

Development of an in vitro blood–brain barrier model—cytotoxicity of mercury and aluminum

Tarja Toimela,^{a,*} Hanna Mäenpää,^{a,b} Marika Mannerström,^a and Hanna Tähti^a

^a *University of Tampere, Medical School, 33014 University of Tampere, Tampere, Finland*

^b *Tampere University Hospital, Tampere, Finland*

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Abstract

In this study, in vitro blood–brain barrier (BBB) models composed of two different cell types were compared. The aim of our study was to find an alternative human cell line that could be used in BBB models. Inorganic and organic mercury and aluminum were studied as model chemicals in the testing of the system. BBB models were composed of endothelial RBE4 cell line or retinal pigment epithelial (RPE) cell line ARPE-19 and neuronal SH-SY5Y cells as target cells. Glial U-373 MG cells were included in part of the tests to induce the formation of a tighter barrier. Millicell CM filter inserts were coated with rat-tail collagen, and RBE4 or ARPE-19 cells were placed on the filters at the density of $3.5\text{--}4 \times 10^5$ cells/filter. During culture, the state of confluency was microscopically observed and confirmed by the measurement of electrical resistance caused by the developing cell layer. The target cells, SH-SY5Y neuroblastoma cells, were plated on the bottom of cell culture wells at the density of 100 000 cells/cm². In part of the studies, glial U-373 MG cells were placed on the under side of the membrane filter. When confluent filters with ARPE-19 or RBE4 cells were placed on top of the SH-SY5Y cells, different concentrations of mercuric chloride, methyl mercury chloride, and aluminum chloride were added into the filter cups along with a fluorescent tracer. Exposure time was 24 h, after which the cytotoxicity in the SH-SY5Y cell layer, as well as in the ARPE-19 or RBE4 cell layer, was evaluated by the luminescent measurement of total ATP. The leakage of the fluorescent tracer was also monitored. The results showed that both barrier cell types were induced by glial cells. Inorganic and organic mercury caused a leakage of the dye and cytotoxicity in SH-SY5Y cells. Especially, methyl mercury chloride could exert an effect on target cells before any profound cytotoxicity in barrier cells could be seen. Aluminum did not cause any leakage in the barrier cell layer, and even the highest concentration (1 mM) of aluminum did not cause any cytotoxicity in the SH-SY5Y cells. In conclusion, BBB models composed of RBE4 and ARPE-19 cells were able to distinguish between different toxicities, and ARPE-19 cells are thus promising candidates for studies of drug penetration through the blood–brain barrier.

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Introduction

Novel techniques in drug and chemical industry have allowed the emergence of a vast number of new drugs and chemicals in the market. There is a need to test these compounds in the most efficient way. In vitro models are becoming more and more valuable in evaluating the safety of chemicals discovered (Ball et al., 1995; Hartung, 2001; Roberts, 2001). One of the essential points is how well these new drugs gain entry into the brain, even when the target of

the drug is not the central nervous system. In vivo, the blood–brain barrier (BBB) isolates the brain from the systemic blood circulation and forms a restrictive barrier against toxins and foreign compounds (Risau and Wolburg, 1990). The BBB is formed by endothelial cells and partly by astrocytes. It is possible to isolate and culture the cell types of the natural BBB, and thus there are several in vitro cell culture models for studying barrier functions. Some of the models mimic the structure and functions of the normal BBB quite well, but the results are not necessarily easy to reproduce and compare. Endothelial microvessel cells isolated for barrier cultures often come from animal sources, usually from bovine or rat brains (Abbott et al., 1992; Audus and Borchardt, 1987; Begley et al., 1996; Raub et al., 1992; Rist et al., 1997). There is a need to frequently

* Corresponding author. University of Tampere, Medical School, 33014 University of Tampere, Medisiininkatu 3, Tampere, Finland. Fax: +358-3-215-6170.

E-mail address: Tarja.Toimela@uta.fi (T. Toimela).

start a new culture if one chooses to use primary cultures, because freshly isolated brain endothelial cells dedifferentiate quickly (Liebner et al., 2000; Panula et al., 1978). Consequently, cultures are different each time. To minimize variation between individual cultures, continuous cell lines, preferably of human origin, are valuable for establishing in vitro barrier models.

In vivo, glial cells form an important part of the blood brain barrier (Prat et al., 2001). In fact, most of the outer area of the endothelial cells is covered with foot processes of glial cells. Glial cells are metabolically active and control the access of many chemicals and metabolites into the brain (Pardridge, 1999). Glial cells induce the formation of a tight barrier cell layer in vivo (Janzer and Raff, 1987). Also in vitro, it has been shown that glial cells are important co-operators in forming the barrier (Abbruscato and Davis, 1999; Arthur et al., 1987; Hayashi et al., 1997; Shivers et al., 1988). High resistance tight junctions in blood–brain barrier models have been achieved by treating brain endothelial cells with a combination of astrocyte-conditioned medium and agents that elevate intracellular cAMP (Rist et al., 1997; Rubin et al., 1991; Yang et al., 2001). Cells treated this way exhibited low rates of paracellular leakage and fluid-phase endocytosis. Either glial cells or astrocyte-conditioned medium are obviously important factors to be included in in vitro barrier models.

In the present study, we compared rat brain endothelial cells with human retinal pigment epithelial (RPE) cells for barrier functions. The advantage of RPE cells is that they are part of the natural blood–retinal barrier and therefore their functions are similar to the BBB of the brain. They are able to form tight junctions when grown in vitro on permeable filters (Ban and Rizzolo, 1997; Rizzolo and Li, 1993). RPE cells are relatively stable and retain many of their characteristics in vitro. Our aim was to study how well RPE cells could be utilized in a novel BBB barrier model. Many RPE human cell lines are available, which makes it possible to build a barrier model constructed totally of human cells.

In developing the barrier systems presented here, the model chemicals chosen were inorganic and organic mercury and aluminum. They are all neurotoxic in their specific ways (Aschner and Aschner, 1990; Nicotera and Rossi, 1993; van der Voet et al., 1991). Inorganic mercury, as a polar ion, cannot easily penetrate the brain through an intact barrier. The neurotoxicity may result from interference with enzymes (Albrecht et al., 1994; Oliveira et al., 1994; Szumańska et al., 1993). Methyl mercury is more of environmental concern. It is almost completely absorbed into the bloodstream and distributed to all tissues. The brain is one of the tissues that accumulate methyl mercury a great deal (Hargreaves et al., 1985; Vahter et al., 1994; Warfvinge et al., 1992). In the mammalian metabolism, methyl mercury can be converted to inorganic mercury (Charleston et al., 1995; Pedersen et al., 1999). Mercury-induced cytotoxicity is well documented in different types of cells (Bohets et al., 1995; Bracken et al., 1984; Brawer et al., 1998; Bucio et al.,

1995; Imura et al., 1980; Kappus and Reinhold, 1994; Olivieri et al., 2000; Walther et al., 2002; Zierold, 2000). Aluminum neurotoxicity is more controversial (Savory et al., 1996). Aluminum has often been connected to neurodegenerative diseases, such as Parkinsonism dementia in Guam, Alzheimer's disease, dialysis encephalopathy, and amyotrophic lateral sclerosis (ALS) (Altschuler, 1999; Meiri et al., 1991; Shin et al., 1995; Yasui et al., 1991). There is ample evidence of aluminum toxicity in different studies, even though aluminum is not considered highly cytotoxic (Campbell et al., 2001; Dominguez et al., 1995; Guo and Liang, 2001; Levesque et al., 2000; Murphy et al., 1993; Sargazi et al., 2001; Suarez Fernandez et al., 1999).

We studied the cytotoxicity caused by inorganic and organic mercury and aluminum in in vitro BBB models. The models were composed of human ARPE-19 cell line or rat brain endothelial (RBE4) cell line grown on a microporous membrane filter forming a restrictive barrier, and neuronal cells as target cells. In the co-culture model glial cells, U-373 MG was grown on the opposite side of the membrane filter. After the exposure, cytotoxicity was evaluated in all cell layers.

Materials and methods

Materials

Cells. Human retinal pigment epithelial cell line ARPE-19 (cat no CRL-2302), human neuroblastoma cell line SH-SY5Y (cat no CRL-2266), and human glioblastoma cell line U-373 MG (cat no HTB-17) were obtained from American Type Culture Collection (ATCC). Rat brain endothelial RBE4 cell line was a generous gift from Dr. Michael Aschner, Winston-Salem, NC, USA.

Materials. Transparent Millicell-CM filters (12 mm \varnothing , pore size 0.4 μ m, membrane material: hydrophilic PTFE) were purchased from Millipore. All cell culturing plastics came from Nunc (Denmark). Black Cliniplate 96-wells, ATP Monitoring Reagent (6415000), and 0.1 M Tris–acetate buffer (6415400) were supplied by Thermo Labsystems (Finland).

Cell culture media. All cell culture media and supplements were obtained from Gibco (UK). Medium for ARPE-19 cell line was 1:1 Dulbecco's Modified Eagle Medium/Ham's F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic solution (AB–AM) containing 100 units/ml penicillin, 100 g/ml streptomycin, 250 ng/ml amphotericin B, and 1.2 g/l final concentration of sodium bicarbonate.

Medium for U-373 MG cell line was Minimum Essential Medium (MEM) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1% AB–AM, 1.5 g/l sodium bicarbonate, and 10% FBS.

SH-SY5Y cell line was grown in 1:1 MEM/Ham's F-12 Kaighn's Modification medium supplemented with 1% AB-AM, 10% FBS, 2 mM L-glutamine, and 0.1 mM nonessential amino acids.

For RBE4 cell line, the medium was 1:1 Alpha MEM/Ham's F10 medium supplemented with final concentration of 1 ng/ml of basic Fibroblast Growth Factor (bFGF), 10% FBS, 1% AB-AM, 25 mM HEPES, and 2 mM L-glutamine.

Methods

Cell culture. SH-SY5Y, ARPE-19, U-373 MG, and RBE4 cells were cultured in standard conditions (at 37 °C with 5% CO₂ in a humidified incubator) according to the instructions of the originator (ATCC or Dr. Aschner). The cells were subcultured after reaching 80% confluency. Passages of 35–38 (ARPE-19), 32–39 (SH-SY5Y), 189–191 (U-373 MG), and 5–7 (after obtaining RBE4) were used in the study.

Measurement of transmembrane resistance. The Ag/AgCl electrodes of Millicell-ERS were sterilized with 70% ethanol for 15 min. After the 15-min equilibration period in cell growth medium, the electrodes were positioned so that one electrode was inside the filter cup and the other in the outside liquid, and the resistance was read. Blank filter cups with no cells were included in the measurement. To get non-drifting readings, the electrodes had to be clamped using a stand. For calculation of the results in $\Omega \text{ cm}^2$, blank values were first subtracted, and then the resultant value was multiplied by the membrane area 0.6 cm² (in 12-mm Millicell CM filters).

Coating of filters. Millicell CM filter inserts (made of PTFE) were coated with 180 μl of rat-tail collagen (2.7 mg/ml in 0.1 N acetic acid/60% ethanol) and left to dry before the subculturing of cells. For a model with glial cells to be grown on the underside of the filter insert, the filter was coated on both sides.

Construction of barrier models. Human ARPE-19 RPE cells or rat endothelial RBE4 cells were placed on the transparent filters at the densities of $3.5\text{--}4 \times 10^5$ cells/filter. The total amount of medium in the filter cups was 400

Table 2

Transepithelial resistances of ARPE-19 and RBE4 barrier cell layers at confluency and the effect of glial cells in the co-culture with barrier cells

Resistance	ARPE-19		RBE4	
	No glia	With glia	No glia	With glia
Mean ($\Omega \text{ cm}^2$)	116.4	354.2	156	384.2
SEM	57.2	128.0	46.6	109.1
<i>t</i> test: no glia vs. with glia	<i>P</i> = 0.1318		<i>P</i> = 0.1417	

ARPE-19 and RBE4 barrier models with and without glial cells were compared statistically with *t* test.

μl . The cultures were incubated in a humidified atmosphere (+37 °C, 5% CO₂). In the glial co-culture model, after 1 day, the membrane was turned upside down, and 20000 glial U-373 MG cells were applied on the filter and let attach for some minutes (taking care not to let the cells dry). The filter was turned over again and the culturing continued, now with cells on both sides of the membrane filter. The development of confluency was microscopically observed and confirmed by the measurement of electrical resistance.

The target cells, SH-SY5Y neuroblastoma cells, were plated separately onto the bottom of the 12-well cell culture plates at the density of 100000 cells/cm², with 1.5-ml growth medium in the well. Filters with confluent barrier cells were placed on top of the SH-SY5Y cells. The medium was changed to one with low serum-content (1%), and the cultures were left to adapt for 1 day before the exposure to test chemicals.

Exposure. Stock solutions for aluminum and mercuric chloride were dissolved in distilled water, and these stock solutions were then sterilized by filtering. Methyl mercury chloride was dissolved in 100% ethanol. Different concentrations of mercuric chloride (0.1–100 μM), methyl mercury chloride (0.1–100 μM), and aluminum chloride (1–1000 μM) were added into the filter cups along with the fluorescent tracer fluorescein. The final solvent concentration did not exceed 0.25% in the filter cups. In control cultures, there were no barrier cells in filter cups; otherwise, the exposure was as explained above. As a positive leakage control, 1 mM EDTA was added into the filter cups to detach the barrier cell layer. Exposure time was 24 h, after which the viability in the SH-SY5Y cell layer, as well as that in the barrier cell layer cells, was evaluated by the luminescent measurement of total ATP. The actual concentrations that reached target wells were measured using the technique of cold vapor atomic absorption spectrophotometry by the Finnish Institute of Occupational Health.

Total ATP measurement. To measure the total ATP content of the cells, 5 μl of ice-cold 10% TCA was added into 100 μl of the culture medium to stop the cellular reactions. The filter cups were moved to unused empty wells and handled separately. In the glial co-culture

Table 1

Actual concentrations of mercury and aluminum in the bottom wells

Concentration (μM)	Mercury (μM)		Methyl mercury (μM)		Aluminum (μM)	
	ARPE-19	RBE4	ARPE-19	RBE4	ARPE-19	RBE4
	Control	0.03	0.03	0.03	0.03	0.2
1 (0.2) ^a	0.20	0.07	0.17	0.14	0.2	0.3
10 (2.1)	0.87	0.78	1.32	1.66	0.5	0.8
50 (10.5)	3.17	3.96	6.14	5.95		
100 (21.1)					0.6	1.5

^a Initial concentrations applied into filter cups, theoretical diluted concentrations in target wells in parenthesis.

model, before adding TCA, the glial cells were detached from the filter with trypsin treatment, and then treated separately.

After TCA treatment, the plates were shaken 600 rpm for 1 min and then put into -75°C freezer. The plates were thawed and frozen three times to release all ATP from the cells. Then, 25 μl of thawed cell suspension was moved to Black Cliniplate 96-well microtiter plate, in triplicates from every well. Then, luciferase reagent (Thermo Labsystems) and Tris–acetate buffer were combined in ratio 1:5 (e.g., 1 + 4 ml), and 100 μl of the combined reagent was added into all wells. Luminescence was measured with Thermo Labsys-

tems Luminoskan Ascent luminometer with the integration time of 1000 ms. Luminescence did not show any significant bleaching during the measurement (decay rate $< 2\%/min$).

Fluorescein leakage. A sample of the growth medium in the bottom wells was collected after the 24-h incubation time. The fluorescence was monitored with Thermo Labsystems Fluoroskan Ascent fluorometer at an excitation wavelength of 485 nm and emission wavelength of 538 nm. All reagents and media included were tested for fluorescence and found negative. The bleaching of fluorescein was minor and did not cause problems during the measurement.

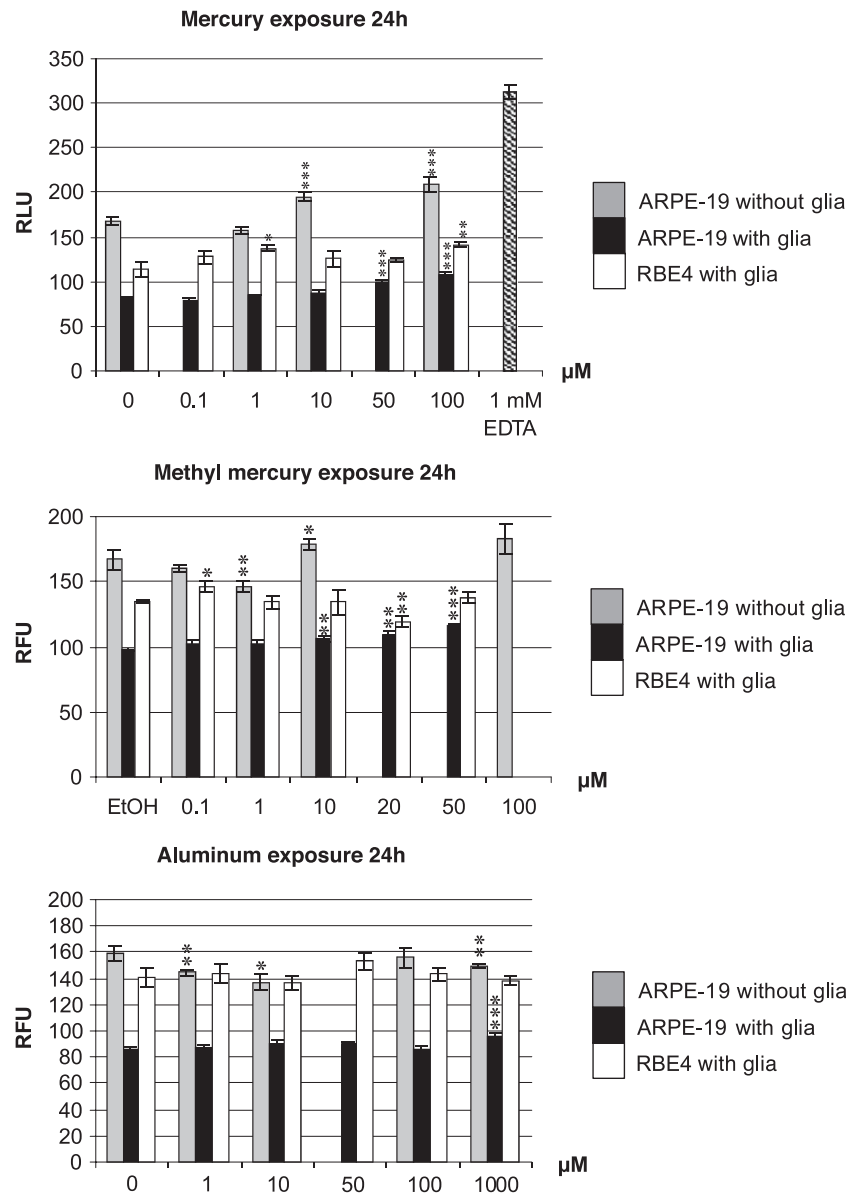


Fig. 1. Leakage of fluorescein after 24 h of exposure to mercury, methyl mercury, and aluminum. Mercury (0.1–100 μM), methyl mercury (0.1–100 μM), and aluminum (1–1000 μM) were applied to upper wells and fluorescein leakage measured (485 nm excitation, 538 nm emission) from the bottom wells after the exposure. RFU = Relative Fluorescence Units. Each point represents the mean \pm SEM of three to four independent experiments. The significances compared to the controls with *t* test are expressed with asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Statistics. Each test was repeated three to four times. The means and SEM of independent cultures were calculated at each concentration. The statistical significances of the differences between controls and exposed cultures were calculated by ANOVA and by Student's *t* test at each test concentration by using Graph Pad Prism 3.0 software.

Results

The purpose of this study was to develop and test a new blood–brain barrier model composed of retinal pigment epithelial cell line ARPE-19. ARPE-19 barrier tests were performed in parallel with a barrier made of brain endothelial cell line RBE4. First, ARPE-19 cells were cultured without glial cells. To improve the method, in the final version, barrier cells were cultured inside membrane filter cups, with the glial cells on the reverse side of the filter and the neuronal SH-SY5Y cells as target cells on the bottom of the wells.

The test chemicals mercury, methyl mercury, and aluminum chloride were added into the filter cups, and the exposure to target cells was only through the barrier cell layer. It is notable that concentrations in the filter cups (concentrations that affect directly the barrier cells) were considerably higher than those that could reach the target cells in the wells. Theoretically, if the test compounds were equally divided without the restriction of the barrier, the dilution would be 4.75 times. In reality, barrier cells are bound to hinder the passage of chemicals.

Concentrations in the wells were generally much lower than those theoretically calculated (Table 1), which indicates the function of barrier cells. There was no apparent difference in the transport of chemicals through the two barrier types. Methyl mercury was more permeant than inorganic mercury, in agreement with the earlier findings. With aluminum, the leakage was not greatly increased at higher concentrations.

Trans epithelial resistance

To measure transepithelial resistance, two electrodes were placed on the opposite sides of the membrane filter, and transepithelial resistance caused by the developing cell layer was measured with Millicell ERS meter. We found that the results at confluency were highly variable (see Table 2). To get any stable results, the electrodes had to be clamped to a certain position. If culturing was still continued after the peak value, the resistance started gradually to decrease, and no higher values were reached. RBE4 cells gave slightly higher values than ARPE-19 cells. Both cells were inducible with glial cells.

Fluorescein leakage

Fluorescein dextrane (MW ~70 000) and fluorescein were tested for a marker of leakage. A two-sided coating

of filters with rat-tail collagen decreased the leakage of fluorescein dextrane to 39%, compared with non-coated filters. Coating affected fluorescein leakage less. After similar coatings, the values were 78% of control. Fluorescein was chosen for a marker to avoid mechanical obstruction of the dye.

The results showed that glial cells made the ARPE-19 barrier more resistant to leakage (gray and black columns in Fig. 1). Without glial cells, the leakage was also highly variable. The level of leakage of ARPE-19 barrier was less than that of RBE4 barrier. The tested concentrations of mercury and methyl mercury caused major damage to the barrier at high concentrations, aluminum toxicity was only apparent in 1 mM concentration.

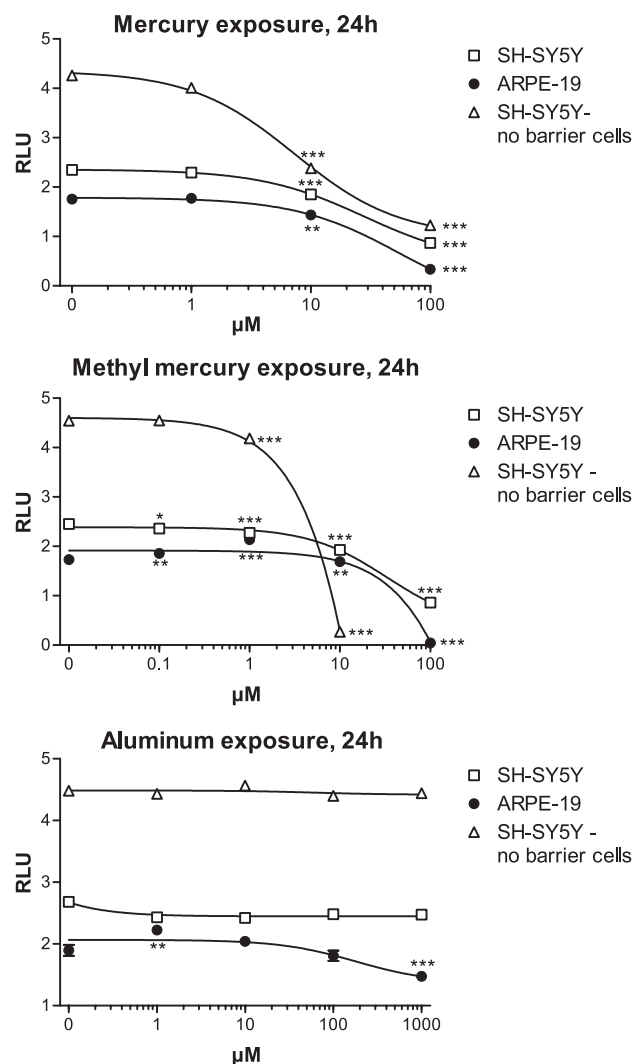


Fig. 2. ARPE-19 barrier model without glial cells. Cytotoxicity of mercury (0.1–100 μM), methyl mercury (0.1–100 μM), and aluminum (1–1000 μM) measured in different cell layers and cytotoxicity in SH-SY5Y cells when no barrier cells were present. Cytotoxicity was evaluated with the measurement of total ATP. Each point represents the mean ± SEM of three to four independent experiments. The significances compared to the controls with *t* test are expressed with asterisks: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. RLU = relative light units.

Cytotoxicity

The amount of intracellular ATP correlates well to cell number and is a sensitive marker of cytotoxicity (Lundin et al., 1986). BBB models with ARPE-19 cells and RBE4 gave quite similar cytotoxicity patterns (Figs. 3 and 4). The total ATP level was different in these two cell lines, but the cytotoxicity started at similar concentrations. In ARPE-19 model without glial cells (Fig. 2), the cytotoxicity appeared earlier than in the models with glial cells. This shows that the barrier is tighter when glial cells are present. Also, when the growths of the target cells, SH-

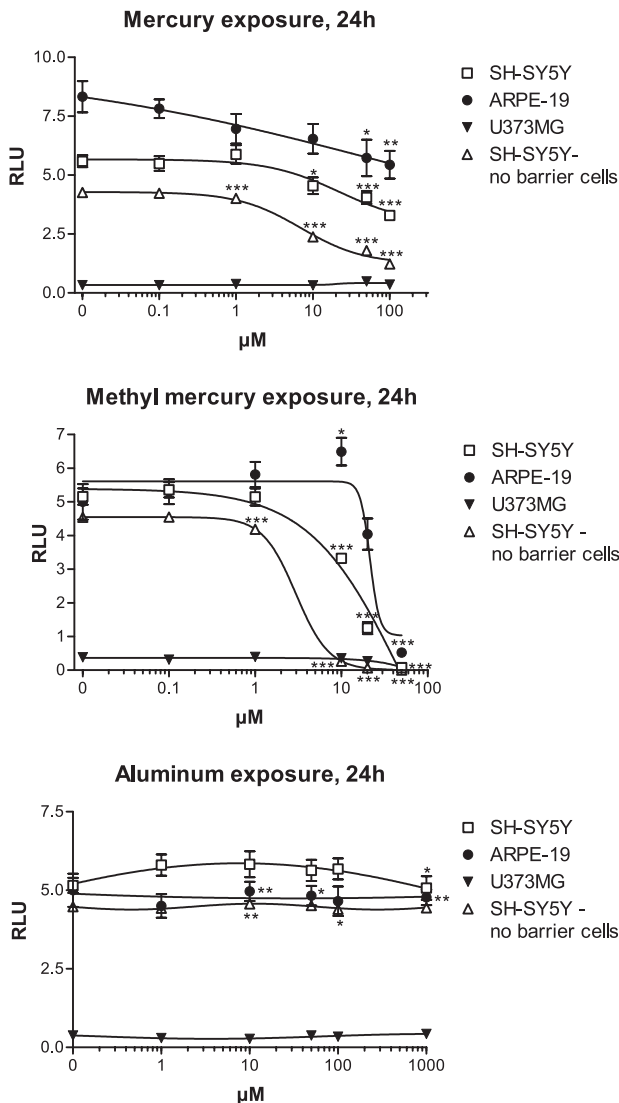


Fig. 3. ARPE-19 barrier model with glial cells. Cytotoxicity of mercury (0.1–100 μM), methyl mercury (0.1–100 μM), and aluminum (1–1000 μM) measured in different cell layers and cytotoxicity in SH-SY5Y cells when no barrier cells were present. Cytotoxicity was evaluated with the measurement of total ATP. Each point represents the mean ± SEM of three to four independent experiments. The significances compared to the controls with *t* test are expressed with asterisks: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. RLU = relative light units.

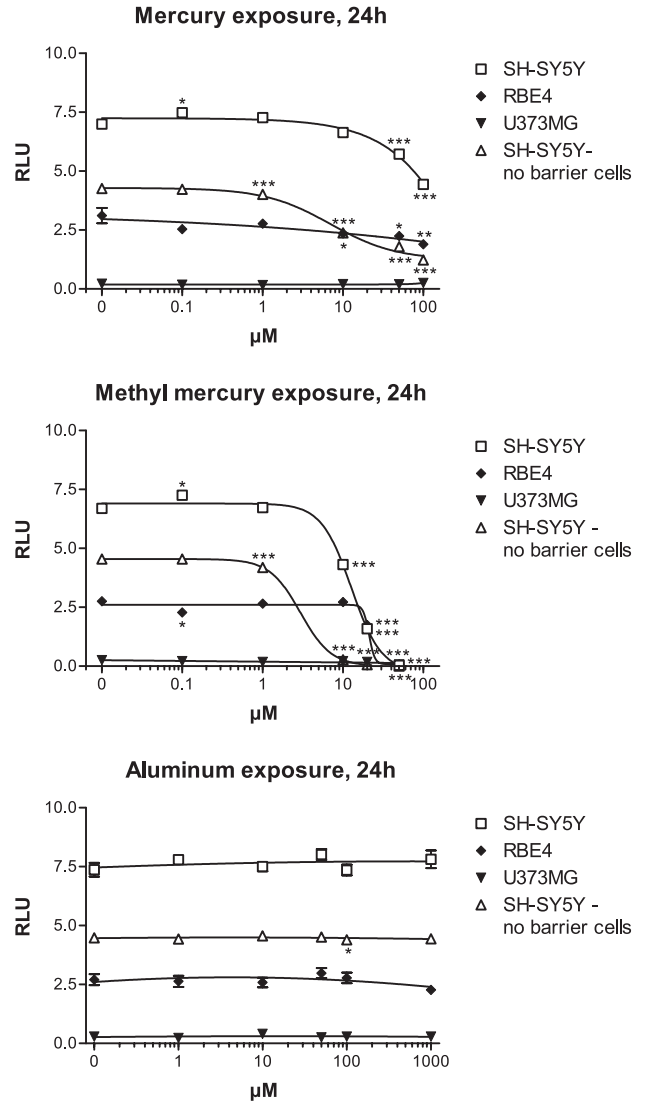


Fig. 4. RBE4 barrier model with glial cells. Cytotoxicity of mercury (0.1–100 μM), methyl mercury (0.1–100 μM), and aluminum (1–1000 μM) measured in different cell layers and cytotoxicity in SH-SY5Y cells when no barrier cells were present. Cytotoxicity was evaluated with the measurement of total ATP. Each point represents the mean ± SEM of three to four independent experiments. The significances compared to the controls with *t* test are expressed with asterisks: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. RLU = relative light units.

SY5Y cells, were compared, clear growth induction was shown when glial cells were present (compare *SH-SY5Y* and *SH-SY5Y-no barrier cells* in Figs. 3 and 4). There was no such induction when only ARPE-19 cells were present (Fig. 2), indicating the effect was indeed caused by glia.

Two mercurial compounds showed different toxicity. Methyl mercury could pass through the barrier earlier and exert an effect on target cells before the barrier was damaged. At 10 μM concentration, ARPE-19 and RBE4 cells were still totally viable, but cytotoxicity was seen in SH-SY5Y cell layer. Inorganic mercury-caused cytotoxic-

Table 3
Comparison of different barrier models on cytotoxicity caused by mercury, methyl mercury, and aluminum

P values	ARPE-19 barrier vs. RBE4 barrier			ARPE-19 barrier with or without glia	
	Neuronal cells	Barrier cells	Glia cells	Neuronal cells	Barrier cells
Mercury	0.5729	0.8251	0.109	0.6241	0.7196
Methyl mercury	0.9814	0.4938	0.7247	0.5278	0.8743
Aluminum	0.2638	0.5234	0.3865	0.0021*	0.551

Results from each cell types were collected and normalized in relation to control values (control = 100%). Data from ARPE-19 cells were compared with *t* test with those of RBE4 cells. Similar comparison was made for data from ARPE-19 cells with and without glial cells.

**P* < 0.01.

ity in the barrier cell layer was followed by toxicity in the target cell layer more precisely. Aluminum failed to give any cytotoxicity in target cells at these concentrations, although some effects could be seen in ARPE-19 cells (Fig. 2).

Statistical analyses

Each barrier model was compared with each other to find out if they give statistically similar results (Table 3). First, the results were normalized so that control values were 100% to make it possible to compare different values. The comparison was made using one-way ANOVA and *t* test. We found no statistical difference between the ARPE-19 and RBE4 barrier models. When two different ARPE-19 barriers were compared, only aluminum exposure showed difference in SH-SY5Y cells.

Discussion

In this study, blood–brain barrier models composed of two different cell types were compared. The aim of our study was to find an alternative human cell line that could be used in barrier models (Takahashi et al., 1990). RBE4 cells are widely used in blood–brain barrier studies (Begley et al., 1996; Durieu Trautmann et al., 1993; Roux et al., 1994). RBE4 cells are able to form a very dense monolayer with high electrical resistance, and they are inducible with glial factors (Yang et al., 2001). Their drawback is that they are of rat origin. Our group has earlier worked with retinal pigment epithelial (RPE) cells (Mäenpää et al., 1997; Mannerström et al., 2001; Toimela and Tähti, 2001; Toimela et al., 1995, 1998). Although RPE cells are not endothelial cells of brain microvessels, one of their main functions is to form barrier structures (the blood retinal barrier) inside the eye. Many of their characteristics suggest they are suitable for forming an *in vitro* barrier. These cells are of neural origin, relatively stable in culture, and form tight junctions when cultured on microporous filters (Giordano et al.,

1997). Pig primary RPE cells are easy to isolate, but more importantly, several human RPE cell lines are available. Because especially human barrier models are valuable, we were interested in testing the suitability of human continuous RPE cell line ARPE-19 for barrier functions (Bridges et al., 2001; Dunn et al., 1996, 1998; Philp et al., 2003). ARPE-19 cell line forms polarized epithelial monolayers on porous filter supports.

There are indications that glial cells induce the formation of a tighter barrier, not only *in vivo* but also *in vitro* (Scism et al., 1999; Stanness et al., 1997). Therefore, we added metabolically active human U-373 MG glial cells to our barrier model (Dunlop et al., 1999; Klegeris et al., 1997; Sultana et al., 1998; Zhou and Skalli, 2000). In our model, glial cells were kept on the other side of the membrane filter separated from barrier cells. The pore size of the membrane was 0.4 μ m, so the cells were not able to invade the filter, but chemical contact was possible. The filter material we chose after several tests was PTFE. We abandoned otherwise suitable polycarbonate filters, mainly because of their poor optical quality. By visual inspection, we could detect if there were plaques without cells. The plaques or empty places could not be detected solely with the measurement of transepithelial resistance. The formation of empty cell-free plaques was initially a problem, because these empty places were not easily filled up during the culture, and the cells tended to curl up at the edges of the plaques, not spreading throughout the empty space. The problem was solved by increasing the initial cell density and by modifying the coating procedure of the filters. There are multiple choices for choosing membrane coating materials (Tilling et al., 1998). We ended up with rat-tail collagen in our tests. There are commercially coated filters available, but our demand for turnable filter cups reduced the choice. We built our model in 12-well plates because turning the filter was impossible in smaller wells. The turning has to be done under medium, otherwise air bubbles will appear inside the cups.

The coating with collagen lowered the permeability of the membrane. This was shown when the transfer of tracer molecules was studied. The purpose of the tracer was to help to evaluate how easily the test compound can go through the barrier cell layer during the exposure. A low molecular weight tracer was chosen to avoid mere physical obstructions of the marker dye, because the molecular weight had a clear effect on the tracer permeability (van Bree et al., 1988), especially when the membrane was coated on both sides. Fluorescein-dextrane of a molecular weight of 70 000 was almost blocked by the coated membrane, even when there were no cells yet. Fluorescein, on the contrary, could go through the coated membrane more easily.

Major problems in verifying the barrier integrity were confronted with the measurement of electrical resistance. Other groups (Hurst and Clark, 1999; Tilling et al., 1998) have reported high transmembrane resistances with different

cells. In our experience, the results were varying and highly dependent on how the measurement was performed. To get sound results, the electrodes had to be stabilized to a certain position in respect to the surface of the liquid and the filter cup. The highest resistance we found for RBE4 cells was $384 \pm 109 \Omega \text{ cm}^2$, and for ARPE19 cells, $354 \pm 128 \Omega \text{ cm}^2$. Glial cells seemed to increase the resistance, in agreement with the findings of other groups (Abbruscato and Davis, 1999; Naassila et al., 1996; Nam et al., 1996; Roux et al., 1994; Tao Cheng et al., 1987).

There are several possibilities as to how the compound can go through the barrier. In this first phase of developing a new barrier model, we merely wanted to see how well our model can differentiate compounds of different toxicity, not the actual route of penetration, may it be paracellular or vesicular. With this in mind, we chose compounds that differed in cytotoxic potency: mercury, methyl mercury, and aluminum. They would penetrate the barrier each in a different way, and as expected, the three compounds showed different toxicity patterns. There was even a difference between the two mercury compounds. Methyl mercury was not very toxic to barrier cells but could go through the barrier and affect the neural cells. On the contrary, inorganic mercury was toxic to both barrier cells and neural cells. Aluminum was not toxic to neural cells, although some toxicity could be seen in barrier cells. The differences between RBE4 and ARPE-19 barriers were not large, which will encourage us to further studies on ARPE-19 cells. Some results were even better for ARPE-19 cells. Glial cells seemed to induce both cell types to form a tighter barrier. This study is continuing with the investigation of the actual penetration mechanisms through the barrier cells, and drugs with different mechanisms will be used. A further aim will probably be to simplify the setting up of the system to make it more suitable for larger-scale toxicity studies.

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