

## Rat Brain Endothelial Cell Lines for the Study of Blood–Brain Barrier Permeability and Transport Functions

Françoise Roux<sup>1,3</sup> and Pierre-Olivier Couraud<sup>2</sup>

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### SUMMARY

1. In vitro models of the BBB have been developed from cocultures between bovine, porcine, rodent or human brain capillary endothelial cells with rodent or human astrocytes. Since most in vivo BBB studies have been performed with small laboratory animals, especially rats, it is important to establish a rat brain endothelial (RBE) cell culture system that will allow correlations between in vitro and in vivo results. The present review will constitute a brief description of the best characterized RBE cell lines (RBE4, GP8/3.9, GPNT, RBEC1, TR-BBBs and rBCEC4 cell lines) and will summarize their recent and important contribution to our current knowledge of the BBB transport functions and permeability to blood-borne solutes, drugs, and cells.

2. In most cases, primary cultures of RBE cells were transduced with an immortalizing gene (SV40 or polyoma virus large T-antigen or adenovirus E1A), either by transfection of plasmid DNA or by infection using retroviral vectors. In one case however, the conditionally immortalized TR-BBB cell line was derived from primary cultures of brain endothelial cells of SV40-T-expressing transgenic rats.

3. All cell lines appear to have an endothelial morphology. The absence of foci formation would mean that the cells are not transformed. The endothelial origin is shown by the expression of Factor VIII-related antigen. Immortalized RBE cells express all the enzymes and transporters that are considered as specific for the blood–brain barrier endothelium, with similar characteristics to those expected from in vivo analyses, but at a significantly lower level. Some RBE cell lines are responsive to astroglial factors, such as RBE4 cells, rBEC4, and TR-BBB cells. None of the immortalized RBE cell lines appear to generate the necessary restrictive paracellular barrier properties that would allow to use them in transendothelial permeability screening.

4. RBE cell lines have been used to demonstrate that transporters such as organic cation transporter/carnitine transporter, serotonin transporter, and the ATA2 system A isoform are expressed in rat brain endothelium. When the transporter is shown to be expressed with the same properties in the immortalized RBE cells as in vivo, regulation studies may be initiated even if the transporter is down-regulated. Pharmacological applications have been proposed with well-characterized transporters such as monocarboxylic acid transporter-1, large neutral amino acid transporter-1, nucleoside carrier systems, and P-glycoprotein. RBE cell monolayers have also been used to investigate the mechanism of the transendothelial

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<sup>1</sup>INSERM U26, Hôpital Fernand Widal, 75010 Paris, France.

<sup>2</sup>Département Biologie cellulaire, Institut Cochin INSERM U567–CNRS UMR8104, 22 rue Méchain, 75014 Paris, France.

<sup>3</sup>To whom correspondence addressed at CNRS UMR7157, Hôpital Fernand Widal, 200 rue du Faubourg Saint-Denis, 75 475 Paris Cedex 10; e-mail: francoise.roux@fwidal.inserm.fr.

transport of large molecules, such as immunoliposomes or nanoparticles, potentially useful as drug delivery vectors to the brain.

5. RBE4 and GP8 cell lines have been extensively used to demonstrate that intercellular adhesion molecule-1 (ICAM-1) engagement in brain endothelial cells triggers multiple signal transduction pathways. Using functional assays, it was established that ICAM-1 signaling is intimately and actively involved in facilitating lymphocyte infiltration.

6. Several RBE cell lines have been described, which constitute tentative *in vitro* models of the rat BBB. The major limitation of these models generally appears to be due to their relatively high paracellular permeability to small molecules, thus limiting their use for permeability studies. The strategies developed for the production of these RBE cell lines will enable the characterization of still more efficient permeability models, as well as the immortalization of human brain endothelial cells.

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**KEY WORDS:** blood–brain barrier; rat; *in vitro* model; cell culture; brain capillary endothelial cell; immortalized cell line; permeability; transporter; efflux pump.

## INTRODUCTION

A great deal of information is now available concerning the structure and function of the blood–brain barrier (BBB) *in vivo*. However, to understand the molecular, membrane, and cellular mechanisms, it is necessary to work on preparations where it is possible to apply the techniques of cell biology and physiology at the single cell level. For this reason, several *in vitro* preparations of brain endothelium have been developed, with the long-term aim of establishing an isolated BBB model.

The BBB is constituted by the brain microvessel endothelial cells (BMECs) linked to each other by tight junctions, with little capacity for pinocytosis and few fenestrations. The BBB is considered as a three-cell archetype comprising the cerebrovascular endothelial cells, the astrocytes, and the supporting pericytes. The role of neurons for the full expression of the BBB phenotype must not be ignored. The important contribution of the astrocytes toward BBB differentiation has been made apparent from the numerous *in vitro* studies of BMECs in culture whose phenotypic expression has shown to be responsive to astrocyte cocultures.

*In vitro* models of the BBB have been developed from cocultures between bovine, porcine, rodent, or human BMECs with rodent or human astrocytes (Cecchelli *et al.*, 1999; Gaillard *et al.*, 2001; Kis *et al.*, 1999; Mégard *et al.*, 2002). However, a number of drawbacks still limit the extensive use of these models in basic research as well as in drug-screening processes: (1) Their use is time consuming and expensive, which may hamper their routine use in nonexpert laboratories, (2) BMECs rapidly de-differentiate *in vitro*, losing the characteristics of BBB endothelial cells after a few passages in culture, which limits their use for biochemical or pharmacological studies, (3) In a primary culture, it is difficult to eliminate all nonendothelial cell contaminants (pericytes, leptomeningeal cells, smooth muscle cells). This is not a major problem for uptake studies, where the results will reflect the activity of the majority endothelial cell type. However, when contaminating cells occupy space within the monolayer, they frequently cause a hole in the layer, because the endothelial cells appear to be unable to grow over them. This becomes a limitation for studies of transendothelial permeability and transport. Cloning or magnetic sorting of BMECs cannot be considered as a solution to that problem when BMEC lifespan is quite limited, which is the case with rat BMECs.

However, since most in vivo BBB studies have been performed with small laboratory animals, especially rats, it is important to establish a rat brain endothelial (RBE) cell culture system that will allow correlations between in vitro and in vivo results.

In order to address these drawbacks, numerous efforts have been made over more than 10 years to establish immortalized RBE cell lines which retain a stable phenotype in culture reminiscent of BBB endothelium in vivo and may thus limit the need for large numbers of animals used for in vivo experiments. The present review will constitute a brief description of the best characterized RBE cell lines (RBE4, GP8/3.9, GPNT, RBEC1, TR-BBBs and rBCEC4 cell lines) and will summarize their recent and important contribution to our current knowledge of the BBB transport functions and permeability to blood-borne solutes, drugs, and cells.

### IMMORTALIZED RBE CELL LINES

A number of academic laboratories have reported the generation of immortalized brain capillary endothelial cell lines, among which the RBE cell lines reported so far are listed in Table I: For a review on cell lines from other species, see Gumbleton and Audus (2001).

### ISOLATION OF THE DIFFERENT IMMORTALIZED RBE CELL LINES

In most cases, primary cultures of RBE cells were transduced with an immortalizing gene (SV40 or polyoma virus large T-antigen or adenovirus E1A), either by transfection of plasmid DNA or by infection using retroviral vectors. In one case however, the conditionally immortalized TR-BBB cell line was derived from primary cultures of brain endothelial cells of SV40-T-expressing transgenic rats.

All the cell lines were selected on the basis of endothelial morphology, growth capacity, and expression of endothelial cell markers.

#### Immortalization by Transduction of the Immortalizing Gene

*RBE4 Cells* (Couraud *et al.*, 2003; Roux *et al.*, 1994)

RBE cells were isolated and cultured by the Hughes and Lantos method (1986) with some modifications (Roux *et al.*, 1989). RBE cells were transfected after two

**Table I.** Immortalized Rat Brain Capillary Endothelial Cell Lines

Cell line name	Immortalized gene	Reference
RBE4	Adenovirus E1A gene	Roux <i>et al.</i> (1994)
GP8/3.9	SV40 T-antigen	Greenwood <i>et al.</i> (1996)
GPNT	SV40 T-antigen	Regina <i>et al.</i> (1999)
RBEC1	SV40 T-antigen	Kido <i>et al.</i> (2000)
TR-BBBs	SV40 T-antigen	Terasaki and Hosoya (2001)
rBCEC4	Polyoma virus large T-antigen	Blasig <i>et al.</i> (2001)

passages by the calcium phosphate coprecipitation procedure with the plasmid pE1A/neo. The plasmid pE1A/neo carries the E1A region of Adenovirus 2 and the neomycin-resistance gene for selection by resistance to G418. After transfection, cells were grown in the selective medium containing 300  $\mu\text{g}/\text{mL}$  of G418.

*GP8/3.9 Cells* (Couraud *et al.*, 2003; Greenwood *et al.*, 1996)

A replication deficient SV40 retroviral vector was transfected into a fibroblast retroviral packaging cell line to create a producer cell line (SVU19.5). The retroviral vector encodes a temperature sensitive (tsa58), non-SV40-origin binding mutant of the large T-antigen and the neomycin-resistance gene for selection by resistance to G418. The conditioned medium of SVU19.5 cells containing retroviral particles was added to primary cultures of RBE cells in the presence of polybrene for 4 h at 37°C. The GP8 parent cell line was isolated through selection in G418.

*GPNT Cells* (Regina *et al.*, 1999)

The GPNT cell line was derived from the parental GP8/3.9 cell line by lipofectin-mediated transfection with pcDNA3-RSV containing the puromycin-resistance gene. An individual clone designated GPNT (for GP8 and the company NeuroTech S.A.) was selected on the basis of its endothelial morphology, growth capacity, and expression of endothelial cell markers. Cells were further incubated in selective media containing 5  $\mu\text{g}/\text{mL}$  of puromycin.

*RBEC1* (Kido *et al.*, 2000)

Primary cultures of RBE cells were immortalized by transfection of recombinant plasmids containing origin-defective SV40 gene, SVori-8–16. Actively growing cells were inoculated onto a 96 well culture plate at low density and the clone designated RBEC1 was selected.

*rBCEC4* (Blasig *et al.*, 2001)

RBE cells were transfected after two passages by the calcium phosphate coprecipitation procedure with the vector pCMVLT2 containing immortalizing genes of polyoma virus: large T-antigen. A proliferation crisis occurred between passages 5 and 11, afterwards, rBCEC4 showed high proliferation at low seeding densities.

**Establishment of Conditionally Immortalized RBE Cell Lines**

*TR-BBBs* (Terasaki and Hosoya, 2001)

Transgenic rats (Tg rats) harboring the temperature sensitive simian virus 40 (ts SV 40) large T-antigen have several advantages for establishing immortalized cell lines. Ts SV 40 large T-antigen gene is stably expressed in all tissues and remains in

the inactive form at 37°C. Cultured cells can be easily immortalized by activation of ts SV 40 large T-antigen at 33°C.

RBE cells isolated from Tg rats were cultured at 37°C during the first 48–72 h to allow cells to attach themselves to the dish and were subsequently cultured at 33°C. Following two or three passages, cells were cloned from a single cell by colony. A total of 11 immortalized RBE cell lines (TR-BBBs) were obtained from three Tg rats. TR-BBB13 exhibited the fastest growth at 33°C (permissive temperature).

### **CRITERIA OF SELECTION AND PHENOTYPIC CHARACTERIZATION OF THE IMMORTALIZED RBE CELL LINES**

Immortalized RBE cell lines were selected on the basis of the following criteria (Couraud *et al.*, 2003):

- They exhibit a nontransformed phenotype.
- They express a whole set of endothelial cell markers.
- They express BBB specific properties, either constitutive or inducible by astrocyte factors.

#### **Evidence of Nontransformed Phenotype**

Immortalized cell lines may be selected if they exhibit a nontransformed phenotype. For example, the RBE4 cell line exhibits such a nontransformed phenotype on the basis of the following observations:

- Formation of regular contact-inhibited monolayers of cells.
- Dependence of proliferation on the presence of appropriate cell substrate (collagen), serum components, and growth factors (basic fibroblast growth factor).
- Absence of proliferation in soft agar.
- Absence of tumors in athymic nude mice following subcutaneous or intrathecal injections.

#### **Expression of Endothelial Cell Markers**

Immortalized brain endothelial cells were selected for their expression of several endothelial cell markers. The endothelial markers which have been characterized for the different immortalized RBE cell lines are listed in Table II. All cell lines appear to have an endothelial, either cobblestone or spindle shaped morphology. The absence of foci formation would mean that the cells are not transformed. The endothelial origin is shown by the expression of Factor VIII-related antigen. In some lines, angiotensin-converting enzyme (ACE) and Griffonia simplicifolia expression, as well as acetylated low-density lipoprotein (AcLDL) uptake have been detected. The expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), as well as the release of nitric oxide (NO), is



enhanced after induction by inflammatory cytokines, such as interleukin-1- $\beta$  (IL-1 $\beta$ ) or tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) (Durieu-Trautmann *et al.*, 1993, 1994; Romero *et al.*, 2003).

### Expression of BBB Specific Properties

A set of BBB specific markers were expressed by the immortalized RBE cells, either constitutively or after induction by astrocyte factors. They are listed in Tables III and IV.

Immortalized RBE cells express all the enzymes and transporters that are considered as specific for the blood-brain barrier endothelium, with similar characteristics to those expected from *in vivo* analyses. For example, Km values determined for the glucose transporter-1 (GLUT1) and large neutral amino acid transporter (LAT1) in RBE4, RBEC1, or TR-BBB cell lines appear quite similar to *in vivo* evaluations (Kido *et al.*, 2000; Regina *et al.*, 1997; Reichel *et al.*, 2000; Terasaki et Hosoya, 2001). Also, the 55-kDa GLUT1 isoform, specific for brain endothelium *in vivo*, is expressed in RBE4 and TR-BBB cells (Hosoya *et al.*, 2000; Regina *et al.*, 2001).

As documented for primary cultures of brain endothelial cells, some RBE cell lines are responsive to astroglial factors, such as RBE4 cells and rBEC4 [increase in  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) and alkaline phosphatase (ALP) activity], and TR-BBB cells [increase in the expression of occludin and *mdr1a* P-glycoprotein (*Pgp*) gene] (Blasig *et al.*, 2001; El Hafny *et al.*, 1996; Hosoya *et al.*, 2002; Roux *et al.*, 1994). Lower sucrose permeability coefficients are obtained when RBE4 cells and rBEC4 cells are cocultured with astrocytes (Blasig *et al.*, 2001; Rist *et al.*, 1997). The expression of Ca<sup>2+</sup>-dependent cell-cell-adhesion molecule E-cadherin is increased in RBE4 cells cultured with astrocyte-conditioned medium (Yang *et al.*, 2001). In cocultures of RBE4 and cortical neuronal cells, neurons induce endothelial cells to synthesize and sort occludin to the cell periphery (Cestelli *et al.*, 2001).

The GPNT cells are more differentiated when they are cultured in the presence of dexamethasone (distribution of actin and cortactin to the cell periphery, decrease in sucrose permeability and increase in *Pgp* activity) (Regina *et al.*, 1999; Romero *et al.*, 2003).

It has to be mentioned, however, that some BBB-specific transporters or enzymatic activities are expressed in most RBE cell lines at a significantly lower level than in brain endothelium *in situ*. As determined by Western or Northern blot analysis, the expression level of GLUT1 and *mdr1a* in immortalized RBE cells, like in primary cultured RBE cells, remains much lower than in brain endothelium *in situ* (Regina *et al.*, 1998, 2001). In TR-BBB cells, the 3-*O*-methyl-D-glucose transport activity appears to be reduced to one-fourth of the 3-*O*-methyl-D-glucose transport activity of GLUT1 at the BBB luminal side *in vivo* (Terasaki and Hosoya, 2001). Similarly, the activity of  $\gamma$ -GT and ALP is significantly decreased when compared with the corresponding activity in isolated rat brain capillaries (in RBE4 cells, 1.9% and 1.15%, respectively; in TR-BBB13, 13% and 11.2%, respectively) (El Hafny *et al.*, 1996; Hosoya *et al.*, 2000). Furthermore, down-regulation of brain endothelium-specific genes, as observed with GLUT1 and *mdr1a* *Pgp* gene, may be accompanied by an up-regulation of nonspecific genes, as observed with glucose transporter-3 (GLUT3)

**Table III.** Expression of BBB Specific Properties

Cell line name (References)	Tight junctions Cytoskeletal differentiation	Tightness of the cell monolayer	Specific BBB enzyme: $\gamma$ -glutamyltranspeptidase	Specific BBB enzyme: ALP
<i>RBE4</i>				
(1) Roux <i>et al.</i> , 1994;	+Astroglial factors = increase in the F-actin content and staining at the cell periphery (3); +neuronal factors = expression and segregation of occludin at the cell periphery (6)	Pe of $^{14}\text{C}$ -sucrose: $12.8 \times 10^{-3}$ cm/min; +cAMP = $6.2 \times 10^{-3}$ cm/min; +astroglial factors = $2.25 \times 10^{-3}$ cm/min (3); Just at confluence: $0.72 \times 10^{-3}$ cm/min (4); Pe of fluorescein: $0.8-2 \times 10^{-3}$ cm/min (5)	Histochemistry: localization in three-dimensional structures above the monolayer Enhanced by bFGF and astroglial factors RBE4 = 0.73 units/mg protein (1, 2)	Histochemistry: idem to $\gamma$ -glutamyltranspeptidase
(2) El Hafny <i>et al.</i> , 1996;				
(3) Rist <i>et al.</i> , 1997;				
(4) Cerletti <i>et al.</i> , 2000;				
(5) Utepbergenov <i>et al.</i> , 1998;				
(6) Cestelli <i>et al.</i> , 2001				
<i>GP8/3.9</i>	ND	ND	ND	ND
<i>GPNT</i>				
Romero <i>et al.</i> , 2003	Expression of ZO-1/occludin; +dexamethasone = increase +translocalization of ZO-1/occludin; actin and cortactin to the cell periphery	Pe of $^{14}\text{C}$ -sucrose: $7.4 \times 10^{-3}$ cm/min; +Dexamethasone = $4.5 \times 10^{-3}$ cm/min	ND	ND
<i>RBEC1</i>				
Kido <i>et al.</i> , 2000	ND	ND	Brain homogenates 3.6 units/mg protein RBEC1 = 4.7 units/mg protein	ND
<i>TR-BBBs</i>				
(1) Hosoya <i>et al.</i> , 2000;	Occludin; claudin-5 (1); Enhanced expression of occludin by astroglial factors (2)	In TR-BBB13: TEER = $99-109$ ohm-cm $^2$ (1);	Immunocytochemistry: localization on apical size Brain capillaries = 38.4 units/mg protein; TR-BBBs = 2.4-4.9 units/mg protein (1)	Brain capillaries = 178 units/mg protein; TR-BBBs = 8.3-23.4 units/mg protein (1)
(2) Hosoya <i>et al.</i> , 2002				
<i>rBCEC4</i>				
Blasig <i>et al.</i> , 2001	ND	Pe of fluorescein: $0.65 \times 10^{-3}$ cm/min, in the presence of astrocytes	Enhanced by astroglial factors	Enhanced by astroglial factors

*Note.* bFGF, basic fibroblast growth factor; cAMP, adenosine 3',5'-cyclic monophosphate; Pe, permeability coefficient; TEER, transendothelial electrical resistance.

Table IV. Expression of BBB Specific Transporters

Cell line name (References)	GLUT1 (55 kDa)	LAT1	Pgp/mdr1a	Transferrin receptor
<i>In vivo results</i>				
(1) Cornford <i>et al.</i> , 1995; (2) Boado <i>et al.</i> , 1999	Km = 6.67 mM (1)	Km (tryptophane) = 31.5 $\mu$ M (2)	Exclusive expression of the mdr1a gene	
<i>RBE4</i>				
Regina <i>et al.</i> : (1) 1997; (2) 1998; (3) 2001; (4) El Hafny <i>et al.</i> , 1997; (5) Reichel <i>et al.</i> , 2000; (6) Huwylter <i>et al.</i> , 1999	Km = 14.3 mM (1) Immunodetection (55 kDa) Down-regulation of GLUT1 Up-regulation of GLUT3 (3)	Km (histidine) = 135 $\mu$ M (5)	Expression of a functional Pgp Down-regulation of Pgp and mdr1a Up-regulation of mdr1b (2, 4)	Immunocytochemistry with OX-26 Binding of OX-26 with high affinity: $K_D = 17.1$ nM (6)
<i>GP8/3.9</i>				
Greenwood <i>et al.</i> , 1996	Immunocytochemistry	ND	Idem	Immunocytochemistry with OX-26
<i>GPNT</i>				
(1) Regina <i>et al.</i> , 1999; (2) Demeuse <i>et al.</i> , 2003	Idem	ND	Idem; +dexamethasone: increase in Pgp activity (1) +puromycin: selective increase of mdr1a mRNA level (2)	Idem
<i>RBE1</i>				
Kido <i>et al.</i> : (1) 2000; (2) 2001b; (3) Tamai <i>et al.</i> , 2000 <i>TR-BBBs</i>	ND	Expression of LAT1/4F2hc; Km (leucine) = 8.92 $\mu$ M (2)	No detection of functional Pgp (3)	Immunocytochemistry with OX-26 (1)
(1) Hosoya <i>et al.</i> , 2000; (2) Terasaki and Hosoya, 2001; (3) Hosoya <i>et al.</i> , 2002	Immunodetection (55 kDa) (1) Km = 9.86 mM; one-fourth of the 3-OMG transport activity in vivo (2)	Expression of LAT1/4F2hc (1)	Immunodetection with C219; expression of mdr1a, mdr1b, and mdr2 mRNA (1) Enhanced expression of mdr1a by astroglial factors (3)	ND

Note. GLUT1, glucose transporter-1; GLUT3, glucose transporter-3; LAT1, large neutral amino acid transporter; mdr, multidrug resistance; mdr1a, mdr1b, mdr2, rodent Pgp genes; 3-OMG, 3-*O*-methyl-D-glucose; Pgp, P-glycoprotein.

and *mdr1b* Pgp gene in RBE4 cells, and with *mdr1b* and *mdr2* Pgp gene in TR-BBB cells (Hosoya *et al.*, 2000; Regina *et al.*, 1998, 2001).

None of the immortalized RBE cell lines appear to generate the necessary restrictive paracellular barrier properties that would allow to use them in transendothelial permeability screening. For example, even if the sucrose permeability coefficient of a RBE4 cell monolayer can be as low as  $0.72 \times 10^{-3}$  cm/min (Cerletti *et al.*, 2000), the disadvantage of this model is the very narrow window for performing permeability studies. Indeed, low permeability for sucrose could be maintained only for a few hours after cells have reached confluence. The effects of culture conditions on sucrose permeability have been investigated with RBE4 cells: sucrose permeability is increased after energy deprivation by *m*-dinitrobenzene (Romero *et al.*, 1997b) and thiamine deficiency (Romero *et al.*, 1997a), after hypoxia/reoxygenation experiments (Utepergenov *et al.*, 1998) or after treatment with menadione which is able to generate free radicals (Lagrange *et al.*, 1999). Alternatively, rBEC4, with a fluorescein permeability coefficient of  $0.65 \times 10^{-3}$  cm/min, may also be useful for pharmacological investigations (Blasig *et al.*, 2001).

## USE OF RBE CELL LINES FOR THE STUDY OF BLOOD–BRAIN BARRIER PERMEABILITY AND TRANSPORT FUNCTIONS

### Use of Immortalized RBE Cell Lines for the Study of Blood–Brain Barrier Transporters

Drug uptake or efflux in vitro assays, using fluorescent or radioactive molecules, could reflect the transport from blood to brain, or from brain to blood, respectively. RBE4 cells, RBEC1 and TR-BBB cells have been used to demonstrate that transporters such as organic cation transporter/carnitine transporter (OCTN2) (Friedrich *et al.*, 2003; Kido *et al.*, 2001a; Mroczkowska *et al.*, 2000), serotonin transporter (Brust *et al.*, 2000), and the ATA2 system A isoform (Takanaga *et al.*, 2002) are expressed in rat brain endothelium (Tables V–VI). However, in vitro data obtained in RBE cell lines have to be confirmed by in vivo experiments or mRNA/protein expression analysis in freshly isolated RBE cells, since over-expression of nonspecific genes such as *mdr1b*, *mdr2*, and multidrug resistance-associated protein-1 (Mrp1) has been observed in RBE cell lines or primary cultures, compared with brain endothelium in situ (Hosoya *et al.*, 2002; Regina *et al.*, 1998; Tamai *et al.*, 2000).

When the transporter is shown to be expressed with the same properties in the immortalized RBE cells as in vivo, regulation studies may be initiated even if the transporter is down-regulated in the primary-cultured and immortalized RBE cells. For example, the involvement of protein kinase A, protein kinase G, protein kinase C, and  $\text{Ca}^{2+}$ /calmodulin-mediated pathways was assessed for the luminal uptake of L-DOPA in RBE4 cells (Sampaio-Maia and Soares-Da-Silva, 2001). Elevation of intra-endothelial cyclic AMP and stimulation of protein kinase C were shown to increase Pgp activity in GP8 cells (Deli *et al.*, 2001). Also, chronic treatment of GP8 and GPNT cells with the selective agent puromycin was found to increase Pgp expression and activity with a selective increase in *mdr1a* mRNA expression (Demeuse *et al.*, 2004).

**Table V.** Representative Transporters at the BBB Studied in Immortalized RBE Cell Lines

Transporters	Substrates	In vitro properties/ in vivo results	Applications
<i>GLUT1</i> Regina <i>et al.</i> , 2001	D-Glucose	Down-regulation; same Km and inhibition profile; 55 kDa isoform	<i>RBE4: regulation studies</i> —increased GLUT1 expression by factors released by glucose-deprived astrocytes
<i>MCT1</i> Kido <i>et al.</i> , 2000	Lactate/monocarboxylic acids	Down-regulation; same Km and pH dependence	<i>RBE1: characterization studies</i> —suitable model for the study of BBB monocarboxylic acid transport: Identification of the transporter of benzoic acid
<i>LAT1/4F2hc (system L)</i> (1) Gomes and Soares-da-Silva, 1999; (2) Sampaio-Maia and Soares-Da-Silva, 2001; (3) Kido <i>et al.</i> , 2001b; (4) Reichel <i>et al.</i> , 2000; (5) Reichel <i>et al.</i> , 2002	Large neutral amino acids: L-DOPA/3-0-methyl-L-DOPA (1, 2, 3); L-histidine/phenylglycine (4); L-leucine (3, 5)	Same Km, inhibition profile and Na <sup>+</sup> independence	<i>RBE4/RBE1: characterization studies</i> —suitable in vitro BBB model for the study of nutrient transfer across the brain capillary endothelium (1, 2, 3). <i>RBE4: regulation studies</i> —L-DOPA transport under the control of calmodulin-mediated pathways (2). <i>Pharmacological applications</i> —development of strategies for CNS drug delivery of phenylglycine-derived therapeutics (4, 5)
<i>System A</i> Takanaga <i>et al.</i> , 2002	Small neutral amino acids: L-proline/L-glycine/ $\alpha$ -methylisobutyric acid	Same Na <sup>+</sup> dependence and inhibition profile	<i>TR-BBB: characterization studies</i> —demonstration that the expression of ATA2 mRNA is predominant. <i>Regulation studies</i> —hypertonic conditions: induction of ATA2 mRNA and activation of $\alpha$ -methylisobutyric acid uptake
<i>Organic cation transporters</i> (1) Friedrich <i>et al.</i> , 2001; (2) Calhau <i>et al.</i> , 2002	MPP( +): 1-methyl-4-phenylpyridinium; 4-(4-(dimethylamino)styryl)- <i>N</i> -methylpyridinium iodide	ND in vivo	<i>RBE4: characterization and regulation studies</i> —Low level of OCT1; no expression of OCT2 and OCT3 (1); apical transporter(s) of MPP(+) in the dephosphorylated state: role of ecto-ALP (2)
<i>OCTN2</i> (1) Mroczkowska <i>et al.</i> , 2000; (2) Kido <i>et al.</i> , 2001a; (3) Friedrich <i>et al.</i> , 2003	Carnitine/organic cation	Na <sup>+</sup> dependence; same Km as the rat intestine or placenta OCTN2	<i>RBE4/RBE1: characterization studies</i> —conclusion that the carnitine/organic cation transporter is functionally expressed in the brain endothelium

Table V. Continued

Transporters	Substrates	In vitro properties/ in vivo results	Applications
<i>OAT3</i> Ohtsuki <i>et al.</i> , 2002	Organic anions; para-aminohippuric acid		<i>TR-BBB: characterization studies</i> —confirmation of the Brain Efflux Index results showing that OAT3 mediates the brain-to-blood transport of indoxyl sulfate and neurotransmitter metabolites
<i>Serotonin transporter</i> Brust <i>et al.</i> , 2000	Serotonin	In vivo: immunohistochemical analysis only	<i>RBE4: characterization studies</i> —conclusion that a serotonin transporter is functionally expressed in the brain endothelium
<i>Choline transporter</i> Friedrich <i>et al.</i> , 2001	Choline	In vivo: in brain, expression of a Na <sup>+</sup> -dependent transporter (CHT1) in RBE4 cells; Na <sup>+</sup> -independent	<i>RBE4: characterization studies</i> —conclusion that a Na <sup>+</sup> -independent choline transporter is functionally expressed in the brain endothelium
<i>Nucleoside carrier systems</i> Chishty <i>et al.</i> , 2002	Adenosine	Involvement of concentrative and equilibrative transport components	<i>RBE4: characterization studies</i> —identification of the transporter of S-adenosylmethionine
<i>TAUT</i> Kang <i>et al.</i> , 2002	Taurine	Same Na <sup>+</sup> /Cl <sup>-</sup> dependence and inhibition profile	<i>TR-BBB: characterization and regulation studies</i> —hypertonic conditions: increase in TAUT mRNA
<i>Histamine H1 and H2 receptor</i> (1) Karlstedt <i>et al.</i> , 1999	Histamine	Sensitivity to inhibitory agents (imipromidine) Requirement of external Na <sup>+</sup>	RBE4: characterization and regulation studies—expression of H1 and H2 receptors (1) Down-regulation of H1 and H2 receptor mRNA expression by dexamethasone (1)
<i>Histamine uptake</i> (2) Huszti <i>et al.</i> , 1997			Stimulation of histamine uptake and binding by mercuric compounds (2)

*Note.* CNS, central nervous system; GLUT1, glucose transporter-1; LAT1, large neutral amino acid transporter; L-DOPA, L-3,4-dihydroxyphenylalanine; MCT1, monocarboxylic acid transporter; OAT, organic anion transporter; OCT1, organic cation transporter; OCTN2, organic cation transporter/carnitine transporter; TAUT, taurine transporter.

**Table VI.** Representative Efflux Transporters at the BBB Studied in Immortalized RBE Cell Lines

Transporters	Substrates	In vitro properties/ in vivo results	Applications
P-glycoprotein (1) Regina <i>et al.</i> , 1998; (2) Hosoya <i>et al.</i> , 2000; (3) Demeuse <i>et al.</i> , 2003; (4) Bendayan <i>et al.</i> , 2002; (5) Regina <i>et al.</i> , 1999; (6) Deli <i>et al.</i> , 2001; (7) Bergmann <i>et al.</i> , 2000; (8) Chishty <i>et al.</i> , 2001; (9) Andersson <i>et al.</i> , 2002; (10) Pham <i>et al.</i> , 2000	Cochicine/ Taxol	Down-regulation of Pgp and mdr1a mRNA Over-expression of mdr1b mRNA Thus, different substrate and inhibitor profile	<i>Characterization studies</i> – RBE4/TR-BBB: <i>Over-expression of</i> mdr1b mRNA (1, 2, 3) Presence of the protein in plasma membrane, in plasmalemmal vesicles and nuclear envelope (4) <i>Regulation studies</i> – GPNT: increase in Pgp activity by dexamethasone (5) – GP8: inhibition by tissue plasminogen activator (6) Increase in Pgp activity by cAMP, protein kinase C (6) <i>Pharmacological studies</i> – RBE4: design of radiotracers with specificity to Pgp (7) Interaction of classical and modern antihistamines with Pgp, determining the presence or absence of side-effects (8) Induction of Pgp after radiotherapy: consequently, poor efficacy of chemotherapy following radiotherapy (9) – GPNT: demonstration that mefloquine is substrate of Pgp (10)
MRP1 (1) Regina <i>et al.</i> , 1998; (2) Hosoya <i>et al.</i> , 2002; (3) Tamai <i>et al.</i> , 2000	Leukotriene C4/Calcein	Over-expression in vitro	<i>Regulation studies</i> – RBE4/TR-BBB: Decrease of MRP1 Expression and activity by astroglial factors (1) – expression and no effect of astroglial factors (2) <i>RBEC1: Pharmacological studies</i> – brain distribution of quinolones is restricted by MRP1 (3)

*Note.* cAMP, adenosine 3',5'-cyclic monophosphate; mdr, multidrug resistance; mdr1a, mdr1b, mdr2, rodent Pgp genes; MRP1, multidrug resistance-associated protein; Pgp, P-glycoprotein.

Pharmacological applications have been proposed with well-characterized transporters such as monocarboxylic acid transporter-1 (MCT1) and MRP1 in RBEC1 (Kido *et al.*, 2000; Tamai *et al.*, 2000), LAT1 (Reichel *et al.*, 2000, 2002), nucleoside carrier systems (Chishty *et al.*, 2002), and Pgp in RBE4 cells (Chishty *et al.*, 2001). In addition, several studies have been carried out to identify the transporter of exogenous substances: MCT1, transporter of benzoic acid in RBEC1 (Kido *et al.*, 2000) or the nucleoside carrier systems, transporter of *S*-adenosylmethionine in RBE4 cells (Chishty *et al.*, 2002). According to Reichel *et al.* (2000, 2002), the RBE4 cell line is a suitable tool for the screening of potential neuropharmaceuticals targeted to specific transport routes, such as LAT1, to enhance central nervous system (CNS) drug delivery.

### **Use of Immortalized RBE Cell Lines for the Study of Drug Delivery Through the Blood–Brain Barrier**

RBE4 cell monolayers have also been used to investigate the mechanism of the transendothelial transport of large molecules, such as immunoliposomes or nanoparticles, potentially useful as drug delivery vectors to the brain. Indeed, transcytosis of immunoliposomes conjugated to a monoclonal antibody to the rat transferrin receptor (OX26) was demonstrated in RBE4 cells in conditions of low sucrose permeability (Cerletti *et al.*, 2000). Incorporation of the Pgp substrate, digoxin, within OX26-immunoliposomes enhanced cellular uptake of digoxin by a factor of 25 in RBE4 cells, showing that Pgp could be bypassed by immunoliposome-based drug delivery systems (Huwlyer *et al.*, 2002). Also, the uptake of fluorescent nanoparticles by RBE4 cells was optimized by polysorbate 80-overcoating (Alyaudtin *et al.*, 2001).

### **Use of Immortalized RBE Cell Lines for the Study of the Mechanisms of Leukocyte Infiltration Through the Blood–Brain Barrier**

Since the early 1980s, a whole set of data have been reported concerning the factors involved in the control of lymphocyte infiltration into the CNS through the BBB (Butcher, 1991). In physiological circumstances, very few immune cells migrate out through the BBB, whereas, in pathological conditions such as multiple sclerosis, human immunodeficiency virus (HIV) and human T-cell leukemia virus type 1 (HTLV-1) infection, activated-T lymphocytes extensively invade the brain parenchyma. However, the molecular mechanisms supporting this process are still poorly understood.

It has previously been demonstrated that the expression of the adhesion molecule ICAM-1 on primary RBE cells is critical to support lymphocyte migration through the endothelia (Greenwood *et al.*, 1995). RBE4 and GP8 cell lines have been extensively used to demonstrate that ICAM-1 engagement in brain endothelial cells triggers multiple signal transduction pathways (Adamson *et al.*, 1999; Etienne *et al.*, 1998; Etienne-Manneville *et al.*, 2000), including the activation of intracellular calcium flux, the phosphorylation cascades, and the reorganization of the actin cytoskeleton. Using functional assays, it was established that ICAM-1 signaling is intimately and actively involved in facilitating lymphocyte infiltration, since pretreatment of the endothelial cells with various inhibitors of ICAM-1-activated signaling pathways significantly reduces the transmigration of activated T-lymphocytes. Other studies on the interaction between the RBE cell line GPNT and the HTLV-I-infected lymphocytes documented the mechanism of viral entry into the CNS (Romero *et al.*, 2000).

## **CONCLUSION**

Following the pioneer RBE4 cells, several RBE cell lines have been described, which stably maintain in culture a number of brain endothelial hallmarks and constitute tentative *in vitro* models of the rat BBB. The major limitation of these models generally appears to be due to their relatively high paracellular permeability to small

molecules, thus limiting their use for permeability studies. However, as a confirmation of F. Joo's early recognition in 1993, in a review of the second decade of in vitro studies of the BBB (Joo, 1993), several RBE cell lines appeared as useful tools, in parallel with primary cultures of RBE cells, for various cellular approaches towards the understanding of BBB biology. Present studies on BBB transporters and efflux pumps, metabolic enzymes, receptors, drug delivery systems or cell infiltration from the blood to the CNS largely benefit from the availability of these cell lines. Moreover, their proliferation capacity and phenotypic stability would make them uniquely appropriate tools for large-scale drug screening, CNS-oriented drug discovery, design of CNS-targeted drug delivery systems, and pharmaceutical development processes. In addition, we can hope that the strategies developed and the know-how acquired for the production of these RBE cell lines will pave the way towards the characterization of still more efficient permeability models, as well as to the immortalization of human brain endothelial cells and the validation of a human BBB in vitro model.

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