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ASSESSMENT OF TRANSPORT BARRIERS USING CELL AND TISSUE CULTURE SYSTEMS

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ABSTRACT

The delivery of the next generation of drugs, particularly polar peptides and proteins, will represent a major challenge to the pharmaceutical scientists. To successfully deliver these potential drugs to specific targets, strategies will have to be developed to circumvent epithelial (*e.g.*, intestinal, buccal, nasal, epidermal) and endothelial (*e.g.*, vascular) cells, which represent both physical and metabolic barriers. This presentation will focus on the possible utility of cultured epithelial and endothelial cells to screen the permeability properties of potential drug candidates, to elucidate transcellular transport mechanisms, to evaluate the potential cellular site of metabolism, and to test strategies to prevent metabolism and/or to enhance the permeability properties of drug candidates. Topics to be discussed include: (a) advantages and disadvantages of cell culture systems; (b) factors important in selecting an appropriate cell culture system which will mimic the *in vivo* biological barrier; (c) characterization and validation of *in vitro* cell culture model systems; (d) factors important in selecting a porous membrane and a diffusion apparatus for transcellular transport studies; (e) the effect of cell culturing conditions on the transport characteristics of cultured cells; and (f) factors important in interpretation of *in vitro* cell culture data.

A major challenge confronting the pharmaceutical scientist in the future will be the selective and efficient delivery of the next generation of drugs. Many of these drugs will be discovered by synthetic chemists through rational drug design or by molecular biologists through recombinant DNA technology. In rational drug design, drug candidates are developed with molecular characteristics that permit optimal interaction with the specific macromolecules (*e.g.*, receptor, enzyme) which mediates the desirable therapeutic effect.[1] However, rational drug design does not necessarily mean rational drug delivery, which strives to incorporate into a molecule the molecular properties necessary for the optimal delivery between the point of administration and the final target site in the body.[2]

DNA technology has for the first time made it possible to produce large quantities of highly pure proteins for pharmaceutical applications.[3] Many of these proteins are endogenous molecules (*e.g.*, insulin) which are very potent and very specific in producing their pharmacological effects. However, delivery of these proteins by routes other than the parenteral route is very problematic because of the inherent physicochemical properties of these molecules (*e.g.*, size, charge, hydrophilic characteristics) and their propensity to undergo metabolism at epithelial barriers (*e.g.*, intestinal mucosa).[4]

In an effort to develop novel strategies for delivery of drug candidates arising from rational drug design and recombinant DNA technology, some pharmaceutical scientists have begun to employ cell culture techniques to study drug transport and metabolism at specific biological barriers.[5-6] These cell culture techniques afford the opportunity (a) to rapidly assess the potential permeability and metabolism of a drug; (b) to elucidate the mechanism(s) of drug transport and the pathway(s) of drug degradation (or activation); (c) to rapidly evaluate strategies for achieving drug targeting, enhancing drug transport and minimizing drug metabolism; (d) to employ human rather than animal tissues; and (e) to minimize time-consuming, expensive and sometimes controversial animal studies.

The objective of this presentation is to describe some of the general factors that should be considered in developing a cell culture model for transport studies and to review in detail the recent progress that has been made in establishing, validating and using cell cultures of an

epithelial barrier *(e.g.*, intestinal mucosa) and an endothelial barrier *(e.g.*, brain microvessel endothelial cells).

General Factors To Consider in Developing a Cell Culture Model System for Drug Transport and Metabolism Studies

In order to successfully mimic a biological barrier with an *in vitro* cell culture system, the selection of the cell line becomes particularly important. The transport and metabolic properties of cultured cells can vary depending on (a) whether the cells are primary cultures, passaged lines or transformed lines; (b) the number of times the cells have been passaged; (c) the phenotypic stability of the cell line; (d) the heterogeneity of the cell line; and (e) the inherent ability of the cell line to undergo differentiation. Once the cell line has been selected, the properties may vary depending on (a) the cell seeding density; (b) whether the cells have reached confluency; (c) the stage of cellular differentiation; and (d) the presence or absence of essential nutrients, growth factors or associated cells that produce trophic factors. During transport experiments the properties may change depending on (a) the composition of the transport media (e.g., concentration of the solute, temperature, pH, presence or absence of a metabolic source of energy or ions, presence or absence of proteins that might bind the solute, presence or absence of competing solutes); and (b) whether the solute is added to the apical or basolateral side of the monolayer. All of these factors need to be carefully optimized and regulated so as to best mimic the biological barrier *in vivo*.

The development of a cell culture system that will mimic a specific biological barrier requires not only an appropriate cell line but also a microporous membrane, which by itself or after treatment with an appropriate matrix material (e.g., collagen) will support cell attachment and cell growth. Ideally, the microporous membrane should also be (a) sufficiently translucent so that the development of the cell monolayer can be verified by microscopic techniques; (b) readily permeable to hydrophilic and hydrophobic solutes; and (c) readily permeable to both low and high molecular weight solutes.

Many microporous membranes for cell culture (e.g., polycarbonate, nitrocellulose) are now commercially available in different surface areas and different pore sizes.[7] Careful selection of the microporous membrane,

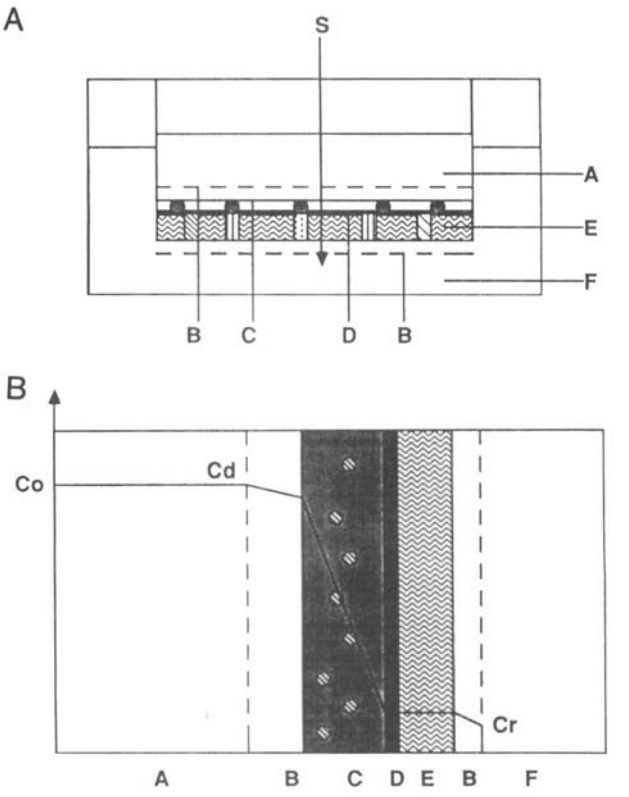


FIGURE 1

Potential barriers to solute transport in a cell culture system grown onto a microporous membrane. Panel A, monolayer cell culture system grown onto a microporous membrane; Panel B, concentration profile for solute (S) with the largest concentration drop produced by the cell monolayer. A, apical side; B, aqueous boundary layer; C, cell monolayer; D, supporting matrix; E, microporous membrane; F, basolateral side; Co, original concentration of solute; Cd, donor side concentration; Cr, receiver side concentration.

including the physical-chemical properties of the membrane, its pore size and surface area, and the nature and thickness of the supporting matrix (*e.g.*, collagen), is critical so as to avoid generating artifactual data in transport experiments. As illustrated in Figure 1, the ideal diffusion characteristics of a cell culture model system occur when the major diffusion barrier is provided by the cell monolayer and not the microporous membrane or the supporting matrix. In conducting transport studies with

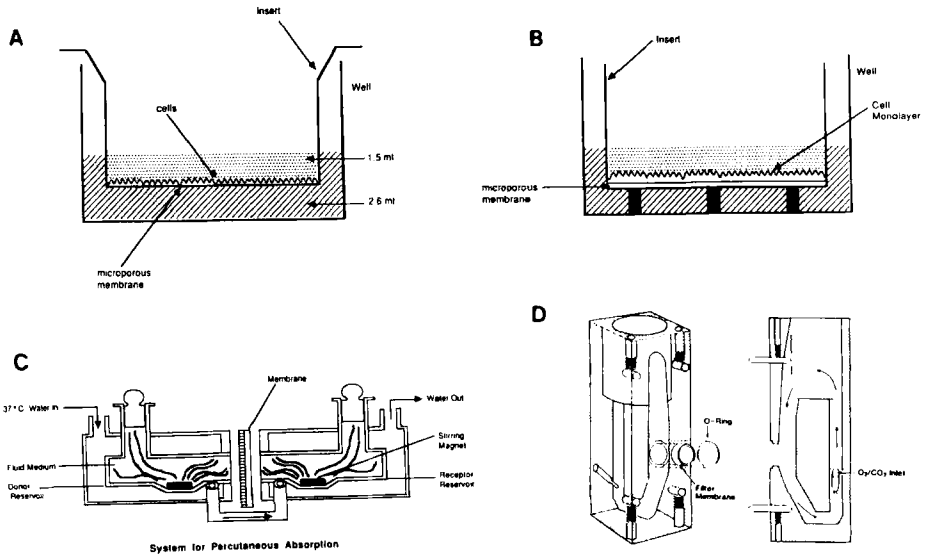


FIGURE 2

Diffusion apparatus used to study solute transport through cultured cells. Panel A, Transwell® arrangement; Panel B, Millicell® arrangement; Panel C, side-by-side diffusion cell with mechanical stirring; Panel D, side-by-side diffusion cell with gas-lift stirring.

cell cultures on microporous membranes, it is essential that control experiments be conducted using the microporous membrane alone and the microporous membrane coated with the supporting matrix. The results of these experiments will assure that the solute is freely permeable through the microporous membrane and the supporting matrix and that the diffusion barrier is provided by the cell monolayer.

Another critical factor, particularly in the study of the transport of lipophilic molecules, is the selection of the diffusion apparatus. Whether the diffusion apparatus is stagnant or stirred can influence the thickness of the aqueous boundary layer on the surface of the cell monolayer (Figure 1) and, thus, the permeability of lipophilic solutes. The types of diffusion apparatus currently employed for studying transport across cell monolayers include: (a) the unstirred cell-insert system (Figure 2a,b); (b) the side-by-side diffusion system stirred mechanically (Figure 2c); and (c) the side-by-side diffusion system stirred by gas lift (Figure 2d). The gas

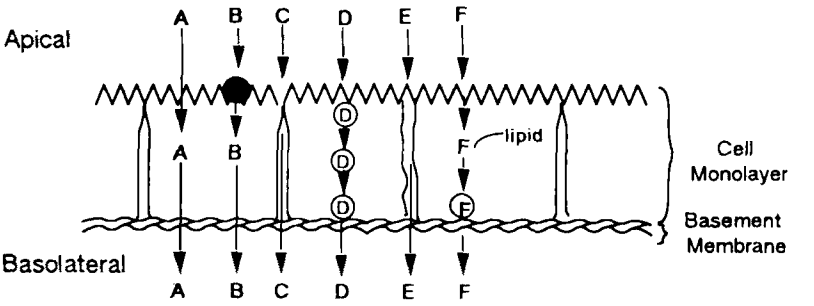


FIGURE 3

Possible pathways for drug transport in a polarized cell monolayer. Pathways: A, passive diffusion; B, active or facilitated diffusion (carrier-mediated); C, paracellular diffusion; D, vesicular-mediated transcytosis (fluid phase, adsorptive, receptor-mediated); E, "altered" paracellular diffusion; F, passive diffusion with incorporation into lipoprotein particles.

lift system was recently developed in our laboratory specifically for conducting transport studies on cell cultures grown in "mini" cell inserts. The stirring provided by the O_2/CO_2 gas lift system produces minimal damage to the cell monolayer and also minimizes the thickness of the aqueous boundary layer.[8]

Thus, in developing a cell culture model system to mimic an in vivo biological barrier, care needs to be taken in selecting the cell line, the microporous membrane, the supporting matrix, the culturing conditions, the conditions for conducting transport studies and the diffusion apparatus. Once these variables have been optimized, the system can be utilized by pharmaceutical scientists for drug transport and metabolism studies.

Since it is possible to readily manipulate the experimental conditions in a cell culture system, these *in vitro* models have tremendous potential to help in the elucidation of the various pathways by which a drug could penetrate a biological barrier (Figure 3). Experiments can be designed to determine whether the permeability of a small solute is via passive diffusion (Pathway A), active or facilitated diffusion (Pathway B) and/or paracellular diffusion Pathway C). For macromolecules, experiments can be designed to determine whether the molecule penetrates the barrier by a

paracellular (Pathway C) or transcellular (Pathway D) mechanism *(e.g.*, fluid phase, absorptive or receptor-mediated transcytosis). These systems are also potentially useful in elucidating the mechanism by which adjuvants enhance intestinal absorption *(e.g.*, Pathway E) and why some drugs partition preferentially into the lymphatic system (Pathway F). Most importantly, these systems may provide scientists with new, basic information about transport mechanisms in biological barriers that will permit them to develop novel strategies for targeting drugs to specific tissue compartments or enhancing drug permeability through now impermeable biological barriers.

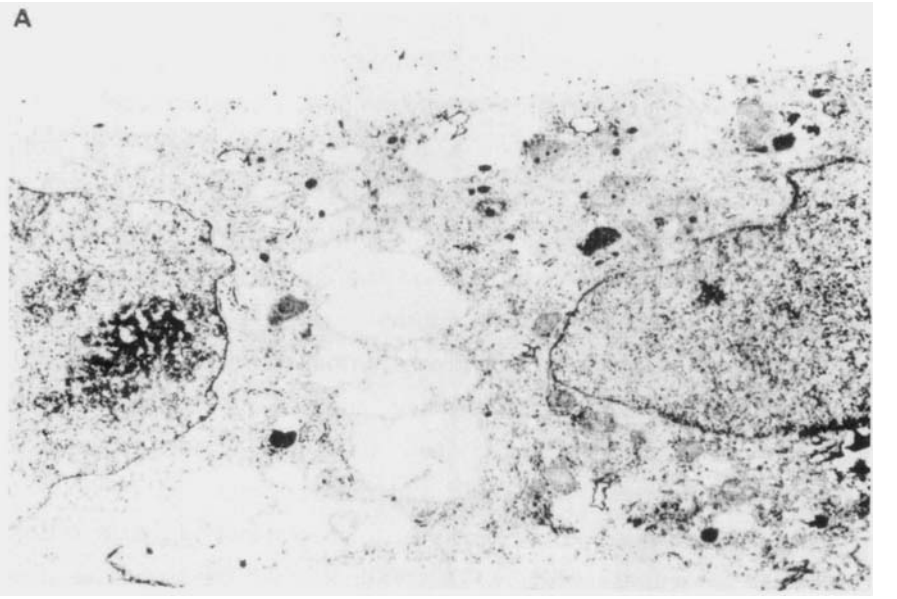
Epithelial Barrier: Intestinal Mucosal Cells

The epithelium of the small intestine, which is the major site of drug absorption, consists of a monolayer of cells having considerable cellular heterogeneity with respect to both morphology and function.[9] The intestinal epithelial cells of primary interest, from the standpoint of drug absorption and metabolism, are the villus cells, which are the fully differentiated cells located at the upper two-thirds of the villi. An *in vitro* cell culture system consisting of a monolayer of viable, polarized and fully differentiated villus cells, similar to that found in the small intestine, would be a valuable tool in the study of drug and nutrient transport and metabolism. Attempts to culture intestinal epithelial cells (*e.g.*, crypt cells) or to establish cell lines derived from enterocytes have not been successful.[10-12]

Recently, alternative approaches have been considered, which include the utilization of some transformed cell lines. Several human colon carcinoma cell lines (*e.g.*, Caco-2, HT-29, SW116, LS174T, SW-480)[13] have been reported to undergo varying degrees of enterocytic differentiation in culture. The most extensively studied cell lines have been the HT-29 and Caco-2. These cell lines, established by Jorgen Fogh[14-15] have received a great deal of attention in recent years because of their ability to express the morphological features of mature enterocytes or goblet cells.[16-18] They have been widely utilized as *in vitro* tools for studying intestinal epithelial differentiation and function.[19-23]

When HT-29 cells are cultured in the presence of glucose, they grow as a multilayer of unpolarized, undifferentiated cells and do not express any characteristics of enterocytes.[16,20] However, when the glucose in the medium is replaced with galactose, they express moderate enterocytic differentiation.[16] Caco-2 cells, on the other hand, undergo spontaneous enterocytic differentiation in culture,[17,21] which starts as soon as the cells achieve monolayer density (*i.e.*, 7 days) and is completed within 20 days. That Caco-2 cells form numerous domes spontaneously after reaching confluence is consistent with their ability to undertake transepithelial ionic transport.[16,20] The ability of Caco-2 cells to achieve a higher degree of enterocytic differentiation than that expressed by the HT-29 cell line and their spontaneous dome formation make this cell line a more relevant *in vitro* model for the investigation of intestinal differentiation and transport processes associated with intestinal cells.[18]

Recently our laboratory reported that Caco-2 cells could be grown onto collagen-coated polycarbonate membranes having a 3.0 μm pore size.[24] Caco-2 cells reached monolayer density 6-7 days after seeding onto collagen-coated polycarbonate membranes. Close examination of cell morphology between days 3 and 15 showed that as the length of time in culture and cell number increased, changes in cell dimensions also occurred. For example, from day 3 to day 16, cell height increased by 489% and cell width decreased by 42%. Electron microscopic examination revealed that by day 3 in culture, adjacent cells had formed occluding junctional complexes (Figure 4A); however, intercellular spaces were prominent. By day 6 in culture the lateral membranes of neighboring cells were strongly interdigitated and contained numerous desmosomes. Occluding junctional complexes were present only at the apical surface of the now functionally polarized cell monolayer. Concomitant with these observations was the progressive formation of a brush border with microvilli becoming more numerous and more organized. After 16 days in culture, Caco-2 cell monolayers consisted of cells ≈30 μm in height that possessed a morphology similar to that described for the simple, columnar epithelium of the small intestine (Figure 4B). Thus, the cells appeared to undergo differentiation from "crypt type cells" to "villus type cells."



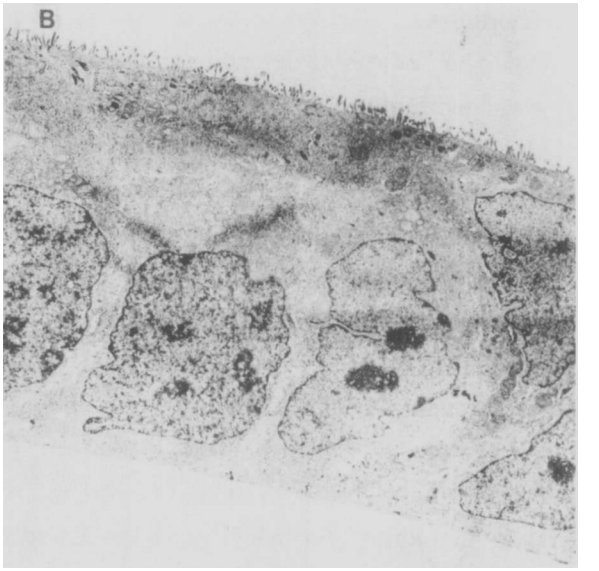


FIGURE 4

Ultrastructural features of Caco-2 cell monolayers grown into microporous membranes. Panel A, at 3 days of culture, cells are nearly confluent as defined by the appearance of occluding functions; however, the lateral membranes of adjacent cells remain associated loosely. Panel B, at 16 days of culture, the monolayer is comprised of cells that have become columnar in shape with a well-defined brush border.

Because epithelial cells have demonstrated an asymmetric distribution of alkaline phosphatase on their luminal surface, the specific activity of this enzyme in brush border membranes and ultrastructural distribution throughout time in culture as a measure of the degree of junctional polarity was determined.[24] Alkaline phosphatase activity increased two- to threefold from day 5 to day 20 in culture. Alkaline phosphatase cytochemistry showed that the majority of the enzyme activity was located at the apical membrane at day 6 in culture.

The Caco-2 cell monolayers grown onto polycarbonate membranes for 10 days exhibited a formidable barrier function, as judged by the leakage (<0.25%/hr) of lucifer yellow CH (mol. wt. 453), polyethylene glycol (mol. wt. 4,000), inulin (mol. wt. 5,000) and dextran (mol. wt. 70,000). Moreover, the lack of apical to basolateral or basolateral to apical diffusion of horseradish peroxidase (mol. wt. 40,000), despite its ability to penetrate the intercellular space after basolateral administration, was in agreement with similar observations by Phillips et al.[25] in rat *in vivo*.

In support of the morphologic and transport studies, monolayer integrity also was evaluated by measuring transepithelial electrical resistance (TEER). TEER values increased from 96.6 ± 22 at day 3 to 173.5 ± 10.9 Ωcm^2 at day 6 and remained constant through 30 days.[24] From these studies we concluded that Caco-2 cells grown onto collagen-coated polycarbonate membranes form a tight monolayer of polarized epithelial cells and thus represent a potential transport model system for the small intestinal epithelium.

Subsequently, pharmaceutical scientists have begun to use Caco-2 cells as a model system of the polarized intestinal epithelium. For example, Caco-2 cells have been used to study: (a) the transport of important nutrients, including bile acids[26] and large neutral amino acids[27] via carrier-mediated processes (Figure 3, Pathway B); (b) the binding, uptake and transport of proteins, including epidermal growth factor (EGF)[28] and cobalamin (Vitamin B12)[29] via receptor-mediated processes (Figure 3, Pathway D); (c) the production and secretion of lipoprotein particles,[30-31] a pathway by which lipophilic drugs can enter the lymphatic system (Figure 3, Pathway F); (d) the uptake of the antibiotic defalexin[32]

by a dipeptide transport system (Figure 3, Pathway B); (e) the transport of drugs *(e.g.*, α-methyldopa[33]) by the large neutral amino acid transport system (Figure 3, Pathway B); and (f) the relative contribution of the paracellular (Figure 3, Pathway C) and transcellular (Figure 3, Pathway A) pathways of absorption of beta-adrenergic antagonists.[34]

Endothelial Barrier: Blood Brain Barrier

A relatively heterogenous, asymmetric monolayer of endothelial cells lining the capillaries regulates the distribution of substances between the blood and the interstitial fluids. In peripheral tissues this cell barrier permits diffusion of low molecular weight water-soluble substances either intercellularly or through fenestra. Blood-borne macromolecules are moved transcellularly by either fluid-phase or adsorptive transcytosis. By contrast, the endothelial barrier separating the blood from the central nervous system (i.e., the blood-brain barrier) is characterized by tight intercellular junctions (i.e., exclude molecules with diameters greater than approximately 20 Å), no fenestra, and an attenuated pinocytosis. Both peripheral and blood-brain endothelial barriers also possess a significant metabolic activity and express a complex glycocalyx that contributes to the selective filtering of substances.[35]

A significant drug delivery problem for the pharmaceutical chemist is the design and development of drugs that will efficiently cross that endothelium forming the most restrictive permeability barrier, the blood-brain barrier. The focus of *in vitro* model development for addressing specifically transendothelial drug delivery problems has therefore, been on systems derived from blood-brain barrier endothelium either in culture systems or as isolated suspensions of capillaries. Therefore, discussion here will be limited to endothelial cell culture systems developed as *in vitro* models of the blood-brain barrier.

Cultures of capillary (i.e., microvessel) endothelial cells have been derived from human, canine,[36] bovine,[37-41] murine,[42-44] porcine,[45] and rat[41,46-48] brain. Generally, either enzymatic or mechanical dispersal followed by either filtration or centrifugation steps are employed to isolate a homogenous population of brain microvessel endothelial cells from the extremely heterogenous population of cells found in brain tissue.

The primary culture system used in our laboratories has been generated from enzymatically isolated bovine brain microvessel endothelial cells (Figure 5A) and has been employed as a model to study BBB transport and metabolism.[39,40] This model has been extensively characterized morphologically, biochemically, and immunohistochemically. Morphologically (Figure 5B), the primary cultures retain tight intercellular junctions, attenuated pinocytosis, and no fenestra.[39,40] In addition, specific BBB enzyme markers (i.e., gamma-glutamyl transpeptidase and alkaline phosphatase), endothelial cell markers (i.e., Factor VIII antigen and angiotensin converting enzyme), catecholamine degrading enzymes (i.e., monoamine oxidases A and B, cytosolic catechol O-methyltransferase, and thermostable phenol sulfotransferase),[39,49-51] acetylcholinesterase, butyrylcholinesterase (A.M. Trammel and R.T. Borchardt, unpublished observations), aminopeptidases,[52] and acid hydrolases[53] are also retained in the model. All morphological and biochemical properties of the *in vitro* bovine BBB model have been observed to be consistent with the present understanding of the BBB *in vivo*.

The overall permeability characteristics of primary cultures of bovine brain microvessel endothelial cell monolayers grown on either polycarbonate or regenerated cellular membranes have been determined in a horizontal side-by side diffusion cell apparatus (Figure 2C). As indicated above, while these primary cultures retain tight intercellular junctions, they are not as complete as *in vivo*. Thus, typically, the permeability of this *in vitro* system is greater than *in vivo*. Correction for "leakiness" has been accomplished in transport studies by normalizing transcellular diffusion for the simultaneous transcellular diffusion of impermeant marker molecules (e.g., sucrose, fluorescein, inulin, dextrans). Subsequently, this *in vitro* model of the BBB has been used to study: (a) the transport of drugs by passive diffusion (Figure 3, Pathway A)[51,54,55]; (b) the transport of important nutrients, including amino acids,[56] choline[57] and nucleosides,[58] via carrier-mediated processes (Figure 3, Pathway B); (c) the transport of drugs by carrier-mediated processes (Figure 3, Pathway B), including baclofen,[59] α-methyldopa[60] and acivicin[61]; (d) the binding, uptake and transport of proteins, including lectins,[62] insulin,[63] modified

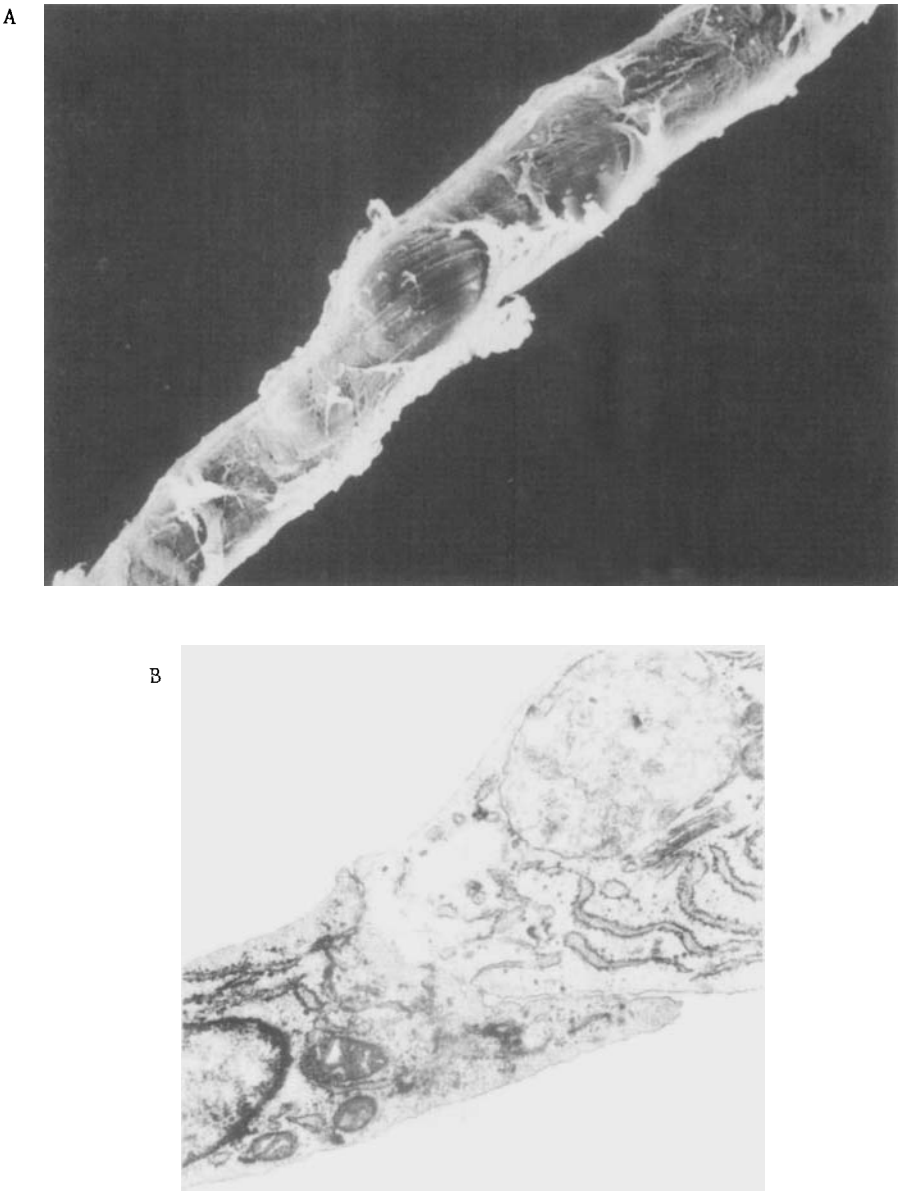


FIGURE 5

Panel A, scanning electron micrograph of bovine brain microvessel isolated by enzymatic dispersion (mag. 640X). Panel B, transmission electron micrograph of a confluent monolayer of brain microvessel endothelial cells in cross section (mag. 20,000X).

albumins,[64] delta sleep-inducing peptide,[65] vasopressin,[66] leu-enkephalin,[67] transferrin,[68] fragments of vasopressin[69] and atrial natriuretic peptide,[70] via receptor-mediated processes (Figure 3, Pathway D), passive diffusion (Figure 3, Pathway A) or paracellular transport (Figure 3, Pathway C).

CONCLUSIONS

The use of cell culturing techniques to study the transport and metabolic characteristics of specific biological barriers to drug delivery is in its infancy. However, as pharmaceutical scientists develop more sophisticated systems, establish the crucial *in vitro-in vivo* correlations to validate the cell culture models, and learn to appreciate the potential advantages of these techniques, it is likely that these systems will find widespread utility in the pharmaceutical industry as tools in drug discovery. In addition, these techniques are likely to be very useful to pharmaceutical scientists interested in elucidating mechanisms of drug transport and in devising novel strategies to enhance drug permeability.

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