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Report

The Application of Bovine Brain Microvessel Endothelial-Cell Monolayers Grown onto Polycarbonate Membranes *in Vitro* to Estimate the Potential Permeability of Solutes Through the Blood–Brain Barrier

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Previously our laboratory (Rim *et al., Int. J. Pharm.* 32:79–84, 1986) described an *in vitro* blood–brain barrier (BBB) model consisting of cultured bovine brain microvessel endothelial cells (BMECs) grown onto regenerated cellulose acetate membranes. However, the utility of this *in vitro* BBB model system was limited because the regenerated cellulose acetate membrane and not the monolayer of bovine BMECs was rate limiting for the permeability of very lipophilic compounds. Therefore, in this study we have evaluated polycarbonate membranes as supports for growing bovine BMECs and for conducting *in vitro* drug permeability studies. Bovine BMECs were cultured on collagen-coated polycarbonate membranes (13-mm diameter, 12-μm pore size) which were then mounted into side-by-side diffusion cells for transport studies. The permeabilities of a series of solutes of varying lipophilicity (progesterone, estrone, testosterone, haloperidol, propranolol, antipyrine, caffeine, urea, acyclovir, ganciclovir, ribavirin, and glycerol) were determined and an excellent correlation ($r = 0.97$) was established between the permeability coefficients of the solutes and their log partition coefficients $(PC)/(MW)^{1/2}$. These results suggest that bovine BMECs cultured onto polycarbonate membranes can be used as an *in vitro* model system for estimating the potential permeability of a solute through the BBB *in vivo*.

KEY WORDS: blood–brain barrier; passive diffusion; brain microvessel endothelial cells; polycarbonate membranes.

INTRODUCTION

Brain microvessel endothelial cells (BMECs) forming the blood–brain barrier (BBB) are joined together by tight intercellular junctions, possess few pinocytotic vesicles, and lack fenestrations. Because of these unique properties, the permeabilities of most small water-soluble solutes, including nutrients, peptides, and drugs, across the BBB are regulated (1–3). Small, water-soluble nutrients (e.g., amino acids, hexoses, amines, purines, nucleosides, and monocarboxylic acids) (2,4) and neuropeptides (e.g., melanocyte-stimulating inhibitory factor, enkephalins, vasopressin) (3,5) are transported across the BBB by specific carrier systems. Additionally, some water-soluble drugs such as α-methyldopa, α-methyltyrosine, melphalan, and L-DOPA are transported across the BBB by the neutral amino acid transport system. However, many bloodborne solutes enter the brain from the blood by passively diffusing through or adsorbing into the lipid membrane of the BMECs. Therefore, rates of passage of these molecules through the BBB depend directly on their lipid solubility and inversely on their molecular size (7,9). Oldendorf (8), Levin (10), and Cornford *et al.* (11) have demonstrated that brain uptake of water-soluble solutes *in vivo* is related to both the octanol/water or octanol/buffer partition coefficient and molecular weight.

Recently, our laboratory reported a similar correlation between a drug's physicochemical properties (e.g., lipophilicity and molecular weight) and its permeability through a monolayer of bovine BMECs grown onto a regenerated cellulose acetate membrane (12). This *in vitro* cell culture model system exhibits histochemical, biochemical, and morphological properties consistent with those of the brain microvessel endothelial cells that constitute the BBB *in vivo* (13–15). However, the utility of the *in vitro* model system originally described by our laboratory (12) was restricted because the permeability of the cellulose acetate membrane itself, and not the monolayer of bovine BMECs, became rate limiting for very lipophilic solutes (e.g., propranolol).

In this study we have evaluated polycarbonate membranes as a support for growing bovine BMECs and the suitability of monolayers of BMECs grown onto polycarbonate membranes for conducting permeability studies of hydrophilic and lipophilic solutes.

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MATERIALS AND METHODS

Materials

[^{3}H]Sucrose (10.1 Ci/mmol), [^{3}H]propranolol (26.6 Ci/mmol), [^{3}H]testosterone (52.6 Ci/mmol), [^{3}H]haloperidol (14.6 Ci/mmol), [^{3}H]hydrocortisone (45 Ci/mmol), [^{3}H]progesterone (45 Ci/mmol), [^{3}H]estrone (42 Ci/mmol), [^{14}C]antipyrine (54 mCi/mmol), [^{14}C]sucrose (498 mCi/mmol), [^{14}C]glycerol (48.7 mCi/mmol), and [^{14}C]caffeine (58 mCi/mmol) were purchased from New England Nuclear (Boston, Mass.). [^{14}C]Thiourea (48 mCi/mmol) and [^{3}H]sodium butyrate (39 mCi/mmol) were purchased from ICN Radiochemicals (Irvine, Calif.). [^{14}C]Acyclovir (53 mCi/mmol) and [^{14}C]ganciclovir (53 mCi/mmol) were generous gifts from Dr. Karen K. Biron at the Burroughs–Wellcome Co. (Research Triangle Park, N.C.). [^{14}C]-Ribavirin (48 mCi/mmol) was a generous gift from the Major M. A. Ussery U.S. Army Research Institute of Infectious Diseases (Ft. Detrick, Frederick, Md.).

Isolation and Culture of Bovine BMECs

Microvessel endothelial cells were isolated from the cerebral gray matter of bovine brain as described by Audus and Borchardt (13). The isolated brain microvessels were stored at −70°C in culture medium containing 10% dimethyl sulfoxide (DMSO) and 20% horse serum. Prior to seeding with brain microvessels, polycarbonate membranes (13-mm diameter, 12-μm pore size) (Nucleopore Co., Pleasanton, Calif.) were placed in 100-mm culture dishes, coated with rat tail collagen and fibronectin, cross-linked with ammonia fumes, and sterilized for 90 min in UV light as described previously by Audus and Borchardt (16). Brain microvessels were seeded at approximately 3×10^6 cells per 100-mm dish and cultured at 37°C with 95% humidity and 5% CO_2. After 10–12 days the cells reach confluency as determined by visual inspection under an inverted microscope. The morphological characteristics of the cells grown onto polycarbonate membranes were consistent with those reported earlier (13–17).

Transcellular Transport Studies

Horizontal Side-Bi-Side diffusion cells (Crown Glass Co., Inc., Somerville, N.J.) were used for transcellular transport studies as described by Rim *et al.* (12). The polycarbonate membrane containing the BMEC monolayers was placed between the two halves of the diffusion cell. The side facing the endothelial cell was designated the donor side and the other side of the diffusion cell was designated the receiver side. The transendothelial assay buffer (122 m*M* NaCl, 25 m*M* $NaHCO_3$, 10 m*M* *d*-glucose, 3 m*M* KCl, 1.2 m*M* $MgSO_4$, 0.4 m*M* K_2HPO_4, 1.4 m*M* $CaCl_2$, 10 m*M* Hepes, pH 7.4) was placed in both the donor and the receiver sides. The temperature was maintained at 37°C using a circulating water bath, and the donor and receiver sides were stirred (600 rpm). The donor chamber was pulsed with the ^{3}H- or ^{14}C-labeled solute (approximately 0.5 μCi) and nonradioactive solute to a final concentration of 100 n*M*. The receiver chamber was sampled (200 μl) at 5-, 10-, 15-, 30-, and 60-min time intervals. When a 200-μl sample was removed from the receiver side, it was replaced with an equal volume of fresh transendothelial buffer. Scintillation cocktail (3a70, Research Products International Corp., Mt. Prospect, Ill.) was added to each 200-μl aliquot and the samples were thoroughly mixed and then counted in a scintillation counter (Beckman Model 6800, Fullerton, Calif.). Raw counts were transformed to disintegrations per minute (dpm) by a standard quench curve. These were then converted to the amount of solute as picomoles and flux rates (k) were calculated from the slopes of the picomoles per milliliter versus minute plots (e.g., Fig. 1) and expressed as picomoles per milliliter per minute. The flux rates (k) of the solutes were corrected for leakiness of the monolayers using [^{3}H]sucrose or [^{14}C]sucrose as described previously by Audus and Borchardt (16). The flux rates (k) were then converted to permeability coefficients (P) according to Eq. (1):

$$P = \frac{kV}{AC_0} \tag{1}$$

where V is the volume (3.0 ml) of the donor chamber, A is the cross-sectional area of the cell surface, and C_0 is the

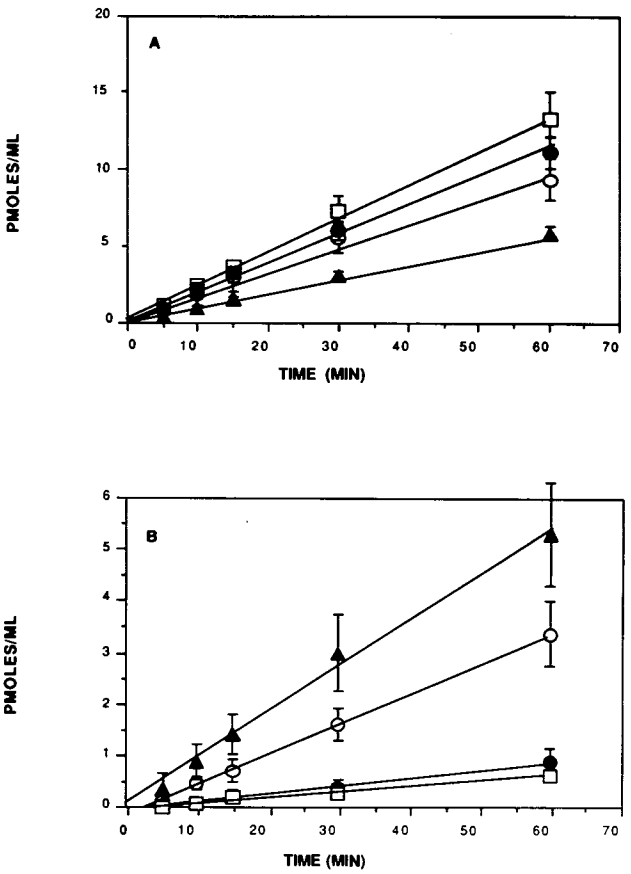

Fig. 1. Time dependence of the flux of solutes across the bovine BMECs grown on polycarbonate membranes. Experimental conditions are described under Materials and Methods. (A) □——□, Testosterone; ○——○, propranolol; ●——●, estrone; ▲——▲, antipyrine. (B) ○——○, Urea; ▲——▲, caffeine; ●——●, acyclovir; □——□, ganciclovir.

initial concentration of the solute in the donor chamber. The concentration of solute in the donor chamber remains at >90% of the initial concentration over the time period for which k was calculated.

Octanol/Buffer Partition Coefficients

Octanol/buffer partition coefficients (PC) were determined for each compound as follows. To a tube containing transendothelial assay buffer (2 ml) and octanol (2 ml), approximately 0.5 μCi of the ^{3}H- or ^{14}C-labeled solute was added. The tube was vortexed for 1 min and then set in a water bath (37°C) for about 2 hr. The tube was vortexed again and this procedure was repeated three times to assure proper equilibrium of the solute between the organic and the aqueous phases. The tube was centrifuged for 30 min to assure complete separation of the solvent phases, after which an aliquot (100 μl) of the octanol phase was removed. After removal of the remaining octanol phase by aspiration, an aliquot (100 μl) of the aqueous phase was removed. The aqueous and octanol phases were counted by liquid scintillation spectrometry and the PCs calculated as follows.

$$PC = \frac{[^{14}C] \text{ or } [^3H] \text{ dpm}_{octanol}}{[^{14}C] \text{ or } [^3H] \text{ dpm}_{buffer}}$$

The procedure described above was done in triplicate for each solute.

RESULTS

Polycarbonate membranes are available from Nucleopore Corp. (Pleasanton, Calif.) in a variety of pore sizes. In preliminary experiments the permeability of [^{3}H]-propranolol through blank membranes having pore sizes of 1, 3, 5, and 12 μm was determined. The highest permeability of [^{3}H]propranolol was observed with the 12-μm-pore size membrane (data not shown). When the 12-μm-pore size membranes were coated with rat tail collagen and treated with fibronectin, they provided a surface conducive to the attachment, growth and development of a confluent monolayer of bovine BMECs. The morphological characteristics (data not shown) of the bovine BMECs grown onto the polycarbonate membranes are identical to those observed when the cells are grown onto regenerated cellulose membranes and on plastic culture dishes (13).

To evaluate the permeability characteristics of the bovine BMECs grown on polycarbonate membranes (pore size, 12 μm), 14 solutes were selected which ranged in octanol/buffer partition coefficients (PC) from log PC = −2.97 (sodium butyrate) to log PC = 3.54 (progesterone). As shown in Fig. 1, the flux of several representative solutes across the monolayers of bovine BMECs grown on polycarbonate membranes increased linearly with time. The flux of these solutes across the monolayers was also concentration dependent but not saturable (data not shown), suggesting a passive diffusion mechanism. For all of the solutes, including those which were highly lipophilic, the cell monolayer rather than the polycarbonate membrane was the rate-limiting barrier.

Knowing the volume of the donor chamber, the cross-sectional area of the cell surface, the initial concentration of the solute, and the flux rates as calculated from the data shown in Fig. 1, the permeability coefficients were calculated for each solute and the results are presented in Table I. When these permeability coefficients are plotted versus the log partition coefficient (PC)/(MW)$^{1/2}$, a linear relationship having a significant positive correlation (r = 0.97) is observed.

DISCUSSION

In order for monolayer cultures of BMECs grown onto porous membranes to be useful as an *in vitro* model of the BBB, the membrane should have the following characteristics: (i) it must provide a surface which will allow for the attachment, growth, and development of a confluent monolayer of BMECs; (ii) it should be sufficiently translucent so that the development of the cell monolayer can be verified by microscopic techniques; (iii) it should be readily permeable to hydrophilic and lipophilic solutes; and (iv) it should be readily permeable to both low and high molecular weight solutes.

In earlier studies, our laboratory employed nylon mesh (Nitex 85 or 103 μm) (13) and regenerated cellulose membranes (Sartorius Filters; nominal MW cutoff, 160 kDa) (12) as porous surfaces for growing BMECs. Both membranes, when coated with rat tail collagen and treated with fibronectin, provided surfaces which allowed for attachment, growth, and development of monolayers of bovine BMECs. However, the utility of the nylon mesh was limited because the surface was not sufficiently flat to allow monolayers to form without leaving very small spaces on the nylon mesh uncovered by cells. Hence, the bovine BMEC monolayers

Table I. Log Partition Coefficients and Bovine BMEC Monolayer Permeability Coefficients

Compound	Log Partition Coefficients[a]	Permeability Coefficients × 10^4 cm/min
Sodium butyrate	−2.97	7.76
Acyclovir	−2.88	6.21
Glycerol	−2.42	5.70
Ganciclovir	−2.07	4.88
Ribavirin	−1.85	4.10
Urea	−1.66	25.8
Thiourea	−0.92	17.0
Caffeine	−0.07	40.3
Antipyrine	0.25	43.6
Propranolol	1.93	68.7
Estrone	2.55	81.2
Testosterone	3.13	89.2
Haloperidol	3.27	97.0
Progesterone	3.54	88.5

[a] Partition coefficients (PC) between octanol and buffer were determined as described under Materials and Methods.

[b] Permeability coefficients (P) through the bovine BMEC monolayer grown on polycarbonate membranes, 12-μm pore size, were calculated using the equation $P = kV/AC_0$, where V is the volume of the donor chamber, k is the flux rate, A is the cross-sectional area of the cell surface, and C_0 is the initial concentration of the solute in the donor chamber.

grown on nylon mesh exhibited unacceptably high levels of [^{14}C]sucrose leakage (13).

Monolayers of bovine BMECs grown on regenerated cellulose membranes have been used by our laboratory to study the passive diffusion [e.g., mannitol (12)] and the carrier-mediated flux [e.g., leucine (16) and baclofen (17)] of low molecular weight (MW) hydrophilic solutes. However, bovine BMECs grown onto regenerated cellulose membranes are not useful for studying the flux of low MW lipophilic solutes [e.g., propranolol (12)] and high molecular weight, hydrophilic solutes (e.g., insulin, unpublished data) because the membrane and not the BMEC monolayer becomes rate limiting.

Therefore, because of the limitations of nylon mesh and regenerated cellulose membranes, we evaluated other porous membranes for culturing bovine BMECs. As described in this study, polycarbonate membranes (12-μm pore size), when coated with collagen and treated with fibronectin, afford a surface which allows the attachment, growth, and development of a confluent monolayer of bovine BMECs. The polycarbonate membranes are sufficiently translucent to permit visualization of the development of the confluent cell monolayer. The polycarbonate membranes are also chemically inert and do not absorb lipophilic solutes! Thus, bovine BMECs grown onto polycarbonate membranes can be used to study the passive, transcellular diffusion of solutes ranging from very hydrophilic (e.g., sodium butyrate; log PC = −2.97) to very lipophilic (e.g., progesterone; log PC = 3.54). As shown in Fig. 2, an excellent correlation was established between the permeability coefficients of the solutes and their log PC/(MW)$^{1/2}$. Since a similar correlation has been observed *in vivo* (7–11), the bovine BMECs cultured onto polycarbonate membranes can be used as an *in vitro* model system for estimating the potential permeability of a solute through the BBB *in vivo*.

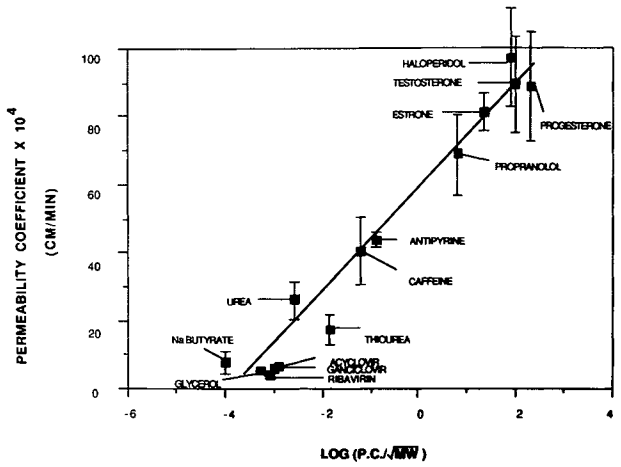

Fig. 2. Relationship between the permeability coefficients of solutes crossing monolayers of bovine BMECs grown on polycarbonate membranes and the log partition coefficient/(MW)$^{1/2}$. See Table I for log partition coefficients and permeability coefficients.

Bovine BMECs grown onto polycarbonate membranes have also been used in our laboratory to study the transcellular diffusion of protein solutes via fluid-phase transcytosis [e.g., bovine serum albumin (BSA) (18)], adsorptive transcytosis [e.g., cationized BSA, glycosylated BSA (18)], and receptor-mediated transcytosis [e.g., insulin, insulin-like growth factors (19)]. This *in vitro* BBB model system has also been used to study the carrier-mediated transcellular transport of amino acid-type drugs [e.g., acivicin (20), α-methyldopa (21)] and the passive, transcellular diffusion of catecholamine prodrugs (22).

In summary, bovine BMECs grown onto polycarbonate membranes appear to provide an *in vitro* model of the BBB suitable for studying the transcellular flux of hydrophilic or lipophilic solutes which penetrate the cell monolayer by passive diffusion, carrier-mediated mechanisms, or transcytotic mechanisms.

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