Safety Testing of Cell-based Medicinal Products: Opportunities for the Monocyte Activation Test for Pyrogens

Thomas Montag, Ingo Spreitzer, Bettina Löschner, Uwe Unkelbach, Egbert Flory, Ralf Sanzenbacher, Michael Schwanig and Christian K. Schneider

Paul-Ehrlich-Institut, Federal Agency for Sera and Vaccines, Langen, Germany

Summary

The European Partnership for Alternative Approaches to Animal Testing (EPAA) pointed out the need to involve authorities throughout the process of validation and legal acceptance of alternatives to animal experiments. The Paul-Ehrlich-Institute (PEI), Federal Agency for Sera and Vaccines, is the national competent authority in Germany which is responsible for the quality and safety of biologicals including blood and cell-based products. This paper is intended to contribute to the discussion concerning the use of alternative methods in safety testing of medicinal products and considers the scientific work of the PEI in this field. From a regulator's perspective, adequate demonstration of safety and quality of medicinal products are of major interest. Additionally, the availability of the products to the patient has to be taken into consideration. It has to be carefully explored whether the respective in vitro method for demonstration of non-clinical safety as part of the non-clinical development programme is able to guarantee safety level comparable to the corresponding experiment in animals. The topics cited above shall be discussed in this paper using the example of the Alternative Pyrogen Test or also called Monocyte Activation Test. The Alternative Pyrogen Test could serve as paradigm to exemplify how an alternative test can provide at least a comparable level of safety estimation in comparison with a conventional animal test. Furthermore, this alternative test creates additional information which cannot be obtained from the animal experiment, and might also open further scientific insight into the mechanisms of pyrogenicity and acute pro-inflammatory reactions in patients. This test method allows the definition of pyrogen limits for medicinal products. Due to its use of relevant cell systems this in vitro test might contribute significantly to safety assessments of advanced medicinal products during the pre-clinical phase.

Zusammenfassung: Sicherheitsprüfung zellbasierter Medizinprodukte: Chancen für den Monozytenaktivierungstest für Pyrogene

Die Europäische Partnerschaft für Alternative Ansätze zu Tierversuchen (EPAA) wies auf die Notwendigkeit hin, die Behörden während des gesamten Prozesses der Validierung und legalen Anerkennung von Alternativen zu Tierversuchen einzubeziehen. Das Paul-Ehrlich-Institut (PEI), Bundesamt für Sera und Impfstoffe, ist die nationale Behörde in Deutschland, die für die Qualität und Sicherheit von Biologika, inklusive Blut und zell-basierte Produkte, zuständig ist. Dieser Aufsatz soll zur Diskussion bezüglich der Nutzung von Alternativmethoden in der Sicherheitsprüfung von Medizinprodukten beitragen und bezieht sich auf die wissenschaftliche Arbeit des PEI in diesem Bereich. Aus der Sicht einer Behörde sind der hinreichende Nachweis von Sicherheit und Qualität von Medizinprodukten von grossem Interesse. Zusätzlich muss die Verfügbarkeit der Produkte für den Patienten berücksichtigt werden. Es muss sorgfältig untersucht werden, ob die entsprechende in vitro Methode zum Nachweis von präklinischer Sicherheit als Teil des präklinischen Entwicklungsprogramms das gleiche Mass an Sicherheit garantiert wie der entsprechende Tierversuch. Die genannten Themen sollen in diesem Aufsatz anhand des Beispiels des alternativen Pyrogentests, des so genannten Monozytenaktivierungstests, diskutiert werden. Der alternative Pyrogentest könnte ein Paradigma darstellen, das beispielhaft zeigt, wie ein Alternativtest sogar zusätzliche Information liefert, die nicht im Tierversuch erfasst wird und auch neue wissenschaftliche Erkenntnisse in die Mechanismen von Pyrogenität und akuter entzündlicher Reaktion in Patienten eröffnet. Diese Testmethode erlaubt die Definition von Pyrogengrenzwerten für Medizinprodukte. Aufgrund der Nutzung von relevanten Zellsystemen könnte dieser in vitro Test signifikant zur Sicherheitsprüfung von neuartigen Medizinprodukten in der präklinischen Phase beitragen.

Keywords: alternatives to animal experiments, Monocyte activation test, alternative pyrogen test, drug safety, advanced therapies, TGN1412, EPAA

Received 15 March 2007; received in final form and accepted for publication 10 April 2007

1 Introduction

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a joint initiative of the European Commission and a number of companies and trade federations that are active in various industrial sectors. The partnership was launched in November 2005 at a major conference "Europe goes alternative" by Commissioners Verheugen and Potocnik and industry representatives. Its purpose is to promote the development of new "3R" methods (replace, reduce, refine animal experiments) as modern alternative approaches to safety testing (EPAA 2005). As one of its main conclusions, the second conference of EPAA in 2006 pointed out the need to involve regulatory authorities throughout the process of validation and legal acceptance of alternative approaches (EPAA, 2006). The Paul-Ehrlich-Institute (PEI), Federal Agency for Sera and Vaccines, is the national competent authority in Germany that is responsible for quality and safety of biologicals including blood and cell based products. This paper is intended to contribute to the discussion concerning the use of alternative methods in safety testing of medicinal products and considers the scientific work of the PEI in this field.

With the advent of novel advanced therapies that typically are highly species-specific, particular challenges arise with regard to non-clinical safety testing. Facing major scientific advancements in the field, the European Medicines Agency's (EMEA) Committee for Medicinal Products for Human Use (CHMP) has recently issued a battery of new guidance documents for public consultation, for example a draft multidisciplinary guideline on human cell-based medicinal products (EMEA, 2006a) or a draft guideline on potency testing of cell based immunotherapy medicinal products for the treatment of cancer (EMEA 2006b). Although through by these upcoming documents individual, more specific guidance is about to become available, one of the principles of ICH guideline S6 (EMEA 1998) might indeed be valid, i.e. that safety should be tested in a relevant species only.

The recent TGN1412 case (Suntharalingam et al., 2006) is a good example of the limitations of conventional animal models to reliably predict acute adverse reactions, including those which are related to cytokine release and its clinical sequelae. TGN1412 is a monoclonal antibody directed against human CD28 with superagonistic properties (Lühder et al., 2003). CD28 is a major co-stimulatory molecule on the surface of T cells, and TGN1412 was developed to bypass physiological T cell activation by the T cell receptor. By this bypass, it was planned to stimulate the typically suppressed immune system of patients with B-CLL (Chronic lymphatic leukaemia, B cell type) to a more activated state. The drug was further developed for the treatment of autoimmune diseases, since data suggested a preferential activation of regulatory T cells by certain concentrations of the antibody, thus promising immunosuppressive properties (Beyersdorf et al., 2005). TGN1412 was tested in six healthy volunteers in March 2006, and all candidates experienced a severe cytokine release syndrome (Suntharalingam et al., 2006), which can also be seen from a biological and mechanistical perspective as a "fever reaction" in its immunological sense. The company TeGenero, that developed this product, submitted considerable information on the characterisation of the non-clinical animal model (cynomolgus monkey) (Hanke, 2006) to the regulatory agencies (the UK Medicines and Healthcare products Regulatory Agency, MHRA, and the German Paul-Ehrlich-Institute). However, the results of non-clinical testing were apparently not predictive for the massive cytokine release that was observed in patients. Since superagonistic antibodies might be considered "high-risk" products (Schneider et al., 2006), but nevertheless be potentially promising medicines, the search for alternative and more predictive *in vitro* tests is of utmost importance. The experimental data published in the report of the UK Expert Scientific Group on Phase One Clinical Trials that were performed with TGN1412 by the UK National Institute for Biological Standards and Control (NIBSC), already point out that simple conventional *in vitro* methods like those that used to be performed previously might not be sufficient (Expert Scientific Group, 2006). Since the first administration of a novel compound to humans represents a critical juncture of non-clinical and clinical development, a rapid and relevant test for novel biomedicinal products, which should in addition be subject to sufficient standardisation, is desirable.

While cell-based medicinal products and other advanced therapies should not be considered high-risk *per se*, this emerging class of medicinal products again demonstrates how difficult it is to find a relevant animal model. Cell-based products are maybe among the most complex medicinal products, since the "compound" consists of the complete live cell, including all surface structures, the cellular activation state, cell viability, and many other factors. This implies that the "pharmacological" interaction for example with tissues carrying the counterparts of cell surface receptors, might be difficult to study reliably in an animal model. Homologous models might become necessary, as discussed also in recent CHMP guidance (EMEA, 2006a), i.e. the animal counterparts of the cell-based medicinal product. However, such models surely have considerable limitations. For example, a homologous model might enable only mechanistic studies, excluding in most cases the evaluation of safety of other components of the product, like process- and product-related impurities etc., which are specific for the actual product for human use, but not necessarily comparable in the animal homologue. For some products, it might be difficult to sufficiently characterise such quality attributes and their potential impact on safety. Further, the immune system of animals has evolved significantly over evolution to *Homo sapiens sapiens*, and there are reports that the human immune system might be more susceptible to T cell activation than even that of chimpanzees (Nguyen et al., 2006).

This highlights that the development of a relevant *in vitro* assay that employs human cells would be of major advantage when it comes to the evaluation of potential acute side effects. For cell-

based products, the main focus of non-clinical testing surely is on mechanistic toxicity. However, since such products are complex, mechanisms of interaction with immune cells might exist that could in some cases trigger immunoactivation.

Even more importantly, some products like cell based products for autologous transfer after *in vitro* modification bear the risk of bacterial contamination. While viral safety is a paramount safety aspect for biotechnological products (EMEA, 2006c) and bacterial contamination is usually adequately controlled, the situation might change for such cell-based products: They have a complex and often multilocal "manufacturing process" (surgical explantation, transfer, *in vitro* propagation and manipulation, transfer back to the patient, administration), bearing the risk of potential bacterial and thus also endotoxin and pyrogen contamination. Since conventional bacterial cultures would take considerably longer time as compared to the time between explantation and re-administration to the patient, alternative approaches are a particular challenge.

From a regulator's view, the safety and quality of medicinal products is of major interest. Additionally, the availability of the products for the patient has to be taken into consideration. It must be carefully explored whether the respective *in vitro* method is able to guarantee a level of safety evidence comparable to the animal experiment. It should be mentioned that the procedures developed in order to replace animal experiments are sometimes considered only as surrogate methods giving limited information.

In this article, we discuss a rapid *in vitro* approach, the Alternative Pyrogen Test also called Monocyte Activation Test (MAT). This approach exemplifies how an alternative test can be able to provide a level of safety information that is at least comparable to a conventional animal test. Furthermore, this alternative test creates additional information which cannot be obtained from the animal experiment. This test might also lead to further scientific understanding of the mechanisms of pyrogenicity and acute pro-inflammatory reactions in patients.

2 Principle of alternative pyrogen test or monocyte activation test

As indicated by the term Monocyte Activation Test (MAT), alternative pyrogen tests employ human monocytes as the detection system. The following monocyte sources have been studied intensively: anticoagulated human whole blood (Hartung and Wendel, 1995), human peripheral blood mononuclear cells (PBMC, consisting of monocytes and lymphocytes) (Poole et al., 1988 and 2003) or monocytic cell lines of human origin (Eperon and Jungi, 1996; Peterbauer et al., 2000; method overviews: Hartung et al., 2001; Hoffmann et al., 2005a).

Typical readouts are pro-inflammatory cytokines, like IL-1ß, IL-6 and TNFα which are usually detected by enzyme-linked immunoassay (EIA, ELISA). The induced proinflammatory cytokines can be detected in the whole blood assay at the earliest after 2-3 hours (plateau reached around 8 h), the typical incubation time is 18-24 hours for practical reasons. The cytokines accumulate during the incubation and are not metabolised *in vitro*. The assays using human whole blood have been further optimised by the development of cryopreservation procedures (Schindler et al., 2004 and 2006). Cryo-preservation of PBMC has also been performed successfully (Brügger, personal communication). The cryopreserved human whole blood (PEI method; described in Schindler et al., 2006) can be stored at -80°C for two years or longer and can be shipped easily on dry ice. Furthermore, donor testing for infection markers can be performed during its production. The availability of safe, standardised and reliable monocyte sources increases the feasibility of these test systems enormously and enables the use of alternative pyrogen testing in routine applications.

The PEI is mainly working with the MAT versions fresh whole blood / cryopreserved whole blood (-80°C) (both typically pooled from several donors). An average experimental setup consists of 200µl diluent (low-Endotoxin RPMI 1640; Cambrex, Verviers, Belgium), 20 µl fresh whole blood / thawed cryoblood and 20 µl of sample, which are pipetted onto a pyrogen-free 96well-plate (Nunc, Wiesbaden, Germany). Each assay includes a negative and positive control (pyrogenfree clinical saline; LPS-spike (25-100 pg/ml) to confirm the suitability of the chosen blood. Furthermore the product is spiked (25-100 pg/ml LPS) to explore potential adverse effects (e.g. toxicity) on the monocytes. A cytokine standard is used to evaluate the ELISA performance.

All glass/plasticware which is used before and during the incubation has to be pyrogen-free. The incubation is performed at 37°C in a cell culture breeder with 5% CO_2. Typically after 18-24h the incubation is stopped, the cell suspensions are transferred onto an ELISA plate/strip. The capture antibody might be anti-human IL-1ß, anti-human IL-6 or anti-human TNFα. Meanwhile the PEI has developed inhouse Di-or Tri-Cytokine-Assays ((IL-ß + IL-6) or (IL-ß + IL-6 + TNFα)), employing mixtures of capture and detection-antibodies (R&D Systems, Wiesbaden, Germany). The purpose of these Di- and Tri-Cytokine-Assays is a safer prediction of pyrogenicity. The non-release of a single pyrogenic cytokine (e.g. IL-1ß) caused by a sample does not necessarily exclude the release of other pyrogenic cytokines like IL-6 or TNFα. In consequence these Di- or Tri-Cytokine-Assays allow an estimation of the summarised pyrogenicity of a sample, but not of the released amount of individual cytokines.

The role of different cytokines and other effectors in fever development has been reviewed extensively in the literature. Interleukin-1ß (IL-1β) was the first cytokine described to be an "endogenous pyrogen" (*Dinarello* et al., 1974 and 1986a). Later on, interleukin-6 (IL-6) and and tumour necrosis factor a (TNFα) were added to the family of "pyrogenic cytokines" (Dinarello et al., 1986b and 1991). The binding of pyrogenic cytokines and/or the binding of ligands to toll-like receptors (TLR) at the blood-brain-barrier are the crucial steps in the induction of fever in vivo (Dinarello, 2004; Blatteis et al., 2000).

The same TLR are present on monocytic cells, inducing monocyte activation

after ligand binding followed by a significant production of pyrogenic cytokines as happens after binding of LPS to the CD14 receptor of these cells. In consequence, when pyrogens are entering the peripheral blood, monocytes are activated and release IL-1β and/or IL-6 and/or TNFα independent of the way the monocytes are activated. Furthermore, intravenous injection of these pyrogenic cytokines leads to fever *in vivo* (Dinarello, 1999 and 2004). Thus, the release of the pro-inflammatory cytokines IL-1β and/or IL-6 and/or TNFα is a predictive and reliable marker for the pyrogenicity of a given sample.

3 In contrast to the animal experiment, the alternative pyrogen tests allow the calculation of pyrogen limits for medicinal products

Pyrogens are substances which induce a fever reaction. Pyrogen tests have historically evolved from the need to test parenteral products for pyrogenic contaminations, resulting for example from bacterial breakdown products after sterilisation. Endotoxin (Lipopolysaccharide) of Gram-negative bacteria is the most important and best characterised pyrogen. Further pyrogenic substances (mainly from bacteria (e.g. Lipoteichoic acid, Lipoproteins, bacterial DNA)) were consequently grouped as "Non-Endotoxin"-pyrogens. This nomenclature reflects the thought that a pyrogen must be necessarily a contamination of microbial origin within the product. It has to be taken into account that a product itself might have pyrogenic entities. Biologicals with their inherent batch-to batch variability are of special interest in drug safety assessment.

The initial aim of the development of alternative pyrogen tests respectively MAT was to replace the "classic" animal experiment, the rabbit pyrogen test (RPT). The principle of the RPT is to inject the test sample into the ear veins of three animals and, thereafter, to monitor their body temperature over three hours. According to the European Pharmacopoeia (*Ph. Eur.*) the RPT is interpreted as follows (*Ph. Eur.* biological test 2.6.8. Pyrogens): If the sum of temperature increase of the three animals remains below a value of 1.15°C, the product passes the test. If the sum of the temperature increases exceeds 2.65°C, the product fails and it has to be considered as pyrogenic. The test defines intermediate temperature ranges which allow repetitions of the test leading to an increase in the number of animals needed (up to 12 rabbits). The respective regulation in the United States Pharmacopoeia (USP) is slightly different. In general, both regulations lead to comparable outcomes (Hoffmann et al., 2005b). Medicinal products failing the RPT have to be discarded which often leads to remarkable financial losses, while in some cases it is doubtful whether such a product would indeed harm the patient or not.

As a qualitative test, the RPT produces the potential results "pass" or "fail". In contrast, the Monocyte Activation Test (MAT) is able to quantify the pyrogenicity of the test sample which opens the opportunity for a safety assessment regarding product properties. This principle has been successfully established since decades in endotoxin testing (Bacterial Endotoxin Test, BET) of medicinal products. The respective monograph of *Ph. Eur.* (*Ph. Eur.* biological test 2.6.14 Bacterial Endotoxins) defines endotoxin limits that are acceptable, i.e. which do not harm the patient, dependent on the application route of the product. The exciting challenge is whether these proven endotoxin limits can be used for the definition of pyrogen limits, i.e. limits for non-endotoxin pyrogens.

Calculating the potential pyrogenicity of a medicinal product, the *in vivo* situation of the recipient has to be taken into consideration. As mentioned above, fever in humans is mediated via the main fever inducing cytokines IL-1β, IL-6 and TNFα. These belong to the pro-inflammatory cytokines and exist in a defined physiological concentration range in the peripheral blood. Injection of pyrogens into the blood stream leads to activation of monocytes which in turn produce these cytokines. Body temperature starts to increase when the concentrations of IL-1β, IL-6 or TNFα exceed their physiological ranges.

In consequence, the fever threshold of human beings is defined by the upper border of the normal range of IL-1β, IL-6 and TNFα in the peripheral blood. Therefore, their concentration measured in the MAT can be used for the calibration of non-endotoxin pyrogens using the experience obtained with endotoxin. This consideration led to the introduction of the so called "endotoxin-equivalent". One endotoxin-equivalent induces in the MAT the same concentration of IL-1β, IL-6 or TNFα as one endotoxin unit (100 pg LPS) does. Thus, the pyrogen limits for medicinal products can be assessed on the basis of the endotoxin limits defined in the biological test of *Ph. Eur.* (European Pharmacopeia 2.6.14 Bacterial Endotoxins).

The safety calculation for a medicinal product shall be exemplified using data from the batch testing of an immunoglobulin intended for intravenous administration (IVIG). The product had been successfully tested ("pass") by the manufacturer as well as by PEI in the RPT. Additionally, the immunoglobulin batch was analysed in MAT by PEI. As can be seen in Figure 1, the undiluted product induces a higher concentration of fever inducing cytokines than the positive control consisting of 25 pg/ml LPS (WHO Standard Endotoxin) in endotoxin-free saline representing the cut-off of the method in this experiment. Thus, the IVIG has to be considered as positive in the MAT. Does this mean that the product is pyrogenic in human beings? The following safety calculation will answer this question.

As can be seen in Figure 1, the dilution 1:2 remains negative in the MAT, i.e. it contains less than 0.25 endotoxin-equivalents per millilitre corresponding to less than 0.5 endotoxin-equivalents in the undiluted product. The entire product volume in the container is 200 millilitres, i.e. the whole vial contains less than 100 endotoxin-equivalents. The pyrogen limit for intravenous administration is defined as 5 endotoxin-units (= 5 endotoxin-equivalents) per kilogram body weight and hour. Considering a patient having a body weight of 70 kilogram, the pyrogen limit would be 350 endotoxin-equivalents per hour. In consequence, the product containing less

than 100 endotoxin-equivalents is safe and can be released without concern. It should be mentioned here that the application of medicinal products to children follows special rules considering their lower body weight.

3.1 Pyrogenicity: Not restricted to contaminations

The calculation of pyrogen limits could also be used for novel medicinal products during pre-clinical testing since this procedure reflects the in vivo reactivity of human beings. In the light of the scientific experiences in the past decade, pyrogenicity should be defined as the potency to activate monocytes independent on the stimulation cause. Beside microbial impurities (mainly Endotoxins (Lipopolysaccharides)) – traditionally understood as pyrogens – a broad range of substances is pyrogenic. For instance, the severe adverse reactions during the first-in-human trial of the Anti-CD28 monoclonal antibody TGN1412 suggest as potential cause a strong monocyte activation from what can be concluded from the clinical data. TNFα was the first cytokine which substantially increased in the peripheral blood namely from 2.8 pg/ml before injection to 1760.1 pg/ml within one hour. Monocytes are considered the main source for TNFα. Furthermore, mainly monocytes are able to react within such a short period whereas lymphocytes might require more time for cytokine production. The latter can be seen from the development of Interferon-gamma (produced mainly by T cells) which remained one hour after administration in its normal range (Suntharalingam et al., 2006). Since the monoclonal antibody TGN1412 was sterile and did not contain endotoxin or other impurities (MHRA Clinical trial final report, 2006), one possible conclusion could be that the preparation itself led to the strong monocyte activation of the patients which was not sufficiently predicted by the available *in vivo* animal data and conventional *in vitro* data. Thus, at least the acute clinical signs could also be interpreted as a pyrogenic reaction in its immunological sense. The exact pathogenesis is currently under investigation (Expert Scientific Group, 2006). While it remains to be proven if the MAT might give indicative results with TGN1412, it might serve as a paradigm that *in vitro* tests could become increasingly important as potential alternative tools to "conventional" animal experiments, at least if further developed and adapted.

4 Safety assessment of novel cell based medicinal products as regards acute reactions

Considering the pyrogen limit calculation cited above, the PEI performed pilot studies in pre-clinical testing of novel cell based products which shall be demonstrated in the following. All of them were dedicated to the use in first-in-human trials. The experiments shown have been performed during the last six years, the different test setups (single cytokine; Di- and Tri-Cytokine; fresh blood, cryoconserved blood; single donor, pooled donors) resemble test variations each of them suitable for product testing.

The first example concerns genetically modified T lymphocytes intended for the treatment of cancer. Interactions between these cells and the monocytes of the patient cannot be excluded. The latter could lead to monocyte activation

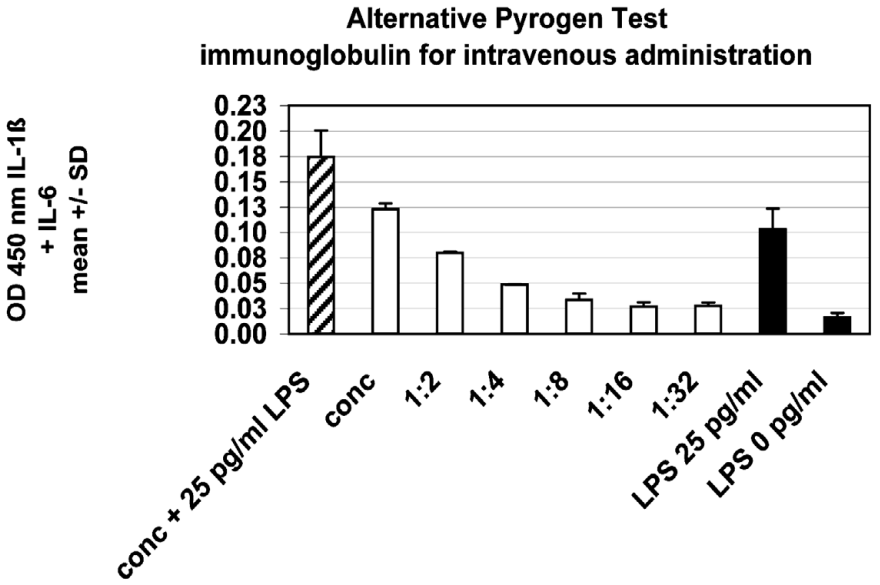

Fig. 1: Monocyte Activation Test (Alternative Pyrogen Test) of an immunoglobulin intended for intravenous administration (IVIG).
The test has been performed as semi-quantitative method using cryo-preserved human whole blood (4 donors; stored for 236 days at -80°C before thawing for this experiment; PEI method). Each sample in duplicates (n = 2). Spiking with 25 pg/ml LPS.
Incubation overnight in a CO_2-incubator.
Readout: Production of Interleukin-1β and Interleukin-6 (Di-Cytokine-ELISA) in the supernatant of blood cell incubation
Cut off in this experiment: reaction on 25 pg LPS (WHO Standard Endotoxin) per millilitre.
Hatched column: undiluted product spiked with 25 pg/ml LPS
White columns: Dilution line of the product, from undiluted (conc) up to 1:32.
Black columns: Positive control, consisting of 25 pg/ml (corresponding to 0.25 E.U./ml) LPS (WHO Standard Endotoxin) in endotoxin-free saline, and negative control (endotoxin-free saline)

followed by acute adverse reactions via release of fever inducing cytokines. Pyrogen testing of human cells in rabbits is impracticable because of immune reactions between rabbit and human cells, and due to lack of relevant interactions between human and rabbit immune cells. Endotoxin testing of cell suspensions is not feasible for several reasons. The cells themselves as well as the plasma content of the preparation strongly interfere with the BET, which leads to inconclusive results. Due to these restrictions, the established methods for pyrogen and endotoxin testing are not applicable in order to obtain the safety assessment of T cell preparations.

Therefore, the MAT was applied for pyrogen testing of the T cell suspensions. Figure 2 shows the results obtained with three different preparations of genetically modified T cells. There was almost no cytokine induction after incubation of the T cells with the blood from four different donors or with the pooled blood of the donors. The amount of IL-1β induced by the cell suspensions appears to be in the range of the negative controls or even lower. Furthermore, the cytokine concentrations induced by the T cells remain clearly below those induced by the positive control consisting of 50 pg/ml LPS (WHO Standard Endotoxin) in endotoxin-free saline. The positive control represents the fever threshold of human beings in the peripheral blood and has been used as cut off in this experiment. Thus, the T cell preparations can be considered to be non-pyrogenic.

The second example deals with peptides intended for the use in monocyte derived dendritic cell tumour vaccines. The manufacturing process of these autologous vaccines requires a brief description. Monocytes are obtained from the patient and transformed into dendritic cells by cultivation in the presence of a suitable cytokine cocktail. Thereafter, the dendritic cells are loaded with peptides the sequences of which are deduced from amino acid sequences of tumour antigens. The peptides are bound to the MHC I receptor on the surface of the dendritic cells like naturally presented antigens. These cells are injected into the patient with the aim of inducing an immune response towards the tumour.

It was not known whether the peptides interact with the monocytes of the patient after injection of the preparation, leading to monocyte activation and thus to acute adverse reactions. Therefore, two peptides with different amino acid sequences were been tested in the MAT. A potential contamination of the peptide preparations by endotoxin was excluded by applying the Limulus assay prior to this experiment. Figure 3 demonstrates that there was no induction of fever inducing cytokines (the figure shows data obtained for TNFα as an example, measurement of IL-1β and IL-6 produced similar results) by both

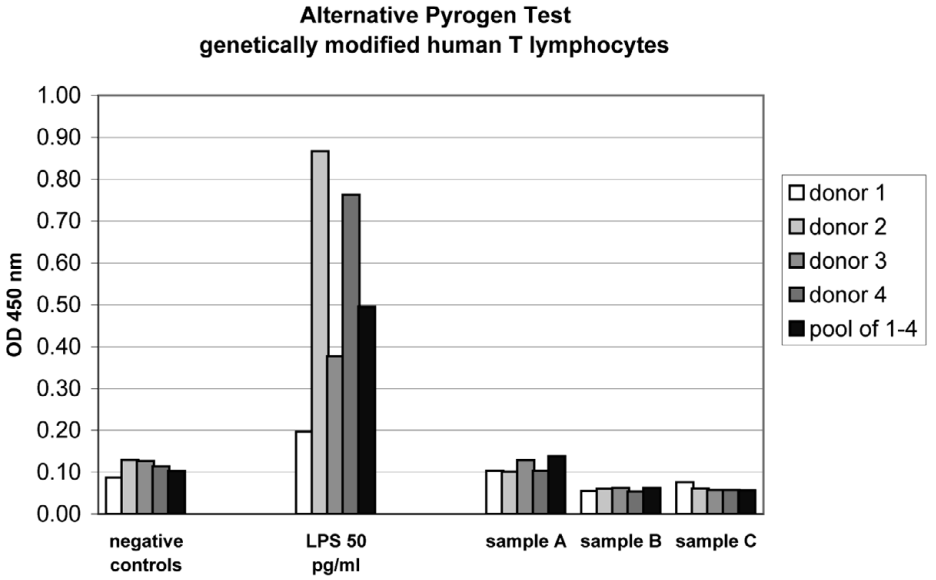

Fig. 2: Monocyte Activation Test (Alternative Pyrogen Test) of genetically modified human T cells (samples A-C) intended for the use in cancer treatment.

The test has been performed with freshly drawn blood samples from 4 different donors and, additionally, with a pool of these 4 blood samples (single values; test repetition was performed with 4 different donors with similar result). In a prior spiking experiment the lymphocyte preparations were not affecting the LPS-recovery (data not shown; separate experiment).
Samples A, B, and C: Three different preparations of human modified T cells:
sample A = 2.6 x 10^6 cells/ml sample
sample B = 3.5 x 10^6 cells/ml sample
sample C = 10.2 x 10^6 cells/ml sample
Incubation overnight in a CO_2-incubator.
Readout: Production of Interleukin-1β in the supernatant of blood cell incubation
Cut off in this experiment: 50 pg/ml LPS (WHO Standard Endotoxin)
Negative controls: endotoxin-free saline
Positive controls: LPS (WHO Standard Endotoxin) in a concentration of 50 pg/ml (corresponding to 0.5 E.U./ml) in endotoxin-free saline

peptides in the test. All dilutions reacted comparably to the negative control consisting of endotoxin-free saline.

The third example concerns human primary hepatocyte preparations which are used for the treatment of patients with severe acute liver failure for short and medium term support of liver function and possibly as a future alternative therapy to liver transplantation. Preliminary studies with the hepatocyte preparations led to the hypothesis that the liver cells within these preparations (in the absence of blood monocytes) are able to produce the fever inducing cytokines IL-1β, IL-6 and TNFα upon contact with endotoxin. In order to explore this question, the hepatocyte preparations were incubated with different concentrations of LPS (WHO Standard Endotoxin) overnight at 37°C in a CO_2-incubator. Thereafter, the potential cytokine content was measured in an in-house Tri-Cytokine-ELISA indicating the summarised production of IL-1β, IL-6 and TNFα. Figure 4 shows the result of this experiment. After incubation with LPS, cells within the hepatocyte preparations produced fever inducing cytokines in a dose dependent manner (in the absence of LPS there was no detectable cytokine production). This result was obtained both in the presence and absence of added blood monocytes. Since the liver cell isolation is accomplished by whole organ perfusion impurities with blood cells (approx. 5%) and non-parenchymal liver cells occur. Independent of the question which cells within these hepatocyte preparations are the source of the inducible cytokines, the final drug product tested was able to release fever inducing cytokines upon LPS stimulation. In the typical MAT-setup (incubation of the sample in the presence of added monocytes) LPS-spikes could be detected. In conclusion, the MAT is feasible to test for pyrogenicity within this hepatocyte preparations.

However, it has to be mentioned that these hepatocyte preparations were concordant with the regulations. They did not induce a cytokine release themselves when tested in the MAT, and have been applied successfully (single case reports) so far.

This result demonstrates exemplarily how complex the effects of cell based medicinal products are. Discussing potential acute adverse reactions during administration of those preparations to the patient, in the past only potential interactions with the monocytes of the recipient have been taken into consideration. Obviously, fever inducing cytokines can be released by cells other than monocytes, too. On the other hand, the above cited procedure for the calculation of pyrogen limits permits a safety assessment of those preparations applying carefully designed *in vitro* methods.

5 Conclusions

Animal experiments in safety testing of medicinal products should be replaced by *in vitro* methods wherever possible. On the other hand, safety and availabili-

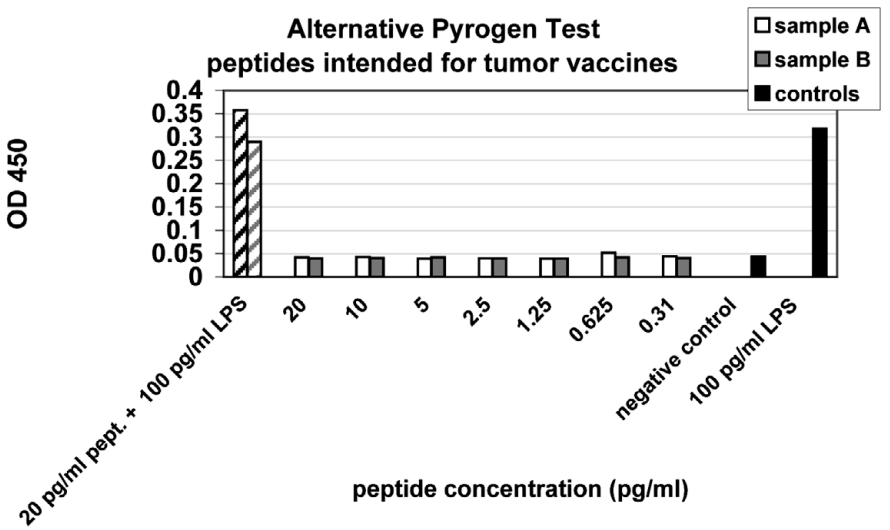

Fig. 3: Monocyte Activation Test (Alternative Pyrogen Test) of two peptide preparations intended for the use in dendritic cell tumour vaccines (see text).
The test has been performed with pooled freshly drawn human whole blood (pool of 4 donors). Single values, the test has been repeated three times with similar results. Spiking with 100 pg/ml LPS
Incubation overnight in a CO_2-incubator.
Readout: Production of TNFα in the supernatant of blood cell incubation. (Measuring of Interleukin-1 beta and Interleukin-6 led to comparable results, data not shown.)
Samples A and B: Two peptide preparations having different amino acid sequences
Hatched columns: 20pg/ml of samples A and B spiked with 100 pg/ml LPS
White columns: sample A
Grey columns: sample B
Black Columns:Negative control (Endotoxin-free saline) and Positive control: LPS (WHO Standard Endotoxin) in a concentration of 100 pg/ml (corresponding to 1 E.U./ml) in endotoxin-free saline

ty of the products have to remain top priorities. Therefore, the alternative method has to be carefully characterised with regard to its relevance in comparison to the corresponding animal experiment.

The authors support the position of EPAA to involve authorities throughout the process of validation and legal acceptance of alternative approaches. The PEI has been engaged in the development and validation of alternative methods for almost two decades. The results of this research could be introduced into several EP regulations. Furthermore, training courses for the industry have been organised by the institute in order to facilitate and to accelerate method transfer. From the experience of the PEI, involvement of authorities in development and validation of alternative methods can contribute substantially to their establishment and distribution.

The Alternative Pyrogen Test is an example that an alternative test can provide at least the same safety level in comparison with the respective animal test. Furthermore, this alternative test is able to produce additional information which cannot be obtained from the animal experiment itself. As an important advantage, the test allows the calculation of pyrogen limits for medicinal products since this procedure reflects the in vivo reactivity of human beings. This approach could also be used for safety assessments regarding potential acute adverse reactions for novel medicinal products during the pre-clinical phase, i.e. before first-in-human clinical trials. This includes, for instance, cell based medicinal products for which no pyrogen testing existed up to now.

Furthermore, the research in alternative pyrogen testing led to a new understanding of pyrogenicity. From a clinical point of view, any substance – independent of its nature – able to activate monocytes should be considered to be potentially pyrogenic since monocyte activation leads to the immediate release of IL-1β and/or IL-6 and/or TNFα as a rather uniform reaction. These cytokines produce acute adverse reactions that can range from induction of fever to life-threatening pyrogenic shock with multi organ failure, depending on the concentration of the stimulus and its intrinsic activity. Such reactions should be considered during the development of new products. Alternative in vitro systems might not only be alternatives to conventional animal experiments, but might even have particular advantages that make them important tools for non-clinical safety testing of novel biomedicinal products. They maybe even further developed to allow for rapid bedside tests to better predict individual responses in a given patient. This might indeed be an advantage, since patients have individual states of their immune system and its activation, depending on various factors like disease, its stage, and comedication. While not necessarily replacing animal experiments in all aspects of non-clinical safety and toxicology testing, *in vitro* tests can add valuable information to the overall non-clinical concept, especially in the critical juncture between non-clinical and clinical development programme. The requirement to perform such tests would have to be decided on a case-by-case basis.

References

Beyersdorf, N., Gaupp, S., Balbach, K. et al. (2005). Selective Targeting of Regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. *J. Exp. Med., 202(3)*, 445-455.

Blatteis, M. C., Sehic, E. and Li, Shuxin

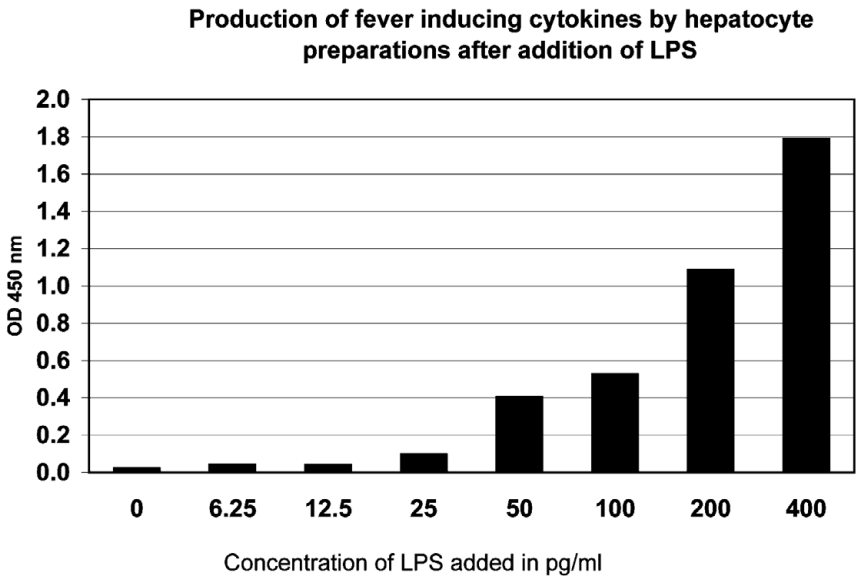

Fig. 4: Production of IL-1β, IL-6, and TNFα by hepatocyte preparation after cultivation in the presence of LPS (WHO Standard Endotoxin) without addition of blood monocytes.

The cells (8.3 x 10^6/ml) have been incubated for 20 hours in a CO_2-incubator. Thereafter, the cytokine concentration has been estimated in the supernantants of the cell cultures. The experiment was repeated twice.

Readout: Summarised production of IL-1β, IL-6, and TNFα in a Tri-Cytokine-ELISA (PEI method).

Negative control: endotoxin-free saline (0 pg/ml LPS)

(2000). Pyrogen sensing and Signaling: Old Views and New Concepts. *Clin. Infect. Dis. 31 (Suppl 5)*, 168-177.

Dinarello, C. A. (1999). Cytokines as endogenous pyrogens. *J. Infect. Dis. Mar. 179 Suppl 2*, 294-304.

Dinarello, C. A. (2004). Infection, fever and exogenous and endogenous pyrogens: some concepts have changed. *J. Endotox. Res. 10*, 201-222.

Dinarello, C. A., Cannon, J. G., Mancilla, J. et al. (1991). Interleukin-6 as an endogenous pyrogen: induction of prostaglandin E2 in brain but not in peripheral blood mononuclear cells. *Brain Res. Oct 25, 562(2)*, 199-206.

Dinarello, C. A., Cannon, J. G., Mier, J. W. et al. (1986a). Multiple biological activities of human recombinant interleukin 1. *J. Clin. Invest. Jun; 77(6)*, 1734-1739.

Dinarello, C. A., Cannon, J. G., Wolff, S. M.,et al. (1986b). Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J. Exp. Med. Jun 1, 163(6)*, 1433-1450.

Dinarello, C. A., Goldin, N. P. and Wolff, S. M. (1974). Demonstration and characterization of two distinct human leukocytic pyrogens. *J. Exp. Med. Jun 1, 139(6)*, 1369-81.

EPAA Conference Europe goes alternative Concluding remarks, Brussels, Belgium, December 18th 2006, http://ec.europa.eu/enterprise/epaa/epaa_conference_concluding_remarks.pdf

Eperon, S.and Jungi, T. W. (1996). The use of human monocytoid lines as indicators of endotoxin. *J. Immunol. Methods. Aug 14, 194(2)*, 121-129.

European Medicines Agency (EMEA) (1998). ICH Topic S6, Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals. http://www.emea.europa.eu/pdfs/human/ich/ 030295en.pdf

European Medicines Agency (EMEA) (2006a). CHMP Draft Guideline on human cell-based medicinal products CHMP/410869/06. http://www.emea.europa.eu/pdfs/human/cpwp/41086906en.pdf

European Medicines Agency (EMEA) (2006c). CHMP Guideline on Virus Safety Evaluation of Biotechnological Investigational Medicinal Products CHMP/BWP/398498/2005-corr. http://www.emea.europa.eu/pdfs/human/bwp/39849805en.pdf

European Medicines Agency (EMEA) (2006b). Guideline on potency testing of cell based immunotherapy medicinal products for the treatment of cancer CHMP/BWP/271475/06. http://www.emea.europa.eu/pdfs/human/bwp/27147506en.pdf

European Partnership for Alternative Approaches to Animal Testing, http://ec.europa.eu/enterprise/epaa/brochure.htm

European Pharmacopoeia 5.0: 2.6.14. Bacterial Endotoxins.

European Pharmacopoeia 5.0: 2.6.8. Pyrogens.

Expert Scientific Group on Phase One Clinical Trials: *Final Report* (2006). The Stationary Office, Norwich, United Kingdom. ISBN-10 0 11 703722 2.

Hanke, T. (2006). Lessons from TGN1412. *Lancet, 368(9547)*, 1569-1570.

Hartung, T., Aaberge, I., Berthold,S. et al. (2001). Novel pyrogen tests based on the human fever reaction. The report and recommendations of ECVAM Workshop 43. European Centre for the Validation of Alternative Methods. European Centre for the Validation of Alternative Methods. *ATLA, Mar-Apr, 29(2)*,99-123.

Hartung, T. and Wendel, A. (1995). Detection of Pyrogens using human whole blood. *ALTEX 12(2)*,70-75.

Hoffmann, S., Peterbauer, A., Schindler, S. et al. (2005a). International validation of novel pyrogen tests based on human monocytoid cells. *J. of Immunol. Method, 298*, 161-173.

Hoffmann, S., Lüderitz-Püchel, U., Montag, T. and Hartung, T. (2005b). Optimisation of pyrogen testing in parenterals according to different pharmacopoeias by probabilistic modelling. *J. Endotoxin Res. 11*, 25-31.

Lühder, F., Huang, Y., Dennehy, K. M. et al. (2003). Topological Requirements and Signaling Properties of T Cell-activating, Anti-CD28 Antibody Superagonists. *J. Exp. Med. 197(8)*, 955-966.

MHRA: Clinical trial final report (2006) http://www.mhra.gov.uk/home/idcplg?IdcService=SS_GET_PAGE&useSecondary=true&ssDocName=CON2023822&ssTargetNodeId=389.

Nguyen, D. H., Hurtado-Ziola, N., Gagneux, P. and Varki, A. (2006). Loss of Siglec expression on T lymphocytes during human evolution. *Proc. Natl. Acad. Sci. USA., 103(20)*, 7765-70.

Personal communication Dr. Peter Brügger, Basel.

Peterbauer, A., Eperon, S., Jungi, T., W. et al. (2000). Interferon-gamma-primed monocytoid cell lines: optimizing their use for in vitro detection of bacterial pyrogens. *J. Immunol. Methods, Jan 13, 233 (1-2)*, 67-76.

Poole, S., Mistry, Y., Ball C. et al. (2003). A rapid 'one-plate' in vitro test for pyrogens. *J. Immunol. Methods, Mar 1, 274(1-2)*, 209-220.

Poole, S., Thorpe, R., Meager, A. and Gearing, A. J. (1988). Assay of pyrogenic contamination in pharmaceuticals by cytokine release from monocytes. *Dev. Biol. Stand. 69*,121-3.

Schindler, S., Asmus ,S., von Aulock, S. et al. (2004). Cryopreservation of human whole blood for pyrogenicity testing. *J. Immunol. Methods. Nov, 294 (1-2)*, 89-100.

Schindler, S., Spreitzer, I., Loeschner, B. et al. (2006). International validation of pyrogen tests based on cryopreserved human primary blood cells. *J. Immunol. Meth., 316*, 42-51.

Schneider, C. K., Kalinke, U. and Löwer, J. (2006). TGN1412 – a regulator's perspective. *Nat. Biotechnol. 24(5)*, 493-496.

Suntharalingam, G., Perry, M. R., Ward, S. et al. (2006). Cytokine Storm in a Phase 1 Trial of the CD-28 Monoclonal Antibody TGN1412. *N. Engl. J. Med. 355*, 1-11.

Correspondence to

Dr. Thomas Montag-Lessing
Paul-Ehrlich-Institut
Federal Agency for Sera and Vaccines
Head of section 1/3 Microbial Safety and Parasitology
63225 Langen
Germany
Tel: +49 6103-778020
Fax: +49 6103-771250
e-mail: month@pei.de