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*Review*

# The Use of Cultured Epithelial and Endothelial Cells for Drug Transport and Metabolism Studies

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In an effort to develop novel strategies for delivery of drug candidates arising from rational drug design and recombinant DNA technology, pharmaceutical scientists have begun to employ the techniques of cell culture to study drug transport and metabolism at specific biological barriers. This review describes some of the general factors that should be considered in developing a cell culture model for transport studies and metabolism studies. In addition, we review in detail the recent progress that has been made in establishing, validating, and using cell cultures of epithelial barriers (e.g., cells that constitute the intestinal, rectal, buccal, sublingual, nasal, and ophthalmic mucosa as well as the epidermis of the skin) and the endothelial barriers (e.g., brain microvessel endothelial cells).

KEY WORDS: epithelial cell cultures; endothelial cell cultures; transport of drugs; cell culture; cell culture, intestinal, rectal, buccal, sublingual, nasal, ophthalmic.

## INTRODUCTION

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 macromolecule (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 made it possible for the first time 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 other than the parenteral route is very problematic because of the inherent physical–chemical properties of these molecules (e.g., size, 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 the technique of cell culture to study drug transport and metabolism in specific biological barriers (5). *In vitro* cultures of the cells have many advantages over conventional techniques, including (a) rapid assessment of the potential permeability and metabolism of a drug; (b) the opportunity to elucidate the molecular mechanism(s) of drug transport or the pathway(s) of drug degradation (or activation); (c) rapid evaluation of strategies for achieving drug targeting, enhancing drug transport, and minimizing drug metabolism; (d) the opportunity to use human rather than animal tissues; and (e) the opportunity to minimize time-consuming, expensive, and sometimes controversial animal studies.

The objective of this article 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 epithelial barriers (cells that constitute the intestinal, rectal, buccal, sublingual, nasal, and ophthalmic mucosa as well as the epidermis of the skin) and endothelial barriers (brain microvessel endothelial cells).

## GENERAL FACTORS TO CONSIDER IN DEVELOPING A CELL CULTURE MODEL SYSTEM FOR DRUG TRANSPORT AND METABOLISM STUDIES

In order to mimic successfully 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 (c) 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

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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 medium (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 (6). Careful selection of the microporous membrane, 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 Fig. 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 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 (Fig. 1) and, thus, the permeability of lipophilic solutes (7). The types of diffusion apparatus currently employed for studying transport across cell monolayers include (a) the unstirred cell-insert system (Figs. 2A, B), (b) the side-by-side diffusion system stirred mechanically (Fig. 2C), and (c) the side-by-side diffusion system stirred by gas lift (Fig. 2D). The gas lift system was recently developed in our laboratory specifically for conducting transport studies on cell cultures grown in "mini" cell inserts (8). 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).

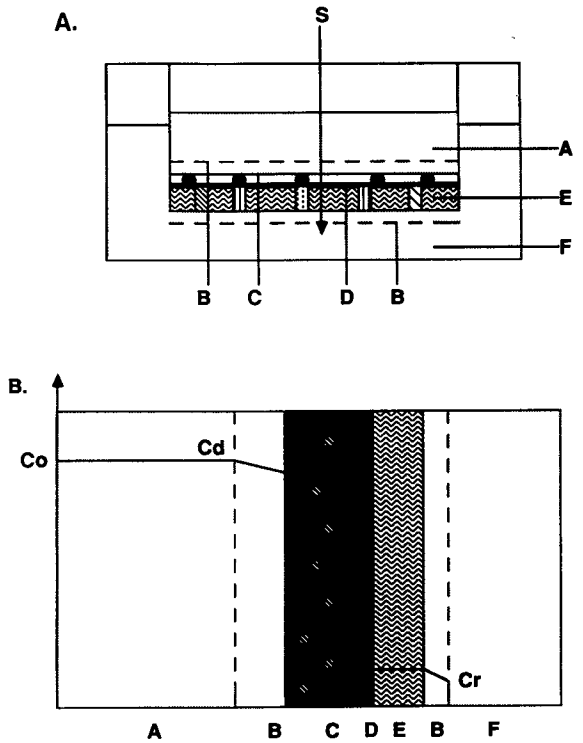


Fig. 1. Potential barriers to solute transport in a cell culture system grown onto a microporous membrane. (A) Monolayer cell culture system grown onto a microporous membrane; (B) concentration profile for solute (S) with the largest concentration drop produced by the cell monolayer. A, apical side; 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.

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 the pharmaceutical scientists for drug transport and metabolism studies.

Since it is possible readily to 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. Experiments can be designed to determine whether the permeability of a small solute is via passive diffusion, active or facilitated diffusion, and/or paracellular diffusion. For macromolecules, experiments can be designed to determine whether the molecule penetrates the barrier by a paracellular or transcellular 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 and why some drugs partition preferentially into the lymphatic system. 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

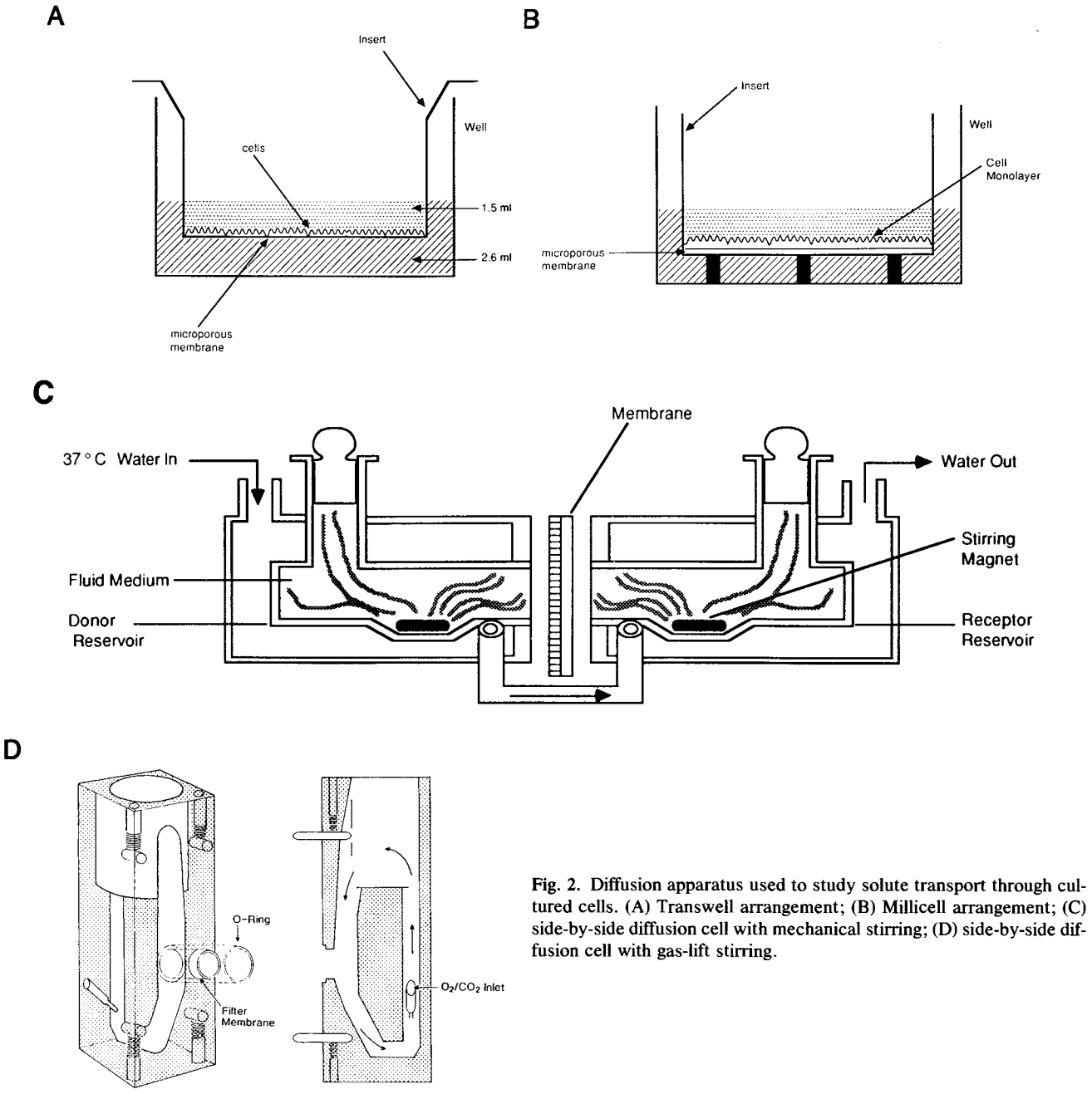


Fig. 2. Diffusion apparatus used to study solute transport through cultured cells. (A) Transwell arrangement; (B) Millicell arrangement; (C) side-by-side diffusion cell with mechanical stirring; (D) side-by-side diffusion cell with gas-lift stirring.

or enhancing drug permeability through now impermeable biological barriers.

## EPITHELIAL BARRIERS

### Intestinal and Rectal Mucosal Cells

Although the oral route is by far the most convenient for systemic drug administration, many important aspects of intestinal mucosal drug absorption are poorly understood. The mucosa of the small intestine is formed by three main layers. Facing the luminal cavity is the intestinal epithelium, a continuous sheet of epithelial cells one cell thick which lines the villi and crypts.

This epithelial layer comprises a cell population exhibiting continuous growth and differentiation *in vivo* (9,10). The continuous loss of differentiated cells at the villus tip is compensated by cell replication at the villus crypt. The presence of tight junctions, which limits the paracellular diffusion of solutes, together with the numerous enzymes present in the brush border region makes the intestinal epithelium the most significant barrier to mucosal drug absorption.

Underlying the epithelium is the lamina propria, a continuous connective tissue space that contains numerous plasma cells, lymphocytes and lymph macrophages, and a few eosinophils, mast cells, fibroblasts, and blood and lymph vessels. There is evidence that the lamina propria with its numerous cells has important immunologic functions (11). In

addition, the lamina propria provides structural support for the epithelium and contains the blood and lymphatic channels responsible for the transport of absorbed material to other regions in the body. The third mucosal layer is the muscularis mucosa, a continuous sheet of smooth muscle 3 to 10 cells thick separating the mucosa from the submucosa. Although the role of the muscularis mucosa is not clearly defined, it is believed that the contraction of this muscular layer may modulate the thickness of the unstirred water layer adjacent to the epithelium and help empty the luminal contents of the crypt (11).

For a substance to be absorbed from the intestinal lumen, it needs first to get across the epithelium and then to enter the lamina propria, where it can reach the blood and lymph vessels which may transport it to other sites in the body (11). Likewise, substances delivered by the intestine to the lumen must also be transported across or secreted by the epithelium.

The permeability properties of the intestinal mucosa have been investigated using a variety of experimental preparations derived from intestinal tissue. Results from these investigations have provided useful information on the influence that physicochemical characteristics such as $pK_a$, lipophilicity, and particle size may have on drug absorption (11). These preparations also have been used to further our understanding of the degradation processes that drugs may undergo in the hostile environment of the gastrointestinal tract.

The realization that experimental preparations of intestinal tissue (e.g., everted sac, everted rings, intestinal loops, etc.) have limited value in studying the cellular events mediating the epithelial uptake and transepithelial transport of drug molecules has led to widespread use of isolated membrane preparations. Brush border and basolateral membrane vesicles have been instrumental in the characterization of important mucosal uptake processes. Indeed, our knowledge of amino acid and sugar transport is due largely to studies carried out using membrane vesicles (12,13). However, these preparations have several disadvantages. First, they lack cellular metabolism, which may limit their utilization in the study of active transport processes. Second, they cannot be used in studying transcellular transport. Third, the extent to which the membrane isolation processes may damage the membranes potentials to carry out enzymatic or carrier functions is an open question.

The utilization of isolated mucosal cells as a model system for the study of epithelial uptake in the intact cell would appear warranted. However, isolated mucosal cells have not been used as widely as membrane preparations, partly because earlier isolation methods did not yield pure cell isolates (i.e., crypt cells vs villus cells) (14). Methods are now available for isolating relatively pure cell populations (i.e., undifferentiated crypt cells vs absorptive villus cells) (15) and these preparations have proven useful in some cellular uptake studies (16). However, the rapid autolysis that isolated cells undergo *in vitro* will greatly limit their application to the study of transport and metabolism. Another disadvantage of using isolated mucosal cells is that the isolation process requires the opening of tight junctions, which in turn causes the destruction of cell polarity. This would make it impossible to determine the specific membrane domain involved in cellular uptake.

An *in vitro* system consisting of a monolayer of viable and polarized intestinal epithelial cells similar to that found in the small intestine would be a valuable tool in the study of drug and nutrient transport and metabolism. Thus, numerous attempts have been made to culture intestinal epithelial cells (17,18) or to establish cell lines derived from intestinal mucosal cells (19). Unfortunately, they have not met with great success. For example, Henle and Deinhardt (17) managed to culture intestinal cells (strain 407), which were derived from jejunal and ileal tissue from a human embryo, for up to 12 passages. However, the cells underwent transformation into a cell type different from that cultured initially. A duodenal cell line established by Lichtenberger *et al.* (18) apparently underwent malignant transformation after which the cells exhibited ultrastructural features of crypt cells. Another study found that rat intestinal cells can be maintained in suspension cultures for up to 14 days with approximately 50% viability, retention of microvillar membrane, and minor changes in aminopeptidases, maltase, sucrase, and amylase activities (19). In the same study, however, when the cells were cultured as a monolayer, they dedifferentiated and were quickly overgrown by fibroblasts (19). Quaroni *et al.* (20) established and cultured an intestinal epithelioid cell line (IEC-6) *in vitro* for up to 6 months. These cells had properties of normal (nontransformed) cells in culture, but their morphological features indicated that they were derived from undifferentiated crypt cells and not from differentiated absorptive cells.

Recently, alternative approaches have been considered, which include the utilization of some transformed cell lines. Several human colon carcinoma cell lines (i.e., Caco-2, HT-29, SW116, LS174T, SW-480) (21) have been reported to undergo varying degrees of enterocytic differentiation in culture. Among them, HT-29 and Caco-2 are the most thoroughly investigated. These cell lines, established by Jorgen Fogh and co-workers (22,23), have received a great deal of attention in recent years because of their ability to express morphologic features of mature enterocytes or goblet cells (24–26). Both Caco-2 and HT-29 cells have been widely utilized as *in vitro* tools for the study of intestinal epithelial differentiation and function (27–31).

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 (24,27). However, when the glucose in the medium is replaced with galactose, they express moderate enterocytic differentiation (24). Caco-2 cells, on the other hand, undergo spontaneous enterocytic differentiation in culture (25,28), 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 (24,27). 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 the transport processes associated with intestinal cells (26).

The development of a model system derived from these cell lines has been facilitated by the increasing availability of tissue culture-treated microporous membranes. These mem-

branes represent a more physiologic environment because it allows the exchange of substances across both the apical and the basolateral membranes rather than just across the apical membrane as is the case with cells grown on plastic dishes.

Caco-2 cells grown on nitrocellulose filters form monolayers of polarized cells with measurable transepithelial electrical resistance (29). Although Caco-2 cells grown on this support can be useful in the study of drug transport, it should be noted that nitrocellulose filters may constitute a diffusion barrier for lipophilic molecules. Moreover, the substantial nonspecific binding found in this type of material may present problems, especially when compounds are used at very low concentrations or in trace amounts. These problems do not seem to be significant with polycarbonate membranes, which also have been used to culture Caco-2 cells (32).

Both polycarbonate and nitrocellulose filters can be obtained with different surface areas and pore sizes. Proper selection of filter diameter and pore size may be important, depending on the particular application. The surface area available for diffusion is a critical factor in the selection of filter size. The pore size (and ratio of pore area to total area) may need to be chosen according to the compounds to be investigated. While larger pore sizes may not represent a diffusion barrier for larger molecules, in some instances, cellular processes may grow through these pores, perhaps making it more difficult to reach polarity or making the monolayers leakier. With the Caco-2 system this was not observed when the membranes were first coated with collagen (32), indicating that such an extracellular matrix may help the cells reach polarity or may partially decrease the pore size. Although Caco-2 cells can be grown to confluence on either collagen-coated or uncoated polycarbonate membranes, they do appear to do better (i.e., faster confluence and higher cell density at confluence) on collagen-coated polycarbonate (32).

Our laboratory has presented evidence that Caco-2 cells grown on collagen-coated polycarbonate membranes with a 3.0-μm pore size reached confluence by 6–8 days in culture (32). After developing measurable transepithelial electrical resistances (TEER) as early as day 3, TEER values reached a plateau, which was consistent with the arrest of cell growth, and remained constant for up to 32 days. Cytochemical localization of the brush border marker enzyme, alkaline phosphatase, indicated that Caco-2 cells were functionally polarized by day 6 (Fig. 3). The monolayers exhibited a formidable barrier function, as judged by the minimal leakage (<0.25%/hr) of Lucifer yellow CH (MW 453), polyethylene glycol (MW 4000), inulin (MW 5000), and dextran (MW 70,000). Moreover, the lack of paracellular diffusion of horseradish peroxidase (MW 40,000), despite its ability to penetrate the intercellular space after basolateral administration (32), was in agreement with similar observations by Neutra and co-workers in the rat *in vivo* (33).

A potential approach to enhancing drug delivery involves the utilization of the body's own carrier systems. This requires the identification and characterization (structural specificity, transport cofactors required, intracellular and transcellular fate of the substrate transported, etc.) of such carriers. Following the characterization of the Caco-2 cell monolayers as a model transport system of the small intestinal epithelium, several studies have been undertaken to determine the suitability of this system for studying specific

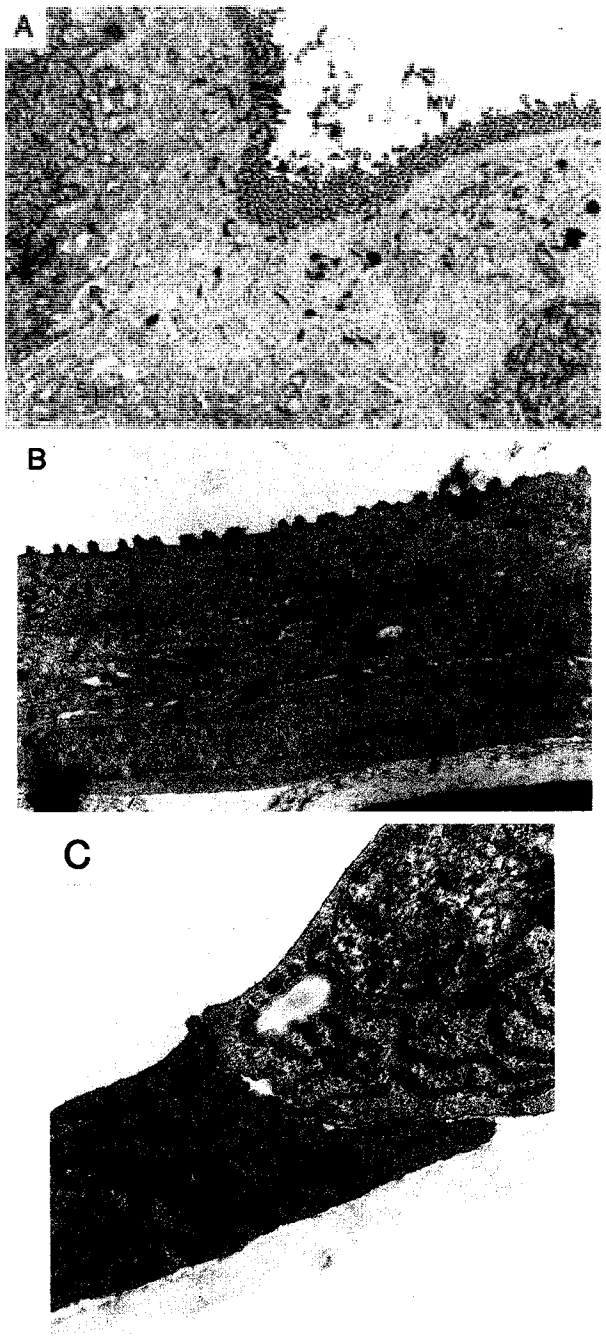


Fig. 3. (A) Alkaline phosphatase distribution in polarized Caco-2 cell monolayers. By day 6 in culture, Caco-2 cells plated on collagen-coated 24.5-mm Transwell inserts had formed a cell monolayer exhibiting tight junctions (arrowheads) and desmosomes (D). Consistent with the achievement of functional polarity, Caco-2 cells expressed alkaline phosphatase activity on the microvilli (MV) region but not in the basolateral membranes or in subcellular organelles. N, nuclei. (B) Transmission electron micrograph of 3-day-old primary culture of hamster pouch buccal epithelium in cross section. Approximately five layers of flattened, stratified epithelial cells exhibiting extensive desmosomal junctions (D) and nuclei (N) are observed at all levels of the primary cultures (66). (C) Transmission electron micrograph of a confluent monolayer of brain microvessel endothelial cells in cross section. Endothelial cells are closely apposed with extensive overlapping intercellular junctions. Fenestra are absent and few pinocytic vesicles are observed (179). (B) 6800×, (C) 20,000×; reduced 55% and 40%, respectively, for reproduction.

carriers found in the small intestine. Evidence has been provided (34) that Caco-2 cells expressed a bile acid carrier, similar in some respects to that found in the enterocyte (35). Bile acid transport in Caco-2 cells increased with time in culture up to 25 days and was greater than 10 times faster from the apical to the basolateral side than in the opposite direction. The main discrepancies, however, were that in Caco-2 cells bile acid transport was not as $Na^+$ dependent as in the enterocyte and that the transport capacity (i.e., $V_{max}$) was approximately two orders of magnitude lower than in the enterocyte (36).

Caco-2 cell monolayers were also used to investigate the intestinal epithelial transport of amino acids. Hidalgo and Borchardt (34) have recently shown active transport of the large neutral amino acid, phenylalanine, in Caco-2 cells. This transport was much faster from the apical to the basolateral side than from the basolateral to the apical. The apical to basolateral transport was $Na^+$dependent. Similar to the large neutral amino acid carrier of the enterocyte, the amino acid carrier of Caco-2 cells showed affinity for the cationic amino acids, lysine and histidine, as indicated by their ability to cause a significant inhibition of phenylalanine transport across Caco-2 cell monolayers. Further evidence in support of a large neutral amino acid was provided by the fact that the anionic amino acids, glutamate and aspartate, and the small amino acid, glycine, failed to inhibit phenylalanine transport (Hidalgo and Borchardt, unpublished results). The amino acid-like drugs, α-methyldopa and L-dopa, reduced the transcellular transport of phenylalanine significantly, indicating that they are probably substrates for the large neutral amino acid carrier.

Polarized Caco-2 cell monolayers were also shown to bind and internalize epidermal growth factor (EGF) via a receptor-mediated process (37). As in human fetal intestine, where development was associated with decreased EGF uptake (38), the binding of EGF was lower in monolayers that had been cultured for longer times (38). While EGF binding did not decrease significantly at 4°C and in the presence of sodium azide, unlabeled EGF, sodium azide, and 4°C abolished internalization completely (37). At this time it is not known whether Caco-2 cell monolayers can undertake transcellular transport of EGF as Neutra and co-workers have reported in the ileum of the suckling rat (38).

A study by Muthiah and Seetharam (39) found that Caco-2 cells take up [$^{57}$Co]cyanocobalamin (Cbl) either free or bound to intrinsic factor. After internalization, Cbl was transferred to transcobalamin II, a protein with a molecular weight of 32,000. Surprisingly, free Cbl was taken up four times more efficiently than that bound to intrinsic factor. Since this study looked only at uptake by cells grown on plastic dishes, it is not clear whether such discrepancy reflects true differences in Cbl transport between Caco-2 cells and enterocytes.

Two recent studies have shown that Caco-2 cells produce (40) and secrete in a polarized manner (41) lipoprotein particles, suggesting that these cultured cells may be useful to study drug delivery to the lymphatic system. Lipoprotein particles enter the lymphatics in the lamina propria and subsequently drain into the inferior vena cava, thus avoiding potential hepatic first-pass elimination. Thus, the Caco-2 model is being used in our laboratory to elucidate the uptake and metabolism of lipid conjugates, hopefully making it possible to identify the chemical properties required for drug targeting to the triglyceride pathway (42).

Cell monolayers may also be used to investigate in detail the barrier properties of the intestinal epithelium. One critical aspect of transepithelial transport is the route through which compounds may traverse the epithelial cell layer. Currently there is evidence that the tight junction is not a static structure as previously thought (43–46). Indeed, recent evidence that tight junctions may regulate paracellular transport of ions and nutrients (47,48) suggests that the paracellular passage may contribute significantly to the overall permeability of the intestinal epithelium to some drugs.

Because of its greater simplicity, tissue culture models may permit the characterization of the unstirred water layer surrounding the epithelial cell layer and its influence on the permeability of lipophilic drug molecules. Recently our laboratory developed a novel side-by-side diffusion cell for studying the transport characteristics of cell culture monolayers (8,49). Cell monolayers would also provide a unique opportunity to study so-called absorption enhancers, since the mechanism by which these enhancers alter the barrier properties of the intestinal epithelium are not fully understood (50).

It is well known that gut wall drug metabolism is partially responsible for the low bioavailability of drugs and peptides. Some of the limitations associated with the utilization of intestinal or mucosal cell preparations in transport studies are also applicable to their utilization in metabolism studies. The usefulness of the Caco-2 cell system in nutrient metabolism studies has been supported by several reports indicating that differentiated Caco-2 cells express differing degrees of the enterocytic enzymes: alkaline phosphatase, aminopeptidase N, sucrase-isomaltase (25), and lactase (51). Sucrase-isomaltase activity in Caco-2 cells was the same as in the ileum of adult or term human fetus (30). A recent study from our laboratory characterized phenol sulfotransferase (PST) activity in Caco-2 cells (Baranczyk-Kuzma *et al.*, unpublished results). It was found that the specific activity and the affinity of the enzyme increased as differentiation progressed. Similarly, the thermal stability of the enzyme was found to increase with time in culture. However, no UDP glucuronyl transferase activity was found in cell monolayers up to 21 days old. Additional characterization of drug metabolizing enzymes in Caco-2 cells will be necessary to determine the potential applications of Caco-2 cells to drug metabolism studies.

The rectal cavity has been investigated as an alternative route for administering drugs exhibiting poor absorption and/or gastrointestinal degradation. Although the surface area of the rectum is only about 200–400 $cm^2$ (52), de Boer and Breimer found that the systemic availability of lidocaine in humans was 2× higher than after oral administration, suggesting that approximately 50% of the rectally administered drug avoided first-pass elimination (53).

Rectal absorption has been investigated using perfusion techniques, microenemas, and suppositories (54–56). In most cases, rectal administration has been utilized in combination with absorption adjuvants (57). The absorption of many small drugs and peptide drugs has been enhanced significantly; however, the processes underlying this enhancement are not well understood (57). The effectiveness of these

enhancers in breaking down the barrier function of the rectal mucosa makes it desirable to elucidate their mechanisms of action and the general biological factors controlling the absorption of drugs across this tissue. Unfortunately, rectal absorption studies have commonly focused on determining the disappearance of drugs from the isolated rectal cavity or their appearance in the systemic circulation, following administration into the rectal compartment. While these protocols may be useful in evaluating the effect of absorption enhancers on the general permeability properties of the rectal mucosa, they provide no valuable information regarding the cellular events taking place during absorption. There is also a lack of information on potential active transport systems that may be relevant for drug transport.

Although cell culture may constitute a powerful technique for performing these types of studies, to date no *in vitro* system of the rectal epithelium based on cell culture has been developed. However, this situation is likely to change as a consequence of the increasing recognition of the potential value of the rectal route of drug administration. Furthermore, the establishment of the moderately well-differentiated human rectal carcinoma cell line, HRT-18 (21), may prove valuable in developing a rectal cell culture model system.

To further our understanding of gastrointestinal and rectal drug absorption will require the characterization of mucosal transport at a molecular and cellular level. Some presently available colon and rectal carcinoma cell lines and others that will probably be established in the near-future will be most valuable in this pursuit.

### Oral Cavity—Buccal and Sublingual Cell Cultures

Convenient and economical systemic delivery of drugs in the absence of first-pass metabolism is a factor stimulating continual consideration of drug delivery systems for the oral cavity (58,59). The stratified squamous epithelial lining of the human oral cavity is an extension of the skin. In great contrast to the skin, however, the epithelium of the oral cavity is continuously hydrated and exhibits considerable regional variation in differentiation. Hydration and the absence of a fully differentiated or keratinized layer in some regions (i.e., buccal, sublingual) have been generally interpreted as evidence for greater permeability of the oral mucosa than for the skin. Regardless of the extent of differentiation, however, there is an intercellular barrier in the superficial zone of oral cavity epithelia across which absorption of polar substances and electrolytes remains limited. Transepithelial movement of relatively lipophilic substances is less restrictive and may occur along plasma membranes as well as through intercellular routes. Since other possible transport mechanisms such as endocytosis and carrier-mediated systems probably occur only in basal cell layers of oral epithelium, efficient transepithelial drug delivery is generally considered to be dependent primarily on the physicochemical characteristics of the substance (58–62). Successful development of an appropriate *in vitro* cell culture model for drug delivery studies depends on generating not only a system that retains the restrictive intercellular epithelial barrier, but one that also retains a similar expression of enzyme systems and other morphological and biochemical properties typical of oral cavity epithelia. For oral epithelia as a whole, literature on fundamental biochemical and cellular characteristics is unfortunately lacking. There is somewhat more information available on the two primary routes, buccal and sublingual, for systemic delivery of drugs in the oral cavity. Of the two regions, recently developed tissue culture systems for drug studies tend to be derived from buccal epithelia of both human and animal origin (63–66). The buccal tissue culture systems can be divided into at least two general types, the first type in which explants are grown in primary culture and then subcultivated (64,65) and the second type in which cells are enzymatically dissociated and grown in primary culture (63,66).

In the first type of tissue culture system, oral keratinocytes derived from human buccal explants have been grown in primary culture. Epithelial cells migrate out from explants placed on suitable growth surfaces, divide, and form a stratified tissue system with typical markers (i.e., tonofibrillar–desmosomal complexes, occasional gap junctions, and the absence of complete keratinization), even on subcultivation (64). The tissue grown in this system is similar but not identical to the parent tissue (64,65). In studies relevant to applications in drug delivery, the explant type of tissue culture system has been used to characterize the toxicity of dental bonding materials. Results suggest that the toxic reactions observed in the culture system parallel those *in vivo* and indicate that an *in vitro* model of human oral epithelia could be employed as an initial toxicity screen (65). The explant-type culture system's transepithelial permeability, metabolic properties, and other biochemical characteristics that may impact on drug delivery have apparently not been examined. This system is relatively easy to establish but may retain nonepithelial cells (e.g., overgrowth of fibroblasts or muscle cells) that may or may not be significant in drug studies.

In the second type of buccal tissue culture system, hamster pouch buccal cells have been enzymatically dissociated and grown in primary culture (63,66). This system has the advantage over explant systems by removing nonepithelial cells from the culture system. By this method a homogeneous population of isolated epithelial cells is seeded onto appropriate growth surfaces, divides, and forms a stratified tissue system with typical markers (i.e., tonofibrillar–desmosomal complexes, occasional gap junctions, and the absence of complete keratinization) (63,66). As for the *in vitro* models derived from explants, these primary cultures are also similar in many respects, but not identical, to the parent tissue. The major difference between the hamster-derived primary cultures and parent tissue is the absence of a completely keratinized epithelium typical of the hamster cheek pouch *in vivo*. In this respect, the cultured tissue more closely resembles the less differentiated or nonkeratinized buccal epithelium of man. Polverini and Solt (63) have used this primary culture system to assess the *in vivo* effects of carcinogen exposure on buccal epithelial cell growth *in vitro*. By examining growth rate and the expression of an enzyme sensitive to carcinogenesis, gamma-glutamyl transpeptidase, this system has been proposed to be a potentially valuable experimental tool in the study of cellular and molecular events occurring in carcinogenesis of oral cavity epithelia (63).

In our laboratories, primary cultures of hamster buccal pouch have been explored for potential applications in drug transport and metabolism studies (66) (Fig. 3B). The overall permeability of the cultured cells has been determined to be greater than in *in vitro* models comprised of excised tissues. For example, the apparent permeability coefficient for water in our primary cultures was $2.0 \times 10^{-4}$ cm/sec (66). The apparent permeability coefficient for water have been estimated at $1.2 \times 10^{-4}$ cm/sec in excised hamster pouch epithelium (66), $0.5 \times 10^{-4}$ cm/sec in excised canine buccal epithelium (67), and $7.5 \times 10^{-7}$ cm/sec in excised porcine buccal epithelium (68). The latter tissues are from 6 to 50 times thicker than the cultured or excised hamster pouch epithelium and this is a significant factor in comparing systems. The primary culture system has been shown to be more exclusive to higher molecular weight substances, completely limiting transcellular passage of dextrans with molecular weights greater than about 18,000. In comparison, excised and cultured rabbit buccal mucosa exclude substances of about 40,000 and 70,000 molecular weight, respectively (69,70). The roles of other physicochemical factors (e.g., pH, temperature, and lipophilicity) in permeation of substances across excised porcine buccal epithelium (71) and hamster pouch epithelium *in vivo* (72) have been recently confirmed in the cultured hamster pouch cells (73).

Several enzyme systems (i.e., alkaline phosphatase, alcohol dehydrogenase, gamma-glutamyl transpeptidase, and aminopeptidases) have been characterized in the primary cultures of hamster buccal epithelium (66,74). Compared to excised hamster (66), human (74), and rabbit (75) buccal epithelium, the primary cultures of hamster pouch epithelium retain similar enzyme activities and affinities for most substrates tested (66,74). Studies also indicate that differences exist between the sensitivities of human and hamster buccal epithelial aminopeptidases to the inhibitors, bestatin and puromycin (74). Though further confirmatory studies are required, these differences in sensitivity to inhibitors may translate to differences in the manner in which peptides are degraded in human tissue as opposed to animal models.

Briefly summarizing, tissue culture models for buccal epithelia, both explant and primary culture types, appear useful for screening substances for metabolic, pharmacologic, and toxicologic factors associated with drug delivery. Both systems require additional refinements to duplicate fully the absolute permeability barrier exhibited *in vivo*. However, the tissue culture models do have potential as useful models for studying the role of physicochemical factors in determining the relative buccal permeability characteristics of a substance.

### Nasal Mucosal Cells

The existence of a large surface area for absorption, extensive underlying vascularization, and the absence of first-pass metabolism are a few of the characteristics that make the nasal cavity a desirable site for drug delivery. Development of the systemic delivery systems, particularly for peptides and proteins, through the intranasal route has been more or less continuously pursued since the beginning of this century (76,77).

Two basic cell types line the nasal cavity, a stratified squamous and a pseudostratified columnar epithelia. The former cell type is an extension of the facial skin and extends from the anterior portion of the nose to the skull entrance of the nasal cavity. This epithelium contains hair and sebaceous glands and presents the first defense against particulate material. Absorption of substances in this area would be subject to restrictions similar to those observed for dermal drug delivery. The second cell type of the nasal cavity, the pseudostratified columnar epithelium, begins at the skull entrance and extends down throughout the tracheobronchial tree. At the skull entrance, the nasal cavity is divided by the septum centrally and convolutes laterally into turbinate projections, substantially increasing the surface area of inside the skull. The absorption area for most drug formulations, and therefore the subject of our discussion here, generally extends from the skull entrance to the nasopharyngeal region of the nasal cavity. Absorption of substances in this area of the nasal cavity is subject to interactions with at least a mucin layer, degradative enzyme systems, and passage across a single columnar epithelial layer before reaching the systemic circulation (78,79).

Development of a nasal cell culture model requires establishment of a complex mixture of cell types. In a representative region of normal nasal mucosa, the turbinate region, the pseudostratified columnar epithelium is the predominant cell type, existing in either ciliated (15–20%) or nonciliated (60–70%) form. Both cell types express numerous microvilli. The role of the cilia is protective and moves the overlying mucous layer containing entrapped particulates to the nasopharyngeal area for expectoration or swallowing. The columnar epithelial cells also express substantial numbers of mitochondria, suggesting a high metabolic activity. A third cell type, the goblet cell, comprises about 5–15% of mucosal cells, depending on age, and are responsible for secretion of carbohydrate-rich constituents of the mucous layer. Columnar epithelial and goblet cells within this single layer of cells form tight intercellular junctions near the apical surface which forces transepithelial diffusion or transport of materials from the nasal cavity to the systemic circulation (79). Minor numbers of other cell types are found in this region which do not extend to the nasal cavity surface and include the progenitor basal cells which differentiate to the above cell types, occasional macrophages, leukocytes, and neurosecretory cells. Occasional submucosal glands can extend to the nasal cavity surface and are responsible for serous and mucous secretions (79).

Several laboratories, including our own, are exploring the potential application of nasal cell cultures in drug delivery research. At least two problems quickly confront researchers in this area. The first problem is the absence of a substantial literature base on the fundamental biochemistry, cell biology, and physiology of nasal epithelium *in vivo*, which is important for validating an *in vitro* model. The second problem is the complexity of the nasal mucosa (e.g., the multiple cell types present), somewhat analogous to the gastrointestinal mucosa. For the purposes of this review we identify three general types of nasal tissue culture systems currently in use. These systems include explants, primary cultures, and cell lines.

The first type of nasal tissue culture system has been developed from either excised rat nasal septal epithelium (80) or excised human (81) and hamster (82,83) tracheal epithelium. In both instances, the tissue explants attached and grew in culture to form a tissue morphologically similar to the parent tissue. Rat septal explants grown in this system have been proposed as a model to study the role of nasal mucosa in the activation of nitrosoamines as nasal-specific carcinogens (80). The hamster tracheal explants have been used in structure–activity studies to screen retinoids for ability to prevent cancer (82) and, more recently, to study the role of cigarette smoking and vitamin A in modulating epithelial cell growth and function (83). As in other explant cell culture systems, the possibility exists that the normally minor numbers of nonepithelial cell types may overgrow in culture and introduce complicating factors into interpretation of experimental results.

The second type of nasal tissue culture system has been developed by the enzymatic dissociation and isolation of human and animal nasal epithelial cells. Several examples of this type of system have been described as originating from tissues extracted from either the turbinate regions of the nasal cavity (84–87) or the tracheobronchial (88–92) regions of the respiratory system. These culture systems are generally comprised of monolayers of nasal epithelial cells exhibiting biochemical characteristics similar to those of the *in vivo* parent tissue. Attempts at duplicating obvious morphological properties (e.g., columnar shape, cilia, microvilli, and extensive intercellular interdigitation) *in vivo,* however, have been most successful in those *in vitro* systems employing tracheal epithelium. This has been disappointing from the standpoint of intranasal drug delivery applications, however, since development of an appropriate tissue culture system from epithelia of the septal or turbinate regions of the nasal cavity seems more applicable. These tissue culture systems are currently being utilized to identify basic functional and biochemical characteristics that distinguish both normal and diseased tissues. For example, recent studies have examined ion permeability, electrical resistance (86,90,92–96), lectin histochemistry (91), hematopoietic growth factor production (87), and proteoglycan synthesis (85) in the various tissue culture systems derived from either normal or cystic fibrosis tissue. The emerging information on the permeability and biochemistry of these primary culture systems should provide an excellent basis for exploring the applications of these models for drug delivery problems.

The third general nasal cell culture type is represented by at least three cell lines. These cell lines are RPMI 2650 (i.e., derived from cancerous human septum) and BT (i.e., derived from normal bovine turbinate), both available from American Type Culture Collection (Rockville, MD), and NAS 2BL [i.e., derived from rat nasal epithelial squamous carcinoma (97)]. Cell lines in tissue culture work can offer convenience and reproducibility in many applications. However, little information on the use of nasal epithelial cell lines in drug delivery studies exists. Preliminary studies in our laboratory have been performed with the BT cell line (M. R. Tavakoli-Saberi and K. L. Audus, unpublished results). These cells will grow to form monolayers on various collagen matrices both on plastic and in Transwell culture systems. In contrast to the typical pseudostratified columnar epithelium, the cells are flattened but express a few cilia and microvilli. Manipulation of culture conditions and growth substrates may improve morphological characteristics, but this remains to be demonstrated. The cells do retain expression of three aminopeptidases with kinetic parameters (M. R. Tavakoli-Saberi and K. L. Audus, unpublished results) similar to those described for rabbit nasal aminopeptidases (75). As indicated above, little published information is available on the potential research applications of these cell lines aside from that mentioned previously and preliminary carcinogenesis studies (97).

The availability of several tissue culture systems now provides the opportunity to conduct much needed basic research on the nasal mucosa. The availability of these models also offers immediate and convenient *in vitro* systems to explore drug effects on nasal epithelial cell growth and function.

## SKIN—KERATINOCYTES OF THE EPIDERMIS

Skin, being the largest and most accessible organ, provides an excellent potential route for drug delivery. It is composed of two layers, the dermis and the epidermis. The dermis is the structural component of the skin and protects the organism against physical trauma. The capillary beds residing in the upper papillary dermis supply the overlying avascular epidermis and are the target site for systemic absorption of transdermally administered drugs. However, before reaching the dermal capillaries, the drug must pass through the epidermis, a keratinizing stratified squamous epithelium which constitutes the primary barrier of the skin. A cell culture model of the epidermis would provide an important alternative to existing *in vitro* model systems (e.g., cadaver skin, snake skin, synthetic membranes) which generally do not contain viable cells.

Epidermal keratinocytes have been cultured from a number of species, including chicken, mouse, rat, cow, dog, and human skin (98). The different culture systems reported all follow the same basic techniques and share many characteristics. The epidermis is removed from full- or split-thickness skin by enzymatic digestion with trypsin (99), dispase (100), or thermolysin (101). Basal keratinocytes are released and then cultured either on plastic or a permeable substrate. The keratinocytes grown on plastic proliferate and partially differentiate to form a multilayered culture. However, these submerged cultures lack many morphologic (98) and biochemical characteristics of the epidermis *in vivo,* proper keratin (102–105) and lipid (106–108) composition in particular. More recently, keratinocytes have been grown on permeable supports and exposed to the air/liquid interface, thereby creating a more physiologic environment. Under these conditions, keratinocytes differentiate to a greater extent and more closely resemble the parent tissue (109–115).

The air/liquid interface culture systems can be divided into three categories according to the permeable substrate used to support the cells: (i) synthetic membranes (109–111), (ii) a dermal equivalent (i.e., a contracted collagen gel containing viable fibroblasts) (112,113), and (iii) dead de-

epidermized dermis (DED) (114,115). Each of these is potentially useful for studying drug transport, metabolism, and/or toxicity, depending on the requirements for a particular model system. For transport studies, it is essential that a stratum corneum, the outer layer of the epidermis, is present morphologically and functionally.

The stratum corneum, the primary barrier of the skin, is composed of terminally differentiated keratinocytes (corneocytes) surrounded by a nonpolar lipid matrix. This matrix is thought to derive from the contents of structures known as lamellar bodies or membrane coating granules found in the outer spinous and granular layers. They contain stacks of lipid bilayers, rich in sphingolipids, free fatty acids, cholesterol, and cholesterol sulfate (116–118). These granules fuse with the membrane at the granular/corneal junction and extrude their contents into the extracellular space where they fuse to form continuous sheets of stacked lipid bilayers (119–121). The biochemical composition and the spatial arrangement of the lipids into intercellular lamellar sheets are crucial for proper barrier function (121–126). Recently, the air/liquid interface keratinocyte cultures have proven to be valuable tools for the study of the relationship between epidermal lipid metabolism and development of barrier function *in vivo*.

Madison *et al.* (110) have described a technique for growing primary cultures of neonatal mouse keratinocytes at the air/liquid interface. Keratinocytes, isolated by trypsinization, were seeded into MilliCell well inserts coated with a reconstituted collagen gel. After 3 days submerged in the medium, the MilliCells were placed atop a stainless-steel grid covered with viable neonatal mouse dermis. The indirect contact between the keratinocytes and the dermis was essential for the degree of differentiation observed. The cultures contained a well-organized viable layer, a distinct granular layer, and a stratum corneum. They were also able to demonstrate by electron microscopy the presence of lamellar bodies in the upper spinous layer, extrusion of the contents at the granular/corneal interface, and stacked lamellar structures between the corneocytes in the outer stratum corneum. The lipid composition of these cultures has recently been enriched (127). When compared to submerged cultures, an enrichment in the stratum corneum lipids, ceramides, and free fatty acids was observed, approaching the content found in neonatal mouse epidermis.

The permeability properties of these cultures has also been examined. Using radiolabeled sucrose as a marker for culture integrity, transport was measured as a function of time in culture (128). Submerged cultures remained intact up to 18–20 days. Although the air/liquid interface cultures possess a stratum corneum, sucrose permeability increased dramatically beginning 3 days after lifting. This leakage was presumably due to degradation of the underlying collagen (129) and retraction of the cells from the outer edge of the MilliCell. This problem was circumvented by Cumpstone *et al.* (130) by the use of a microcapillary diffusion apparatus with a diffusional area of 0.02 $cm^2$. The water permeability of the neonatal mouse skin and the air/liquid interface cultures was compared using tritiated water. After 10 days, the permeability of the cultures was relatively constant up to 30 days but was two- to threefold higher than that of the neonatal skin ($\sim 7 \times 10^{-2}$ versus $\sim 2 \times 10^{-2}$ cm/hr). It was also found that the diffusion of water was dependent on the cross-sectional area of the capillary for the cultures but not for the neonatal skin and that the permeability was variable across different sites on the same culture well. This indicates that the stratum corneum formed was nonuniform and/or incompletely developed.

Based on this report, it is difficult to evaluate the utility of this culture system as a model for epidermal drug transport. The method described by Madison *et al.* (110) used BALB/c mice and the transport was done with CD1 mouse keratinocytes. Since no histology or electron microscopy was reported to demonstrate the condition of the cultures, it is possible that the data obtained could be explained by suboptimal cultures and/or incomplete differentiation of the CD1 keratinocytes. The permeability properties of larger, more lipophilic compounds also need to be determined before any conclusions are drawn.

The other air/liquid interface keratinocyte culture system for which permeability properties have been reported utilizes human keratinocytes grown on DED (131,132). DED is cadaver skin incubated in phosphate-buffered saline at 37°C for 3–5 days to remove the epidermis and then irradiated or repeatedly frozen to kill any remaining viable cells (17). This tissue retains the basal lamina which lines the dermal–epidermal junction and has proven to be a suitable substrate to induce keratinocyte differentiation (114,133–136). Human keratinocytes isolated from adult breast epidermis and grown as a primary culture on DED at the air/liquid interface showed a striking resemblance to intact epidermis. They also exhibit many of the ultrastructural markers of the epidermis, including hemidesmosomal attachment to the basement membrane and intra- and extracellular lamellar bodies (114,133). Similar results were obtained using either neonatal foreskin (131,136) or adult breast (132) keratinocytes initially cultured on lethally irradiated 3T3 cells according to the procedure described by Rheinwald and Green (137) and seeded onto DED at the air/liquid interface in the second or third passage. The lipid composition of these cultures was compared to epidermis and to parallel cultures grown submerged in the culture medium (136). Exposure to the air/liquid interface caused a decrease in phospholipid content and an increase in ceramides and free fatty acids. This is consistent with the increased differentiation observed; however, there were still minor discrepancies when compared to epidermis.

Using this technique, Mak *et al.* (131) measured the water permeability of the cultures with the microcapillary diffusion apparatus mentioned above. After 3 weeks at the air/liquid interface, the permeability of the cultures to water ($\sim 11 \times 10^{-2}$ cm/hr) was five- to sixfold higher than for neonatal foreskin and twofold higher than for adult skin. This permeability was reduced somewhat by lowering the relative humidity of the culture chamber from 100 to 75%. Nitroglycerine permeation across adult keratinocytes grown on DED showed similar results (132). Flux of the drug was approximately threefold higher through the cultures than through excised adult skin or isolated stratum corneum (11.4 versus 3.7 and 3.3 $\mu g/cm^2$/hr, respectively).

As was the case with the mouse keratinocyte cultures described above, the transport data are too preliminary to conclude how well the barrier properties of these human

keratinocyte cultures compare with those of intact human skin.

In addition to those already mentioned, there are two other types of air/liquid interface human keratinocyte cultures that may be useful for permeability studies in the future. One is the neonatal foreskin keratinocytes grown on collagen-coated nylon filters described by Williams *et al.* (111). In addition to the air/liquid interface, stratum corneum formation is enhanced by supplementing the media of the lifted cultures with lipid precursors. The other type is the living skin equivalent reported by Bell *et al.* (112). In this case, adult keratinocytes are grown on a dermal equivalent. This is formed by incorporation of fibroblasts into a collagen gel. After the fibroblasts have reorganized and contracted the collagen gel, the keratinocytes are added. These cultures have been used primarily to study skin transplantation and the problem of graft rejection (113).

Although the characterization of the *in vitro* epidermal barrier is only beginning, the keratinocyte cultures are also being used to examine cutaneous drug toxicity and metabolism.

Vaughan *et al.* (138) have cultured neonatal rat keratinocytes on uncoated nylon membranes (Puropor). Grown at the air/liquid interface, the cultures resemble intact epidermis and form a stratum corneum-like structure. While several ultrastructural features of the epidermis were present, the cultures have not been characterized to the degree of those described earlier. They are currently being used to elucidate the molecular mechanism(s) of sulfur mustard toxicity (139).

Cutaneous drug metabolism has also been studied in keratinocyte cultures to a limited degree. The enzyme system that has received the greatest attention is the cytochrome P450-dependent monooxygenase system. Primary cultures of neonatal mouse keratinocytes and human keratinocytes retain the activities of aryl hydrocarbon hydroxylase (AHH), 7-ethyoxycoumarin *O*-deethylase (7-ED), and epoxide hydrolase (140–143). As *in vivo,* the activities of AHH and 7-ED were induced when the cultures were pretreated with benz[a]anthracene or benzo[a]pyrene for 24 hr. When neonatal mouse keratinocytes were incubated with benzo[a]pyrene, the oxidized metabolites formed by the monooxygenase system were subsequently converted to the glucuronide and sulfate metabolites, indicating the presence of phase II metabolism *in vitro* (143,144).

Due to the current interest in the transdermal delivery of peptides, aminopeptidase activities have been quantitated in both neonatal mouse (145) and human keratinocytes (low calcium and the HaCaT cell line) (146). In both species, the aminopeptidase activities of the intact epidermis and of the cultured cells were comparable. Leucine, tyrosine, and lysine but not aspartic acid aminopeptidase activities were detected in both neonatal mouse and human keratinocytes.

Although the isolation and growth of keratinocytes have become routine, there are still many unanswered questions concerning regulation of keratinocyte differentiation and the eventual formation of the stratum corneum. The cell culture models described here have been used to address these basic issues of epidermal physiology. However, the by-products of these studies are culture techniques which reproduce many of the features of the *in vivo* epidermis: techniques which, if applied to drug transport and metabolism studies, could provide information not readily obtainable from other model systems or from *in vivo* studies.

## OPHTHALMIC—CORNEAL CELLS

Drug delivery by the ophthalmic route is generally concerned with delivering a therapeutic substance across the corneal barrier to intraocular tissue target sites. Consequently, approaches to meet the continuing needs of ocular pharmacology have been directed at improving intraocular bioavailability by extending topical ocular contact, modifying corneal permeability properties, and designing prodrugs. In part, the success of these approaches will depend on further characterization of the biochemical and/or molecular level properties of the corneal barrier (147,148). Although the ophthalmic route can also be considered for systemic delivery of drugs with poor oral bioavailability (147,148), for our purposes, only those tissue culture models relevant to the corneal barrier to intraocular drug delivery are mentioned.

The corneal tissue barrier to drug delivery is delimited by stratified epithelium anteriorly and a monolayer of endothelium posteriorly, with a substantial stromal layer in between (149). The anterior epithelial barrier is about five cells thick and accounts for approximately one-tenth of the total corneal thickness. These epithelial cells have extensive intercellular interdigitations and an abundance of desmosomal type junctions and contain a large amount of glycogen. The stromal layer of the cornea comprises the bulk of the corneal thickness and consists of laminated collagen fibers, keratocytes (i.e., stromal cells) interposed with the collagen fibers, and sulfated polysaccharides. Two very thin acellular layers, Bowman's membrane and Descemet's membrane, separate the stroma from the epithelial basement membrane anteriorly and the endothelial basement membrane posteriorly, respectively. The endothelial barrier of the cornea consists of a monolayer of closely apposed, overlapping, interdigitating cells that express tight intercellular junctions and possess a high metabolic capacity (149). To reach intraocular target sites, a topically applied drug must be able to traverse epithelial and endothelial tissues that present hydrophobic transport barriers to water-soluble substances, associated enzymatic barriers, and the stroma, which is a hydrophilic transport barrier to relatively lipophilic substances (149,150).

Cell culture systems derived from corneal cells appear to have been first described at least three decades ago by Stocker *et al.* (151). Numerous cell culture systems originating primarily from rabbit and human cornea have been described since (152). A sampling of recent publications in this research area suggests that either mechanically or enzymatically isolated populations of corneal epithelial (152–155), endothelial (152,156–163), and stromal (152) cells have emerged as the most popular methods for establishing homogenous tissue culture systems. Cells grown in these systems retain biochemical and morphological features that are similar but, again, not identical to the parent cell types (152,154,159–162,164). At least one cell line, from normal rabbit cornea (SIRC), is also available from American Type Culture Collections (Rockville, MD). However, this cell line

has not been characterized biochemically and morphologically and has been described as fibroblast-like.

Both corneal epithelial and corneal endothelial cell cultures have been employed in studies that are directly relevant to drug delivery problems. Important uses of corneal epithelial cell cultures have included the study of β-adrenergic responsiveness and wound repair (165,166), effects of chemical irritants on cell growth (155), and chemical induction of plasminogen activator secretion (154). The success in quantitating the sensitivity of the corneal epithelial cell cultures to chemical irritants suggests that these *in vitro* systems may eventually replace the Draize test (154,155,167). Details of the metabolic activity and transcellular permeability properties have apparently not been addressed in these corneal epithelial cell culture systems.

Decidedly more research has been conducted on the corneal endothelial cell culture systems relative to the corneal epithelial cell culture systems described above. The characteristics of cell growth (160–162), effects of steroids and adrenergic drugs on cell growth (158,163), utility in transplantation (161), basement membrane secretion (168), cyclic nucleotide content (169), surface glycoproteins (170), and ATPase saturation kinetics (159) of the corneal endothelial cell cultures have been explored recently. Although limited, these studies could form the basis for extensive investigation of the transport and metabolic functions of the endothelial barrier in intraocular drug metabolism and transport.

Several corneal cell cultures have been developed. While these systems remain poorly defined both biochemically and morphologically, they offer potentially valuable resources for the pharmaceutical chemist to elucidate further cellular/molecular characteristics of the corneal transport and metabolic barriers directly impacting on efficient intraocular drug delivery.

## ENDOTHELIAL BARRIERS

A relatively heterogeneous, asymmetric monolayer of endothelial cells lining the capillaries regulates the distribution of substances between the blood and the interstitial fluids (171). In peripheral tissues this cell barrier permits diffusion of low molecular weight water-soluble substances either intercellularly or through fenestra. Bloodborne macromolecules are moved transcellularly by either fluid-phase or adsorptive transcytosis. In 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 (172–174).

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 is 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 (175), bovine (176–180), murine (181–183), porcine (184), and rat (180,185–187) brain. Generally, either enzymatic or mechanical dispersal followed by either filtration or centrifugation steps is employed to isolate a homogeneous population of brain microvessel endothelial cells from the extremely heterogeneous population of cells found in brain tissue. Combinations of enzymatic and mechanical isolation techniques have also been used. In general, those techniques utilizing enzymatic dispersal to isolate brain capillaries are preferred. While possibly easier and quicker, drawbacks in using mechanical isolation techniques include lower cell yields, reduced overall cell viability, and the induction of primary metabolic defects in isolated cells. Mechanically induced alterations in isolated cells may also be responsible for introducing variability in the culture systems (188–190).

Other brain cells may contribute to the properties expressed by this specialized endothelial cell population forming the blood–brain barrier (BBB). Recent evidence indicates that astrocytes may influence specific BBB marker enzymes (191) and intercellular junctions (173,192) of endothelia comprising the BBB. Tight intercellular junctions are sufficiently developed in primary cultures, at least in the bovine system, to prevent transcellular passage of ionic lanthanum, a marker for tight intercellular junctions. However, the extent and configuration of the tight intercellular junctions in primary culture are still not identical to those of *in vivo* junctions (173). These observations have stimulated further research on astrocyte factors in several laboratories, including our own (193), that may improve permeability characteristics of both passaged and primary *in vitro* BBB models. Under certain coculture conditions, astrocytes may secrete factors that reduce the permeability of primary cultures of bovine brain microvessel endothelial cell monolayers by about 50% (193). Although astrocytes have been targeted as providing influence on BBB endothelial differentiation, pericytes and smooth muscle cells also regulate endothelial cell function (194,195). Contributions of other cell types, pericytes and smooth muscle, to the BBB remain to be addressed (173).

In drug delivery studies, an *in vitro* BBB model retaining tight intercellular junctions and metabolic activities similar to the *in vivo* situation is crucial in applying the system to study permeability characteristics of the BBB. Primary cultures of brain microvessel endothelial cells isolated either enzymatically or mechanically retain many characteristics of the parent tissue including the morphology, specific BBB enzyme markers, few pinocytic vesicles, no fenestra, and tight intercellular junctions (175–187). Attempts to generate cell lines or passaged cell culture systems have been generally limited by the inability to retain specific endothelial and BBB markers or tight intercellular junctions (173). Thus, at present, the primary culture systems are most useful in addressing BBB drug delivery problems *in vitro*.

The primary culture system used in our laboratories has been generated from enzymatically isolated bovine brain mi-

crovessel endothelial cells and has been employed as a model to study BBB transport and metabolism (178,179). This model has been extensively characterized morphologically, biochemically, and immunohistochemically. Morphologically (Fig. 3C), the primary cultures retain tight intercellular junctions, attenuated pinocytosis, and no fenestra (178,179). 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) (178,196–198), acetylcholinesterase, butyrylcholinesterase (A. M. Trammel and R. T. Borchardt, unpublished observations), aminopeptidases (199), and acid hydrolases (200) 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 of primary cultures of bovine brain microvessel endothelial cell monolayers grown on either polycarbonate or regenerated cellular membranes has been characterized in a horizontal side-by side diffusion cell apparatus. Permeation of substances across the monolayers has been shown to be related to both lipophilicity and molecular weight in a manner consistent with the BBB *in vivo* (6,201–203). 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). Consequently, the *in vitro* model can be used to study carrier-mediated transcellular transport of various substances. Using this model, the carrier-mediated transport of amino acids (204), choline (205), epinine esters (198,202), and nucleosides (206) has been characterized. Transcellular transport of leucine, for example, was shown to be saturable, competitive with other amino acids, and energy independent in the *in vitro* model. In addition, the kinetic parameters for leucine transport *in vitro* (204) appear to be in good agreement with true kinetic parameters of the *in vivo* BBB (207). The transport of several amino acid drugs, including, baclofen (208), α-methyldopa (209), and acivicin (210), by the amino acid carrier has also been explored in this system.

The potential importance of therapeutic peptides and proteins in the future has provided incentive to researchers to define basic kinetic parameters and conditions of transendothelial transport mechanisms. Primary mechanisms for moving larger molecules across the BBB endothelium include attenuated fluid-phase and adsorptive transcytosis. By qualitative and quantitative measure of Lucifer yellow uptake and transport, fluid-phase endocytosis and subsequent transcytosis have been shown to be attenuated in the BBB model system, consistent with corresponding activity at the BBB *in vivo* (211). On the other hand, evidence for a more active adsorptive pathway for lectins has been demonstrated in the *in vitro* model (212). BBB adsorptive endocytosis and transcytosis *in vivo* is poorly defined, thus comparisons to the model have not been possible. The transport of larger molecules such as insulin (213), modified albumins (e.g., native, cationized, or glycosylated) (214), delta sleep-inducing peptide (215), vasopressin (216), and leu-enkephalin (217) has been or is currently under investigation with the model system. In this respect, other researchers have also begun to use this model to characterize the transcytosis of transferrin (218) and fragments of vasopressin (219). Insulin, modified albumins, and transferrin appear to undergo either specific or nonspecific adsorptive transcytosis as defined in the model (213,214,218). Smaller peptides, vasopressin, delta sleep-inducing peptide, and vasopressin fragments, cross the monolayers passively (blood-to-brain) via paracellular pathways (215,216,219). Recent studies indicate that certain opioids increase the permeability of the BBB *in vivo* (220). Similarly, we have observed that nanomolar concentrations of leu-enkephalin alter the permeability of the monolayers as demonstrated by increased permeability to sucrose (217). Further exploration of the possible role of the μ opioid receptor in mediating the effects of leu-enkephalins on BBB permeability is under way. Currently, our laboratories are developing an *in vitro* primate BBB model as an alternative to the intact animal of choice in AIDS studies to assess transport mechanisms of antiviral CD4 peptide fragments directed at the central nervous system component of AIDS.

The *in vitro* model system has been used to investigate other factors regulating the permeability characteristics of the BBB. For instance, occupation of apical angiotensin II binding sites on brain microvessel endothelial cell monolayers stimulates fluid-phase endocytosis but reduces transcellular permeability by about 80%. The effects of angiotensin II appear to be mediated by prostaglandin release and alterations in membrane fluidity (221–224). Along with morphological characteristics (211), the apical sensitivity of the cultured cells to angiotensin II peptides provides strong evidence that the cells remain polarized in primary culture. In related efforts, atrial natriuretic peptide (ANP) receptors have been identified in this model (225). ANP receptors may also regulate BBB permeability to water and electrolytes. Environmental substances such as aluminum, a common ingredient in many over-the-counter products, can also be shown to modulate both permeability and growth characteristics of the BBB *in vitro* (226,227). Studies focused on the regulation of BBB permeability have immediate implications for pharmacological approaches to hypertension, stroke, inflammation, cerebral trauma, and injury. Drug delivery applications of this research requires further consideration, however, the ability to modulate BBB permeability through specific receptors has more appeal than nonspecific hyperosmotic treatments proposed for anticancer therapy in the central nervous system.

The primary cultures of bovine brain microvessel endothelial cell monolayers offer an excellent model for addressing drug delivery across the BBB. As outlined above, the *in vitro* BBB model has been and can be used to address a variety of problems ranging from carrier-mediated transport, simple diffusion, and metabolism to factors modulating BBB permeability. This model continues to evolve much like the other epithelial cell culture systems described above. For instance, future manipulations in tissue culture conditions, such as the introduction of an as yet unidentified astrocyte

factor, will move the *in vitro* system closer to the *in vivo* situation relative to restrictive BBB permeability properties. We anticipate that this model will become and continue to be a widely recognized, humane, and economical alternative for the pharmaceutical chemist in screening BBB permeability of newly developed central nervous system-directed drugs.

## 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 vivo–in vitro* 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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