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Development of a Humanized In Vitro Blood–Brain Barrier Model to Screen for Brain Penetration of Antiepileptic Drugs

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Summary: *Purpose:* A biotechnologic breakthrough for the study of drug permeability across the blood–brain barrier (BBB) would be the use of a reproducible in vitro model that recapitulates the functional, structural, and pathologic properties of the BBB in situ. We developed a humanized dynamic in vitro BBB model (DIV-BBB) based on cocultures of human microvascular endothelial cells (HBMECs) from "normal" and drug-resistant epileptic brain tissue with human brain astrocytes (HAs) from epilepsy patients or controls.

Methods: HBMECs and HAs were cocultured for 28 days in polypropylene capillaries. HBMECs were exposed to physiologic levels of shear stress generated by intraluminal flow. Permeability to [^{3}H]sucrose, [^{14}C]phenytoin, and [^{14}C]diazepam was measured in control and drug-resistant DIV-BBB with and without pretreatment with the MDR1 inhibitor XR9576. BBB integrity was monitored by transendothelial electrical resistance measurements (TEERs). Cell growth and viability were assessed by measurement of glucose consumption and lactate production.

Results: $P_{Sucrose}$ and TEER values did not depend on the origin of the endothelium used (epileptic or normal). $P_{Phenytoin}$ was 10-fold less (1.54×10^{-6} cm/s) in drug-resistant BBB models than in controls (1.74×10^{-5} cm/s). MDR1 blockade with XR9576 was effective (3.5-fold increase) only in drug-resistant cultures. $P_{Diazepam}$ in control and drug-resistant DIV-BBB was not affected by XR9576 and did not depend on the epileptic or control origin of endothelia. The overall contribution of epileptic glia to pharmacoresistance was negligible.

Conclusions: These results show that, for the substances used, the humanized DIV-BBB recapitulates the physiologic permeability properties of the BBB in vivo and is also capable of mimicking a drug-resistant BBB phenotype. **Key Words:** Drug delivery—Pharmacogenomics—Drug resistance—Drug discovery—Lead optimization.

Multiple drug resistance is characterized by insensitivity to a broad spectrum of drugs with different chemical nature (Dombrowski et al., 2001; Abbott et al., 2002; Marroni et al., 2003b; Loscher, 2005), presumably acting on different receptors and/or by different mechanisms. Increased expression of drug-efflux transporters such as MDR1 has been found in brain tissue surgically resected from patients with medically intractable epilepsy (Dombrowski et al., 2001). Expression of these drug-extrusion mechanisms could justify the pharmacokinetic feature of pharmacoresistance (Oby and Janigro, 2006). This seems likely because evidence exists to support that overexpression of drug efflux transporters at the blood–brain barrier (BBB) such as P-glycoprotein (P-gp) may reduce levels of antiepileptic drugs (AEDs) in epileptogenic brain tissue (Tishler et al., 1995; Marchi et al., 2004, 2005; Oby et al., 2006).

Despite the numerous animal models of epilepsy developed so far, a significant impediment to the fast and effective development of AEDs has been the paucity of in vivo models that mimic the patterns of multiple drug resistance in humans with epilepsy (Loscher, 2002a). Ideally, a perfect model of AED drug resistance would encompass a therapeutic target comparable to the one to be reached in patients, and mechanisms that either "hide" the target or otherwise prevent the drug from reaching the target itself. Similar considerations apply to an ideal in vitro model of pharmacoresistance. However, in vitro experiments allow the use of resected human tissue, where the efficacy of a given drug treatment can be studied in isolation from other confounding factors. By using cortical brain slices from drug-refractory epilepsy patients, we were able to demonstrate that pharmacokinetic mechanisms such as increased levels of multiple drug resistance transporters at the BBB correlate with reduced levels of AEDs in the epileptic

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focus of multiple-drug–resistant patients as compared with the surrounding brain tissue. Therefore hampered drug distribution may be an essential contributor to pharmacoresistance (Oby et al., 2006).

We and others developed a strategy to study the passage of drugs from serum into the brain based on a coculture of endothelial and glial cells under dynamic conditions (Stanness et al., 1996; Janigro et al., 1999, 2000; Cucullo et al., 2002). This dynamic in vitro-BBB model has been used in our laboratory to demonstrate (a) the influence of glia on endothelial cell differentiation (Stanness et al., 1997; Cucullo et al., 2002, 2005c); (b) the feasibility of using the DIV-BBB to study the effects of flow on endothelial cell metabolism and gene expression (Cucullo et al., 2002; Desai et al., 2002a; Krizanac-Bengez et al., 2003); and (c) the expression of multiple drug resistance genes in endothelial cells from human epileptic brain (Dombrowski et al., 2001).

The modeling that we used in the past was, however, limited to studies on either "normal" human brain or brain endothelium and glia of animal origin. This has several limitations, chiefly the lack of mechanisms of drug extrusion by MDR1 or other proteins that can mimic those present at the human drug-resistant BBB. Little is known about how pathologic expression of MDR1 in patients affects the passage across the BBB of AEDs and other drugs that are substrates of these proteins.

The exact mechanisms of reluctant distribution of AEDs in multiple-drug-resistant epileptic brain is also confounded by the fact that in epilepsy patients, the BBB appears to be impaired, allowing passage of normally excluded molecules (for a review, see Oby and Janigro, 2006). It is thus possible that this impaired selective permeability may be an additional, albeit paradoxic, mechanism of multiple drug resistance in epileptic brain. Thus modeling of drug permeation across the epileptic BBB may also encompass the use of strategies aimed at "opening" the BBB.

The purpose of our study was thus twofold: on the one hand, we wanted to develop a human model of the BBB in vitro that expresses multiple drug-resistance mechanisms normally present in human epileptic brains. To achieve this, we used endothelial cells and glia isolated from either normal or epileptic brain, the latter having been previously extensively characterized in terms of drug-extrusion molecule expression (Dombrowski et al., 2001). In addition, we studied how the normal and epileptic BBB affect the transport of polar and nonpolar molecules under conditions of BBB failure. These studies were designed to mimic as much as possible the pharmacokinetic properties of the vasculature of the human epileptic, multidrug-resistant brain.

METHODS

Isolation of epileptic human brain microvascular endothelial cells (epi-HBMEC) from surgical specimens

Endothelial cell (EC) cultures were established from cerebral cortex biopsies of patients who underwent temporal lobectomies for intractable epilepsy (Dombrowski et al., 2001; Marroni et al., 2003a) (see Table 1). Tissue was resected en bloc when possible, and care was taken to minimize trauma to the tissue during and after resections. Brain resections were collected in an ice-cold solution mimicking cerebrospinal fluid composition (aCSF solution: 120 m*M* NaCl, 3.1 m*M* KCl, 1.0 m*M* $MgCl_2$; 6 H_2O, 26.0 m*M* $NaHCO_3$, 1.25 m*M* KH_2PO_4, 10.0 m*M* dextrose, 1.0 m*M* $CaCl_2$) and bubbled with 5% CO_2/95% O_2. After gentle trituration, tissue was homogenized in 5% Dissection Media (DM: 1 L contains 4.6 ml of 7.5% $NaHCO_3$, 20 ml of 1 *M* HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin, 100 ml Medium 199 with Hank's salts and glutamine (x10), sterile water (5% serum added when needed). The homogenate was then centrifuged (1,200 rpm/800 *g* for 7 min.), and the supernatant aspirated. The pellet was suspended in DMEM containing no serum to a final volume of 27 ml. Then 3 ml of protease solution (5 mg/ml) was added, and the solution was incubated in a shaking water bath for 1 h at 37°C. The homogenate was then transferred to a centrifugation tube. The solution was rinsed to a final volume of 40 ml (for this step and subsequent steps, 5% DM was used). After centrifugation (1,200 rpm/800 *g* for 7 min.), the supernatant was aspirated, and the pellet was suspended in 25 ml of 15% dextran (average MW, 71.000; Sigma, St. Louis, MO, U.S.A.; D-1537). The tube was vortexed for ≥2 min and then spun at 5,000 rpm (3,450 *g*) for 10 min (brake = 1) at 4°C. The pellet was then suspended in a solution containing 9 ml of 5% DM and 1 ml of collagenase, 10 mg/ml. The solution was incubated in a shaking water bath for 2 h at 37°C and then spun (1,200 rpm/800 *g* for 7 min.) and the supernatant aspirated. The pellet, suspended in 2 ml of 5% DM, was then added to 35 ml of a percoll gradient solution. The solution was centrifuged at 2,500 rpm (860 *g*) for 10 min. (brake = 0) at 4°C. The gradient section below the fat ring was collected, diluted with 5% DM, and spun (1,200 rpm/800 *g* for 7 min.). The pellet was finally suspended in endothelial growth medium (see later), and cells were then seeded in fibronectin-coated flasks or in multiwell plates, incubated at 37°C for ≥1 h and washed with PBS.

Isolation of human brain astrocytes (HAs) from surgical specimens

Astrocyte cultures were established from human cerebral cortical tissue of patients who underwent temporal lobectomies. These cells were morphologically and

molecularly characterized and then stored under liquid nitrogen for future use. Primary cultures of human brain astrocytes were obtained as described elsewhere (Booher and Sensenbrenner, 1972). In brief, brain resections were collected in an ice-cold solution mimicking CSF composition bubbled with 5% CO_2/95% O_2. This solution consisted of (in m*M*): 120 NaCl, 3.1 KCl, 3 $MgCl_2$, 1 $CaCl_2$, 1.25 KH_2PO_4, 26 $NaHCO_3$, and 10 dextrose. Tissue was homogenized after gentle trituration and incubation in phosphate buffered saline (PBS) containing trypsin (0.2%)/DNASE (1 mg/ml, Sigma-Aldrich, St. Louis, MO, U.S.A.) for 20 min at 37°C. After centrifugation (200 *g* for 5 min) and filtration through a 70-μm nylon sieve, cells were seeded in appropriated poly-L-lysine–coated flask or in 6- or 24-multiwell plates. The culture medium consisted of Dulbecco's modified essential medium (DMEM) supplemented with 10% FBS and 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

After reaching confluence, cultures were agitated overnight at 37°C. Cytosine arabinoside and L-leucine methyl ester (Sigma-Aldrich) were added to obtain a highly purified astrocyte population (Meyer et al., 1991). A week later, astrocytes reached confluence and were used for the experiments described herein. Immunologic characterization, performed with rabbit polyclonal antibodies that recognized the glial marker glial fibrillary acidic protein (GFAP; Dako Corporation, Carpinteria, CA, U.S.A.) showed that ~95% of living cells (as visualized by DAPI) were astrocytes (data not shown).

Cell culture

Normal human brain microvascular endothelial cells (HBMECs, Cat. 6100), and human astrocytes (HAs, Cat 1800) were purchased from ScienCell Research Laboratories (San Diego, CA, U.S.A.). HBMECs were initially expanded in 75-cm^2 flasks precoated with fibronectin (3 $\mu g/cm^2$) with the appropriate endothelial growth medium consisting of 1.5 g/100 ml of MCDB 105 (Sigma, Cat. M6395), 10% of fetal bovine serum (FBS; ScienCell Research Laboratories, Cat. 0025), 15 mg/100 ml of endothelial cell growth supplement (ECGS; Cat. 1052), 800 units/ml of heparin (Sigma, Cat H3393), 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate.

HAs were grown in Poly-D-Lysine precoated flasks (3 $\mu g/cm^2$) with DMEM-F12 supplemented with 2 m*M* glutamine, 5% fetal bovine serum (FBS), 100 units/ml of penicillin G sodium, and 100 μg/ml of streptomycin sulfate. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Cellular growth was monitored every day by inspection with phase-contrast microscopy.

DIV-BBB setup

The DIV-BBB model used for the experiments described herein was purchased from Spectrum (Cat. 400-025; Spectrum Laboratories, Inc., Rancho Dominguez, CA, U.S.A.). Each module consists of 50 hollow polypropylene fibers embedded in a clear plastic chamber (see Table 2 for details). The porosity of the hollow fibers allows gas and nutrient exchange between the two compartments (luminal/extraluminal) but does not permit cells to pass. Both luminal and abluminal areas are accessible by ports in the circuit connected with a media reservoir and a pulsatile pump apparatus. Gaseous exchange (O_2, and CO_2) occurs through gas-permeable silicone tubing that connects the cartridge and the media reservoir. Four electrodes are positioned in pairs in the luminal and extraluminal chamber, allowing faster and more accurate transendothelial electrical resistance (TEER) measurements by real-time computerized monitoring systems (see later). The pump (CellMax QUAD Artificial Capillary Cell Culture System, Spectrum Laboratories, Inc., Los Angeles, CA, U.S.A.) generates a pulsatile flow of medium through the lumen of the artificial capillaries, allowing diffusion of nutrients out to the extraluminal space (ECS) through the 0.5-μm transcapillary pores at a controllable rate (flow rates of 1 to 50 ml/min, corresponding to shear-stress levels of ~1 to 200 dyne/cm^2). The flow pattern generated consists of a complex waveform, and a substantial decrease of pressure occurs at the end of the capillaries, giving these models the ability to reproduce the hemodynamic condition in vivo (Desai et al., 2002b). Metabolic products are similarly removed from the cartridge. The entire apparatus resides in a water-jacketed

TABLE 1. *Patient characteristics*

Age (yr), gender	Pathology	MDR1 MRNA fold increase
34, M	TLE with sclerosis	3.12
28, M	TLE with sclerosis	4.92
41, F	TLE w/o sclerosis	5.24
8, F	TLE w/o sclerosis	3.02
14, F	TLE with sclerosis	4.98

Clinical and experimental data of the patients (three adults and two children) whose specimens were used for this study. The increased expression levels of MDR1 mRNA (Dombrowski et al., 2001; Marroni et al., 2003a) in the same tissues also are reported.

TABLE 2. *Properties of the DIV-BBB: volume, surface area, number of capillaries*

Dimensions	135 × 9 × 9 mm
Number of capillaries	50
Capillary length	13 cm
Pore size	0.5 μm
Wall thickness	150 μm
Inner diameter	330 μm
Diameter	650 μm
Capillary surface area	128.5 cm^2
Luminal area	67.35 cm^2
ECS volume	1.4 ml
Lumen volume	0.5 ml
Cell inoculation (ECS)	3–4 million
Cell inoculation (lumen)	6 million

incubator with 5% CO_2 and was sampled in a sterile manner by placing it inside a laminar flow hood. The hollow fibers were precoated with fibronectin (3 μg/cm^2) intraluminally and with poly-D-lysine (3 μg/cm^2) extraluminally to enhance cell attachment and proliferation.

To study and compare the induction of a functional BBB, we used two different cocultures setups: (a) Epi: HBMECs plus HAs (epileptic modules); and (b) control-HBMECs plus HAs (control modules). Human endothelial cells were seeded intraluminally ($\approx 4 \times 10^6$/cartridge) and allowed to adapt to flow for 7–15 days alongside their extraluminal astrocytic ($\approx 6 \times 10^6$/cartridge) counterparts. To facilitate cell adhesion we used an initial flow rate of 1 ml/min for the initial 48 h after cell inoculation and then adjusted the flow to 4 ml/min (i.e., 4 dyne/cm^2). The entire apparatus resided in a water-jacketed incubator with 5% CO_2 at 37°C. Media samples were taken on regular basis and analyzed to assess the glucose consumption and lactate production.

Cell metabolism: lactate production and glucose consumption

In conjunction with TEER monitoring, indicators of cell metabolism were used to monitor cell growth and the establishment of a functional BBB in vitro in the DIV-BBB. Depletion of the main carbohydrate component of the growth medium (glucose) and accumulation of metabolically produced lactic acid are used as indicators of cell growth (Stanness et al., 1997). For this purpose, luminal and extracapillary space were sampled at routinely every 2–3 days. The calculations for glucose consumption (mg/day) and lactate production rates (mg/day) are based on medium replacement, volume of nonreplaced medium, and previous values. Glucose-consumption rate was calculated based on the concentration of glucose in fresh and unreplaced medium in the system, according to the following equation:

$$\frac{(V_n \times G_n) + (V_o \times G_p) - (V_{total} \times G_c)}{T_c - T_p} \quad (1)$$

where V represents added volumes of medium (ml), G is the glucose concentration (mg/ml), T is time of sampling (in fractions of days; c and p indicate the current and previous samples, respectively), n represents fresh medium added after previous sampling, whereas o represents old, unreplaced medium. Lactate production rate (mg/day) was calculated similarly:

$$\frac{(V_t \times L_c) - (V_n \times L_n) + (V_u \times L_p)}{T_c - T_p} \quad (2)$$

(L) refers to the concentration of lactic acid in mg/ml. Dual-channel immobilized oxidase enzyme biochemistry (YSI 2700 SELECT; YSI Inc., Yellow Springs, OH, U.S.A.) was used to measure lactate and glucose in the cell-culture medium. Data obtained with the described equation were then converted to μM/day.

DIV-BBB TEER measurement system

The TEER measurement provides a quick and easy evaluation of the integrity of the DIV-BBB (Cucullo et al., 2002; Cucullo et al., 2005a; Santaguida et al. 2006). We used a newly developed TEER measurement device (see www.flocel.com), which uses electronic multiplexing to measure multiple cartridges in quick succession and assesses the integrity and viability of tissue-culture bilayers rapidly and reliably. The device uses a Universal Serial Bus (USB) interface to a PC computer. To sample, the excitation voltage (0.06 V) is applied across the excitation electrodes inserted in each cartridge, and the microcontroller computes the resistivity and capacitance per cm^2 of the barrier from physical parameters. The values of capacitance are calculated by comparison of the voltage and current waveforms. The delay from peak to peak of the two waveforms is proportional to the capacitance value, which is expressed as arch tension. TEER was measured continuously from the initial setup throughout the course of each experiment.

Drug permeability: uptake of [^{14}C]-phenytoin, [^{14}C]-diazepam, and [^{3}H]-sucrose

Boluses (0.5 ml each) of the radioactive tracers [^{14}C]-phenytoin (PerkinElmer, Boston, MA, U.S.A., Cat. NEC-246), [^{14}C]-diazepam (Amersham, Piscataway, NJ, U.S.A., Cat. CFA-591), and [^{3}H]-sucrose (Amersham, Cat. TRA-332) were injected upstream (before the capillary) into the lumen, and the diffusion into the extracapillary space was monitored over time while maintaining a 1 ml/min intraluminal perfusion rate. A total of 1 μCi per compound was used. The role played by intraluminal shear stress in brain microvessel endothelial cell differentiation, as well as maintenance and induction of a BBB phenotype and integrity, is now well recognized. Note, however, that the reduction of flow rate from 4 to 1 ml/min (equivalent to a reduction of shear stress from 4 to 1 dyne/cm^2) for a short period (1 h) does not affect BBB integrity. This was previously demonstrated by our group (Desai et al., 2002a; Marroni et al., 2003a) and by others (Mashour and Boock, 1999). Samples were taken from ECS and lumen (100 μl each), as previously described (Stanness et al., 1997), at time zero (immediately after the injection) and at 1, 5, 10, 15, 30, and 60 min after the injection. Note also that luminal samples were collected downstream from the cartridge (where the media leave the capillary to enter the silicon tubing). Samples (replaced with equal volumes of medium) taken from the lumen and the ECS were introduced into vials with 4 ml of Ready Protein Beckman scintillation cocktail (Packard Ultima Gold; ECN, Costa Mesa, CA, U.S.A.).

Radioactivity was counted with an LS 6500 scintillation counter (Beckman). Permeability was calculated by

graphic integration of drug concentration in the lumen and in ECS over a 60-min period. Permeability for a given compound was calculated as described elsewhere (Davson and Segal, 1996). In brief, the permeability values were calculated by using an equation derived from a differential equation based on Fick's Law.

$$\frac{dM_{ECS}}{dt} = PA(C_{lumen} - C_{ECS}) \quad (3)$$

The equation has been modified based on the characteristics of the DIV-BBB. In this equation, dM_{ECS} represents the amount of solutes (moles) entering the extracapillary space (ECS) over time; P is the permeability coefficient; A is the surface area of capillaries; C_{lumen} is the concentration of solute at the luminal space, and C_{ECS} is the concentration of solute in the ECS.

From Fick's Law, dividing by the ECS volume (V_{ECS}), we obtain the differential equation for the solute concentration in the ECS:

$$\frac{dC_{ECS}}{dt} = \frac{PA}{V_{ECS}} \times (C_{lumen} - C_{ECS}) \quad (4)$$

where the transfer coefficient K can be expressed as follows:

$$\frac{PA}{V_{ECS}} = K \quad \text{thus} \quad \frac{dC_{ECS}}{dt} = K(C_{lumen} - C_{ECS}) \quad (5)$$

By integration of this differential equation between the limits of time zero and t, we obtain

$$\int_0^t dC_{ECS} = K \int_0^t (C_{lumen} - C_{ECS})$$

where the ratio of the line over the area is

$$K = \frac{\int_0^t dC_{ECS}}{\int_0^t (C_{lumen} - C_{ECS})} \quad (6)$$

Now, by solving the defined integral equation, we obtain

$$K = \frac{C_{ECS}(t) - C_{ECS}(0)}{[AUC_{lumen}]_0^t - [AUC_{ECS}]_0^t} \quad (7)$$

K is, however, linked to the permeability by the following formula, previously described

$$\frac{PA}{V_{ECS}} = K, \quad \text{thus} \quad P = \frac{K \times V_{ECS}}{A}.$$

At this point, P can be calculated because K is a coefficient obtained experimentally, and the other parameters are derived from the characteristics of the model and therefore have known values:

V_{ECS} = 1.4 cm; A (capillary surface area) = 128.5cm^2

To obtain the p value in cm/s, we have to divide by 60, thus obtaining the final equation specific for the DIV-BBB:

$$P[cm/\sec] = \left[\frac{(C_{ECS}(t) - C_{ECS}(0))}{([AUC_{lumen}]_0^t - [AUC_{ECS}]_0^t)}\right] \times \frac{1.4}{128.5} \times \frac{1}{60} \quad (8)$$

Permeability of the specific compound was calculated by integrating the area under the ECS and lumen data points (AUC) according to the final equation (5), where C_{ECS} (t) and C_{ECS} (0) are the extraluminal space concentrations of compound × at time zero and time (t). Note that equation 7 (or 8) implies that the diffusion of the drug or tracer is independent of the units of measurement used. In other words, the dimensions of the drug or tracer are canceled out in the equation, and the same permeability values (expressed in cm/s) will be obtained when using M or cpm values. In addition, the driving force for drug transfer is not, in these equations, concentration dependent. In other words, the integral of luminal and ECS values vary only as function of permeability or time. Thus use of tracer amounts is a legitimate way to measure drug formation (see also Davson and Segal, 1996).

Similar measurements were then performed after the culture medium was replaced with a fresh one containing the MDR1 inhibitor XR9576 (tariquidar, 200 n*M* final concentration) (Mistry et al., 2001). The system was allowed to stabilize for 1 h before proceeding with the permeability experiment.

BBB opening by hyperosmolar mannitol

Infusion of 10 ml of growth medium containing mannitol (1.6 *M*) was used to breach the BBB (Rapoport, 2000). The solution was prepared under sterile conditions and injected intraluminally at a perfusion rate of 1 ml/min. TEER was monitored during the course of the experiment to assess for BBB failure and recovery. Drug-permeability measurements were taken as described earlier.

Statistical analysis

The statistical analysis was performed by Origin 7.0 (OriginLab, Northampton, MA, U.S.A.). For parametric variables such as TEER, permeability values, glucose consumption, and lactate production, differences between populations were analyzed with analysis of variance (ANOVA). Values were determined to be significantly different when $p < 0.05$. We used four DIV-BBB modules/group because previous experiments provided sufficient power to demonstrate statistical significance for positive findings.

RESULTS

The purpose of this study was to establish whether a humanized DIV-BBB cell-culture system can be used to mimic the in situ pharmacokinetic properties of the human BBB in normal brain or in patients affected by

drug-refractory epilepsy. We wished to demonstrate preliminary evidence that a novel in vitro humanized BBB model may be useful to study drug permeation under therapeutically realistic conditions. In this model, we studied electrical resistance, metabolism, permeability to a well-established paracellular marker (sucrose), a highly permeable compound (diazepam; DZP), and an antiepileptic drug (phenytoin; PHT), which is a substrate for MDR transport. Sucrose is commonly used as a marker of the paracellular pathway and has been almost universally acknowledged to be a good marker of BBB integrity. Furthermore, sucrose, as TEER, does not depend on metabolism of the cell or specific transporters into or out of the brain. The other marker chosen for these experiments (PHT) represents an MDR1 substrate, and differences in permeability between the epileptic and control models can be used as indicators of drug resistance. The third marker chosen, DZP, is not transported to our knowledge by any multidrug-resistance molecules but moves across the BBB by passive diffusion.

In the first set of experiments, we tested the feasibility of coculturing epileptic and "normal" HBMECs with normal human astrocytes. In particular, we wished to understand if these cells can establish a viable in vitro BBB. This was achieved by monitoring TEER, metabolism of the cells growing in the DIV-BBB, and by measuring permeability to the tracer of paracellular leakage [^{3}H]-sucrose. Note that under dynamic (flow) conditions, endothelial cells develop a morphology that closely resembles the endothelial phenotype in situ (Ott and Ballermann, 1995), contributing to much higher TEER values and drug-permeability values, closely resembling in vivo conditions. This model has better predictive value than do static cultures (Santaguida et al., 2006).

Permeability values to this marker (Stanness et al., 1997) were respectively $3.92 \times 10^{-7} \pm 0.61$ cm/s in control BBB modules and $4.37 \times 10^{-7} \pm 0.53$ cm/s ($n = 4$) in cocultures with epileptic endothelial cells. These values are similar to sucrose permeability in vivo (Davson and Segal, 1995). We found no differences ($p < 0.05$) between permeability values of control and epileptic DIV-BBB modules (Fig. 1A). This demonstrates that human endothelial cells exposed to dynamic growth conditions form a BBB phenotype in vitro regardless of the "control" or "epileptic" origin of endothelial cells used.

A fundamental property of the BBB is the formation of an impediment to the passage of polar molecules and selective permeability to ions and other nutrients/substances. An index of this endothelial "tightness" is the TEER (Stanness et al., 1997). Note that the development of high TEER ($>$1,100 ohm/cm^2) was not statistically different ($p < 0.05$) between control and epileptic cocultures (Fig. 1B). Taken together, the results so far presented demonstrate that endothelial cells from epileptic brain are capable of forming a viable and functional BBB that, in terms of tightness and permeability to polar molecules, does not differ from the "normal" counterparts.

The assessment of glucose consumption/lactate production provides useful information on the metabolic pathway (anaerobic versus aerobic) of endothelial–glial cocultures grown under dynamic conditions. A previous study (Desai et al., 2002a) demonstrated that exposure to shear stress induces changes in the expression pattern of several metabolic enzymes including upregulation of NADH-producing enzymes [Krebs cycle dehydrogenases and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] accompanied by a simultaneous decrease in NADH-depleting pathways [e.g., lactate dehydrogenase (LDH)] and diminished production of lactate (Desai et al., 2002a). Thus the assessment of an aerobic metabolism is a hallmark of the establishment of a functional BBB. The results in Fig. 1C show that both normal and epileptic HBMECs differentiate in a BBB phenotype. In both cases, the ratio between glucose consumption and lactate production was $\approx$1, indicating increased propensity toward an aerobic metabolic pattern. We found no significant metabolic differences between epileptic and normal endothelium ($p < 0.05$).

CNS drugs in general and AEDs in particular are intentionally designed to be lipophilic. Lipophilicity improves passage across the BBB and subsequently increases drug distribution into the brain. We first tested the capacity of DZP, a highly lipophilic and commonly used AED, to permeate across a humanized in vitro BBB. Fig. 2A shows the permeability values of DZP in control BBB ($4.75 \times 10^{-3} \pm 0.65$ cm/s) or in BBB consisting of epileptic endothelium ($2.93 \times 10^{-3} \pm 0.71$ cm/s). These data indicate that the in vitro BBB established in our model has a permeability to DZP almost identical to that observed in vivo. In addition, although numerous AEDs have been shown to be substrates of MDR1 (Potschka et al., 2004; Schmidt and Loscher, 2005; Volk and Loscher, 2005; van Vliet et al., 2006), DZP moves across the BBB by passive diffusion (Yamazaki et al., 2001). Our permeability data are in agreement with this finding because (a) no significant difference was found in DZP permeability ($p > 0.05$) between control and epileptic DIV-BBBs (Fig. 2A); and (b) pretreatment with the MDR1 blocker XR9576 did not produce any significant effect on DZP permeability (see Fig. 2B).

BBB opening after exposure to hyperosmotic mannitol is commonly used to enhance chemotherapeutic drug penetration of the BBB to treat patients with metastatic or primary brain tumors (Rapoport, 2000; Brown et al., 2004). Hyperosmotic opening of the BBB is mediated by vasodilatation and shrinkage of cerebrovascular endothelial cells, with widening of the interendothelial tight junctions to an estimated radius of 200 Å, thus facilitating the paracellular passage of substances across the microvascular brain endothelium (Rapoport, 2000; Brown et al.,

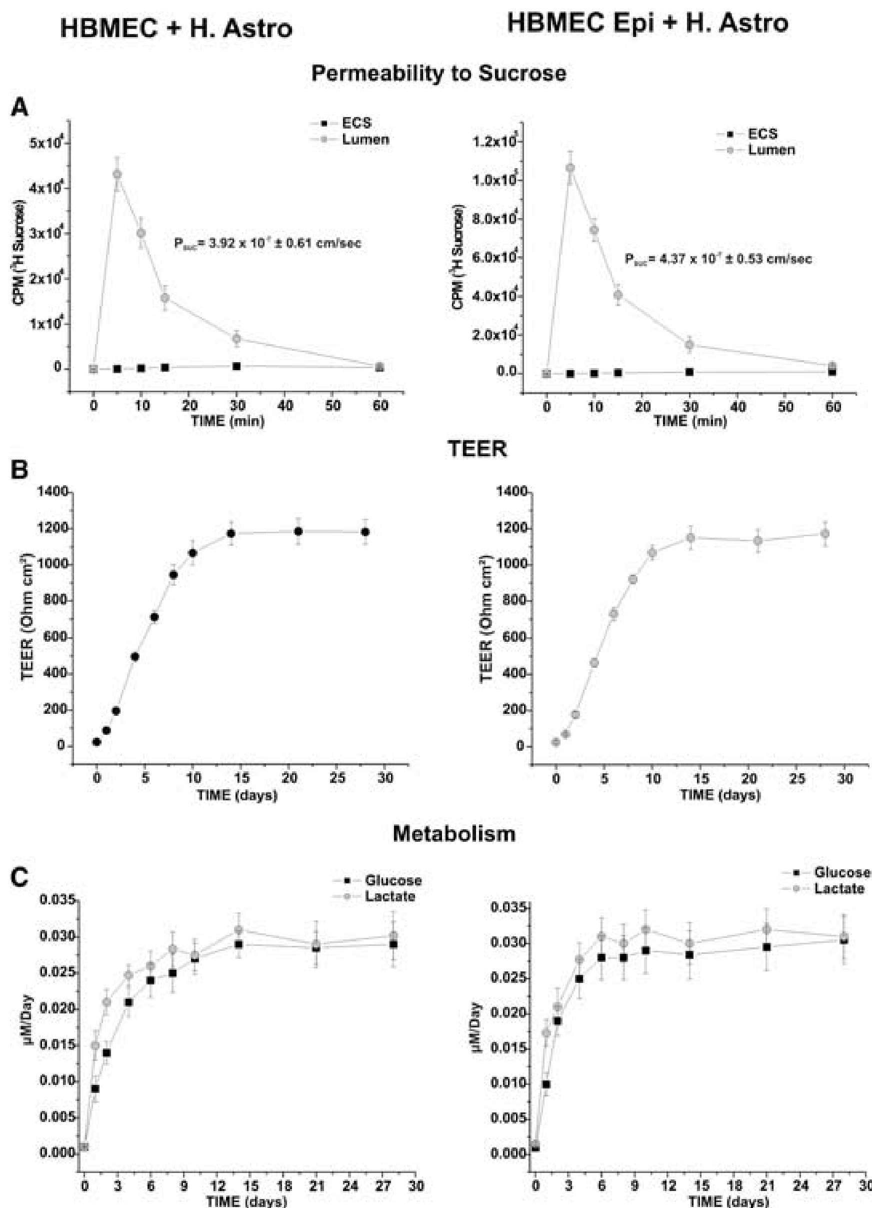

FIG. 1. Human brain microvascular endothelial cells (HBMECs) from control and epileptic brain cocultured with human astrocytes (HAs) and exposed to flow to develop blood–brain barrier (BBB) properties. **A:** Permeability measurements of [^{3}H]-sucrose in control and epileptic in vitro BBB models. Both models develop a very tight barrier to this well-established paracellular marker. No statistically significant differences in sucrose permeability (P_{suc}) were observed. **B:** HBMECs develop a very tight barrier characterized by a stable, high (>1,100 ohm/cm^2) transendothelial electrical resistance (TEER). No statistical differences were observed between control and epileptic dynamic in vitro BBB (DIV-BBB) modules. All the experiments were repeated in quadruplicate (n = 4), and permeability values are given as ±SEM. **C:** Glucose consumption and lactate production in both control and epileptic cocultures demonstrate an almost identical, predominantly anaerobic, metabolic pathway. No statistical differences between control and epileptic DIV-BBB were observed.

2004). Opening of the BBB in vitro by hypertonic mannitol solution demonstrated an almost complete loss of selective permeability to DZP. As expected, the effect was identical ($p < 0.05$) regardless of the epileptic ($2.82 \times 10^{-2} \pm 1.2$ cm/s) or normal ($2.33 \times 10^{-2} \pm 0.80$ cm/s) origin of endothelial cells (Fig. 2C).

These results suggested that (a) The DIV-BBB system can effectively reproduce the permeability characteristics on the BBB in vivo; (b) disruption of normal or epileptic BBB can be achieved with hyperosmotic mannitol; (c) the integrity and tightness of the BBB in epileptic brain was similar to that observed in normal brain; and (d) permeability to DZP was not affected by blockade of MDR1 by XR9576. However, the latter finding could be the result of insignificant MDR1 expression in the epileptic model. To rule out this hypothesis, we repeated the same experiments with PHT, which is at least in part an MDR1 substrate.

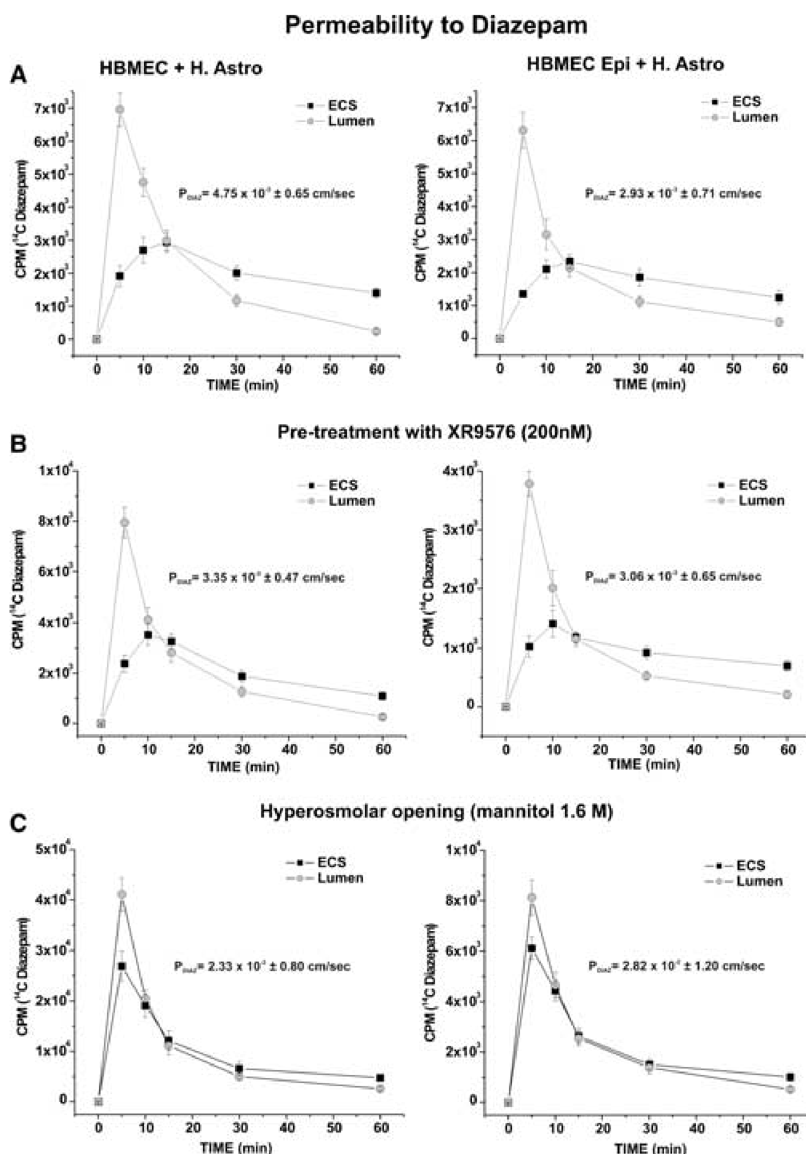

FIG. 2. Diazepam permeability in control and epileptic dynamic in vitro blood–brain barrier (DIV-BBB): a side-by-side comparison of humanized models. **A:** No significant difference was observed between permeability of diazepam (P_{DIAZ}) in control and epileptic DIV-BBB. **B:** Pretreatment with the MDR1 inhibitor XR9576 did not affect the permeability to diazepam regardless the expression of MDR1. **C:** BBB opening after exposure to hyperosmotic mannitol facilitates the paracellular passage of diazepam across the microvascular brain endothelium. This effect was identical regardless of the epileptic or nonepileptic origin of the vascular endothelium.

In contrast to the results previously shown with DZP, permeability values of PHT in control ($1.74 \times 10^{-5} \pm 0.44$ cm/s) or epileptic endothelium ($1.54 \times 10^{-6} \pm 0.76$ cm/s) differed ($p < 0.05$) by an order of magnitude (Fig. 3A). In addition, although pretreatment with XR9576 did not affect PHT permeability in control, epileptic cocultures demonstrated ≈3.5-fold increase in PHT permeability after exposure to the MDR1 blocker (Fig. 3B). Similar to that previously observed with DZP, the permeability to PHT was increased after the osmotic opening of the BBB with mannitol (Fig. 3C). No statistical differences between control and epileptic in vitro BBB were observed.

To assess epileptic astrocytes' contribution to pharmacoresistance, we established parallel DIV-BBB models in which normal HBMECs were cocultured with astrocytes isolated from normal or epileptic brains. TEER was identical in both modules regardless of the origin of astrocytes (data not shown). Once the BBBs were formed, we performed permeability experiments to sucrose, PHT, and DZP (Fig. 4). Note that no differences in permeability were seen regardless of the astrocytes' origin (epileptic or normal).

DISCUSSION

The most important outcome of this research is the development of an in vitro model of pharmacoresistance to AEDs based on coculture of human endothelium with glia. We propose to further the development of this model and extend this research to other AEDs. Our results support the use of this model for drug screening, but several issues remain to be addressed. These are listed in the following paragraphs.

Figure 5 summarizes the results of this study. The use of a humanized DIV-BBB models allowed permeability measurements with a close correlation with that measured

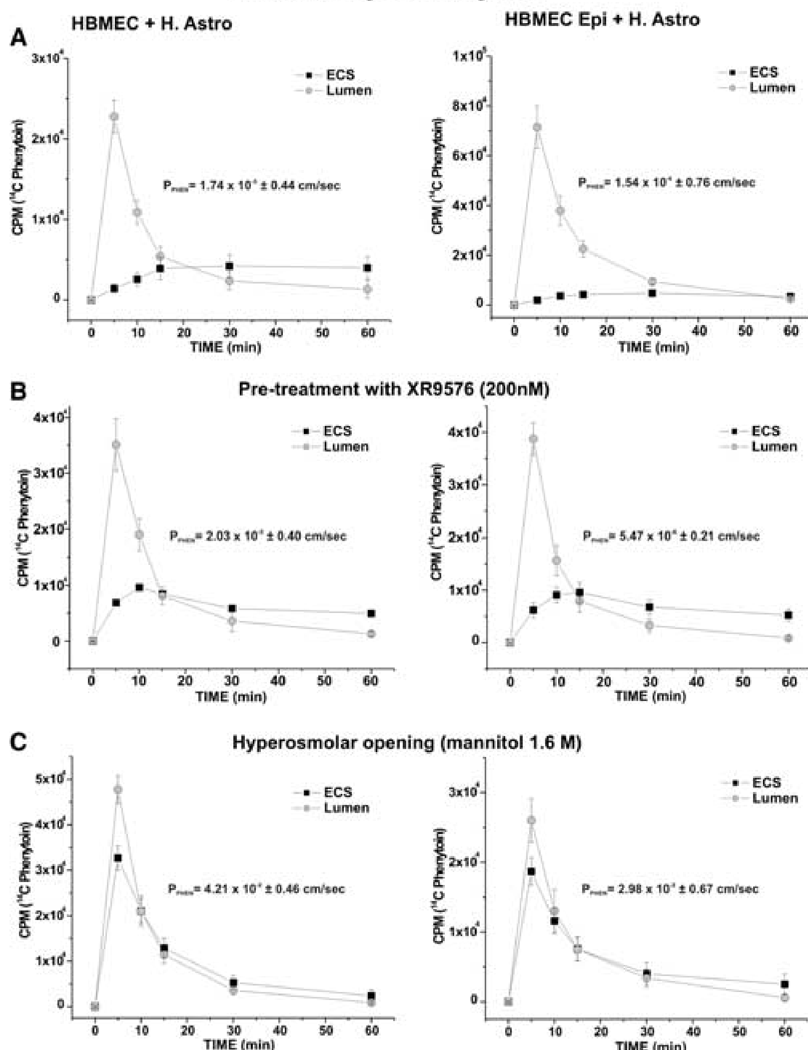

FIG. 3. Phenytoin permeability in control and epileptic dynamic in vitro blood–brain barrier (DIV-BBB). **A**: Phenytoin is 10-fold less permeable in epileptic than in control BBB ($p < 0.05$). **B:** The 3.5-fold increase in phenytoin permeability (P_{PHEN}) was observed in epileptic in vitro BBB pretreated with XR9576 ($p < 0.05$). **C:** Similar to diazepam, the osmotic BBB opening with mannitol increased phenytoin permeability regardless of the epileptic or nonepileptic origin of the endothelium.

in vivo (S = 0.83; Fig. 5A). These data demonstrate that the humanized DIV-BBB represents an excellent system to assess drug permeability with a degree of accuracy comparable to what one expects at the in situ human BBB. The contribution of pathologic expression of multiple drug resistance transporters is clearly maintained in our model. This is demonstrated by the significant difference in PHT permeability between control and epileptic cocultures. This difference was partially abolished by MDR1 blockade with XR9576 (Fig. 5B). Osmotic opening of the BBB by hyperosmolar mannitol as a mean to improve drug permeability was successfully achieved in the DIV-BBB (Fig. 5B), regardless the origin of the brain microvascular endothelium used (from normal or epileptic brain) or the lipophilicity of the compounds tested.

We intentionally used PHT as a prototype of an AED that undergoes active extrusion by multiple drug resistance molecules. Several studies have pointed out that PHT is indeed an MDR1 substrate (Potschka and Loscher, 2001; Rizzi et al., 2002), but other results suggest a minor role (Maines et al., 2005; Awasthi et al., 2005). Our results have shown a small but significant ($p < 0.05$) effect of the MDR1 blocker XR9576, suggesting that at least a small portion of drug resistance to PHT may be due to MDR1 overexpression. Because the P-gp inhibitor had no effect on the permeability of PHT in normal brain, which still expresses a constitutive low level of MDR1, it appears that only significant MDR1 increases above "normal" levels are required to promote measurable drug extrusion. However, given the broad range of genes encoding for multiple drug resistance proteins that are expressed by epileptic brain endothelial cells isolated from surgical specimens (Dombrowski et al., 2001), the contribution of other transporters may also play a role (e.g,, RLIP76) (Awasthi et al., 2005).

Regardless of the mechanism of drugs extrusion, one remarkable result was that in presumably non–multiple drug-resistant brain, PHT permeability was identical to

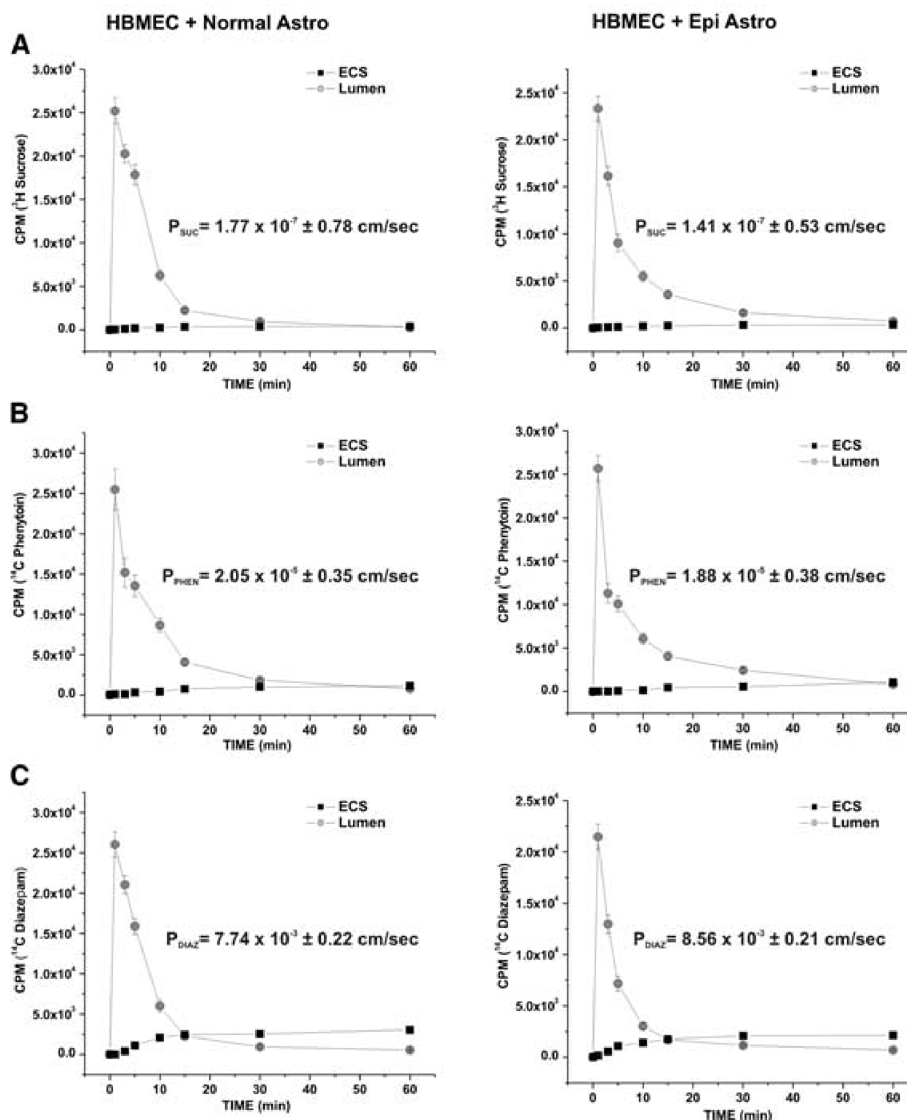

FIG. 4. Epileptic astrocytes do not contribute to endothelial pharmacoresistance. No significant differences were observed between permeability to sucrose (P_{suc}), phenytoin (P_{PHEN}), and diazepam (P_{DIAZ}) in dynamic in vitro blood–brain barrier (DIV-BBB) established by coculturing brain microvascular endothelial cells (HBMECs) with normal or epileptic astrocytes.

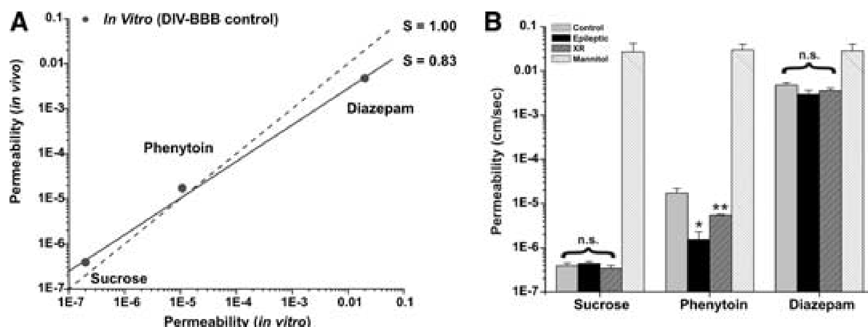

FIG. 5. Correlation between permeability in vivo and in vitro. **A:** Permeability to sucrose, phenytoin, and diazepam in control dynamic in vitro blood–brain barrier modules (DIV-BBB) versus published in vivo data (Davson and Segal, 1996; Patsalos et al., 1996; Walker et al., 1996; Cucullo et al., 2005b). Dashed line, Idealized relation if the data in vivo were identical to that in vitro. Note how permeability obtained in the DIV-BBB accurately reflects the in vivo scenario. **B:** Summary of permeability of the compounds tested across the in vitro BBB comprising either normal or epileptic brain microvascular endothelial cells under different experimental conditions.

that predicted by its oil/water partition coefficient. In contrast, tissue from drug-resistant epilepsy patients provided endothelial cells forming a BBB with significantly lower PHT permeability. Based on our tissue-screening experiments (Dombrowski et al., 2001), we expect that this behavior may be consistently seen in similar endothelial cells. Another interesting finding is that functional expression of drug-resistance mechanisms present in freshly isolated brain persists in cultured and passaged cells. The contribution of glial cells and intraluminal flow to this phenomenon is currently being investigated. Preliminary results (data not shown) suggest that flow per se does not cause induction or downregulation of MDR1 in brain endothelial cells. The role of glia remains unknown, but because normal endothelial cells have a very low expression of MDR1 in comparison to their epileptic counterpart, if any, the role of glia is related to maintenance of expression rather than induction. This also is supported by the fact that permeability to PHT in DIV-BBB established with normal HBMECs was not affected by the origin (from normal or epileptic brain) of glial cells (Fig. 4). This suggests that astrocytes have a minor contribution to the establishment of pharmacoresistance and that overexpression of MDR1 in astrocytes might instead play a role in cell survival (Marchi et al., 2004).

Several reports suggest that the epileptic BBB is altered both in terms of permeability and functional expression of glucose transporters (for a review, see Gronlund et al., 1996; Cornford et al., 2000). Our in vitro results suggest that the intrinsic capacity of endothelial cells isolated from epileptic brain is not different from their "normal" counterparts. Both cells promptly and reproducibly responded to flow and glia as expected, based on our previous work (Cucullo et al., 2005a). Thus it appears that factors other than intrinsically altered endothelial cell properties are involved in the described leakiness of the BBB. We did, however, test the capacity of our system to react to factors known to alter BBB permeability. We focused on the clinically relevant use of hyperosmotic agents to demonstrate that (a) BBB permeability can be transiently altered in the DIV-BBB; (b) these changes follow a time course comparable to that observed in vivo (Marchi et al., 2003); and (c) drug permeability is massively increased after disruption of the endothelial tight junctions.

The fact that a leaky BBB, as suspected to be in epilepsy patients, allows greater passage of drugs appears fairly obvious and yet is in sharp contrast with the clinical reality. Increasing evidence suggests that AED penetration in human epileptic brain is decreased (Oby et al., 2006) despite an apparent increased permeability. Thus other factors, including perhaps perivascular edema or different pKa values in epileptic brain, may be involved.

As soon as new AEDs have been introduced into the market, hope that drug resistance will be overcome is rapidly overtaken by lack of significant improvement in multiple drug resistant epilepsy patients. The antiepileptic activity of these drugs was, for the most part, defined by acute seizure models such as subcutaneous pentylenetetrazol seizure tests and rat kindling. The clinical evidence to date would suggest that none of these models, albeit useful, is likely to identify those therapeutics that will effectively manage the refractory seizures. Data from this and other laboratories (reviewed in Loscher, 2002b; Oby and Janigro, 2006) suggested that the BBB plays a crucial role in drug resistance. We envisage that the transendothelial permeation properties of novel compounds obtained by using a "drug resistant" humanized BBB model are more likely representative and predictive of the brain uptake of such compounds by the "epileptic" brain.

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