Culturing Cells Without Serum: Lessons Learnt Using Molecules of Plant Origin

*Patricia Pazos, Monica Boveri, Alessandra Gennari, Juan Casado, Fernando Fernandez and Pilar Prieto**

ECVAM, Institute for Health & Consumer Protection, Joint Research Centre, European Commission, I-Ispra

Summary

To successfully grow cells in serum-free medium is an interesting challenge to cell biology. The use of such media for in vitro cell culture work would be a key contribution to the 3Rs concept, enabling the avoidance of the use of animals and animal products at all stages of the experiment. In addition, numerous problems related to virus infections transmitted by animal serum would be avoided, thus increasing reproducibility. Prolifix is a new reagent of plant origin. It contains a molecule (GCR 1003) that has an activity similar to that of the mitogenic molecules in serum and could be suitable to substitute serum in culture medium. Two epithelial cell lines, LLC-PK1 and Caco-2, were progressively adapted to a special culture medium containing 10% Prolifix in the absence of serum. After adaptation, cell cultures were characterised. We found that these reagents of plant origin could be promising alternatives to serum. However, more work and effort is needed to improve cell adaptation, cell attachment, growth rates as well as freezing/thawing protocols.

Zusammenfassung: Serumfreie Kultivierung von Zellen: Erfahrungen mit pflanzlichen Ersatzstoffen

Die erfolgreiche Kultivierung von Zellen in serumfreiem Medium ist eine interessante Herausforderung in der Zellbiologie. Die Verwendung von solchen Medien für die in vitro Zellkulturtechnik würde einen Hauptbeitrag zum 3R Konzept liefern, welches die Vermeidung des Gebrauchs von Tieren und tierischen Produkten auf allen Ebenen fordert. Zusätzlich könnten zahlreiche Probleme der Übertragung von Viren mit dem Serum verhindert und dadurch die Reproduzierbarkeit erhöht werden. Prolifix ist ein neues Reagens pflanzlicher Herkunft. Es beinhaltet ein Molekül (GCR 1003), dessen Aktivität ähnlich der mitogenen Moleküle im Serum ist und könnte daher als Serumersatz in Kulturmedien dienen. Um dies zu prüfen wurden zwei verschiedene Zelllinien, LLC-PK1 und Caco-2, stufenweise an ein spezielles Kulturmedium, welches 10% Prolifix in Abwesenheit von Serum enthielt, adaptiert. Nach Adaptierung wurden die Zelllinien charakterisiert. Wir stellten fest, dass dieses Reagens pflanzlicher Herkunft eine vielversprechende Alternative zu Serum sein könnte. Jedoch sind weitere Experimente erforderlich, um Zelladaptation, Zellhaftung, Wachstumsraten und auch das Einfrier-/Auftauprotokoll zu verbessern.

Keywords: serum, replacement, in vitro, epithelial cells, plant components

1 Introduction

Serum is used in cell culture medium formulations to support cell survival and cell growth by providing hormones, nutrients, binding proteins and attachment factors (Barnes and Sato, 1980; Gstraunthaler, 2003). Despite these beneficial effects, researchers have made great efforts during the last decades to avoid serum in their culture media because of the also well-documented negative aspects associated with the use of serum in cell culture work. The positive and negative aspects of the use of serum have been summarised in a recent report from a workshop on fetal bovine serum held in Utrecht in 2003 (van der Valk et al., 2004). There are clear scientific and ethical reasons that question the use of serum in media formulations. It seems paradoxical that *in vitro* methods are constantly being favoured because they promote the 3Rs concept (Russell and Burch, 1959) but most of them still require the use of animal serum.

However, the replacement of serum is not an easy task. Several serum-free formulations are on the market but not all are suitable for all cell types. Additional problems linked to the production of cell-derived products arise (van der Valk et al., 2004) and therefore more efforts are required to develop serum-free cell culture media.

A new reagent of plant origin free of all human or animal proteins, Prolifix, contains a molecule (GCR 1003) that has mitogenic activity similar to that of mitogenic molecules in serum. Although this reagent contains patented components in its formulation, which posed additional problems, it seemed a promising alternative to serum formulations.

The work presented here shows the results of a pilot study performed at ECVAM (European Centre for the Validation of Alternative Methods) laboratories to evaluate the usefulness of molecules of plant origin (Prolifix) to replace fetal bovine serum (FBS) in cell

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* corresponding author

culture medium for two epithelial cell lines, the porcine renal proximal tubular epithelial cell line LLC-PK1 and the human colorectal adenocarcinoma cell line Caco-2.

After the adaptation process both cell lines retained their epithelial morphology, although there were some differences in terms of barrier integrity and response to toxic insult in comparison to cells grown in serum-containing medium. The advantages and problems that arose are discussed and some recommendations are outlined.

2 Materials and methods

2.1 Cell culture

LLC-PK1 cells (porcine renal proximal tubular cell line) and Caco-2 cells (human colorectal adenocarcinoma cell line) were obtained from the American Tissue Culture Collection (ATCC). LLC-PK1 cells were grown on Unicell 0.4 µm polycarbonate 24-well culture plates (Whatman Inc., Clifton, NJ, USA) at a cell density of 370.000 cells/cm^2 at 37°C with humidified, 5% CO_2 atmosphere, in Dulbecco's modified Eagle's medium (DMEM) containing 5 mmol/l D-glucose (Sigma, St. Louis, MO, USA) and supplemented with 2 mM L-glutamine, 10.000 U/ml penicillin, and 10 mg/ml streptomycin (Life Technologies) and 10% Prolifix S8 (Biomedia, France) or 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA). 0.3 ml and 0.5 ml of media were placed in the apical and basolateral compartments of each insert, respectively. Caco-2 cells were grown in DMEM (Life Technologies) supplemented with 10% Prolifix S6 (Biomedia, France) or 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, and 10.000 U/ml penicillin, and 10 mg/ml streptomycin. Cells were seeded at a cell density of 500.000 cells/cm^2 on Transwell-Clear 0.4 µm polyester membrane inserts in 24-well culture plates (Corning-Costar, Cambridge, MA). 0.3 ml and 1 ml of media were placed in the apical and basolateral compartments of each insert, respectively. For cell culture maintenance both cell lines were grown in conventional 75 cm^2 culture flasks (Corning-Costar) at 37°C in a humidified, 5% CO_2 atmosphere. Medium was changed every three days.

2.2 Cell adaptation to Prolifix

LLC-PK1 and Caco-2 cell lines were progressively adapted to grow in medium supplemented with 10% Prolifix S8 and S6, respectively. Cells were subcultured in medium containing decreasing amounts of FBS (7.5%, 5%, 2.5%, 1%, 0%) and increasing amounts of Prolifix (2.5%, 5%, 7.5%, 9%, 10%) and were cultured in each adapting condition for at least two passages.

2.3 Morphological analysis

Cells were observed with a phase contrast inverted microscope (Olympus CK40, Japan). Formation of domes in both cell lines under the different culture conditions was checked and documented (digital camera, Nikon Coolpix 990).

2.4 Immunocytochemistry

Junction formation was followed by immunofluorescent observation of the distribution of ZO-1, a protein specific for tight junctions, and the junction-associated protein actin. LLC-PK1 and Caco-2 cells were fixed in 4% paraformaldehyde at 4°C for 1 h, then permeabilised with 0.01% Triton X-100. Non-specific binding sites were blocked with 10% goat serum for 30 min at room temperature. Cells were then incubated with a mouse monoclonal anti-ZO-1 antibody (1:500 diluted in PBS, Histo-Line Laboratories, Milan, Italy), followed by Alexa-Fluor 488-conjugated goat anti-mouse IgG antibody (diluted 1:200 in PBS, Molecular Probes, Eugene, OR, USA) or directly stained with Alexa-Fluor 488-phalloidin (diluted 1:100 in PBS, Molecular Probes). Cells were examined under a confocal laser-scanning microscope (MP 2000, Biorad).

2.5 Treatment

$CdCl_2$ (Sigma) was dissolved in water. Working solutions were freshly diluted in medium. For all the experiments, both the apical and the basolateral side of the epithelial monolayer were exposed for 24 h to $CdCl_2$ (0 µM-50 µM) at 37°C. LLC-PK1 cells were treated on day 4 after seeding and Caco-2 cells on day 21 after seeding.

2.6 Cytotoxicity assays

Cell toxicity was measured by assessing the release of lactate dehydrogenase (LDH) into the medium, as described by Tyson and Green (1987). Following $CdCl_2$ treatment, culture medium from both the apical and the basolateral compartments of each cell culture insert was collected and LDH determined. LDH activity was calculated as the rate of decrease in the absorbance at 340 nm resulting from the oxidation of NADH (Sigma) in the presence of pyruvate (Sigma), by using a spectrophotometer (Beckman, Palo Alto, Ca, USA).

2.7 Barrier integrity

The barrier integrity of epithelial monolayers was evaluated by measuring two parameters, the trans-epithelial electrical resistance (TEER) and the paracellular permeability (PCP). TEER measurements were performed by using a chamber electrode (24-Endohm-Multi™ chamber and Endohm-6 chamber for LLC-PK1 and Caco-2 cells, respectively) in combination with the epithelial voltohmmeter EVOM™ purchased from World Precision Instruments (WPI Inc., New Haven, CT, USA). For LLC-PK1, TEER was measured by inserting the top electrode into each well as described previously (Gennari et al., 2003). For the Caco-2 cell line, the single cell culture insert of the 24-well culture plate was transferred to the Endohm chamber, which was then closed with the top electrode as described previously (Boveri et al., 2004). Experimental TEER values were expressed as ohms (Ω) x cm^2. TEER was measured before and after $CdCl_2$ treatment. The Unicell membrane surface area was 0.385 cm^2 and the Transwell insert membrane area was 0.332 cm^2 for all calculations.

Fluorescein isothio-cynate (FITC)-inulin (Sigma) was used to monitor the paracellular transport across the epithelial monolayer. FITC-inulin was added to the apical side at a final concentration of 5 µg/ml together with $CdCl_2$. At the end of the treatment period, the increase of fluorescence (in arbitrary fluorescence units, AFU) in the basolateral compartment was assessed using a fluorometric reader (Fluoroskan L Ascent, Labsystem, Helsinki, Finland).

2.8 Statistics

Results were expressed as means ± SD. Data were from triplicate samples from at least three independent experiments. The statistical significance was determined by two-way analysis of variance followed by Dunnett's test. $p < 0.05$ was considered statistically significant.

3 Results

3.1 Morphological analysis

Figure 1 shows the typical morphology of LLC-PK1 and Caco-2 cells grown on plastic supports in medium supplemented with Prolifix (A and B) and in medium supplemented with FBS (C and D). In all cases, dome formation, which is characteristic of transporting epithelia grown on plastic supports, was clearly observed. Cells grown in medium supplemented with Prolifix showed fewer domes as compared to cells grown in medium supplemented with FBS.

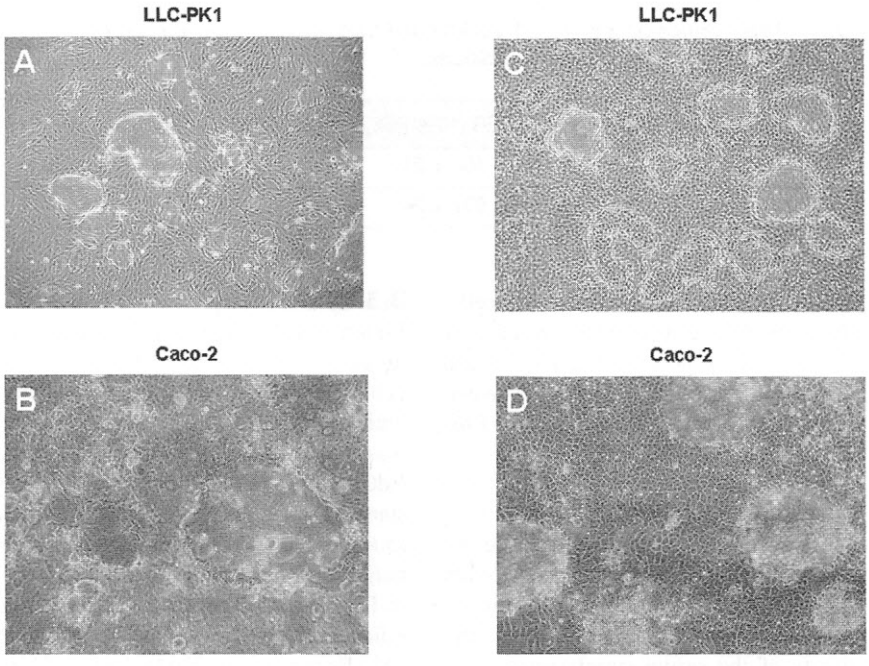

Fig. 1: Phase-contrast micrographs of domes in Caco-2 and LLC-PK1 cells cultured on plastic supports and grown in medium supplemented with Prolifix (A, B) and FBS (C, D).

3.2 Characterisation of barrier properties

The two main functions of an epithelium are the transport and the barrier functions. Solute transport by the transcellular and/or the paracellular routes creates a trans-epithelial potential difference, and the electrical resistance depends on the tightness of sealing between the cells (Wills et al., 1996). The barrier formation was checked by monitoring TEER after culturing both cell lines on porous supports under the different medium conditions. As can be seen in Table 1, LLC-PK1 cells reached similar stable TEER values under both culture conditions (100 ± 21, 90 ± 34 in FBS and Prolifix, respectively). However, TEER values of Caco-2 cells cultured in Prolifix-supplemented medium were 76% lowered than when cells were grown in FBS.

3.3 ZO-1 distribution

ZO-1 is one of the best characterised proteins in the cytoplasmic plaque of the tight junctions (Anderson and Stevenson, 2000). Figure 2 shows the effects of serum supplement, Prolifix (A and B) and FBS (C and D), on ZO-1 distribution in LLC-PK1 and Caco-2 cells. Prolifix and FBS induced a similar distribution of

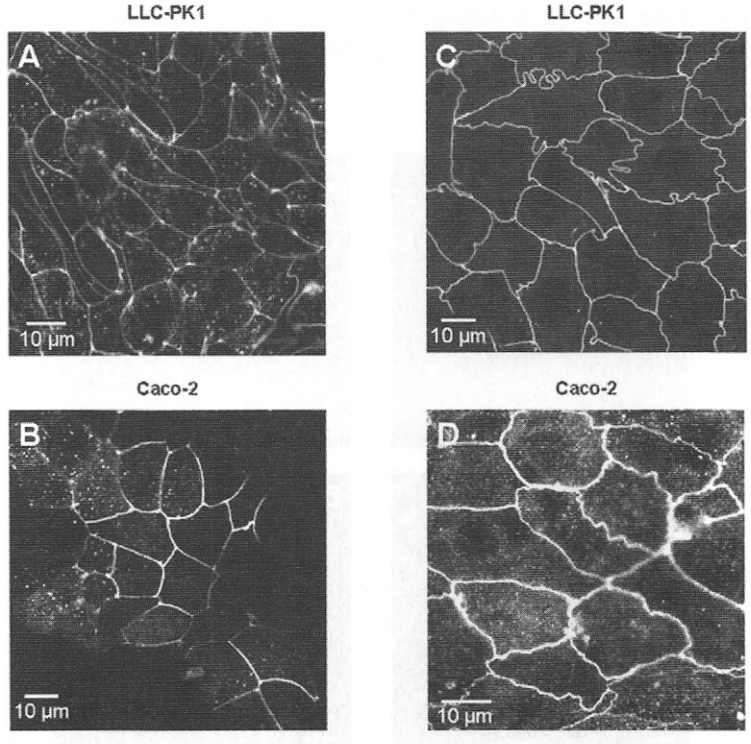

Fig. 2: Confocal fluorescence microscopy of ZO-1 staining in LLC-PK1 and Caco-2 cells cultured on permeable supports.
A and B represent cells grown in Prolifix-supplemented medium and C and D show cells grown in FBS-supplemented medium. In B, the signal appears to go out of the focus in some cells contained within the image as not all junctions are on the same focal plane.

Tab. 1: TEER values (Ω x cm^2) in LLC-PK1 and Caco-2 cells grown on permeable supports under different serum conditions.

Cell line	TEER (10% FBS)	TEER (10% Prolifix)
LLC-PK1	100 ± 21	90 ± 34
Caco-2	637 ± 24	148 ± 36

ZO-1 in LLC-PK1 and Caco-2 cells. However, cells grown in the presence of FBS (C and D) showed invaginations and extrusions that are less evident when cells were grown in the presence of Prolifix (A and B).

3.4 Actin distribution

The cytoskeleton plays a role in the establishment of epithelial polarity (Wills et al., 1996). Actin filaments are components of cytoskeleton. Figure 3 shows the effects of the serum supplements, Prolifix (A and B) and FBS (C and D), on actin distribution in LLC-PK1 and Caco-2 cells. In both cell lines and under both culture conditions, actin was distributed in the cortical cytoplasm all around the periphery of the cells. Some differences in signal intensity were observed.

3.5 Cytotoxicity

Figure 4 shows the cytotoxic effect induced by $CdCl_2$ in LLC-PK1 and Caco-2 cells grown in medium supplemented with Prolifix. A significant LDH leakage was observed in Caco-2 cells at 5 µM $CdCl_2$; in LLC-PK1 cytotoxicity only started at the highest concentration tested (50 µM). In contrast, previous results obtained in our laboratory growing the cells in FBS showed that $CdCl_2$ induced significant cytotoxicity in Caco-2 cells at 25 µM (Boveri et al., 2004). LLC-PK1 responded similarly under both culture conditions (Gennari et al., 2003).

3.6 Alteration of barrier integrity

After treatment of Caco-2 and LLC-PK1 cells with $CdCl_2$ (0-50 µM) for 24 h, barrier integrity was monitored by measuring TEER and PCP. The results are shown in Figure 5. The damage induced by cadmium at epithelial barrier level was greater when cells were grown in medium supplemented with reagents of plant origin. For example, Caco-2 cells were more sensitive to cadmium both in decreasing TEER and increasing PCP. In addition, in the presence of FBS, both cell lines behaved similarly regarding both endpoints, however in the presence of Prolifix, Caco-2 cells were more sensitive.

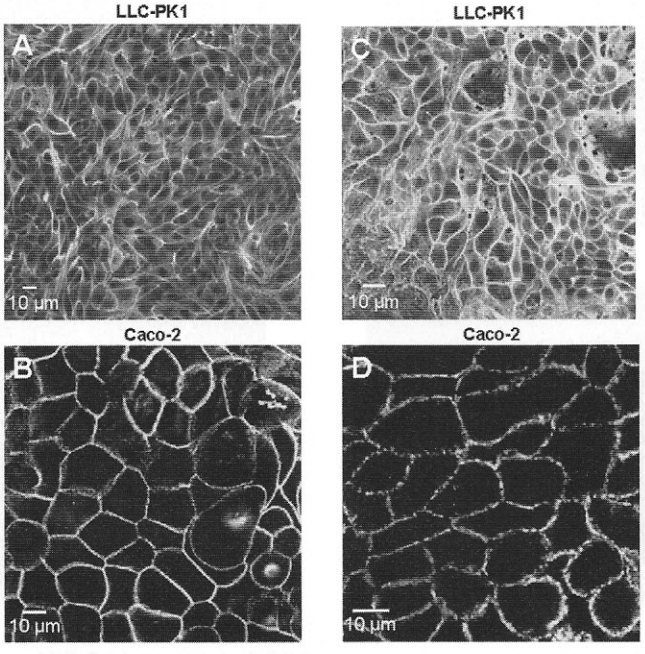

Fig. 3: Confocal fluorescence microscopy of actin microfilament staining in LLC-PK1 and Caco-2 cells cultured on permeable supports.
A and B represent cells grown in Prolifix-supplemented medium and C and D show cells grown in FBS-supplemented medium.

4 Discussion

The use of animal serum in cell culture is still a matter of contention and is of concern both to the scientific community and to animal welfare groups (Defrancesco, 1998; Jochems et al., 2002; van der Valk et al., 2004). There are ethical but also scientific reasons that have led scientists to look for alternatives to serum-supplemented media. In response to these concerns, companies have started to produce serum-free medium formulations and/or supplements to substitute for animal serum (www.Focusonalternatives.org.uk). Among the products available on the market, reagents derived from plants completely avoid the use of animal components. Prolifix is one of these reagents and it was the aim of our work to evaluate its usefulness to replace FBS in cell culture medium. We tested its use on two widely used epithelial cell lines, LLC-PK1 and Caco-2.

The use of Prolifix required an adaptation process during which the FBS concentration was gradually reduced and the Prolifix concentration was increased until a value 10% Prolifix was reached. Once the cells were cultured in the presence of Prolifix they grew slower (doubling time 72 h) compared to cells grown in the presence of FBS (doubling time 40 h). Serum provides micronutrients and growth factors, which may be absent in the serum-free medium used (Butler and Jenkins, 1989). In addition, it is known that epithelial cells require hormones to grow in serum-free media (Bjare, 1992). However, the exact composition of the supplemented medium used in this work was unknown since there were patented components in the medium formulation.

Cell detachment was always performed by trypsinisation, which complicated the manipulation of cells grown in Prolifix-supplemented medium since extra washes with PBS were required in order to completely remove the trypsin. This procedure could increase the risk of contamination during handling.

The formation of domes is considered a good indicator of a transporting epithelium when cells are grown on plastic supports (Gstraunthaler, 1988). The phase contrast morphological analysis performed showed that both cell lines were able to attach and to differentiate, forming a monolayer that maintained its transporting capacities. However, it is worth noting that the number of domes was higher when cells were grown in the presence of FBS. The integrity of the barrier was further analysed by measuring an endpoint, TEER, which is recognised as a valid parameter to assess barrier function of epithelial monolayers grown on permeable filters (Powell, 1981; Gstraunthaler et al., 1990; Pfaller and Troppmair, 2000; Kim, 2002). TEER values in LLC-PK1 cells did not differ whether cells were grown in medium supplemented with FBS or Prolifix. However, Caco-2 cells grown in the presence of Prolifix showed a lower TEER value than in the presence of FBS, indicating that Caco-2 cells were more sensitive to the presence of FBS than LLC-PK1 cells under our experimental conditions.

Besides the measurement of TEER as a good indication of barrier integrity, we examined the distribution of ZO-1, a specific tight junction protein, and actin, a component of the cytoskeleton which is associated with tight junctions (Anderson and Stevenson, 2000), in both cell lines under both culture conditions. Although a similar distribution of ZO-1 and actin in Caco-2 and LLC-PK1 cells was found, both proteins were less well focused to the cell-cell contact when cells were grown in Prolifix as compared to cells grown in FBS.

Previous work carried out in our laboratory has demonstrated the damage induced by $CdCl_2$ in both epithelial cell lines used when cultured in medium supplemented with FBS (Gennari et al., 2003; Boveri et al., 2004). We wanted to investigate how Prolifix influenced the effects of this environmental pollutant in both cell lines. $CdCl_2$ was more toxic when the cell lines were grown in medium supplemented with Prolifix than in medium supplemented with FBS. The effects on PCP were in agreement with the effects observed in TEER and also confirmed previous results obtained in our laboratory (Gennari et al., 2003; Boveri et al., 2004) and by others (Duff et al., 2002) showing that PCP is a less sensitive endpoint compared to TEER. Moreover, in Caco-2 cells, cytotoxicity was already significant at 5 µM, in contrast to cells grown in FBS where cytotoxic effects only started to occur at 25 µM $CdCl_2$ (Boveri et al., 2004). In LLC-PK1 cytotoxicity was observed at 50 µM under both culture conditions (Gennari et al.,

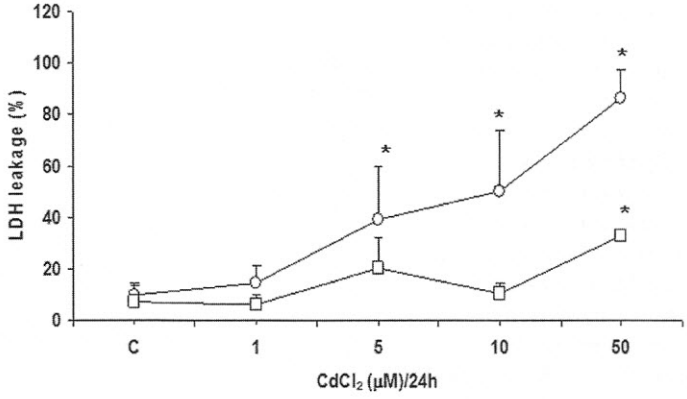

Fig. 4: LDH leakage after $CdCl_2$ treatment.
Caco-2 (circle) and LLC-PK1 (square) cells cultured on permeable supports and grown in medium supplemented with Prolifix were exposed to increasing concentrations of $CdCl_2$ (0-50 µM) for 24 h, then release of LDH into the medium was measured. Each point represents the mean ± SD of three independent experiments. *p < 0.05 versus control.

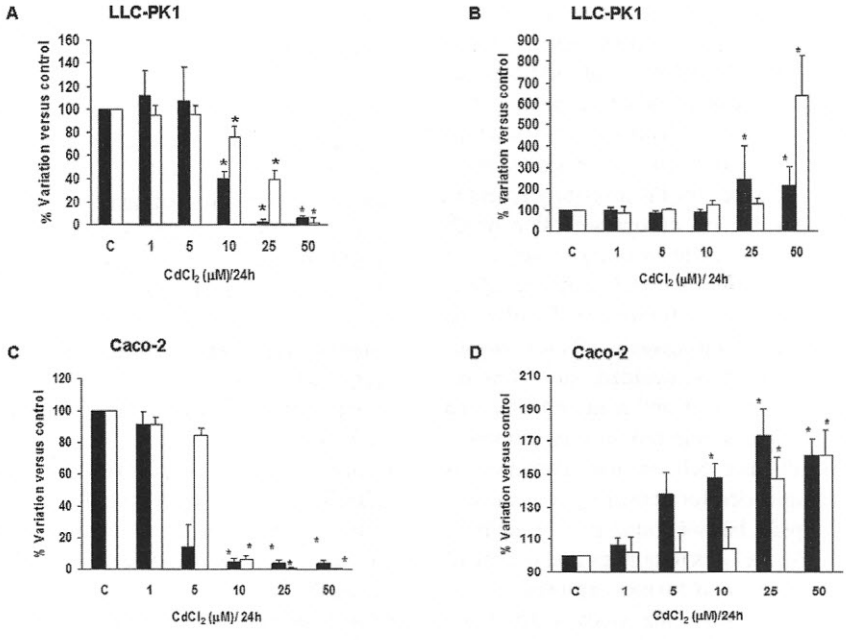

Fig. 5: Effects of $CdCl_2$ on TEER (A, C) and PCP (B, D) in Caco-2 and LLC-PK1 cells cultured on permeable supports.
Black bars represent cells grown in Prolifix-supplemented medium. White bars represent cells grown in FBS-supplemented medium. Each point represents the mean ± SD of three independent experiments. *p < 0.05 versus control.

2003). It is well known that proteins in serum can bind to chemicals thereby reducing their effective concentrations and therefore this could explain the differing toxic effects observed with Prolifix and FBS in the medium. Furthermore, the barrier characterisation showed clear differences when cells were cultured with Prolifix which could explain the different sensitivity observed.

Briefly, the results obtained demonstrate that Caco-2 and LLC-PK1 epithelial cells can be grown in medium supplemented with reagents of plant origin and in the absence of FBS. Cells can grow both on plastic and on permeable supports without coating. In addition, they develop a typical epithelial morphology and they maintain their transporting capacity as shown by the presence of domes. In both cases, an epithelial barrier is formed, although in the case of Caco-2 cells the barrier is less tight as compared to Caco-2 grown in the presence of FBS. All these data demonstrate that the use of reagents of plant origin allows the culturing of cells without FBS.

However, it should be pointed out that there were a number of problems resulting from the use of Prolifix. Firstly, the growth rates were slowed in both cell lines, which could pose a problem when the cultures are intended for screening purposes. Therefore, the addition of growth factors or other supplements to overcome these effects should be investigated. Secondly, it was difficult to freeze cells adapted to Prolifix-supplemented medium. Hence, cryopreservation needs further refinement in order to achieve a good survival rate and thus provide a stock of cells for future use. Thirdly, the use of trypsin to detach cells for subculturing should be avoided, since this requires extra steps and handling time and could increase the risk of contamination and influence cell survival. Papaine was recommended for detaching cells instead of trypsin, but we found it to be ineffective. We feel it is important that the use of these reagents is further explored.

In conclusion, the study carried out with the reagent of plant origin, Prolifix, gave us the opportunity to learn about the advantages and disadvantages of replacing FBS in the culture medium. Since other reagents derived from plant extracts are on the market (e.g. Epi-Life) we believe the results shown here will be useful for companies that are interested in producing these types of reagents in the future and for researchers who would like to culture their epithelial cells in serum-free medium. The results on the whole are promising, although more effort is clearly needed in order to optimise cell adaptation, cell attachment, growth rates, and freezing/thawing protocols.

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Correspondence to

Pilar Prieto, PhD
ECVAM, Institute for Health & Consumer Protection, JRC
Via Enrico Fermi 1
I-21020 Ispra (VA),Italy
phone +39-0332-785534
fax +39-0332-785336
e-mail: maria.prieto-pilar@jrc.it