In vivo*, enzymatic activities of cerebrovascular endothelial cells against endogenous molecules or xenobiotics are important to protect brain from toxic damage.

In animals, measurement of BBB functions is both difficult and costly to perform, therefore its reliable *in vitro* model would be of great value to study transport, absorption and toxicity mechanisms in consequence of chemicals exposure.

We have developed an *in vitro* BBB system, in which an immortalised rat brain endothelial cell line (GPNT) is co-cultured, on microporous membrane filter, with primary rat cortical astrocytes (either fetal or neonatal) or retinal Muller glia, seeded in the opposite side of the filter. That is, the direct interaction between astrocytes and endothelial cells, crucial for the induction of *in vivo* BBB properties, is maintained. The choice to use at least one continuous cell line, has been taken to reduce animal numbers and to simplify experimental procedures.

We have characterised our BBB model, and preliminary results show that it matches some *in vivo* BBB features, such as: cell morphology, revealed by SEM analysis, presence of tight-junctional complexes, analysed by ZO-1 and occludin immunostaining, P-gp expression and activity, determined by cytofluorimetric assay, low paracellular permeability to (H)-mannitol and slight increases of Trans-Endothelial Electrical Resistance.

Further studies are in progress to elucidate metabolic properties of our BBB model, in order to better characterise it as a possible useful tool for toxicological evaluation.

**Poster**

**Impact of excipients and solubilisers on the *in vitro* gastrointestinal permeation of marker molecules**

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The aim of the present work was to determine the impact of excipients used in oral dosage forms and solubilisers on the transport of drugs across Caco-2 monolayers without damaging membrane integrity or influencing permeability. For this purpose we tested seven excipients/solubiliser: PG, PEG 400, Tween 80, Cremophor EL, TPGS 1000, HP-ß-CD and BSA. For the permeation studies the low permeability marker mannitol, digoxin (substrate for Pgp), phenylalanine as example for an uptake-transport and propranolol as high permeability marker were chosen.

Permeation studies on Caco-2 cell monolayers were carried out on Transwell® clear filters with an area of 1.13 cm² and 0.4 µm pore size. The transport (21-30 days after seeding) was performed in triplicate. The solutions of excipients/solubilisers with radiolabelled test substances was added to the donor compartment and KRB Buffer (pH 7.4) with 2% BSA to the receiver compartment.

The ratio of the Papp values from the basolateral side to the apical cell side depends on the transport mechanism. For mannitol the ratio of permeability coefficient is ≈ 1, for digoxin >>1 and for phenylalanine <<1.

The highest influence on mannitol transport was established for PEG 400 and PG. Cremophor EL, Tween 80 and TPGS 1000 inhibit the eflux-pump Pgp. The other solubilisers show different effects on digoxin transport. For all tested solubilisers only low effects on phenylalanine transport were observed. Therefore the individual interactions between the test substances and the excipients/solubilisers play a key role in oral absorption process.
A major step in drug development is the assessment of the inherent potential risk that the respective compound might bear when used in man. Neurotoxicity testing poses considerable difficulties to realistic in vitro modelling. The currently used in vivo methods are expensive and time consuming. Besides the toxicity of the compound one has also to consider other side effects that might not be directly toxic but could have deleterious consequences. These side effects include impact on cell proliferation and differentiation.

Organotypic hippocampal slice cultures (OHC) combine the accessibility of an in vitro system and the retained 3D structure of the respective tissue found in vivo. OHC also greatly reduce the number of experimental animals and time needed.

We have established two injury models of neurotoxicity: Excitotoxic injury induced by glutamate and ischemic injury induced by oxygen-glucose-deprivation. Quantification of pyramidal neuronal death is performed by densitometric assessment of propidium iodide (PI) incorporation. Proliferating cells are labelled with BrdU and subsequently detected by immunohistochemistry and confocal laser microscopy. Neurogenesis is identified by immuno-histochemical double-labelling (BrdU/neuronal marker: Doublecortin, beta-III tubulin, NeuN).

Neuronal cell death and neurogenesis was characterised under normal and injury conditions. Our data demonstrate the existence of neurogenic zones in this rodent brain tissue culture. Thus, this OHC-based in vitro assay represents an excellent model system for simultaneous (one sample-three endpoints) detection of compound effects on neuronal viability, proliferation and differentiation in the CNS.

Poster
Organotypic hippocampal slice cultures: A novel in vitro assay system for simultaneous analysis of neurotoxicity, proliferation and neurogenesis in the same sample

Olga Chechneva
Leibniz Institute for Neurobiology, Project Group Neuropharmacology, Magdeburg, Germany

Lecture
Modelling long-term repeat-dose toxicity: Challenges faced

Richard Clothier
University of Nottingham, Faculty of Medicine and Health Sciences, School of Biomedical Sciences, Nottingham, UK
Poster

**Evaluation of chemically induced neurotoxicity in primary culture of rat Cerebellum Granule Cells (CGCs) using specific neuronal and cytotoxic endpoints**

*Sandra Coecke, Valentina Pilotto, Agnieszka Kinsner, Joanne Gartlon, Erwin van Vliet, Thomas Hartung and Anna Price*

ECVAM, Institute for Health and Consumer Protection, Joint Research Center, Ispra, Italy

*In vitro* systems have been extensively used to study the mechanisms of neurotoxicity at the cellular and molecular level, however, their application in hazard and human health risk assessment has not been explored to any great extent. The challenge to use *in vitro* systems for neurotoxicity screening is to differentiate cytotoxicants from neurotoxicants.

In these studies, we have exposed primary neuronal-glial culture of rat cerebellar granule cells (CGCs) and the non-neuronal cell line of the mouse fibroblast (BALB/3T3) to the neurotoxic (trimethyltin, aluminium chloride, methylmercury, acrylamide, colchicine, paraquat, parathion and chloroquine) and non-neurotoxic compounds (triethylenemelamine, paracetamol, cycloheximide) for 72 hrs. To evaluate whether tested chemicals had different potency of toxicity in neuronal versus non-neuronal cells IC20, IC50 and IC80 of all tested compounds was determined in culture of CGCs and compared with BALB/3T3 cell line. The specific neuronal- and glial-endpoints (neurofilament or glial fibrillary acid protein quantification using ELISA) were applied and compared with general assays for cytotoxicity (mitochondrial membrane potential, reactive oxygen species and ATP quantification) and cell viability (alamar blue assay) to discriminate between neurotoxicity and cytotoxicity.

The results suggest that:

1) IC50 values of chemicals tested in the culture of CGCs and BALB/3T3 correlated well with rat LD50 values at the higher concentrations, whereas at the lower concentrations IC50 of CGCs correlated better than those of BALB/3T3.

2) Neuronal specific endpoints for neurotoxic chemicals in primary culture of CGCs were more sensitive than cytotoxic assays.

3) The neurotoxic compounds produced characteristic dose-response profiles that differed from cytotoxic chemicals.

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**Poster**

**Alternatives to animal serum for cell culture – 2005**

*Erwin Falkner1,2, Helmut Appl2, Claudia Eder1,2, Udo Losert4, Harald Schoeffl2 and Walter Pfaller1*

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Background: Aim of our work was an up to date study on aspects of animal serum usage for cell cultivation tasks and alternative approaches of nutrition medium supplementation. The final report and up to date product guide should support researchers in making their best choices for specific *in vitro* studies.

Methods: Data sheets, product descriptions, cell culture manuals and published papers on the subject were gathered/analysed by literature survey and contacting scientists working in the field at universities and industry research centres.

Results: A growing number of alternatives exists for cell lines and primary cultures derived from a broad range of tissues: e.g. chemically defined media, complementations of non-serum origin, non-animal derived proteins and also optimised sampling/processing protocols and production systems/bioreactors for serum-free usage of all scales. A literature search structured for cell type/field of application was performed and paper abstract/citation-shortcuts were collected. The final document including the newly updated product guide 01/2005 is available at http://www.zet.or.at. Further updates are scheduled every 6 months.

Conclusion: In recent years various easily available alternatives to animal sera have been reported and are on sale. For optimal cell performance *in vitro*, to limit costs and, last but not least, following animal welfare considerations, the authors advise the gathering of exact informations to make qualified choices possible.
A natural blood clot contains 95% of red blood cells, 5% platelets, less than 1% and numerous amounts of fibrin strands. In comparison to a PRP (Platelet rich plasma) blood clot containing 4% of red blood cells, 95% platelets and 1% of white cells. Several components in the blood clot were recognised being a part of the natural healing process if added to the wounded tissues or surgical sites and have the potential to accelerate the healing. It was also shown to increase the bone formed from 19 to 25% when measured at 4 and 6 months. The specific components of the PRP are the platelets derived growth factor (PDGF) and the Transforming growth factor b (TGF b). Both of them are contained in the a granules of the platelets. Fibronectin and vitronectin are also the components of the PRP. They are the cell adhesion molecules found in plasma and fibrin itself. The experiments presented herein were aimed to isolate, characterise and to test in vitro on different cell cultures the growth promoting material from sheep's blood clot. The sheep’s blood was collected and allowed to form the clot. Afterward the whole content was centrifuged at 2500 RPM for 20 minutes and the supernatant was aspirated off. The sediment (“clot”) was quickly washed with the sterile PBS (Phosphate buffer saline) pH=5.8 for 10 minutes, and centrifuged at 2500 RPM for 25 minutes. The supernatant (fraction I) was collected and frozen. To the remaining “clot” the PBS pH=7.2 was added and left for 1 hour at +4°C. After the centrifugation of the suspension at 2500 RPM for 25 minutes, the supernatant (fraction II) was collected and frozen. To the sediment (“clot”) the PBS pH=7.4 was than added for 18 hours (fraction III) and for 5 days (fraction IV). All the fractions were sterilised by 0.2 membrane filtration. The content was analysed by PAG-SDS electrophoresis. The porcine ocular fluid was obtained from the whole eye’s fluid content filtered through the gauze and centrifuged at 3000 RPM/30 minutes at +4°C. The clear supernatant was collected, filtered through the 0.44 mesh filter and stored at -30°C. The cell growth promotion/inhibition activity in the comparison to the SR-2.055P (serum replacement based on porcine ocular fluid) and FCS (foetal calf serum) was tested on the chicken embryonal fibroblasts, WISH, HAC-3/T2 (human amniotic cell lines), PLA-2 (adult pig kidney cell line), IPEC-J2 (porcine intestinal endothelial cell line) and WiREF (Wistar rat embryonal fibroblastoid cell line). Fraction I shows the growth inhibition and toxicity, while other fractions shows the growth promotion, but different according to the group of cells used in the test. The optimal content was 5-8% in Eagle’s medium. In this range up to 90% value of the SR-2.55P could be obtained.
**Posters**

**High-dose atrazine affects ovarian steroidogenesis and oocyte maturation but not pre-implantation embryo development in mouse studies**

*Erik Gobbers¹, Kelly Lemeire¹, Valérie Van Merris¹, Johan Smitz² and Rita Cortvrindt*¹

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Introduction: Studies suggest that the herbicide atrazine disturbs fertility by altering the pituitary secretion of luteinising hormone and prolactin. We used a follicle bioassay to investigate whether atrazine affects the ovary directly. Embryotoxicity of atrazine is observed in animal models. We evaluated its specific effect on pre-implantation embryo development by the mouse embryo assay (MEA).

Materials and methods: Preantral mouse ovarian follicles were cultured during 13 days, up to ovulation and were chronically exposed to 1, 10 or 100 µM atrazine (60 follicles/dose). One-cell mouse embryos were cultured under oil in the presence of the same doses (40 embryos/dose).

Results: No effects were observed on folliculogenesis, but 100 µM atrazine affected oocyte maturation: only 26±18% of the oocytes was able to extrude a polar body (versus 90±9% in the control). Estradiol and testosterone production were increased at the 3 dose levels, but only estradiol was significantly different from the control on day 12 (70.1±31.5 µg/l versus 23.0±10.3 µg/l). The 100 µM dose increased pre-ovulatory progesterone significantly on day 12 (38.1±17.6 µg/l versus 5.2±1.6 µg/l in the control), whereas hCG-induced progesterone was elevated at the 3 dose levels (significant when exposed to 1 and 100 µM: 451±43 µg/l and 494±76 µg/l versus 311±71 µg/l in the control). In the MEA blastocyst formation and hatching capacity were normal irrespective of the atrazine dose level.

Conclusion: Atrazine seems to affect the ovarian function only directly at high dose levels, as was evidenced by disturbed oocyte maturation and steroidogenesis, whereas pre-implantation embryo development was not influenced.

**Posters**

**Adaptation of cell lines to chemically defined minimal (cdm) media and evaluation of a protocol for cryo-preservation of cells cultured in absence of animal derived components**

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Our research attempts to replace foetal bovine serum (FBS) by supplementing the cell culture media with alternative additives. Removing FBS from cell culture media will have broad benefit to both scientist and to animal welfare groups. Researchers will benefit by the downstream processing and registration of cell products for pharmaceutical use. Eliminating the brutal practice of collecting blood from bovine foetuses will reduce animal cruelty.

In the present project we adapted several cell lines frequently used by biotechnologists and molecular biologists to cdm-media. We followed the growth behaviour before and during the adaptation phase. After a stringent quality control and authentication, the cell lines are now available for the scientific community by the European Collection of Cell Cultures (ECACC).

Cells cultured without FBS are in general more sensitive to chemical agents and mechanical stress. The proteins in FBS, consisting mainly of albumins, create a balanced viscosity in the cells’ environment and bind added reagents, as well as toxic metabolites. These facts are especially important in the process of cryo-conservation as it is often done using a mixture of culture medium, FBS and DMSO as anti-freeze agent.

The main part of our presentation deals with the evaluation of a freezing protocol designed for cells cultured in absence of FBS. The mechanism of the freezing process and the results obtained by varying the cooling rate using different freezing media are discussed. We compare simple “home-made” media with commercially available freezing media.

This project was sponsored by FFVFF, Zurich, Switzerland (www.ffvff.ch).
Technological developments in cell culture techniques have lead to the production of increasingly organotypic organ cultures. MatTek currently produce 3-dimensional, multi-differentiated of a variety of organs, including the human bronchi. The EpiAirway® bronchial cultures not only present an organotypic phenotype, but also provide the opportunity for representative aerosol exposure as they are grown at the air:liquid interface. These are currently utilised for a variety of purposes, but the aim of this study was to investigate their efficacy as a toxicological tool.

EpiAirway® cultures were exposed to a range of known respiratory toxins and a variety of their responses assessed. These included TEER, MTT, protein exudation, histopathology and cytokine analysis. From this a toxicological response profile was created and compared previous studies into alternative lung models. Morphological analysis showed that the cultures were representative of bronchial epithelium, and that damage occurred indiscriminately across cell types. Biochemical results showed immediate, transient release of pro-inflammatory cytokines concurrent with a similarly transient loss of TEER. This was followed by dose-dependent change in TEER and a dose- and time-dependant decrease of cellular viability. The toxicological profile was significantly attenuated following co-exposure to antioxidant-rich, surrogate, epithelial lining fluid (sELF).

Overall, the cultures displayed significant morphological and biochemical correlation with both in vitro and in vivo models, including primary Type II cell monocultures (Richards et al., 1990). This study has served to iterate the potential value of air-liquid interface cultures in modelling the human respiratory tract in vitro.

A high-throughput in vitro model of human tracheal/bronchial epithelium (EpiAirway™) for preclinical safety and efficacy testing of pharmaceuticals

Patrick Hayden, Mitchell Klausner*, George Jackson, John Sheasgreen and Joseph Kubilus
MatTek Corporation, R&D, Ashland, USA

A growing need exists for high throughput in vitro models that can provide rapid, reliable safety and efficacy screening in preclinical drug development. MatTek has recently adapted EpiAirway, an in vitro model of human tracheal/bronchial epithelium, to a 96-well high-throughput screening (HTS) format compatible with robotic manipulation. HTS EpiAirway (AIR-196) is derived from normal human cells cultured at the air/liquid interface in 96-well microporous membrane plates to produce three-dimensional organotypic cultures. AIR-196 exhibits a pseudostratified structure and displays a differentiated mucociliary phenotype with barrier properties similar to native tracheal/bronchial or nasal epithelium, including development of transepithelial electrical resistance (TEER), conferred by functional tight junctions. Over the course of several consecutive culture lots, the intraplate and interlot barrier function reproducibility of the AIR-196 cultures was determined by measuring TEER. Average baseline TEER readings of all wells in a given 96-well plate (intraplate average) ranged from 388.6 to 445.0 Ohm * cm². The average coefficient of variation between wells on the same plate (intraplate variability) was 19.5 %, while the variability between plates (interlot variability) was 6.8 %. The utility of AIR-196 for drug formulation developed was demonstrated by looking at the peptide induced permeation enhancement of FITC-dextran (MW=4000) through the tissue. Optimal peptides reversibly decreased TEER values to <15% of controls, did not induce cytotoxicity (viability >95%), and increased permeation by 3 fold. Thus, the HTS EpiAirway will find utility for drug permeation studies as well as in vitro irritation/toxicity screening, high content cell testing, and molecular biology assays.
Infection in humans with the larval stage (metacestode) of fox tapeworm *Echinococcus multilocularis* causes alveolar echinococcosis (AE). The disease is fatal if not treated appropriately. The *in vitro* culture model for the continuous proliferation of *Echinococcus multilocularis* metacestodes was established, which has made this parasitic stage experimentally accessible, and has since then provided a unique model to study a number of aspects of the parasite biology and its interaction with the host. This model has served as an unlimited resource of antigen for both diagnosis as well as experimental studies, and at the same time, most importantly, it has enabled us to avoid the extensive use of laboratory animals for a number of studies. We have shown, that this model is highly suitable for first-round *in vitro* drug screening assays, and we developed an easy-to-use and reliable test to determine parasite viability, which allows to investigate large numbers of potentially interesting compounds. During such studies, we identified the 5-nitrothiazole analogue nitazoxanide as a novel potential drug for anti-echinococcal chemotherapy. Following very limited experimentation in laboratory mice, this drug will be evaluated in a clinical study involving human AE patients, starting towards the end of this year.

**Poster**

**In vitro culture of Echinococcus multilocularis metacestodes as alternative to animal use**

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*Neospora caninum* is a protozoan parasite, belonging to the *apicomplexa*, and is a pathogen of considerable veterinary importance. It causes neuromuscular disease in dogs and represents the most important cause of abortion in cattle. Infection with the proliferative and disease-causing stage of this intracellular parasite, the *tachyzoites*, leads to destruction of tissues and immunopathological events. *Tachyzoites* can be cultured *in vitro* in the presence of host cells. The cystic stage, the *bradyzoites*, will form tissue cysts that can persist within an infected animal for many years, leading to chronic infection. These tissue cysts have long been experimentally accessible only through extensive and time consuming animal experimentation, with a large number of laboratory mice being required for these studies. We have now succeeded to develop an *in vitro* culture model for the production of *N. caninum* tissue cysts. We have applied this model to study the cell biology of stage conversion, and to characterise the *bradyzoite* stage and its interaction with the host. More recently, we have adapted the *N. caninum tachyzoite* culture model to screen for anti-parasitic drugs. Parasite proliferation is easily measured through a quantitative real time PCR assay developed in our group, and these assessments are complemented by light- and electron-microscopical observations. Drug efficacy studies using a series of selected compound, nitazoxanide, have been carried out, and allowed to us to determine the structure-function relationship of defined alterations of this molecule.

**Poster**

**In vitro development of tissue cysts of Neospora caninum and use of in vitro culture in structure-function analysis of anti-parasitic drugs**

*Andrew Hemphill, Nathalie Vonlaufen and Marco Esposito*

Institute of Parasitology, University of Bern, Bern, Switzerland
Poster

Cytomic assays for \textit{in vitro} toxicity assessment

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Classical \textit{in vitro} toxicity assessment involves the measurement of either cell proliferation or gross cell death. With the dawn of genomics and the rising of proteomics these classical assays seem antiquated. However, newer safer more reliable cell death and cell proliferation assays are available. Also, fluorescent and luminescent based assays which can measure such sensitive endpoints as inner mitochondrial membrane potential and hydrogen peroxide production are now on the market. It is possible to combine many of these assays together in one experiment thereby generating as much data as possible, saving time, cost and biological material. Using the chronic nephrotoxin cyclosporine A as an example we discuss the applicability of a wide variety of assays to the determination toxicity \textit{in vitro}.

The human proximal tubular cell line HK-2, was treated with various concentrations of CsA for 24 or 72 hours. On live cells resazurin reduction, cell cycle analysis, mitochondrial membrane potential and thymidine (BrdU) up-take were determined. In supernatants glucose, lactate, LDH, adenylate kinase and hydrogen peroxide was measured. In cell lysates caspase-3 activity was determined.

Using these assays we could determine CsA induced effects at concentrations below cell death. CsA at sub-lethal concentrations increased ROS, altered glucose metabolism, increased mitochondrial membrane potential and was anti-proliferative. Multi-parametric cytomic analysis when used appropriately can be very useful in toxicity assessment \textit{in vitro}.

Lecture

Engineering human hepatoma cells with key transcription factors as a mean to generate metabolically competent human hepatic cell models

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Replacement of laboratory animals by alternative human-derived \textit{in vitro} models is a goal in human pharmacology and toxicology. Within this context, the knowledge of the biotransformation pathways of a compound is a necessary step for the development of better and safer drugs. Primary cultured human hepatocytes, as they produce a metabolic profile of a drug very similar to that found \textit{in vivo} and respond to inducers, have become the gold standard in \textit{in vitro} studies. However, because of their restricted accessibility, other cell models (e.g. hepatoma cells) have been considered. Unfortunately, hepatic cell lines express only marginal levels of drug-metabolising enzymes (i.e. CYPs; cytochrome P450s) and are not a real alternative. To understand why human hepatoma cells do not express CYP genes, we undertook a detailed analysis and found evidences showing that the levels of several key transcription factors and co-regulators clearly differ from those found in adult hepatocytes. Based on these observations we postulated that the re-expression of one (or more) of these transcription factors in hepatoma cells could lead to an efficient transcription of CYP genes. The feasibility of this hypothesis was demonstrated by genetic engineering of hepatoma HepG2 cells with different factors (e.g. C/EBP, HNF3) and strategies (chromatin remodelling), followed by the analysis of the expression of human CYPs. Tailored re-expression of activators and co-activators missing in hepatoma cells lead to the transcription of relevant CYP genes. Our results open a promising new experimental strategy to metabolically upgrade human hepatoma cells for human drug metabolism and toxicity studies.
As evident with the recent high profile recalls, drugs entering the market are still vulnerable to variations in efficacy in patient groups and to additional side effects under a specific predisposition. This calls for further extension and higher precision of drug testing. Standardised in vitro systems based on miniaturised histotypic tissue cultures can substantially increase the predictive value of preclinical drug testing. A novel miniaturised modular bioreactor technology was developed on the basis of fully disposable culture ware. Heart of the culture ware is a unique micro culture cassette ensuring long term histotypic cultivation of human and rodent primary tissues. A bioreactor prototype with a cell culture volume of 0.5 ml was developed and tested specifically for survival of leucocytes. Furthermore human lymphatic tissues generated in this type of bioreactor were subjected to treatment with different substances during culture. Microenvironment, tissue reactivity and metabolic parameters were monitored. In addition the micro culture cassettes with cultured cells where embedded into Technovit 7100 and histological staining of cross sections was used to evaluate micro architecture within the cell culture compartments. Observed results clearly indicated that the novel bioreactor platform provides a perfect basis for high content drug testing on miniaturised human lymphatic tissues in vitro. Thus the development may contribute to predictive immunogenicity testing of drugs avoiding both animal testing and risky early trials in man.

Poster

A novel miniaturised perfusion bioreactor for predictive immunogenicity testing of drugs

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Poster

Human vaginal-ectocervical tissue model (EpiVaginal™) to test the irritation of contraceptive and vaginal-care products

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Vaginal-ectocervical (VEC) tissues were reconstructed using normal human VEC epithelial and VEC+dendritic cells. Both tissues mimic native in vivo tissue in that they have basal, parabasal, glycogenated intermediate, and the superficial cell layers. To test the utility of the tissues, contraceptives, microbicides, anti-itch agents, and other vaginal-care products (VCP) were topically applied. To mimic the heterosexual HIV infection, the tissue models were topically exposed to HIV-1 viruses. Quality control (QC) testing on each batch of tissue utilised Triton X-100 (1%) and water as positive and negative controls, respectively. The MTT assay was used to determine the exposure time necessary to decrease the tissue viability to 50% (ET50) for the positive control and 20 VCP.

QC testing showed the tissues to be highly reproducible; the average intra-lot coefficient of variation (CV) was <10% and ET-50s averaged 1.10 hr±0.27 (n=15 lots). Also, the VEC tissue model discriminated between the mildness of VCP. ET50 values ranged between 3.5-7.0 hr for contraceptives, 6.9->18 hr for anti-itch creams, and 1.7-2.7 hr for feminine washes. Released cytokines and gene expression levels showed that IL-1α, IL-1β, IL-6, and IL-8 were associated with toxicity induced by VCP. In addition, the VLC tissue was infectible with macrophage-tropic and T-cell tropic HIV-1 strains.

The VEC tissue models will serve as useful, highly reproducible, non-animal tools to assess the irritation of VCP. The in vitro tissue model will also enable studies pertaining to HIV infection, microbicides and drug absorption.
Three-dimensional models of the human oral epithelia, exhibiting a buccal or gingival phenotype have been developed using normal human oral epithelial cells cultured in serum free medium. The buccal tissue (ORL-200) contains 8-12 cell layers with cells becoming increasingly squamous toward the apical surface have been developed. No evidence of cornification is present in histological slides and immuno-staining shows the expression of cytokeratin K13 human beta-defensins in the suprabasal layers. These features are characteristic of buccal epithelium. The gingival tissue (GIN-100) has 9-13 layers of viable, nucleated cells and is partially cornified at the apical surface. Lipid analysis revealed that, of the ceramides important in the barrier of epidermis, only ceramide 2 in was present in ORL-200, a result that matches human buccal tissue. GIN-100 showed the presence of the three least polar ceramides, C1, C2, C3, in a ratio of 1.0:8.2:4.5, respectively. When exposed to the surfactant Triton X-100 (1%), an exposure time of 52±20 minutes (n=31) reduces the viability of ORL-200 to 50% as determined by an MTT assay. For GIN-100, an exposure of >8 hours is required to damage the tissue to the same extent. In addition, MTT assay and cytokine release results from ORL-200 tissue have been used to provide industrial and academic researchers with a quick, reproducible method for evaluating the irritation potential of oral care excipients and products. The methodology correlates well to human irritation results, and can provide a reliable alternative to animal testing.

Human oral keratinocytes primary culture: A comparative study between two isolation techniques

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The need to recover mucosal oral defects is routinely present in maxillofacial and oral interventions, when the surgeon frequently confronts himself with the lack of available tissue. Cultivation in vitro of oral mucosal keratinocytes into transplantable autologous epithelia could be a convenient source of tissue. The aims of the present work were to test and compare the efficiency of the enzymatic or the direct explant technique methods for keratinocytes isolation from oral mucosal fragments and to evaluate the feasibility of the obtaining transplantable grafts. A comparative study between the enzymatic and the direct explant techniques for mucosal keratinocytes isolation will give enough subsidies, so as to improve results in vivo for short and long time periods and at the same time a better quality in the obtained cells. Keratinocytes were extracted using both isolation techniques, from oral mucosal fragments donated from healthy human subjects undergoing dental surgeries (research project approved by the Ethical Research Committee-IPEN, under Licence N° 087/CEP-IPEN/SP).

The cells were cultivated over a feeder-layer of previously irradiated murine fibroblasts in appropriate keratinocyte culture medium. Once sub confluent, the primary cultures were amplified into subsequent cultures. Thus, cell life span could be estimated.

The oral keratinocytes cultivation and their subsequent expansion and cultures were possible and successful, reaching a compatible number of duplications like human skin keratinocytes. Human oral keratinocytes are possible to be cultivated in vitro and these cell cultures could also be employed as an in vitro model for cell/drug interaction, avoiding unnecessary animal testing.
Poster

A novel technique for the continuous determination of Trans-Epithelial Electrical Resistance (TEER)

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Trans-Epithelial Electrical Resistance (TEER) measurement is an established method to quantify barrier function in epithelial and endothelial cells. TEER has been demonstrated to be a sensitive endpoint for determining the toxicity of compounds to epithelial cells in vitro. The aim of this study is to develop a TEER measurement technique to monitor barrier function of epithelial cells under continuous perfusion conditions.

The porcine renal epithelial cell line, LLC-PK1, were cultivated on microporous growth supports in a perfusion apparatus (EpiFlow®). A newly designed TEER unit was implemented into the perfusion device and resistance measurements were monitored over the life of the culture. Additionally cells were intoxicated with a previously established toxic concentration of CdCl2 (15 µM). Lactate dehydrogenase (LDH) concentration in the out-flow medium was also measured.

During formation of the epithelial monolayer, TEER increases up to a steady state value, as expected. CdCl2 exposure resulted in a gradual collapse of TEER, which correlated to an increase LDH release.

We have successfully developed a TEER unit which can be used for the continuous monitoring of barrier function in perfusion cell culture. This is a powerful, non-invasive online measuring tool of monolayer integrity.

Lecture

A novel perfusion cell culture system for acute, repeat dose and long term toxicity testing

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In vitro models for long-term toxicity assessment, defined as a recurring exposure to compounds over a prolonged period of time, are still underrepresented in the drug evaluation and chemical testing processes. A possible reason for this is that the classical approach to cell culture is not readily suitable to long term repetitive applications. One avenue to overcome the limitations of cell culture for long term testing is to use perfusion culture systems. Such systems allow continuous or repeat dose application of toxins to the cell or tissue culture. Also biomarkers of vitality can be measured in the out flowing medium such as enzyme release, consumption of medium components or the production of metabolites. In addition there is evidence that cells cultured under these conditions often more closely match the phenotype of their in vivo counterpart. Thus perfusion culture systems are likely to be more relevant to the assessment of chemical induced toxicity than static cultures. We describe one such system which was developed primarily with epithelial cells in mind. This system provides a separate apical and basolateral compartment allowing the continuous monitoring of transepithelial electrical resistance through-out the experiment. Moreover this novel perfusion culture system enables the co-culturing of several cell types with or without contact between the different cell types. This is of importance when generating more relevant models for target organ toxicity testing over prolonged intervals, since many chronic toxic effects may be mediated by the release of inflammatory mediators from neighbouring cells.
Poster

Novel approaches for in vitro investigations concerning eczematous dermatitis and skin pigmentation

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During the last decade, a variety of models (2D, 3D) have been developed to provide useful methods for investigation of skin physiology and pathophysiology, respectively. The aim of this study was to develop reliable co-culture systems in vitro, which allow us to study questions concerning eczematous dermatitis and skin pigmentation.

To achieve a wide standardisation and a reproducibility of the results immortal human keratinocytes are used in both systems and depending on the focus these cells were co-cultured either with activated T cells or with melanocytes.

In case of eczematous dermatitis the model enables the reproduction of several clinical hallmarks of chronic inflammatory skin disease: T cell induced keratinocyte apoptosis (TUNEL-assay), reduced expression of the adhesion molecule E-cadherin, increased expression of intercellular adhesion molecule-1 and upregulation of neutrophin-4. The protein expressions were analysed using the Western Blot technique.

Regarding the skin pigmentation the developed co-culture model enables the assessment of regulators of pigmentation by measuring the overall melanin content spectrophotometrically and observing visual changes in cell morphology. The well-known skin lighteners kojic acid and arbutin as well as the pigmentation enhancer IBMX showed the expected effects on the melanin content.

In summary, both co-cultured systems offer new and valuable tools for in vitro investigations of eczematous dermatitis or skin pigmentation. Besides they offer test systems for either therapeutics possibly influencing inflammatory skin diseases or formulations affecting skin pigmentation.

Poster

A novel collagen vitrigel scaffold that can facilitate a three-dimensional culture for reconstructing epithelial-mesenchymal models

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Conventional three-dimensional culture systems in comparison with two-dimensional ones have some demerits in that 1) the culture process is complicated, 2) the observation of cells by a phase-contrast microscope is difficult or impossible, 3) the reproducibility of the distribution of seeded cells is not always excellent, and 4) the aseptic handling for the medium change or for the co-culture of secondary cells is also hard. Opaque egg white or fish eyeballs prepared by boiling can be converted into thin, transparent, and rigid materials like glass by evaporating the moisture and this phenomenon is known as the vitrification of denatured proteins. We applied the vitrification technology to a type-I collagen gel and converted it into a rigid material like glass. We attempted to rehydrate the glass-like material and succeeded in preparing a novel stable state of collagen gel that is a thin and transparent membrane with excellent gel strength. We named it “collagen vitrigel”. Further, the framework-embedded collagen vitrigel scaffold that can be easily reversed by forceps was prepared by inserting a nylon membrane ring in the collagen solution prior to the gelation. Anchorage-dependent cells can be cultured on both surfaces of the scaffold by the manipulation of two-dimensional cultures and consequently it resulted in reconstructing a three-dimensional organoid. An intestinal epithelial-mesenchymal model was reconstructed by co-culturing fibroblasts on the opposite side of the monolayered Caco-2 cells on the scaffold. These results demonstrate that the framework-embedded collagen vitrigel scaffold can provide an excellent three-dimensional culture system for reconstructing epithelial-mesenchymal models.
Lecture
Development of culture technologies reflecting in vivo conditions

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Various scaffolds have been prepared from natural, synthetic, and hybrid materials for maintaining the activity of functional cells, for regulating cell behaviour, and for reconstructing organoids [1]. I have developed five culture technologies reflecting in vivo conditions by utilising unique scaffolds; 1) the preparation of a multicellular hetero-spheroid composed of mesenchymal cells and epithelial cells utilising a scaffold made of the mixture of collagen and a thermo-responsive polymer [2], 2) the preparation of a three-dimensionally reconstructed multicellular mass (3-DRMM) with a medium accumulating or circulating system utilising naturally branched scaffolds made of rice fibrous roots or cotton-gauze, respectively [3], 3) a concept for organ engineering that can remodel an organ into an organoid by a continuous three-step perfusion to convert extracellular matrices (ECMs) into a collagen scaffold [4], 4) a concept for cellomics study to culture cells on a scaffold made of animal tissue/organ sections for histopathology (TOSHI) that conserves the microarchitecture and component of the original tissue in vivo [5], and 5) the simple reconstruction of an epithelial-mesenchymal model utilising a nylon ring membrane-embedded collagen vitrigel, a thin collagen gel membrane [6].

References:

Lecture
Use of chromatin remodelling as a new way to create differentiated primary hepatocyte cultures

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Background/Aims: The level of histone acetylation, determined by the balance between histone acetyltransferase activity and histone deacetylase (HDAC) activity, is crucial for gene transcription. As such, the HDAC inhibitor Trichostatin A (TSA) has been shown to interfere with proliferation, differentiation and apoptosis in tumour cells, including hepatoma cell lines. Here, we investigated the effects of TSA and its metabolically more stable hydroxamic acid derivative, 5-(4-dimethylaminobenzoyl)-aminovaleric acid hydroxamide (4-Me2N-BAVAH) on cell cycle progression and survival in EGF-stimulated cultures of primary rat hepatocytes.

Methods: DNA replication was measured using [methyl-3H]-thymidine incorporation. Cell cycle, apoptosis and differentiation markers were analysed by western blotting. Albumin secretion was determined via ELISA.

Results: It was found that 1µM TSA and 50µM 4-Me2N-BAVAH completely abolished DNA synthesis and this was accompanied by induced histone H4 acetylation. In addition, in the presence of the HDAC inhibitors, the S-phase marker cdk1 was down-regulated, together with G1 cyclin D1. However, in the case of TSA, the decrease of cyclin D1 was dependent on the time of onset of treatment. For both TSA and 4-Me2N-BAVAH, the expression of p21 was not altered. In contrast to hepatoma cells, TSA and 4-Me2N-BAVAH also reduced spontaneous apoptosis as evidenced by a reduction of procaspase-3 cleavage and a decreased expression of pro-apoptotic Bid. Moreover, especially in the case of the TSA analogue, increased albumin secretion, CYP2B and Cx32 expression, together with decreased Cx26 and Cx43 expression were observed.

Conclusion: Our results show that besides the induction of an early p21-independent cell cycle arrest, TSA and in particular the TSA analogue 4-Me2N-BAVAH potentiate the anti-apoptotic effect of EGF in primary hepatocytes, and positively affect hepatocyte functioning. Therefore, we propose HDAC inhibitors as an innovative way to create functional long-term cultures of primary hepatocytes (PCT/EP2004/0012134).
Poster

**Novel cell culture techniques revise the role of the sebaceous gland in human skin – the fantastic future of a skin appendage**

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Sebaceous glands produce sebum been responsible for seborrhea and acne; seemingly, a limited and less attractive field to work in. In addition, the new EU Cosmetic Directive caused great concern to the cosmetic industry because of missing adequate human models. Therefore, it is of major importance for dermatological and cosmetic research that a silent revolution occurred during the last years, which assisted the revision of sebaceous gland role in human skin: The development of sebaceous gland cell culture techniques and the establishment of human sebaceous cell lines (patent DE19903920). This skin appendage has turned to be an organelle with major involvement in skin homeostasis: Sebaceous lipid fractions are responsible for the three-dimensional organisation of skin surface lipids and the integrity of skin barrier and sebum transports antioxidants to the skin surface. Moreover, sebaceous PAF acetylhydrolase-II was found to protect the skin against oxidative stress and, especially, epidermal keratinocytes against UVB irradiation, and the sebum-specific fatty acid C16:1∆6 to exhibit innate antimicrobial activity. New fascinating data were acquired indicating that sebocytes express pro- and anti-inflammatory properties, present a regulatory program for neuropeptides, synthesise cholesterol *de novo* and use it in its own steroidogenic program, and selectively control the action of vitamins, hormones and xenobiotics on the skin. Sebaceous glands were shown to be “the brain of the skin” and to fulfill all requirements making skin an independent peripheral endocrine organ. Several further sebaceous gland functions are currently under investigation. Novel cell culture techniques made the future of sebaceous glands look fantastic!
The Tg.AC transgenic mouse is a genetically initiated, tumour promoter–sensitive epidermal tumourigenesis model that is being evaluated as an alternative to the traditional two-year bioassay. The model requires fewer animals and shorter duration of exposure. Tg.AC mice develop epidermal papillomas in response to treatment with dermally applied carcinogens. The US National Toxicology Program (NTP) selected four chemicals to study in the Tg.AC mouse model by dermal route of exposure as a part of its effort to validate the model as a replacement for two-year dermal carcinogenicity studies. The chemicals studied were dicyclohexylcarbodiimide (DCC), diisocarbodiimide (DIC), trimethylolpropane triacrylate (TMPTA) and pentaerythritol triacrylate (PETA). The studies on TMPTA and PETA have been completed. Topical application of TMPTA and PETA at dose levels ranging from 0.75 to 12 mg/kg to Tg.AC mice for six months showed dose-dependent increases in squamous cell papillomas at the site of application, with decreases in the latency of their appearance in mice receiving 3 mg/kg or greater. Papillomas were accompanied by a few squamous cell carcinomas, along with hyperplastic and inflammatory lesions. The results of these studies were presented to the NTP Peer Review Panel as definitive studies of carcinogenicity in lieu of two-year bioassay but were not accepted by the Panel. Currently, the NTP is conducting 2-year studies on TMPTA in rats and mice to identify its carcinogenic potential. Our experience suggests that additional mechanistic data is needed to gain acceptance of the Tg.AC mouse model as an alternate to the traditional animal bioassay.

The Tg.AC transgenic mouse model as an alternative to the two-year bioassay: The US National Toxicology Program experience

Rajendra Chhabra
NIEHS/NIH, Environmental Toxicology Program, Research Triangle Program, USA
Lecture

Non-genotoxic carcinogens: Mechanistic perspectives for alternatives

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Non-genotoxic carcinogens are thought to act via disturbance of the balance between cell death and cell proliferation, leading to clonal expansion of pre-neoplastic cells. Precise mechanisms that lead to this disruption are varied and this hinders the development of short term and in vitro tests to predict non-genotoxic carcinogenic potential. Biomarkers indicative of a chemical’s ability to influence cell survival and proliferative advantage may be helpful in the interpretation of rodent carcinogenicity that may occur in the absence of genotoxicity, and may aid human risk assessment. The dose response relationships and species differences in response are critical parameters in this process. Biomarkers can be relatively non-specific relating to apoptosis and proliferation in target organs but may be optimised and more refined by interrogation of the modulation of specific molecules involved in key signalling processes. Both the modulation of connexin-mediated gap junction intercellular communication and the status of DNA methylation have shown particular promise as relevant biomarkers in conjunction with functional studies as above. For example, the rodent non-genotoxic hepatocarcinogens Wy-14,643, 2,3,7,8-tetrachlorodibenzo-p-dioxin, methapyriline and hexachlorobenzene and the rat kidney carcinogens chloroform and p-dichlorobenzene all disrupt gap junction plaques containing connexin 32 in the target organs in the absence of toxicity and they all cause proliferation at the carcinogenic dose. Furthermore, compounds that deplete glutathione such as chloroform and carbon tetrachloride have the ability to induce a secondary genotoxicity through oxidative stress but only at high concentrations. Transcriptomic analyses have identified further novel potential biomarkers associated with species- and tissue-specific non-genotoxic carcinogenesis such as the activation of pathways involved in the control of cell cycle.

Poster

ECVAM Key Area Carcinogenicity and Genotoxicity: Summary of ongoing activities

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The ECVAM key area on carcinogenicity and genotoxicity has been reorganised with the initiation of several activities related both to validation and its applied research.

The carcinogenicity/genotoxicity taskforce was set up in January 2004. During the first meeting the experts agreed on the need to validate the cell transformation assay (CTA), as this is the only developed in vitro test which has the potential to detect both genotoxic and non-genotoxic carcinogens. In the area of genotoxicity, it was decided to validate the micronucleus test (MNT) in vitro based on existing data.

During the second taskforce meeting (November 2004) there was a consensus that, in addition to ongoing activities, it would be necessary to focus on the reduction and refinement of genotoxicity tests, since considerable in vivo testing is still required for confirmation of the genotoxic prediction.

Two workshops on CTA and MNT in vitro, respectively, have been organised in April 2004. In these workshops, it was agreed to prevalidate the CTA on both Balb/c 3T3 and SHE cell systems and to initiate a retrospective validation on the MNT in vitro.

The prevalidation studies on CTA are ongoing.

The retrospective validation of MNT in vitro is foreseen to be submitted to the ECVAM Scientific Advisory Committee (ESAC) for peer review in summer 2005.

In support to the validation activities, extensive research work is carried out in the ECVAM laboratories with the aim of developing a strategy which integrates the CTA, the MNT and the Comet assay in vitro.

The independent validation of the different tests will lead ultimately to the development of a refined strategy for genotoxicity and carcinogenicity, which has to be as safe as the current one but would consume less animals.
Lecture
Validation of toxicogenomics-based tests, a new generation of alternatives
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Toxicogenomics-based methods are being widely applied in toxicology and biomedical research. Since data are already being generated using these technologies, it is both timely and important to address the critical validation issues now with the aim of establishing a foundation that will facilitate future regulatory acceptance of scientifically valid toxicogenomics-based test methods. Addressing such issues early on, will also facilitate early buy-in and confidence in the technologies by the regulatory arena in its quest for new and improved methods by which to help ensure human health, protect the environment, and demonstrate responsiveness to animal welfare issues.

For that reason, the European Centre for the Validation of Alternative Methods (ECVAM), the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program (NTP) Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) have started to investigate the specific considerations necessary for adequate validation of toxicogenomics-based test methods. Experience in validation of conventional alternative test methods has led to an understanding that the validation approach will have to be adapted to the evaluation of methods based on toxicogenomics. The toxicogenomics field is rapidly evolving; therefore the validation process should accommodate the anticipated changes in the technology and must not be at the expenses of innovation. Moreover, other international Organizations as the OECD and the WHO/IPCS are currently drafting activity programs related to the possible use of toxicogenomics-based test methods for hazard and risk assessment purposes.

Poster
Modelling squamous metaplasia in an in vitro bronchial cell culture model: A flow cytometric analysis
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The development of squamous metaplasia in an in vitro bronchial cell culture model is being investigated as a tool to measure the effects of repeat exposure to airborne pollutants. Squamous metaplasia describes a potentially reversible event that may serve to protect the tracheobronchial lining from the effects of inhaling airborne pollutants but has also been linked to squamous cell carcinoma. The analytical methods used to assess pathological changes that occur in animals following repeated inhalation of toxic compounds are subjective and can not be quantified. Therefore it is difficult to obtain the reliable and reproducible data that is essential for the validation of a toxicity test. However, techniques and methods of analysis not practical in animal models can be combined in in vitro models. Squamous metaplasia is characterised by morphological and molecular changes including the replacement of the pseudostratified mucociliary epithelium with a stratified squamous epithelium and the expression of involucrin, filaggrin, a change in the cytokeratin profile, transglutaminase I and the formation of a cross linked envelope. Flow cytometry has been used to illustrate: Changes in the expression of these proteins to assess their contribution in response to different inducers; transglutaminase catalysed fluorescein cadaverine incorporation as a measure of activity; and annexin V binding as a measure of apoptosis, a form of programmed cell death that shares many characteristics in common with the squamous pathway. These investigations will lead to the establishment of a battery of end points signifying the initiation and development of squamous metaplasia.
Lecture

Animal carcinogenicity studies: Alternatives to the bioassay

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Traditional animal carcinogenicity tests take around three years to design, conduct and interpret. Consequently, only a tiny fraction of the thousands of industrial chemicals in use have yet been tested for carcinogenicity. Despite the cost of hundreds of millions of dollars, millions of skilled personnel hours, and millions of animal lives, several investigations have revealed animal carcinogenicity data to be lacking in human specificity (ability to distinguish human from animal carcinogens, where different), which severely limits its human utility. Causes include documented scientific inadequacies of the majority of carcinogenicity bioassays, and numerous serious biological and mathematical obstacles, which render attempts to accurately extrapolate human carcinogenicity assessments from animal data profoundly difficult, if not impossible. Proposed modifications have included the elimination of mice, the use of genetically-altered or neonatal mice, decreased timeframes, initiation-promotion models, greater incorporation of toxicokinetic and toxicodynamic assessments, quantitative structure-activity relationship (computerised) expert systems, in vitro assays, cDNA microarrays for detecting genetic expression changes, limited human clinical trials, and epidemiological research. Advantages of non-animal assays when compared to bioassays include superior human specificity results, greatly reduced timeframes, and greatly reduced demands on financial, personnel and animal resources. Inexplicably, however, regulatory agencies have been frustratingly slow to adopt alternative protocols. In order to minimise cancer losses to society, a substantial redirection of resources away from excessively slow and resource-intensive rodent bioassays, into the further development and implementation of non-animal assays, is strongly justified and urgently required.

Poster

Animal carcinogenicity studies: Poor human predictivity

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The regulation of human exposures to potentially carcinogenic chemicals constitutes society’s most important use of animal carcinogenicity data. Environmental contaminants of greatest U.S. concern are listed in the Environmental Protection Agency’s (EPA) Integrated Risk Information System (IRIS) chemicals database. However, of the 160 IRIS chemicals lacking even limited human exposure data but possessing animal data as of January 1, 2004, we found that in most cases (58.1%; 93/160) the EPA considered the animal data inadequate to support a classification of probable human carcinogen or non-carcinogen. For the 128 chemicals with human or animal data also assessed by the World Health Organization’s International Agency for Research on Cancer (IARC), human carcinogenicity classifications were compatible with EPA classifications only for those 17 having at least limited human data (p=0.5896). For those 111 primarily reliant on animal data, the EPA was much likelier than the IARC to assign carcinogenicity classifications indicative of greater human risk (p<0.0001). The IARC is a leading international authority on carcinogenicity assessments, and the significant differences in human carcinogenicity classifications of identical chemicals between the IARC and the EPA indicate that: (i) in the absence of significant human data the EPA is over-reliant on animal carcinogenicity data, (ii) as a result, the EPA tends to over-predict carcinogenic risk, and (iii) the true human specificity, and hence utility, of animal carcinogenicity data is even poorer than indicated by EPA figures alone.
Due to a paucity of human exposure data, risk classification and the consequent regulation of exposures to potential carcinogens has traditionally relied heavily upon animal tests. However, several investigations have revealed animal carcinogenicity data to be lacking in human specificity (the ability to distinguish human from animal carcinogens, where different). In order to investigate the reasons, we surveyed the 160 chemicals possessing animal but not human exposure data within the U.S. Environmental Protection Agency chemicals database that had received human carcinogenicity assessments as of January 1, 2004. We found a wide variety of species used, with rodents being predominant; a wide variety of routes of administration used, and a particularly wide variety of organ systems affected. The likely causes of the poor human specificity, and hence utility, of rodent carcinogenicity bioassays include (i) the profound discordance of bioassay results between rodent species, strains and genders, and further, between rodents and human beings; (ii) the variable and substantial stresses caused by handling and restraint and the stressful routes of administration endemic to carcinogenicity bioassays, with consequent effects on hormonal regulation, immune status and carcinogenesis predisposition; (iii) the differences in transport mechanisms and rates of absorption between test routes of administration and other important human routes of exposure; (iv) the considerable variability of organ systems in response to carcinogenic insults, between and within species, combined with the inability of commonly-used predictors of human carcinogenicity, such as the number of organ systems or sex-species groups effected, or fatalities, to withstand careful scrutiny; and (v) the inherent predisposition of chronic high dose bioassays towards false positive results, due to the overwhelming of physiological defences, and the unnatural elevation of cell division rates during ad libitum feeding studies. Such factors render attempts to extrapolate accurate human carcinogenicity assessments from animal data profoundly difficult, if not impossible.

Poster

Animal carcinogenicity studies: Obstacles to human extrapolation

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Lecture

Humane endpoints and in vitro alternatives for sensitised genotoxicity screening

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Human risk-assessment for chemical compounds is often based on non-human model systems with arbitrary safety factors for extrapolation. In addition, large amounts of animals are used for this safety testing. Therefore, we propose to devise a human-based in vitro model system with a genome stability marker (lacZ-transgene) for mechanistic genome stability studies, and evaluate that model as a tool for realistic human risk assessment to genotoxic stressors. To increase the sensitivity of the human in vitro cell culture system, we will mimic the DNA-repair deficient phenotypes of specific knockout mice by knocking down DNA-repair proteins using an emerging innovative powerful tool called “RNA interference”.

The DNA repair deficient phenotypes of two mouse models will be mimicked in our human system. The first knockout mouse model having a complete defect in nucleotide excision repair (NER), Xpa, was created in our own lab. The second mouse model, sensitive for genotoxic agents termed “clastogens” (genotoxic agents creating double-strand breaks), is deficient for Rad54 and its homologue Rad54B, two genes involved in DNA double-strand break repair (DSBR). Both mouse models are highly sensitive to genotoxic agents. Moreover, crosses of these mice with reporter mice carrying lacZ genes revealed mice able to detect genotoxic properties of chemical compounds in a wide variety of tissues. It is our expectation that comparing mutation frequency induction in vivo with in vitro and between species (human / mouse), will reveal whether in vitro assays are suitable for reliable safety measurements, and whether human cell systems will form a basis for a more accurate human risk-assessment.

This work was supported by ZonMw.
The Bhas promotion assay is a cell culture transformation assay designed as a sensitive and economical method for detecting tumour-promoting activities of chemicals. In order to validate transferability and applicability of this assay, an inter-laboratory collaborative study was conducted with the participation of fourteen laboratories. After confirmation that these laboratories could obtain positive results with tumour promoters 12-O-tetradecanoylphorbol-13-acetate (TPA) and lithocholic acid (LCA), 12 chemicals were assayed under masked conditions. Each chemical was tested in four laboratories. For eight chemicals, all four laboratories obtained consistent results, and for two of the other four chemicals only one among the four laboratories showed inconsistent results. Thus, the rate of consistency was high. During the study, several issues were raised. Each issue was analysed step-by-step and resulted in protocol revision of the original assay. Among these issues were the importance of careful maintenance of mother cultures and the adoption of test concentrations for toxic chemicals. In addition, it is suggested that there are three different types of chemicals showing positive promoting activity in the assay. Those designated as T-type induced extreme growth enhancement, and included TPA, mezerein, PDD and insulin. LCA and okadaic acid belonged to the L-type category in which transformed foci were induced at concentrations showing growth-inhibition. In contrast, progesterone, catechol and sodium saccharin (M-type) induced foci at concentrations with little to slight growth inhibition. The fact that different types of chemicals similarly induce transformed foci in the Bhas promotion assay may provide clues for elucidating mechanisms of tumour promotion.

This study was supported by a Grant-in-Aid from the Japan Chemical Industry Association.
The Syrian hamster embryo (SHE) assay is actually the most predictive in vitro alternative to the 2 year rodent carcinogenesis (LeBoeuf et al., 1996). The assay is based on the ability of chemical/physical/biological agent to induce morphological transformation of SHE cells, which correlates with the potential to induce carcinogenesis. It is worthwhile noting that this assay possesses the ability to identify genotoxic as well as non-genotoxic carcinogens.

Hundreds (circa 400) of chemical/physical agents have been tested in the so called “low pH” version of the SHE assay with a sensitivity of 86%, a specificity of 83% and an 83% overall concordance with the rodent bioassay. Despite its good performances, the SHE assay has suffered a lot of criticism. This can be partly justified by the fact that the molecular events underlying the morphological transformation have not been fully elucidated. Furthermore, the visual scoring of the colonies (which is tedious and subjective) is far more the greatest weakness of this assay.

We have successfully set up and validated this assay in our laboratory. We are now committed in seeking molecular markers of morphological transformation. In doing so, we are confident that this will ease the scoring of the transformed phenotype and make it more objective. Preliminary steps of this research project addressed proteins involved in cell cytoskeleton (actin) and in cellular gap communication (connexin 43).

Poster

The Syrian hamster embryo assay: An in vitro alternative to the rodent bioassay

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The cell transformation assay using BALB/3T3 cells is able to detect tumour promoters as transformation promoters. TPA, okadaic acid (OA), orthovanadate (VA) and p-nonylphenol (NP) significantly enhanced the cell transformation. To develop a short term assay identifying tumour promoters by detection of altered gene expressions, alterations in the gene expressions in BALB/3T3 cells exposed to these non-genotoxic transformation promoters were revealed using fluorescent mRNA differential display analysis and confirmed by RT-PCR. Elevated expressions of the following genes were induced: Ass1, Ly6e and Nudt9 by TPA and OA, Plat and Lgals3bp by OA, Ssb and Sned1 by NP. Decreased expressions of the following genes were induced: Thbs1 and an EST (BY594155) by TPA and OA, Vim by OA and NP, AI458795 and Sparc by TPA, Rhl3 by OA, ND1 by NP. TPA and OA caused common changes in the expression of several genes suggesting the existence of common actions on the cells between TPA and OA. However, the time courses of these changes were different between TPA and OA. No common gene was regulated by four non-genotoxic transformation promoters. It would be difficult to develop a simple assay method for tumour promoters utilizing detection of increased and decreased gene expressions. These data will contribute to clarifying the mechanisms of promotion by non-genotoxic chemicals during cell transformation and carcinogenesis.

Poster

Differentially expressed genes in BALB/3T3 cells with exposure to non-genotoxic chemicals which promote cell transformation

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Lecture

Detection of non-genotoxic carcinogens using ras-transfected Bhas 42 cells

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In vitro cell transformation tests can simulate the process of two-stage animal carcinogenesis, and propose a useful screening method for the detection of chemicals with carcinogenic activities. As formation of transformed foci is the consequence of the complex process of cell malignisation, the tests can be anticipated to be useful for the detection of not only tumour initiators but also tumour promoters and non-genotoxic carcinogens. In spite of this expectation, none of in vitro cell transformation tests have been accepted as a routine screening method, because the tests are thought to be laborious and time-consuming compared with the routine genotoxicity tests.

Ohmori et al. have developed an in vitro cell transformation assay for tumour promoters using Bhas 42 cells. The cells are v-Ha-ras-transfected BALB/c 3T3 cells. This transformation assay is a very sensitive method and has many advantages, such as shortened experimental period, use of less materials and simplicity of the procedure. Recently, in addition to the promotion assay we developed an assay method for the evaluation of initiating activity using these cells. When typical tumour initiators, were examined, transformed foci were induced in initiation assay but not in promotion assay. On the contrary, typical tumour promoters, were negative in initiation assay but positive in promotion assay.

Thus, Bhas initiation and promotion assays were suggested to be a highly sensitive screening method for the detection of chemicals with different mechanisms of transforming potential.

Lecture

The effect of the histone deacetylase inhibitor Trichostatin A on gap junctional intercellular communication in primary cultures of adult rat hepatocytes

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Background/aims: Trichostatin A (TSA), a histone deacetylase inhibitor, is known to induce cytodifferentiation. The latter is partly controlled by gap junctional intercellular communication (GJIC). Hepatocellular gap junctions are composed of connexins Cx32 and Cx26, whereas Cx43 only becomes detectable upon dedifferentiation. Considering (i) the involvement of GJIC in cytodifferentiation, and (ii) the positive effects of TSA on this process, we raised the question whether TSA might affect hepatocellular GJIC.

Methods: Freshly isolated rat hepatocytes were cultivated and exposed to TSA. In another set of experiments, hepatocytes were isolated while TSA was present in the perfusion medium. Drug exposure was continued during consequent cultivation. RT-PCR was used to study Cx gene expression. Cx protein expression and localisation were investigated by means of immunoblotting and immunocytochemistry, while GJIC and albumin secretion were measured by using the scrape loading/dye transfer assay and ELISA, respectively.

Results: TSA induced the expression of Cx32, and downregulated the Cx26 production, the latter becoming located within the perinuclear compartment. TSA also promoted the appearance of Cx43, an effect that was only seen at the translational level. Overall, this resulted in enhanced GJIC and albumin secretion. Finally, the starting time of drug exposure was found to be a key parameter for the extent of the biological TSA outcome.

Conclusion: (i) TSA favours GJIC in primary cultures of rat hepatocytes, thereby suggesting a potential role for this compound in the optimisation of hepatocyte-based in vitro models. (ii) Cx proteins are likely to fulfil differential roles in the control of hepatocellular homeostasis.
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