A Conditionally Immortalized Epithelial Cell Line for Studies of Intestinal Drug Transport

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ABSTRACT
A new cell culture model that better mimics the permeability of the human small intestine was developed for studies of passive drug transport. The intestinal epithelial cell line, 2/4/A1, conditionally immortalized with a temperature-sensitive mutant of the growth-promoting oncogene simian virus 40 (SV40) large T, was grown on permeable supports. The cells grew at 33°C, where the oncogene is fully active, but stopped growing and entered a differentiation program at 39°C, where the oncogene is inactive. Significant cell death was observed at 39°C and, therefore, growth conditions under which 2/4/A1 cells survive during the differentiation process were developed. Cells grown on extracellular matrices which contained laminin at an intermediate temperature of 37°C formed viable differentiated monolayers with tight junctions, an increased expression of brush border enzymes, and a paracellular permeability that was comparable to that of the human small intestine. The permeability of 17 structurally diverse drugs gave a sigmoidal relationship with the absorbed fraction of the drugs after oral administration to humans. The relationship was compared with those obtained with the well established Caco-2 model and after in vivo perfusion of the human jejunum. The transport of drugs with low permeability in 2/4/A1 monolayers was comparable to that in the human jejunum, and up to 300 times faster than that in Caco-2 monolayers. The transport of drugs with high permeability was comparable in all models. These results indicate that 2/4/A1 monolayers are promising alternatives to Caco-2 monolayers for studies of passive drug transport.

The good correlation between passive transcellular drug transport in Caco-2 monolayers and drug transport seen in vivo has made it possible to use cultures of this cell line in screening for drug candidates and in studies of structure-absorption relationships (Artursson and Borchardt, 1997). However, Caco-2 cell monolayers are not a perfect model of all transport routes in the normal intestinal enterocyte. Caco-2 and similar cell lines (such as HT29) form monolayers with a very low paracellular permeability, up to 100 times lower than that of the human small intestine (Artursson et al., 1993). This has been attributed to the colonic origin of the cells (Grasset et al., 1984). This makes it difficult to study structure-absorption relationships for drugs that are transported via the paracellular route.

Because new drugs discovered by combinatorial chemistry and high throughput pharmacological screening generally are larger and have more groups that form hydrogen bonds than conventional drugs (Lipinski et al., 1997), it is believed that the paracellular route contributes significantly to their transepithelial transport. The development of a cell culture model of the intestinal epithelium that better mimics the in vivo permeability of the paracellular route is, therefore, highly desirable. Previous attempts to develop such cell culture models have been only partly successful. HT29–18-C1, a monolayer-forming epithelial cell line of colonic origin, is only moderately more leaky than Caco-2 cells (Wils et al., 1994; Collett et al., 1996). A more leaky small intestinal epithelial cell line, IEC-18, forms multilayers in cell culture and displays a discontinuous paracellular barrier (Duizer et al., 1997). Cocultures of absorptive and goblet cell lines with a higher paracellular permeability have been established but express an abnormal phenotype in cell culture (Wikman Larhed and Artursson, 1995). Recently, several approaches have been taken to develop intestinal epithelial cell lines of a more normal phenotype by immortalization of isolated normal human intestinal epithelial cells (Quaroni and Hochman, 1996). It is necessary to immortalize the cells because normal intestinal epithelial cells are programmed to survive for only a few days. In one attractive approach, a temperature-sensitive gene switch strategy was used (Paul et al., 1993). By express-
ing a temperature-sensitive mutant of the growth-promoting simian virus 40 (SV40) large T oncogene in normal intestinal epithelial cells, the cells can proliferate at a lower permissive temperature where the oncogene is fully active. When the temperature is increased, the SV40 large T mutant is inactivated, and the cells stop dividing and may enter a differentiation program (Jat and Sharp, 1989). Unfortunately, the inactivation of SV40 large T may also induce cell death in the transfected cell lines, making them unsuitable for transport studies (Yanai and Obinata, 1994). No successful approach to overcome this problem in intestinal epithelial cells has been presented.

In this study, we have developed a new cell culture model for studies of passive transcellular and paracellular drug transport based on the intestinal epithelial cell line 2/4/A1, conditionally immortalized with a temperature-sensitive mutant of SV40 large T (Paul et al., 1993). We describe growth conditions in which 2/4/A1 cells survive and develop into differentiated monolayer cultures with a paracellular permeability that closely mimics that observed in the human intestine in vivo. We performed an initial characterization of the temperature-dependent differentiation of 2/4/A1 cells and investigated the permeability of the paracellular route. Finally, we investigated the applicability of the cell culture model in studies of a series of structurally diverse (passively transported) drugs, and compared our results with drug transport data obtained in Caco-2 cell monolayers, in the perfused human intestine, and after oral administration to humans.

**Materials and Methods**

**Drugs and Radiolabeled Markers.** Alpenolol hydrochloride, atenolol, fluorescein-conjugated dextran (mol wt 50,000), lactulose, metolazone, metoprolol tartrate, sodium-fluorescein, phenazone, phosphonoformic acid (foscanet), pindolol, propanolol hydrochloride, d-rafﬁnose, suﬁlasalazine, sulpiride, and terbutaline were purchased from Sigma (St. Louis, MO). Olsalazine was a gift from Dr. Wenche Rolfsen, Pharmacia & Upjohn (Upsala, Sweden). Lucifer yellow was purchased from Molecular Probes (Eugene, OR). [14C]Phosphonoformic acid, [3H]lactulose, and [14C]PEG (mol wt 4000) were purchased from Morave Biochemicals (Brea, CA), American Radiolabeled Chemicals, Inc. (St. Louis, MO), and Amersham (Arlington Heights, IL), respectively. [14C]Creatinine, [14C]mannitol, and d-[3H]rafﬁnose were obtained from NEN Life Sciences (Boston, MA).

**Cell Culture.** Culture media and supplements were purchased from Gibco/BRL Life Technologies AB (Taby, Sweden) unless otherwise stated. 2/4/A1 cells originating from fetal rat intestine were immortalized conditionally with a pZipSVtsa58 plasmid containing a wise stated. 2/4/A1 cells originating from fetal rat intestine were contaminated ever second month throughout this study. In brief, 2/4/A1 cells were seeded at a density of 440,000/cm² on uncoated permeable polycarbonate filters with a 0.45-μm pore size, 12 mm in diameter (Transwell Costar). Cells at passage numbers 95 through 105 were used.

The 2/4/A1 and Caco-2 cultures were tested negative for mycoplasma contamination every second month throughout this study.

**Cell Attachment Assay.** The attachment of 2/4/A1 cells to different extracellular matrices was studied according to the method of Benya et al. (1991). In brief, cells were cultured on the various extracellular matrices in plastic chambers at 33, 37, or 39°C for 24 h. The cells were then washed three times with PBS at pH 7.4, trypsinized, and counted in a hemocytometer. Cell counts were expressed as number of cells/cm².

**DNA Staining and Analysis of Cell Death.** 2/4/A1 cells were seeded on plastic chamber slides coated with ECL and cultured at 33, 37, or 39°C. After 24 h, the medium containing nonattached cells was replaced with fresh medium. After 4 days in culture, the cells were washed with PBS, fixed with 3% paraformaldehyde in PBS, and permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min. After washing with PBS, the cells were stained for 60 min with 1.0 μg/ml propidium iodide (Molecular Probes) in PBS. After staining, the cells were washed with PBS and mounted, with Dako fluorescent mounting medium (12 μg/ml) according to the manufacturer’s specifications. 2/4/A1 cells were seeded at 30,000/cm² on plastic chambers and at 100,000/cm² on permeable filter supports.

Caco-2 cells originating from a human colorectal carcinoma were obtained from American Type Culture Collection (Rockville, MD) and cultured as described in detail previously (Artursson et al., 1996). In brief, Caco-2 cells were seeded at a density of 440,000/cm² on uncoated permeable polycarbonate filters with a 0.45-μm pore size, 12 mm in diameter (Transwell Costar). Cells at passage number 95 through 105 were used.

**Immunofluorescence.** Cells grown on ECL-coated permeable supports were washed with PBS and fixed with 3% paraformaldehyde in PBS at pH 7.4 for 10 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min. The F-actin distribution was visualized by direct fluorescence with rhodamine-conjugated phalloidin (5 U/ml; Molecular Probes) according to the manufacturer’s instructions. Tight and adherence junction protein distributions were studied by indirect immunofluorescence with rabbit polyclonal antibody to ZO-1 protein (1:1000) (Zymed Laboratories Inc., San Francisco, CA) and a mouse monoclonal Ig human to E-cadherin (5 μg/ml) (Transduction Laboratories, Lexington, KY), respectively. The SV40 large T antigen was stained with mouse monoclonal Ig SV40 large T antigen antibody (1:1000) (Oncogene Science, Uniondale, NY). The cells were incubated with the primary antibodies for 1 h at room temperature, washed with PBS, and incubated with secondary fluorescein isothiocyanatelabeled antibodies against rabbit or mouse Ig (1:100 in PBS) (Amersham) for 30 min at room temperature. The samples were washed with PBS, mounted in Dako fluorescent mounting medium, sealed, and examined under a confocal laser scanning microscope (Leica TCS 4D; Leica LT, Heidelberg, Germany).
Electron Microscopy. 2/4/A1 and Caco-2 cells cultivated on permeable supports as described above were fixed in 2.5% glutaraldehyde and then immersed in 1% osmium tetroxide and dehydrated with ethanol. Thin sections were stained with uranyl acetate plus lead citrate and examined by transmission electron microscopy in a Philips 420 electron microscope (Philips, Eindhoven, the Netherlands).

Brush Border Enzyme Assays. For assay of the brush border-associated enzymes alkaline phosphatase, aminopeptidase N, and sucrase-isomaltase, 2/4/A1 cells were cultured in ECL-coated plastic chambers at 33, 37, or 39°C. After 4 to 6 days the cells were washed with PBS, scraped off with a rubber policeman, and homogenized with an Ultra Turrax homogenizer (IKA-Works, Inc., Cincinnati, OH) for 20 s. Alkaline phosphatase was measured by using 4-nitrophenolphosphate as substrate (Granett; Merck, Darmstadt, Germany), and aminopeptidase N was measured by using l-leucine-4-nitroanilide as substrate (Janes and Hafkenscheid, 1984). Sucrase-isomaltase was assayed by the one-step method of Messier and Dahlqvist (1966). Protein was assessed with the Coomassie blue protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Electrophysiological Measurements. The transepithelial electrical resistance (TER) of 2/4/A1 monolayers grown on ECL-coated permeable supports was measured at 37°C with an Endohm tissue resistance measurement chamber connected to an Evohm resistance meter (World Precision Instruments, Sarasota, FL). The cell culture medium was replaced by Hank’s balanced salt solution (HBSS) containing 25 mM HEPES at pH 7.4 preheated to 37°C. The 2/4/A1 monolayers were equilibrated for 20 to 25 min before TER measurements. The resistance of ECL-coated filters without cells was subtracted from each TER value.

Permeability to Hydrophilic Marker Molecules. Transport studies with hydrophilic marker molecules were performed on 2/4/A1 and Caco-2 cells which were cultivated on permeable supports as described above. All experiments were carried out at 37°C in HBSS under “sink” conditions, as described previously (Artursson et al., 1996), with 2/4/A1 monolayers that were 1 to 10 days old and Caco-2 monolayers that were 21 to 35 days old.

All solutions were preheated to 37°C, and the experiments were performed at 37 ± 1°C on a custom-built heating plate (Detron, Linköping, Sweden). The cell monolayers were washed with preheated HBSS and equilibrated in the same buffer for 20 to 25 min before the transport experiments. The filters were then transferred to wells containing 1.2 ml of fresh preheated HBSS. HBSS (0.4 ml) containing radiolabeled or fluorescent paracellular marker molecule was added to the donor chamber. The following markers were used: [14C]mannitol (mol wt 182), sodium-fluorescein (mol wt 376), Lucifer yellow (mol wt 450), [14C]PEG (mol wt 4,000), and fluorescein-conjugated dextran (mol wt 50,000). The marker molecules were used at concentrations of 5,000 or 10,000 Bq/ml for 2/4/A1 monolayers or 10,000 or 20,000 Bq/ml for Caco-2 monolayers ([14C]-labeled markers), or at 1 mg/ml (fluorescent markers). Samples were taken from the apical solutions to measure the initial donor concentration. For four regular time intervals, the inserts were moved to new wells, and samples were taken from the basolateral solution. All samples were analyzed directly as described below.

Permeability of Drugs in 2/4/A1 Monolayers. Drug transport experiments were performed in 2/4/A1 monolayers that were 3 to 8 days old, grown at 37°C under the same conditions as described above (Artursson et al., 1996). The TER of the cells and their permeability to the paracellular marker molecules were constant over this period.

HBSS (0.4 ml) containing the drug to be tested was added to the donor chamber at the following concentrations: propranolol (100 μM); metoprolol, phenazone, alprenolol, pindolol, terbutaline, metolazone, atenolol, sulpiride, sulfasalazine, and lactulose (1 mM); PEG 4000 (1 mg/ml) spiked with 10,000 Bq/ml [14C]PEG 4000, creatinine, foscarnet, mannitol, and lactulose; or raffinose (1 mM) spiked with 5,000 Bq/ml [14C]creatinine, [14C]foscarnet, [14C]mannitol, [3H]lactulose, or [3H]raffinose, respectively. Samples were withdrawn from the receiver side at regular time intervals: every 3 min for phenazone and alprenolol; every 5 min for metoprolol, propranolol, pindolol, and terbutaline; and every 10 min for all other compounds, except PEG 4000, which was sampled every 20 min. The samples were replaced with fresh preheated HBSS. Samples were taken from the donor solutions to measure the initial donor concentration. Samples containing unlabeled drugs were kept at −20°C pending HPLC analysis, whereas samples containing radiolabeled drugs were analyzed immediately in a liquid scintillation counter.

To obtain drug permeability coefficients that were unaffected by the unstirred water layer, the filters were agitated on a calibrated plate shaker (IKA Shüttler MTS 4) at two different stirring rates, 100 rpm and 500 rpm (see below). The paracellular marker [14C]PEG 4000 was used to assess the integrity of the monolayers exposed to the drugs at both stirring rates; no increase in [14C]PEG 4000 permeability was observed under these conditions. All transport experiments were carried out within 60 min, with the exception of the PEG 4000 experiments, which were carried out in 120 min.

The possible effect of the ECL coating on the permeability of the permeable supports was investigated by comparing the diffusion of [14C]mannitol across cell-free supports with or without the ECL coating. No retardation in diffusion of [14C]mannitol was observed, which showed that the ECL barrier to diffusion of mannitol could be neglected.

Analytical Methods. Radioactive samples were analyzed by using a liquid scintillation counter (Packard Instruments 1900CA TRICARB; Canberra Packard Instruments, Downers Grove, IL). Fluorescein-labeled samples were analyzed with a fluorescence plate reader (FL500; Bio-Tek Instruments Inc., Winooski, VT).

Unlabeled samples were analyzed with reversed-phase HPLC. The system consisted of a Perkin-Elmer Isocratic LC pump 250, a Perkin-Elmer Advanced LC Sample Processor ISS-200, a Spectra Physics UV100 detector, and the integration software program Chromatography Station for Windows. The analytical column was a Beckman UltraspHERE ODS (250 × 5.6 mm) with a mean particle size of 5 μm. The mobile phase was composed of freshly prepared phosphate buffer (pH 3.0, 60 mM KH2PO4, and 8 mM H3PO4) and acetonicitrile in the following proportions: 90:10 for atenolol, sulpiride, and terbutaline; 75:25 for metoprolol and pindolol; 70:30 for alprenolol and phenazone; 65:35 for propranolol and metolazone. A mobile phase composed of phosphate buffer (pH 5.0, 33 mM KH2PO4 and 0.47 mM Na2HPO4) and methanol was used for the analysis of sulfasalazine and osalazine. The proportions used were 45:55 and 55:45, respectively. Flow rates were 0.6 to 1.0 ml/min and injection volumes were 100 to 200 μl. The wavelength was set to the most suitable UV absorption maximum for each compound.

Calculations. The apparent permeability coefficient (Papp cm/s) was determined according to the following equation:

\[ P_{app} = \frac{K \cdot V}{A} \]  

(1)

where \( K \) is the steady-state rate of change in concentration in the receiver chamber (C1/C0) versus time (t), C1 is the concentration in the receiver compartment at the end of each time interval, C0 is the initial concentration in the apical chamber at each time interval (mol/ml), \( V \) is the volume of the receiver chamber (ml), and A is the surface area of the filter membrane (cm²).

The cellular permeability (Pc) was calculated by determining the apparent permeability of the drugs in 2/4/A1 monolayers at two different stirring rates (V). \( P_{app} \) was calculated from the slope of the linear relationship \( V/P_{app} \) and \( V \) as described previously (Artursson et al., 1996).

\[ \frac{V}{P_{app}} = \frac{1}{K} + \left( \frac{1}{P_{c}} + \frac{1}{P_{f}} \right) \cdot V \]  

(2)
where $P_f$ is the calculated permeability coefficient of the filter support and $K$ is a constant.

The sigmoidal equation:

$$FA = \frac{100}{1 + \left(\frac{P_f}{P_{50}}\right)^\gamma}$$

was fitted to the drug transport data. $FA$ is the fraction of a drug absorbed in humans after oral administration, $P_{50}$ is the $P_f$ at an $FA$ value of 50%, and $\gamma$ is a slope factor. The equation was fitted to the data by minimizing the unweighted sum of square residuals.

**Statistics.** The experiments were repeated at least twice, applying four monolayers each time unless otherwise stated. The results were expressed as mean values ± S.D. One-way ANOVA was used to compare means. A 95% probability was considered significant. The root mean square error (RMSE), i.e., the standard error of regression, and the coefficient of determination ($r^2$) were used to measure how well the sigmoidal equation fits the observations.

## Results

### Characterization of Growth and Differentiation of 2/4/A1 Cells at Different Temperatures

**Extracellular Matrix.** At all temperatures, significantly higher numbers of 2/4/A1 cells attached to fibronectin, laminin and ECL-coated supports than to the uncoated supports ($p < .05$) (Fig. 1). No significant improvement in cell attachment was observed for supports coated with collagen I and IV ($p > .05$). Because preliminary results indicated that fibronectin reduced the survival of 2/4/A1 cells (data not shown), the laminin-containing ECL-coated supports were selected for all additional studies.

**Expression of SV40 Large T Antigen.** Immunofluorescence microscopy showed that SV40 large T antigen was expressed in all cells to a varying degree, and that the antigen was relatively evenly distributed in all cell nuclei at 33°C (Fig. 2A). At 37°C, all cells still showed expression of SV40 large T, but the staining was fainter (Fig. 2B). At 39°C only some fragments of the nuclei were stained in an irregular pattern (Fig. 2C). These results indicate a gradual loss of immunoreactivity of the mutated SV40 large T antigen with increasing temperature, consistent with the reported temperature sensitivity of this antigen (Jat and Sharp, 1989).

**Cell Death.** Morphological analysis with light microscopy showed that the 2/4/A1 cells formed continuous multilayers after 4 days of culture on ECL at 33°C (data not shown). At 37°C, the cells formed monolayers within 24 h and remained as intact monolayers for at least 10 days in culture (see below). 2/4/A1 cells, seeded at 39°C onto ECL, initially formed monolayers comparable to those observed at 37°C. However, during the first 24 h, the monolayers of these cells developed defects, and within the following 24 h in culture, cell detachment was observed. The cell detachment increased over the next 4 days in culture, after which < 50% of the surface area of the support was covered with cells (data not shown).

Examination of the cell nuclei after propidium iodide staining showed that at 33°C, the 2/4/A1 cells grew and divided at a high cell density, consistent with the formation of multi-

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**Fig. 1.** Attachment of 2/4/A1 cells to plastic (P), collagen I (C I), collagen IV (C IV), fibronectin (F), laminin (L), and ECL at 33°C (A), 37°C (B), and 39°C (C) on ECL. Cells were fixed in formaldehyde, and the SV40 large T antigen was stained with a mouse monoclonal antibody and a secondary fluorescein isothiocyanate-labeled antibody. The immunostaining of the antigen decreased with increasing temperature, consistent with the temperature sensitivity of the antigen. Images were obtained by confocal laser scanning microscopy. Bar, 10 μm.

**Fig. 2.** Immunofluorescence staining of temperature-sensitive SV40 large T antigen in 2/4/A1 cells after 4 days of culture at 33°C (A), 37°C (B), and 39°C (C) on ECL. Cells were fixed in formaldehyde, and the SV40 large T antigen was stained with a mouse monoclonal antibody and a secondary fluorescein isothiocyanate-labeled antibody. The immunostaining of the antigen decreased with increasing temperature, consistent with the temperature sensitivity of the antigen. Images were obtained by confocal laser scanning microscopy. Bar, 10 μm.

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1999 Drug Transport in a New Intestinal Epithelial Cell Line 1215

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layers observed in the light microscope (Fig. 3A). The cell nuclei of 2/4/A1 cells grown at 37°C were more evenly distributed, supporting the finding that the cells formed monolayers at this temperature (Fig. 3B). The cell nuclei had a normal oval morphology at both 33 and 37°C. In contrast, many of the cells grown at 39°C displayed condensed and fragmented nuclear DNA, indicating that many cells died at this temperature (Fig. 3C).

Development of Tight and Adherence Junctions. The tight junction protein ZO-1 was present in 2/4/A1 cells grown at all three temperatures. Its distribution was less well ordered in cells grown at 33°C (Figs. 4A and 5A) than in those grown at 37°C. At 37°C, ZO-1 formed a continuous network (Fig. 4B) located at the apical region of the cell-to-cell junctions (Fig. 5B). In contrast, after cultivation of 2/4/A1 cells at 39°C, the ZO-1 network became discontinuous, indicating that the cell-to-cell contacts had loosened (Fig. 4C). Similarly, the protein E-cadherin, which is associated with adherence junctions, was present at all temperatures. E-cadherin formed a diffusely distributed dot-like network at 33°C (Figs. 4D and 5C). At 37°C the dot-like network was more marked and was located closer to the apical cell membrane and the cell junctions (Figs. 4E and 5D). At 39°C, the E-cadherin network at the cell junctions was not as distinct as that observed at 37°C (Fig. 4F). Actin filaments were found at the cell borders, but they also appeared as stress fibers at 33°C (Fig. 4G). At 37°C, actin had redistributed to the perijunctional area of the terminal web (Fig. 4H). At 39°C the actin network indicated broadening of extracellular spaces and defects in the monolayer (Fig. 4I). These findings indicate that 2/4/A1 cells grown at 37°C form monolayers consisting of polarized epithelial cells with typical junctional complexes, containing ZO-1 at tight junctions and E-cadherin at areas of cell-cell contact.

Transmission electron microscopy confirmed the findings of the light and fluorescence microscopy. At 33°C, 2/4/A1 cells grew as multilayers which were 2 to 4 cells thick (data not shown), whereas at 37°C, monolayers of cuboidal cells connected by tight junctions were observed (Fig. 6A). Cells grown at 39°C showed the morphology of dying cells, with irregular and shrunken cell nuclei and defects in the cell monolayers (data not shown). At all temperatures, the surface of the apical cell membrane had fewer microvilli than did the well differentiated human colorectal carcinoma cell line Caco-2 (Fig. 6B).

Brush Border Enzyme Activities. Brush border enzymes have been shown to localize at the apical cell membrane of 2/4/A1 cells in a temperature-dependent fashion (Paul et al., 1993). In this study the activities of brush border enzymes alkaline phosphatase, aminopeptidase N, and sucrase-isomaltase were assayed in cell homogenates prepared from 2/4/A1 cells grown at 33, 37, and 39°C (Table 1). The activity of alkaline phosphatase was significantly higher at 37°C than at 33°C, indicating that 2/4/A1 cell monolayers had partially differentiated at this temperature (p < .05). All enzyme activities increased in the subpopulation of cells that remained adherent at 39°C.

Integrity and Permeability of 2/4/A1 Monolayers

The studies of the temperature-dependent differentiation showed that 2/4/A1 cells grown on ECL form monolayers of viable cells only at the intermediate temperature of 37°C. Therefore, 2/4/A1 cells grown at this temperature were selected for additional functional studies on the integrity and permeability of the cell monolayers. Monolayers of the well differentiated human intestinal epithelial cell line Caco-2 were used as controls.

TER. The TER in 2/4/A1 cells increased with time and reached a plateau value of 25 ± 2.9 Ω·cm² after 4 days in culture (Fig. 7A). This TER was maintained for at least 8 days. By comparison, the TER in Caco-2 cell monolayers reached a plateau of 234 ± 12 Ω·cm² and maintained this value for at least 40 days in culture (Fig. 7B), which is in agreement with previous findings (Artursson, 1990). Thus, the TER of confluent 2/4/A1 monolayers was approximately 9-fold lower than the TER of Caco-2 monolayers.

Permeability to Hydrophilic Markers. Preliminary experiments showed that cell monolayers obtained over a wide number of passages (passages 18–43) had a permeability comparable to that of the hydrophilic marker molecules. However, monolayers obtained from passage numbers >43 showed a slight increase in the paracellular permeability,
suggesting that the 2/4/A1 cells may obtain slightly different properties at these higher passage numbers. For the sake of brevity, only results obtained from cells cultivated at passage numbers 30 to 43 are presented here.

The permeability of 2/4/A1 monolayers to the hydrophilic marker molecules decreased during the first 2 days in culture and then remained at a constant low level for at least 10 days (data not shown). During this period (2–10 days) the permeability depended on the size of the marker molecule and was comparable to that obtained for hydrophilic markers in the human small intestine (Chadwick et al., 1977; Artursson et al., 1993) (Fig. 8A). The permeabilities of the low molecular weight marker molecules mannitol, fluorescein, and Lucifer yellow were significantly different from each other \((p < .05)\), with the exception of that between fluorescein and Lucifer yellow \((p > .05)\). There was no significant difference in permeability between the high molecular weight marker molecules PEG 4000 and dextran \((p > .05)\). The apparent permeability coefficients \(P_{app}\) ranged from \(15.5 \pm 2.09 \cdot 10^{-6} \text{ cm/s}\) for the smallest marker molecule, mannitol (mol wt 182), to
Fig. 6. Transmission electron micrographs of tight junctions and the intercellular spaces of 2/4/A1 (A) and Caco-2 cells (B). 2/4/A1 and Caco-2 cells were cultivated on permeable supports at 37°C for 4 and 21 days, respectively. Thin sections were stained with uranyl acetate plus lead citrate. 2/4/A1 cells form tight junctions (arrow) and relatively straight lateral spaces at 37°C but display few microvilli at the apical surface (A). Caco-2 cells form tight junctions (arrow) with tortuous lateral spaces and display a more differentiated morphology with numerous microvilli (B). Original magnification, 32,000× (A) and 11,000× (B).

Table 1
Alkaline phosphatase, aminopeptidase N, and sucrase-isomaltase activities in 2/4/A1 cells grown for 6 days at 33, 37, and 39°C.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>33°C</th>
<th>37°C</th>
<th>39°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>56.5 ± 2.8</td>
<td>79.8 ± 10.4</td>
<td>86.8 ± 12.3</td>
</tr>
<tr>
<td>Aminopeptidase N</td>
<td>34.3 ± 11.7</td>
<td>45.9 ± 5.7</td>
<td>54.3 ± 8.9</td>
</tr>
<tr>
<td>Sucrase-isomaltase</td>
<td>30.8 ± 12.2</td>
<td>44.5 ± 9.6</td>
<td>51.7 ± 13.4</td>
</tr>
</tbody>
</table>

*Values are expressed as U/g protein, mean ± S.D. of four determinations done in triplicate.
*Note that the results at 37°C were obtained from a subpopulation of the cells because >50% of the cells had detached from the support at the indicated time point.
*Results significantly different (p < .05) from the results obtained at 33°C.

Fig. 7. Time-dependent development of TER of 2/4/A1 (A) and Caco-2 (B) cell monolayers. The TER of 2/4/A1 monolayers increased with time and reached a plateau of 25 ± 2.9 (2 cm² after 4 days in culture. This TER was maintained for at least 8 days. The TER in Caco-2 cell monolayers grown on uncoated permeable supports (B) reached a plateau of 234 ± 14 cm² after 17 days and maintained this value for at least 40 days in culture. Measurements were performed in HBSS at 37°C. Each point represents the mean ± S.D. of four experiments.

0.40 ± 0.03 × 10⁻⁶ cm/s for the largest marker molecule, dextran (mol wt 50,000), or 38-fold. By comparison, for Caco-2 monolayers, the $P_{app}$ values ranged from 0.24 ± 0.02 × 10⁻⁶ cm/s for mannitol to 0.05 ± 0.01 × 10⁻⁶ cm/s for dextran (Fig. 8B) or 5-fold. In the Caco-2 monolayers, the $P_{app}$ values of low molecular weight marker molecules were significantly different from each other (p < .05), with the exception of the one between mannitol and fluorescein (p > .05). No significant difference in permeability of the two high molecular weight marker molecules was observed. Thus, the permeability of confluent 2/4/A1 monolayers to the hydrophilic marker molecules depends more strongly on molecular weight than that of Caco-2 monolayers. The $P_{app}$ values were, however, significantly higher than in the Caco-2 monolayers (8–62 times).

Fig. 8. Molecular weight-dependent permeability ($P_{app}$) of 2/4/A1 (A) and Caco-2 cell monolayers (B) to the hydrophilic marker molecules mannitol (mol wt 182) (M), fluorescein (mol wt 376) (F), Lucifer yellow (mol wt 450) (L), PEG (mol wt 4000) (P), and dextran (mol wt 50,000) (D) at 37°C. Each bar represents the mean ± S.D. of six experiments.

Passive Drug Permeability. The relationship between the permeability of 2/4/A1 cell monolayers to 17 structurally diverse model drugs and published absorption data in humans after oral administration was investigated. The model drugs (Table 2) were chosen to cover a wide range of absorption (FA = 0–100%) and a wide range of physicochemical properties after oral administration. Stringent inclusion criteria were used to select the model drugs. These included: 1) the availability of reliable data on the absorbed fraction in humans; 2) clear indications that the drugs were predominantly absorbed by a process that is independent of concentration; and 3) complicating factors, such as solubility problems and presystemic metabolism, which were either negligible or had already been accounted for in the determination of the absorbed fraction of the drug. The permeability data obtained in 2/4/A1 monolayers were compared with permeability data obtained in the human jejunum (Lennernäs et al., 1996) and in Caco-2 cell monolayers (Lennernäs et al., 1997; Palm et al., 1997; Stenberg et al., in preparation). The cellular permeability ($P_C$) of the 17 model drugs in 2/4/A1 monolayers were related to the fraction absorbed in humans by a sigmoidal relationship (RMSE = 12.5%, $r^2$ = 0.91) (Fig. 9). The $P_C$ values ranged from 0.47 ± 0.06 × 10⁻⁶ cm/s for PEG 4000 (FA = 0%) to 286 ± 12.3 × 10⁻⁶ cm/s for alprenolol (FA = 96%) or a factor of 608. The corresponding relationship between published human jejunal permeability coefficients ($P_{jej}$) of 9 drugs and the fraction absorbed (Lennernäs et al., 1997) was identical with that observed for the 2/4/A1 monolayers (Fig. 9).

In agreement with previous observations (Artursson and Karlsson, 1991), a strong sigmoidal relationship to the fraction absorbed in humans is also observed for drug permeabilities across Caco-2 monolayers (RMSE = 6.66%, $r^2$ = 0.97) (Fig. 9). The $P_C$ values for the completely and rapidly absorbed drugs were comparable in 2/4/A1 and Caco-2 monolayers. For example, the $P_C$ values of alprenolol were 286 ± 12.3 × 10⁻⁶ cm/s in 2/4/A1 and 242 ± 14 × 10⁻⁶ cm/s in
TABLE 2
Summary of absorption, permeability, and physicochemical properties of the investigated drugs

<table>
<thead>
<tr>
<th>Substance</th>
<th>FA (%)</th>
<th>P&lt;sub&gt;c&lt;/sub&gt; 2/4/A1</th>
<th>P&lt;sub&gt;all&lt;/sub&gt; Human</th>
<th>Molecular Weight</th>
<th>Charge</th>
<th>ClogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metoprolol</td>
<td>102 ± 5</td>
<td>191 ± 8.37</td>
<td>150 ± 10.7</td>
<td>267</td>
<td>±</td>
<td>1.20</td>
</tr>
<tr>
<td>Naproxen</td>
<td>100 n.r.</td>
<td>n.d.</td>
<td>1000 ± 44.1</td>
<td>250</td>
<td>41.4</td>
<td>2.86</td>
</tr>
<tr>
<td>Propranol</td>
<td>97 ± 7</td>
<td>254 ± 12.3</td>
<td>560 ± 9.9</td>
<td>188</td>
<td>0</td>
<td>0.41</td>
</tr>
<tr>
<td>Phenazone</td>
<td>96 n.r.</td>
<td>286 ± 6.9</td>
<td>249 ± 4.6</td>
<td>29</td>
<td>±</td>
<td>2.65</td>
</tr>
<tr>
<td>Pindolol</td>
<td>92 ± 11</td>
<td>67.4 ± 7.17</td>
<td>428 ± 1.67</td>
<td>41.3</td>
<td>±</td>
<td>4.67</td>
</tr>
<tr>
<td>Creatinine</td>
<td>70 (60–80)</td>
<td>49.2 ± 1.24</td>
<td>29 ± 3.5</td>
<td>113</td>
<td>±</td>
<td>1.77</td>
</tr>
<tr>
<td>Metolazone</td>
<td>64 ± 23</td>
<td>17.0 ± 0.60</td>
<td>366 ± 0.72</td>
<td>225</td>
<td>±</td>
<td>0.48</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>60 (25–80)</td>
<td>41.4 ± 0.72</td>
<td>30 ± 0.24</td>
<td>266</td>
<td>±</td>
<td>0.11</td>
</tr>
<tr>
<td>Sulpiride</td>
<td>54 ± 17</td>
<td>23.8 ± 1.67</td>
<td>341 ± 1.67</td>
<td>346</td>
<td>±</td>
<td>1.11</td>
</tr>
<tr>
<td>Mannitol</td>
<td>36 ± 20</td>
<td>17.1 ± 1.67</td>
<td>341 ± 1.67</td>
<td>346</td>
<td>±</td>
<td>1.11</td>
</tr>
<tr>
<td>Foscarnet</td>
<td>26 (1–89)</td>
<td>15.5 ± 2.09</td>
<td>182 ± 0.72</td>
<td>128</td>
<td>±</td>
<td>0.79</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>15 ± 5</td>
<td>15.4 ± 2.74</td>
<td>n.r.</td>
<td>398</td>
<td>±</td>
<td>3.83</td>
</tr>
<tr>
<td>Enalaprilat</td>
<td>10 n.r.</td>
<td>11.0 ± 0.29</td>
<td>10 ± 0.72</td>
<td>348</td>
<td>±</td>
<td>0.79</td>
</tr>
<tr>
<td>Olsalazine</td>
<td>2.3 ± 0.8</td>
<td>9.99 ± 1.02</td>
<td>392 ± 0.72</td>
<td>342</td>
<td>±</td>
<td>4.50</td>
</tr>
<tr>
<td>Lactulose</td>
<td>0.6 ± 0.3</td>
<td>16.4 ± 1.08</td>
<td>342 ± 0.72</td>
<td>0.50 ± 0.03</td>
<td>±</td>
<td>-5.69</td>
</tr>
<tr>
<td>Raffinose</td>
<td>0.3 (0.1–0.9)</td>
<td>6.55 ± 0.53</td>
<td>n.r.</td>
<td>504</td>
<td>±</td>
<td>-8.09</td>
</tr>
<tr>
<td>Inogatan</td>
<td>0 n.r.</td>
<td>n.d.</td>
<td>3 ± 0.06</td>
<td>439</td>
<td>±</td>
<td>-0.01</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>0 n.r.</td>
<td>0.47 ± 0.06</td>
<td>n.d.</td>
<td>4000</td>
<td>±</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* FA: Fraction absorbed after oral administration to humans, expressed as mean values ± S.D. The values were the percentage of each drug absorbed after oral administration to humans, expressed as mean values ± S.D (Palm et al., 1997). The values are the percentage of each drug absorbed after oral administration to humans, expressed as mean values ± S.D. when S.D. was available. Mean values and ranges (given within parentheses) are given for creatinine, mannitol, raffinose, and terbutaline. See Palm et al. (1997) and Lennernäs et al. (1997) for original references.

The P<sub>c</sub> values across 2/4/A1 monolayers were determined as described in Materials and Methods.

The P<sub>c</sub> values of phenazone were 254 ± 10.7 × 10<sup>-6</sup> cm/s and 267 ± 7 × 10<sup>-6</sup> cm/s in 2/4/A1 and Caco-2 monolayers, respectively. However, the P<sub>c</sub> values for the incompletely absorbed drugs were lower for Caco-2 than for 2/4/A1. The values were 40 times lower for sulfasalazine (FA = 12%) and 300 times lower for foscarnet (FA = 17%). The P<sub>c</sub> values in Caco-2 monolayers varied by a factor of approximately 5000, whereas the P<sub>c</sub> values in 2/4/A1 monolayers varied by a factor of approximately 600. The sigmoidal relationship for 2/4/A1 cells is, thus, steeper. Despite this difference, the distinction between completely and sparingly absorbed drugs was identical in the two models. Drugs with P<sub>c</sub> > 55 × 10<sup>-6</sup> cm/s in both models had a FA > 90%, whereas drugs with P<sub>c</sub> < 10 × 10<sup>-6</sup> cm/s and < 0.05 × 10<sup>-6</sup> cm/s in 2/4/A1 and Caco-2 monolayers, respectively, had a FA < 10%.

**Discussion**

The 2/4/A1 cell line is the first example of a transformed intestinal epithelial cell line that at least partly recaptures the well-organized cellular processes which control proliferation and differentiation of the intestinal epithelium (Paul et al., 1993). This makes this cell line interesting as a potential alternative to high-resistance Caco-2 cell monolayers in studies of drug transport. Previous studies have shown that 2/4/A1 cells form monolayers at 39°C with a TER similar to that found in the excised human intestine (Paul et al., 1993). This makes this cell line interesting as a potential alternative to high-resistance Caco-2 cell monolayers in studies of drug transport. Previous studies have shown that 2/4/A1 cells form monolayers at 39°C with a TER similar to that found in the excised human intestine (Paul et al., 1993). However, in our hands, the 2/4/A1 cells underwent massive cell death at 39°C, which made it impossible to establish intact cell monolayers at this temperature. At 39°C, cells showed morphological signs of apoptosis (condensed or fragmented nuclei) (Yanai and Obinata, 1994). DNA fragmentation, which is usually associated with apoptotic cell death, was also seen when the nuclear DNA was subjected to agarose electrophoresis (DNA-laddering) or in situ labeling with terminal deoxynucleotidyl transferase in the presence of fluorescein-labeled dUTP (data not shown). The cell death at 39°C could not be prevented by modifications of the culture...
medium or of the extracellular matrix. Therefore, we had to establish new conditions for the culturing of this cell line.

The cell death at the nonpermissive temperature in cell lines transacted with the temperature-sensitive mutant of the SV40 large T antigen has been attributed to binding of wild-type p53 tumor suppression protein to SV40 large T at 33°C. The tumor suppression protein is released when the antigen is inactivated at 39°C (Yanai and Obinata, 1994). However, SV40 large T is a multifunctional antigen that also binds the retinoblastoma (pRb) tumor suppression protein (Fanning and Knippers, 1992). The exact mechanism of the cell death in 2/4/A1 cells at 39°C will therefore be difficult to elucidate. Nevertheless, we speculated that at an intermediate temperature, the SV40 large T would remain sufficiently active to bind the tumor suppression proteins, and sufficiently inactive to allow the cells to enter a differentiation program. Indeed, at 37°C the 2/4/A1 cells formed differentiated monolayers with moderately enhanced brush border enzyme activity, polarized distributions of the junctional protein ZO-1, F-actin, and E-cadherin, and a TER comparable to that of the small intestine in vivo. Furthermore, cells grown at 37°C did not grow as multilayers when the temperature was shifted back to 33°C, suggesting that terminal differentiation had been induced at 37°C (data not shown). From these findings, we believed that 2/4/A1 monolayers grown at 37°C would be well suited for studies of intestinal drug permeability.

The striking temperature-dependent recruitment of E-cadherin, F-actin, and the tight junction protein ZO-1 to the junctional complex at 37°C is strong evidence that the 2/4/A1 cells are organized into polarized monolayers. Discontinuities in the ZO-1 and F-actin rings have previously been related to increased paracellular permeability of intestinal epithelial cells (e.g., Lindmark et al., 1995). The finding of such discontinuities in cell monolayers grown at 39°C agrees with the observed cell death at this temperature. The observation that ZO-1 and F-actin formed continuous perijunctional rings at 37°C therefore indicated that 2/4/A1 cells formed an intact epithelial barrier. These conclusions were further supported by observations in the electron microscope, that 2/4/A1 cells grown at 37°C formed cell monolayers of cuboidal cells connected by tight junctions. Furthermore, the intercellular spaces in 2/4/A1 cells appear to be more in vivo-like than in Caco-2 cell monolayers. The TER in 2/4/A1 cells was comparable to that reported for the small intestine in various species (Powell, 1987), which agrees with our findings.

Monolayers of 2/4/A1 cells did not show clear morphological properties of a differentiated intestinal epithelial phenotype. The cells were cuboidal in shape, rather than columnar, and the microvilli of the apical cell membrane were few and less developed than in Caco-2 cells or in vivo. This is not surprising, because 2/4/A1 cells originate from the fetal intestine isolated at 18 days of gestation, a time when the rodent intestine is not fully differentiated (Emami et al., 1990). This is in contrast to the human fetal intestine, which is fully differentiated at the corresponding time point (Quaroni and Beaulieu, 1997).

No vectorial transport of celioprol [a substrate for MDR-1 in intestinal epithelial cells (Karlsson et al., 1993)] or ciprofloxacin [a substrate for an efflux system distinct from MDR-1 in intestinal epithelial cells (Griffiths et al., 1993)] was observed in 2/4/A1 cells, which suggests that these efflux systems are either not expressed or not functional in 2/4/A1 cells (data not shown). This anomaly will be advantageous for the study of passive drug transport in vitro because the high expression of MDR-1 and other efflux systems in many epithelial cell lines, including Caco-2, complicates the study of passive drug transport (Palm et al., 1998). This results in an exaggeration of the role of efflux proteins as barriers to drug absorption in vitro as compared to in vivo (Sandström et al., 1998). However, the 2/4/A1 cells were cultivated for a relatively short time period, and it is possible that the efflux mechanisms will appear in 2/4/A1 cell monolayers after longer cultivation times.

The permeability to the hydrophilic marker molecules was more similar to that found in vivo in the 2/4/A1 than in Caco-2 monolayers. For instance, mannitol had a P<sub>c</sub> value of 15.5 ± 2.09 × 10<sup>−6</sup> cm/s in 2/4/A1 cell monolayers compared to 35 ± 17 × 10<sup>−6</sup> cm/s after perfusion of the human jejunum calculated from Laker et al. (1982); and creatinine had a P<sub>c</sub> value of 49.2 ± 1.24 × 10<sup>−6</sup> cm/s in 2/4/A1 cell monolayers compared to 29 ± 16 × 10<sup>−6</sup> cm/s after perfusion of the human jejunum (Lennerås et al., 1997). These findings are supported by preliminary calculations of the average radius of the aqueous pores by the Renkin function (Curry, 1984). Such calculations give an average pore radius of 9 Å for 2/4/A1 monolayers and 5 Å for Caco-2 cells, which are to be compared to the 8 to 13 Å recently reported for human jejunum in vivo (Fine et al., 1995). Importantly, the 2/4/A1 monolayers discriminated the permeability coefficients of these markers according to molecular weight in a way comparable to that in vivo (Artursson et al., 1993). Moreover, the permeabilities of the investigated markers differed by a factor of nearly 40 in 2/4/A1 cells, whereas they varied only by a factor of 5 in Caco-2 cells. This supports the suggestion that 2/4/A1 cells may provide a more discriminating model for the paracellular route than Caco-2 cells.

One major application of cell culture models is the screening of intestinal epithelial permeability of libraries of new drug analogs that have been generated through combinatorial chemistry and high-throughput pharmacological screening (Artursson and Borchardt, 1997). Advantages of 2/4/A1 cell monolayers in this regard would include not only the fact that their permeability is more similar to that of the small intestine, but also their shorter cultivation time (4 days on permeable supports for 2/4/A1 cells compared to 21 days for Caco-2 cells). The drug transport experiments are also shorter, especially for sparingly absorbed hydrophilic drugs (minutes in 2/4/A1 monolayers as compared to hours in Caco-2 monolayers), and the analytical procedures are easier (less sensitive methods are required because more compound is transported). The experiments with the hydrophilic marker molecules showed that the sigmoidal relationship between the permeability of the 2/4/A1 monolayers to a set of structurally diverse drugs and the absorbed fraction of the drugs after oral administration to humans was shifted toward higher permeability than that of the Caco-2 cells. However, the permeabilities of the rapidly and completely absorbed drugs were comparable in the two models, which indicates that the transcellular routes were equally accessible in the two monolayer cultures. The more shallow relationship observed for Caco-2 cells probably results from the tighter paracellular route of these cell monolayers, which
gives a larger contribution of the transcellular route to the observed permeability for the incompletely absorbed drugs. Whereas this may be an advantage in studies of passive transcellular drug transport and for the ranking of permeabilities of relatively well absorbed compounds, it makes it difficult to rank sparingly absorbed drugs that are significantly transported by the paracellular route. Importantly, the ranking of all drugs was exactly the same in the two models. Most encouragingly, the relationships between drug permeability in 2/4/A1 cells and the absorbed fraction, and the corresponding relationship for human jejunal drug permeability and absorbed fraction were superimposable. Thus, the rat origin of the 2/4/A1 cells did not significantly impair its application in predictions of human intestinal permeability.

In conclusion, we have established conditions for the cultivation of the intestinal epithelial cell line 2/4/A1 as viable and intact monolayers on matrix-coated permeable supports. The 2/4/A1 monolayers mimicked the human jejunal permeability better than Caco-2 cells and are well suited for rapid screening of intestinal drug absorption. The high permeability of the paracellular spaces in 2/4/A1 cells is similar to that found in vivo, which makes this cell line a valuable tool for studies of the paracellular barrier to drug transport.

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References


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Drug Transport in a New Intestinal Epithelial Cell Line

1221