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Research Article

Characterization of an *In Vitro* Blood–Brain Barrier Model System for Studying Drug Transport and Metabolism

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Bovine brain microvessel endothelial cells have been isolated and grown in culture to monolayers. These endothelial cell monolayers have been characterized morphologically with electron microscopy, histochemically for brain endothelium enzyme markers, alkaline phosphatase and γ-glutamyl transpeptidase, and by immunofluorescence to detect Factor VIII antigen, an exclusive endothelial antigen. Results of these studies indicate that the cells forming the monolayers are of endothelial origin and possess many features of the *in vivo* brain endothelium responsible for formation of the blood–brain barrier. This *in vitro* blood–brain barrier model system was used in experiments to determine the permeability of the cultured monolayer to sucrose, leucine, and propranolol. Leucine rapidly moved across the monolayers of this *in vitro* system and tended to plateau after approximately 10 min. In contrast, the rates of sucrose and propranolol movement were linear during a 1-hr observation period, with the rate of propranolol movement across the monolayer greater than that of sucrose. The ability to detect differences in the permeability of the monolayers to leucine, propranolol, and sucrose with radioactive tracers suggests that this *in vitro* model system will be an important tool for the investigation of the role of the blood–brain barrier in the delivery of centrally acting drugs and nutrients.

KEY WORDS: blood-brain barrier; brain microvessel endothelium; endothelium; drug delivery; primary culture; monolayers.

INTRODUCTION

The structure responsible for formation of the blood–brain barrier is the brain microvessel endothelial cell (1,2). These endothelial cells are bound together by tight intercellular junctions that, combined with the presence of few pinocytotic vesicles, form a barrier that effectively restricts the movement of most polar molecules and proteins from blood to brain (1,3,4).

Recently developed methods now permit the isolation and culture of a homogeneous population of cerebral microvessel endothelial cells to form an *in vitro* blood–brain barrier model (5,6). This *in vitro* blood–brain barrier model system presents the opportunity for one to investigate, at the biochemical level, the mechanisms responsible for regulating the permeability of the blood–brain barrier. A potentially important application of this model system is to study the drug transport and metabolic mechanisms present in these cells, in order to obtain a basic understanding of the role of the blood–brain barrier in regulating drug delivery to the brain.

This report describes the successful development and characterization of an *in vitro* blood–brain barrier model and the use of the model system in preliminary transendothelial-cell transport experiments. The endothelial cells of this model system retain characteristics of brain endothelial cells *in vivo* and therefore represent a useful *in vitro* system for examination of the transport and metabolic properties of the blood–brain barrier.

MATERIALS AND METHODS

Isolation and Culture of Bovine Brain Microvessel Endothelial-Cell Monolayers

Bovine microvessel endothelial cells were isolated by the method of Bowman *et al.* (5,6) with minor modifications. Briefly, two or three bovine brains were obtained from a local slaughterhouse 30–40 min after death and transported to the laboratory in ice-cold serum-free minimum essential medium (MEM; KC Biological, Inc., Lenexa, Kans.) buffered with 50 m*M* Hepes, pH 7.4, and containing 100 μg/ml penicillin G, 100 μg/ml streptomycin, 50 μg/ml polymyxin B, and 2.5 μg/ml amphotericin B (Sigma Chemical Co., St. Louis, Mo.). Meninges and large surface vessels were removed from the cerebral cortex of the brains and discarded. The gray matter of the cerebral cortex was cut away with a sterile razor blade and minced into 1- to 2-mm cubes. Approximately 250 ml of wet minced gray matter was collected and placed in a 500-ml sterile plastic bottle. The gray matter was suspended to a final volume of 500 ml in MEM containing dispase (Boehringer–Mannheim Biochemicals, Indianapolis, Ind.) at a final concentration of 0.5% and placed in

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a shaking water bath for 3 hr at 37°C. After the dispase treatment, the cell suspension was centrifuged at 1000*g* for 10 min then the pellets were resuspended in 500 ml of 13% dextran (Sigma Chemical Co., St. Louis, Mo.; avg. MW 70000) and centrifuged at 5800*g* for 10 min. Cell debris, fat, and myelin, which float on the dextran, were discarded and the crude capillary pellets resuspended in 20 ml of MEM containing 1 mg/ml collagenase/dispase (Boehringer–Mannheim Biochemicals, Indianapolis, Ind). The suspension of brain capillaries was placed in a shaking water bath for 5 hr at 37°C. After the incubation period, the capillary suspension was diluted up to 50 ml with fresh MEM and centrifuged at 1000*g* for 10 min. The resulting capillary pellet was resuspended in 8 ml of fresh MEM. Aliquots (2 ml) of the suspension were layered over 50 ml of a preestablished 50% Percoll (Sigma Chemical Co., St. Louis, Mo.) gradient (6) in each of four 75-ml centrifuge tubes and then centrifuged at 1000*g* for 10 min. Approximately 120–150 million endothelial cells per isolation were collected from the Percoll gradients, washed with MEM by centrifugation at 1000*g* for 10 min and resuspended in culture medium containing equal parts of F-12 Ham nutrient mix (KC Biological, Inc., Lenexa, Kans.) and minimal essential medium, 10 m*M* Hepes, 13 m*M* sodium bicarbonate, pH 7.4, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 50 μg/ml polymyxin B, 2.5 μg/ml amphotericin B, 100 μg/ml heparin (Sigma Chemical Co., St. Louis, Mo.), and 10% plasma-derived horse serum (HyClone Laboratories, Logan, Utah). Cell viability was determined to be approximately 90% by trypan blue exclusion. For convenience, most of the isolated endothelial cells were frozen (−70°C), for later use, in culture medium supplemented with 10% dimethyl sulfoxide (DMSO) and 20% plasma-derived horse serum.

Endothelial cells were counted by crystal violet nuclei staining, seeded onto culture surfaces at a density of 50,000 cells/cm^2, and allowed to attach and grow to a complete monolayer in the presence of 95% humidity, 5% CO_2, at 37°C. Culture media were changed after the first 3 days in culture to media not containing polymyxin B. All growth surfaces were coated with rat-tail collagen prepared and applied by the method of Michelopoulis and Pitot (7) followed by treatment with 10 μg/cm^2 human fibronectin (Boehringer–Mannheim Biochemicals, Indianapolis, Ind.) for 1 hr.

Characterization of Bovine Brain Microvessel Endothelial-Cell Monolayers

To facilitate characterization of the endothelial-cell monolayers, monolayers were grown on collagen-coated, fibronectin-treated glass coverslips in 100-mm plastic petri dishes. Isolated microvessel endothelial cells and monolayers were prepared for examination by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described by Bowman *et al.* (6). Alkaline phosphatase activity in monolayers and in freshly isolated endothelial cells was demonstrated histochemically with Sigma Kit No. 85 (Sigma Chemical Co., St. Louis, Mo.). γ-Glutamyl transpeptidase in the endothelial-cell monolayers was determined histochemicaly by the method of Glenner *et al.* (8). γ-Glutamyl transpeptidase activity in isolated endothelial cells was assayed biochemically as described by Caspers and Diglio (9). Cell protein was determined by the method of Lowry *et al.* (10). The method of Jaffe *et al.* (11) was employed to test for the presence of Factor VIII antigen.

Transendothelial Transport Studies

Endothelial cells were seeded onto collagen- and fibronectin-coated 15 × 15-mm nylon-mesh squares (Nitex 85 or 103 μm; Tetko Inc., Elmsford, N.Y.) and allowed to grow to confluence. The nylon-mesh squares were then attached across a 9-mm hole cut through a 12-mm-thick, 22 × 22-mm, plexiglass block to form a chamber, with the bottom formed by the nylon mesh covered by an endothelial-cell monolayer. The plexiglass supports with attached legs were set into six-well plates, with 0.45 ml buffer added to the chamber in the plexiglass square and 7 ml buffer added to the plate well containing the plexiglass support. The buffer in the plate well bathes the sides and bottom of both the plexiglass block and the nylon mesh and forms the bottom chamber. The six-well plates containing the plexiglass supports were placed in a shaking water bath at 37°C to maintain a constant temperature and to mix the contents of the system. Labeled compounds [about 1–2 μCi of ^{3}H-leucine, 120 Ci/mmol (ICN Chemical & Radioisotope Division, Irvine, Calif.), or ^{3}H-propranolol, 20 Ci/mmol (Amersham, Arlington Heights, Ill.), along with 0.1–0.2 μCi of ^{14}C-sucrose, 540 mCi/mmol (Research Products International Corp., Mount Prospect, Ill.)] were added in 0.05-ml aliquots to the upper chamber (within the plexiglass support) and 0.5-ml aliquots were removed from the lower chamber at various times. The 0.5-ml aliquot from the bottom chamber was placed in a scintillation vial with 10 ml of scintillation cocktail and then assayed by scintillation spectrometry. Aliquots (0.5 ml) of fresh buffer were added back to the lower chamber to maintain a constant volume. The assay buffer consisted of 150 m*M* sodium chloride, 4 m*M* potassium chloride, 3.2 m*M* calcium chloride, 1.2 m*M* magnesium chloride, 15 m*M* Hepes, pH 7.4, 5 m*M* glucose, and 1% (w/v) bovine serum albumin.

RESULTS

The SEM in Fig. 1 shows a single, enzymatically isolated, bovine brain microvessel. The endothelial cells retain tight intercellular junctions despite the enzymatic treatment; therefore, the final isolate appears as clumps of short segments of microvessels. Removal or disruption of the basement membrane by collagenase exposes the endothelial-cell membrane surface, giving the microvessel a rough appearance as is also seen in Fig. 1. The enzymatic treatment facilitates the release of contaminating pericytes and neuronal attachments, and it provides for a better separation of a homogeneous microvessel endothelial-cell isolate on the Percoll gradient. Microvessel isolates in this study were free from contaminating cells as observed under the light and electron microscope.

The microvessel endothelial cells suspended in culture medium seeded onto growth surfaces attach and grow out from the original microvessel. Figure 2 shows a group of brain microvessel endothelial cells with the typical cobblestone appearance after 5 days in culture. As pictured in Fig. 2, the cells are generally fusiform in shape, with the occa-

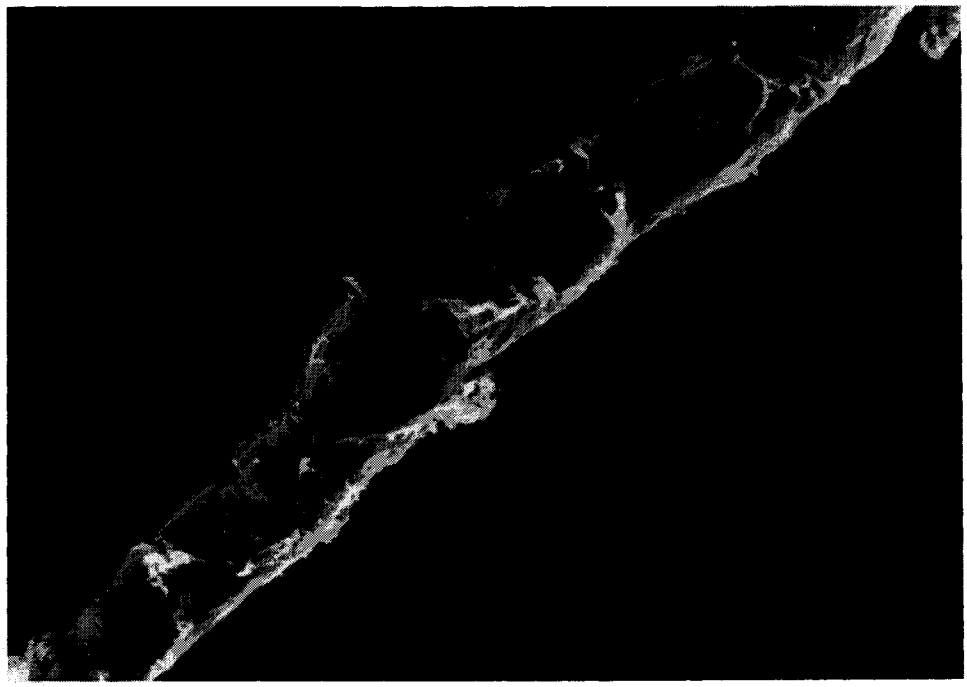

Fig. 1. Scanning electron micrograph of an enzymatically isolated bovine brain microvessel. 640×; reduced 25% for reproduction.

sional appearance of polygonal-shaped cells. Seeded at a density of approximately 50,000 cells/cm^2, a complete monolayer was observed in 8 to 9 days.

SEM of the surface of a complete endothelial-cell monolayer (Fig. 3) reveals the intimate association of the cells and raised folds in areas of possible cell junctions. In cross section through the monolayers, frequent cell overlap and junctional complexes and few pinocytotic vesicles are observed. The TEM in Fig. 4 is an example of a junctional complex between two endothelial cells.

Microvessel endothelial cells, freshly isolated or grown to monolayers in culture, stain histochemically positive for alkaline phosphatase as indicated by the dark blue perinuclear granules observed in these cells (not shown). The spe-

Fig. 2. Scanning electron micrograph of a group of brain microvessel endothelial cells after 5 days in culture. 175×; reduced 25% for reproduction.

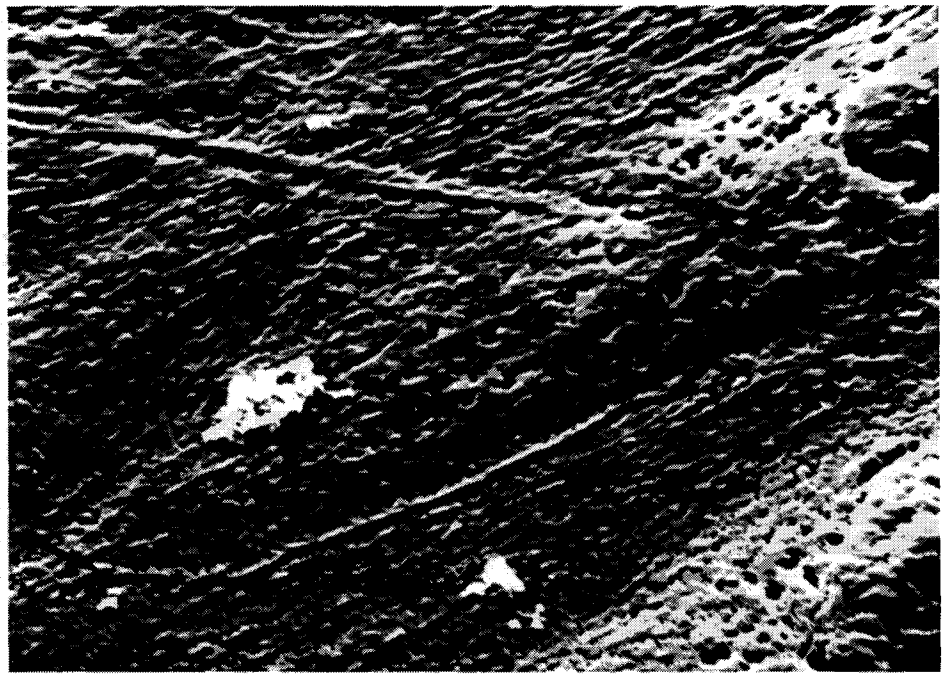

Fig. 3. Scanning electron micrograph of the surface of an endothelial-cell monolayer. Cell nuclei appear as large, oval, raised areas (lower and upper right corners) on the monolayers. Arrows indicate possible areas of junctional complexes. 3000×; reduced 25% for reproduction.

cific activity of γ-glutamyl transpeptidase in freshly isolated microvessel endothelial cells is approximately 48 ± 3 nmol/min/mg protein and is about three times the activity observed in whole cerebral cortex homogenate. The endothelial cells also retain γ-glutamyl transpeptidase activity in primary cultures where characteristic reddish-brown perinuclear staining is observed in the endothelial cells (not shown).

Factor VIII antigen is observed in the cells of these monolayers after treatment with rabbit antiserum to Factor VIII and as indicated by perinuclear fluorescence observed in endothelial cells subsequently exposed to fluorescein-conjugated IgG (not shown). In contrast, cells treated with normal rabbit serum, as a control, do not fluoresce following exposure to the fluorescein-conjugated IgG.

To look at transendothelial transport, the cells were

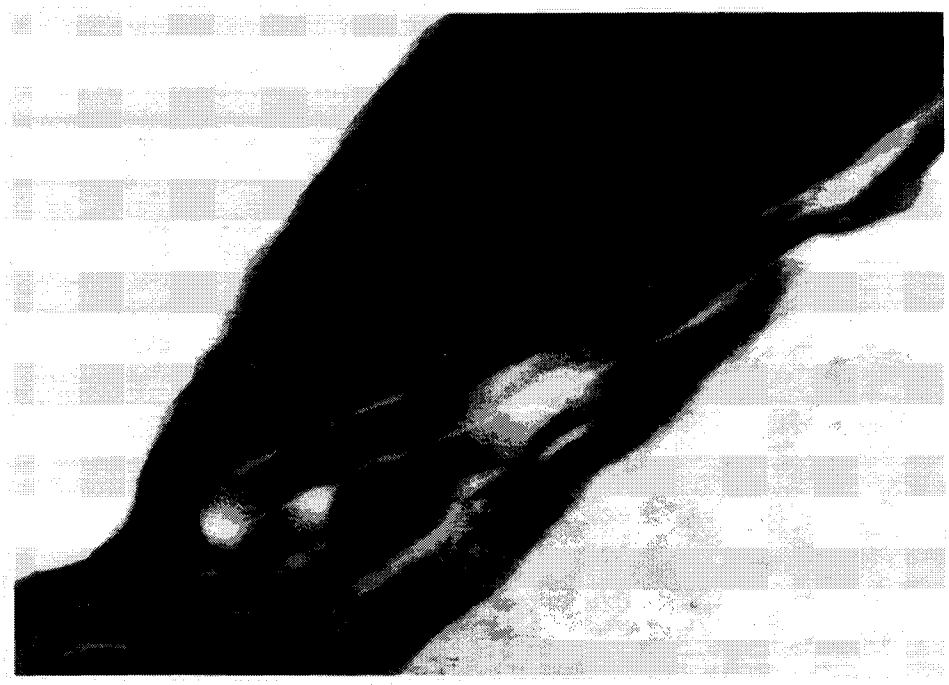

Fig. 4. Transmission electron micrograph of a junction between two endothelial cells. 70,000×; reduced 25% for reproduction.

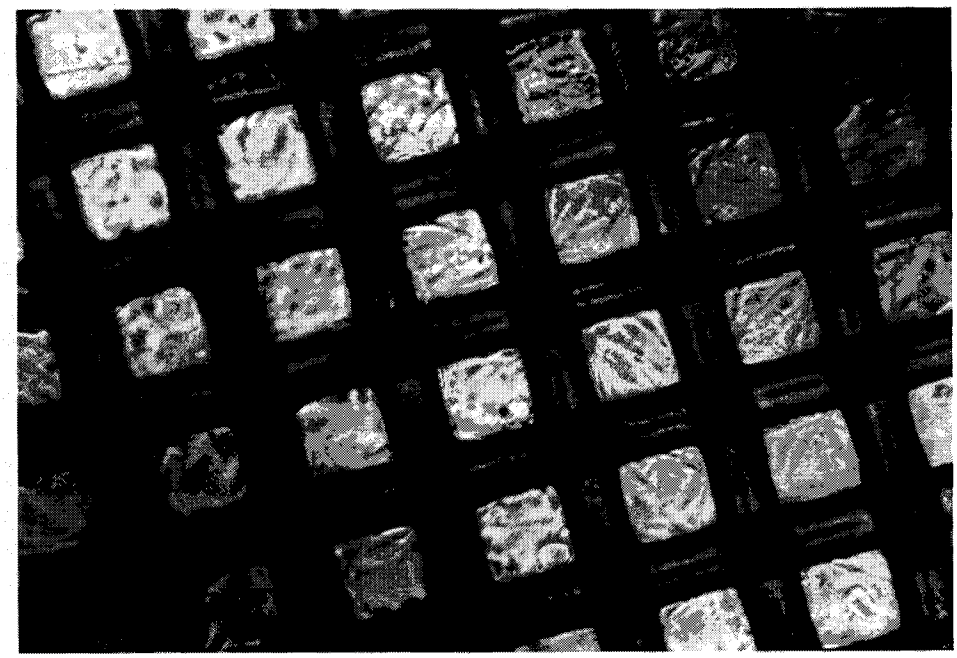

Fig. 5. Light micrograph of a monolayer of brain microvessel endothelial cells grown on nylon mesh. 100×; reduced 25% for reproduction.

grown on collagen-coated, fibronectin-treated, nylon mesh as shown in Fig. 5. The movement of leucine, propranolol, and sucrose across endothelial-cell monolayers was followed over a period of 1 hr and the data are expressed as the total radioactivity in the bottom chamber (counts per minute; cpm) observed at a particular sample time divided by the total cpm added to the system, times 100%. As shown in Fig. 6, sucrose and propranolol movement across the monolayer is linear during the observation period. Leucine movement, however, as also shown in Fig. 6, is much more rapid initially and tends to plateau after aproximately 10 min. ^{14}C-Sucrose diffusion across the same monolayer used in the leucine experiments was monitored simultaneously and also found to be approximately as shown in Fig. 6. These compounds move at equivalent rates across collagen-coated nylon mesh in the absence of a monolayer to 60–70% of the total cpm after 1 hr (data not shown). As determined by trypan blue exclusion, there was no decline in the viability of the endothelial cells of these monolayers over the time period of these experiments.

In other experiments, sucrose movement across the monolayers could be shown to be increased following a 5-min exposure of the monolayers to a 1.6 *M* solution of arabinose. The change in sucrose movement across the monolayer as a result of the hyperosmotic insult could be reversed by returning the monolayers to normal culture conditions for 6 hr (data not shown).

DISCUSSION

Several methods exist for the isolation of cerebral microvessel endothelial cells at the present time (5,6,12–15). The microvessels isolated in this study are morphologically consistent with the microvessels described in those reports. In addition, the microvessels are metabolically functional as shown by the histochemical demonstration of alkaline phosphatase and the biochemical demonstration of γ-glutamyl transpeptidase in this study. Both enzymes are considered to be specific for brain microvessel endothelium (16,17). The threefold enrichment of γ-glutamyl transpeptidase and the high viability of microvessel isolates in this study are also in good agreement with previous reports for enzymatically isolated brain microvessels (5,6,14).

In culture, the microvessel endothelial cells attach and spread into a continuous sheet of overlapping cells that appear to be morphologically consistent with the monolayers described by Bowman *et al.* (5,6). Our histochemical studies demonstrate that, in primary culture, the monolayers retain both alkaline phosphatase and γ-glutamyl transpeptidase activity. The activity of these enzymes in the monolayers has also been confirmed and quantitated biochemically by Baranczyk-Kuzma *et al.* (18). As indicated above, these enzymes are considered to be specific enzyme markers for brain microvessel endothelium and have been demonstrated

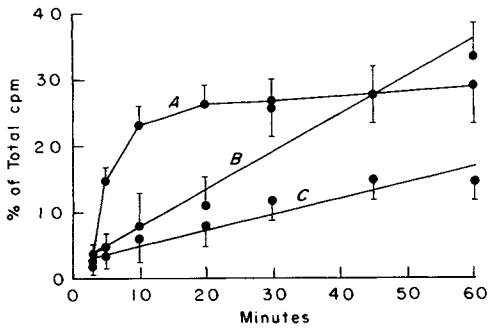

Fig. 6. Time course of transendothelial movement of (A) leucine, (B) propranolol, and (C) sucrose. Data points represent the mean ± SE with $N = 4$.

in other endothelial-cell monolayers derived from cerebral microvessels (15). The presence of Factor VIII antigen, an antigen found exclusively in endothelial cells (19), further demonstrates the endothelial origin of these cells. The results of these morphological, enzyme marker, and Factor VIII antigen studies indicate that the cells *in vitro* possess many characteristics that are observed in the blood–brain barrier *in vivo*.

Since the cultured cerebral microvessel endothelial cells form a monolayer with characteristics of the blood–brain barrier *in vivo*, as shown above and by others (5,6,12,13,15), experiments were conducted in this laboratory to test this *in vitro* model system's applicability for drug delivery studies. The results obtained show that sucrose, a compound that does not readily cross cell membranes, slowly penetrates the endothelial-cell monolayers of this model system. Sucrose movement across these monolayers is presumed to be through either intercellular spaces or areas of the diffusion barrier where the monolayer was incomplete. The slow diffusion of sucrose across these monolayers is similar to that observed in the *in vitro* model system described by Bowman *et al.* (5). Bowman *et al.* (5) were also able to alter reversibly the diffusion of sucrose across the monolayers by exposing them to hyperosmotic solutions and calcium-free solutions. Further investigations by Bowman *et al.* (5) and Dorovini-Zis *et al.* (20) reveal that hyperosmotic solutions open the tight intercellular junctions, effecting an increased permeability to sucrose. Preliminary experiments in this laboratory also suggest that sucrose permeability in this system can be reversibly changed by a hyperosmotic arabinose solution. Transient enhanced permeability after treatment with hyperosmotic solutions is also characteristic of the blood–brain barrier *in vivo* (21).

The surface of nylon mesh is not sufficiently flat to allow monolayers to form without leaving very small spaces on the nylon mesh uncovered by cells. Hence, significant leakage of sucrose across the monolayers was observed in these studies. Differences in the monolayer permeability to the three molecules used in this study are discernible and consistent with the present knowledge of the permeability characteristics of the three molecules. However, a flat-surfaced, permeable-growth surface which would allow for the formation of a more complete and, therefore, a more restrictive monolayer is desirable and has been developed. Brain microvessel endothelial cells seeded onto regenerated cellulose membranes (nominal molecular weight cutoff, 160,000), for example, form a complete monolayer that allows only negligible leakage of sucrose over up to 40 min after introducing sucrose into the test system (22). Monolayers grown on regenerated cellulose membranes, then, will permit the characterization of drug and nutrient transport in the absence of significant leakage because of areas on the growth surface not covered by cells.

Propranolol, a lipophilic molecule, diffuses across the monolayers of this system at a greater rate than sucrose. Propranolol diffuses across the membranes of the endothelial-cell monolayer passively as well as through areas of the diffusion barrier where the monolayer may be incomplete. That propranolol moves across the monolayer at a greater rate than sucrose is suggested by the results obtained in experiments where mixtures of ^{3}H-propranolol and ^{14}C-sucrose were used to determined the permeability of a monolayer. Propranolol diffusion across the blood–brain barrier *in vivo* has been reported to be a saturable process of a very low affinity (apparent K_m = 9.8 m*M*) and high capacity (V_{max} = 5.7 μmol/min/g) (23). Additional experimentation is required to determine if a similar system may exist in this *in vitro* model.

The large neutral amino acid transport system of the blood–brain barrier is a bidirectional, facilitated transport system that is important for both amino acid (24) and drug (25,26) transport. In this *in vitro* model system, leucine, representing a large neutral amino acid, moved across the monolayers very rapidly and tended to plateau after 10 min. The observation that leucine movement plateaus after 10 min is consistent with the presence of an equilibrative amino acid carrier system in the monolayers. Further investigation will be required to verify this observation. Although the percentage of leucine at the plateau may seem lower than expected, this result can perhaps be explained in part by the significant incorportion of leucine into proteins of these cells (22,27), as well as by distribution to other intracellular pools. Previous studies have shown that the ratio of intracellular and extracellular concentrations of leucine in microvessel endothelial cells is approximately 1.0 (28). Therefore, an equilibrium must be reached not only across the monolayer but also with the intracellular domain. Our results are in good aggreement with the observations of leucine uptake in both the microvessel (28) and the monolayer (27) *in vitro* model systems, where leucine uptake approached equilibrium after 10 min.

To conclude, we have described an *in vitro* blood–brain barrier model system that retains many features characteristic of the blood–brain barrier *in vivo*. Among these features are a similar morphology and the presence of specific enzyme markers and Factor VIII antigen. Moreover, this model system also appears to be a promising *in vitro* system for investigation of the transport mechanisms for drugs and nutrients based upon differences in the transendothelial movement of leucine, propranolol, and sucrose observed in the system. Currently, transport by passive and facilitated mechanisms and the metabolic properties of this model system are under investigation in this laboratory.

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REFERENCES

1. T. S. Reese and M. J. Karnovsky. *J. Cell Biol.* 34:207–217 (1967).
2. M. W. Brightman and T. S. Reese. *J. Cell Biol.* 40:648–677 (1969).
3. M. Bradbury. In *The Concept of a Blood Brain Barrier*, Wiley & Sons, New York, Chichester, 1979.

4. S. I. Rapoport. In *Blood Brain Barrier in Physiology and Medicine,* Raven Press, New York, 1976.
5. P. D. Bowman, S. R. Ennis, K. E. Rarey, A. L. Betz, and G. W. Goldstein. *Ann. Neurol.* **14**:396–402 (1983).
6. P. D. Bowman, A. L. Betz, J. S. Wolinsky, J. B. Penney, R. R. Shivers, and G. W. Goldstein. *In Vitro* 17:353–362 (1981).
7. G. Michelopoulis and H. Pitot. *Exp. Cell Res.* **94**:70–78 (1975).
8. G. G. Glenner, J. E. Folk, and P. J. McMillan. *J. Histochem. Cytochem.* **10**:481–489 (1962).
9. M. L. Caspers and C. A. Diglio. *Biochim. Biophys. Acta* **803**:1–6 (1984).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. *J. Biol. Chem.* **193**:265–275 (1951).
11. E. A. Jaffe, L. W. Hoyer, and R. L. Nachman. *J. Clin. Invest.* **52**:2757–2764 (1973).
12. L. E. DeBault, L. E. Kahn, S. P. Frommes, and P. A. Cancilla. *In Vitro* **15**:473–487 (1979).
13. P. Phillips, P. Kumar, S. Kumar and M. Waghe. *J. Anat.* **129**:261–272 (1979).
14. S. K. Williams, J. F. Gillis, M. A. Matthews, R. C. Wagner, and M. W. Bitensky. *J. Neurochem.* **35**:374–381 (1980).
15. M. Spatz, J. Bembry, R. F. Dodson, H. Hervonen, and M. R. Murray. *Brain Res.* **191**:577–582 (1980).
16. M. Orlowski, G. Sessa, and J. P. Green. *Science* **184**:66–68 (1974).
17. B. M. Djuricic and B. B. Mrsulja. *Brain Res.* **138**:561–564 (1977).
18. A. Baranczyk-Kuzma, K. L. Audus, and R. T. Borchardt. *J. Neurochem.* (in press).
19. E. A. Jaffe. *N. Engl. J. Med.* **296**:377–383 (1977).
20. K. Dorovini-Zis, P. D. Bowman, A. L. Betz, and G. W. Goldstein. *Brain Res.* **320**:383–386 (1984).
21. S. I. Rapoport, W. R. Fredricks, K. Ohno, and K. D. Pettigrew. *Am. J. Physiol.* **238**:R421–431. (1980).
22. K. L. Audus and R. T. Borchardt. *J. Neurochem.* (in press).
23. W. M. Pardridge, R. Sakiyama, G. Fierer. *Am. J. Physiol.* **247**:R582–R588 (1984).
24. W. M. Pardridge, R. J. Wurtman and J. J. Wurtman (eds.), In *Nutrition and the Brain,* Raven Press, New York, 1977, pp. 141–203.
25. A. F. Sved, I. M. Goldberg, and J. D. Fernstrom. *J. Pharmacol. Exp. Ther.* **214**:147–151 (1980).
26. J. G. Nutt, W. R. Woodward, J. P. Hammerstad, J. H. Carter, and J. L. Anderson. *N. Engl. J. Med.* **310**:483–488 (1984).
27. P. A. Cancilla and L. E. DeBault. *J. Neuropathol. Exp. Neurol.* **42**:191–199 (1983).
28. A. L. Betz and G. W. Goldstein. *Science* **202**:225–227 (1978).