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Constitutive Expression of P-Glycoprotein in Normal Lung Alveolar Epithelium and Functionality in Primary Alveolar Epithelial Cultures

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ABSTRACT

The multidrug resistant (MDR) transporter P-glycoprotein (P-gp) is constitutively expressed in normal tissues, where its spatial distribution defines it as an important element reducing the systemic exposure and tissue access of potentially harmful xenobiotics. We sought to determine whether P-gp is functionally expressed within alveolar epithelium of lung, in particular within the predominant cell type of this barrier, the alveolar epithelial (AE) type I cell. By immunohistochemistry, MDR-1/mdr-1 P-gp was localized to luminal membranes of AE type I epithelium within normal human and rat lung tissue. Using a primary rat cell culture model affording study of AE type II to AE type I differentiation, we observed increased expression (reverse transcription-polymerase chain reaction (RT-PCR), Western blot, and immunoflow cytometry techniques) of mdr-1a and mdr-1b P-gp in the cultures as they adopted an AE type I phenotype; freshly isolated AE type II cells were negative for mdr-1/P-gp. The functionality of P-gp within the AE cultures was demonstrated by a flow cytometric accumulation-retention assay using rhodamine-123 as substrate, and also by the polarized transport of vinblastine across confluent AE type I monolayers (basal-to-apical permeability was 3-fold that of apical-to-basal permeability), which was found to be comparable with the P-gp transport barrier presented by Caco-2 cell monolayers. The implications of localizing P-gp within alveolar epithelium is of significance to studies of fundamental respiratory cell biology as well as to further clarifying the nature of the barrier to xenobiotic transfer from alveolar airspace to pulmonary interstitium and capillary blood.

P-Glycoprotein (P-gp) is a member of the ATP-binding cassette superfamily of membrane transport proteins that mediates the vectorial movement across cell membranes of a wide range of physicochemically diverse solutes (Stouch and Gudmundsson, 2002). In humans, two P-gp-related genes have been cloned and subsequently termed MDR1 and MDR3 (for review, see Ambudkar et al., 1999). The MDR1/P-gp gene product is recognized in particular to actively efflux from a cell a diverse range of cytotoxic drugs, a characteristic that is an important facet in the multidrug resistant (MDR) cell phenotype. Current evidence suggests that the MDR3/P-gp gene product does not contribute to an MDR phenotype.

In rodents, three P-gp-related genes have been identified and designated mdr-1a, mdr-1b, and mdr-2. Sequencing of the mdr1a and mdr1b gene products has shown them to correspond to the human MDR1, and as expected both mdr-1 genes encode for distinct functional multidrug transporters (Endicott et al., 1991). Cloning of the cognate rodent mdr-2 gene has shown it to be homologous to the human MDR3 (Endicott et al., 1991).

The MDR1 and MDR3 gene products are constitutively expressed in normal tissues, however, the full scope of their biological functions in normal tissues remains to be fully determined. Among other sites the MDR3/P-gp has been localized to the biliary domain of the hepatocyte cell membrane where it functions as a phospholipid transporter, facilitating the selective translocation of phosphatidylcholine into the outer leaflet of the liver canalicular membrane (Deleuze et al., 1996). The MDR1/P-gp is present at a number of anatomical barriers where one of its main functions is to reduce the systemic exposure and specific tissue access of potentially harmful compounds (for review, see Schinkel, 1997).

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ABBREVIATIONS. P-gp, P-glycoprotein; MDR, multidrug resistant; AE, alveolar epithelial; RT-PCR, reverse transcription-polymerase chain reaction; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; MDCK, Madin-Darby canine kidney; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; FACS, fluorescence-activated cell sorting; TEER, transepithelial electrical resistance; A-B, apical to basal; B-A, basal to apical; HRP, horseradish peroxidase.

The lung alveolar septa represent the gaseous exchange region of the lung and comprise mainly the thin cellular barriers of alveolar epithelium and the less restrictive pulmonary capillary endothelium. To facilitate efficient gaseous exchange the alveolar epithelium occupies a total surface area approximately 40-fold greater than that of the epithelium lining the lung conducting airways (from trachea to terminal bronchioles). This large alveolar epithelial surface is comprised essentially of two cell types, the squamous alveolar epithelial (AE) type I cell and the cuboidal AE type II cell. The latter AE type II cell is more numerous and undertakes a range of functions, including, among others, the synthesis and secretion of pulmonary surfactant. Although less abundant, the thin (<0.3 μm in its peripheral attenuated regions) but larger squamous AE type I cell constitutes approximately 95% of the total alveolar epithelial surface area (Crapo et al., 1982). Beyond serving as a cellular conduit for gaseous exchange it is increasingly clear that the AE type I cell, in effectively being the limiting barrier to solute movement between alveolar airspace and capillary blood, possesses the capacity for a wide range of functions, including solute transporter activity, ion transport and fluid homeostasis, and macromolecule uptake (for review, see Crandall and Matthay, 2001).

Given a spatial pattern of constitutive MDR-1/P-gp expression within barriers critical to xenobiotic exposure then on teological grounds the functional expression of MDR-1/P-gp (or its species equivalent) within alveolar epithelium should be anticipated. The aim of this research is to determine whether P-gp is functionally expressed within alveolar epithelium, and in particular within the alveolar type I epithelial cell that forms the limiting barrier to solute transport. The implications for localizing P-gp within this barrier would be significant to studies of fundamental respiratory cell biology as well as to further clarification of the nature of the barrier to xenobiotic transfer from alveolar airspace to pulmonary interstitium and capillary blood. In this study, we report the microanatomical immunolocalization of MDR1/mdr1 P-gp expression to alveolar epithelium within intact normal human and rat lung tissue, with clear localization evident on the luminal membranes of AE type I epithelium. Using a well characterized in vitro approach to study AE differentiation, namely, a rat primary culture model of AE cells (Dobbs et al., 1988; Cheek et al., 1989; Danto et al., 1992; Campbell et al., 1999), we went on to show the increased expression (confirmed by RT-PCR, Western blot, and immunoflow cytometry techniques) of mdr1/P-gp in these primary cultures as the AE culture model progressed toward an AE type I phenotype; freshly isolated rat AE type II cells seemed negative for mdr1/P-gp The functionality of P-gp within the AE primary cultures was established by a flow cytometric accumulation-retention assay using rhodamine-123 as substrate, and also by the polarized transport of vinblastine across confluent AE monolayers grown on semipermeable supports.

Materials and Methods

Immunohistochemical Localization of mdr-1/MDR-1 P-Glycoprotein in Rat and Human Alveolar Epithelium within Intact Normal Lung Tissue. Paraffin wax blocks of in vivo rat lung were prepared as described previously (Newman et al., 1999). Briefly, under terminal anesthesia lung tissue was fixed by a whole-body perfusion method, whereby the pulmonary vasculature was perfused with highly purified 1% monomeric 0.1 M phosphate-buffered glutaraldehyde (pH 7.4) (TAAB Laboratories Equipment Ltd., Berks, UK) for a total of 15 min at normal physiological hydrostatic pressure. After perfusion fixation, small specimens of peripheral lung tissue (approximately 1–5 mm^3) were dissected from the whole fixed lungs in situ and immersed in the same fixative for a further 2 h. The tissue blocks were then embedded in paraffin wax using an automated tissue processor (Shanndon, Cheshire, UK).

Human lung tissue for immunohistochemistry comprised archival formalin-fixed human lung specimens, embedded in paraffin wax (obtained from the Department of Histopathology, Llandough Hospital, Cardiff, UK), and fresh human lung tissue exhibiting normal morphology that was obtained from pneumonectomy specimens. The latter involved material from three patients undergoing surgery for lung cancer with the normal tissue taken from sites as distal as possible from the tumor tissue itself. The fresh human tissue was processed as described for rat except for omission of the vasculature perfusion step.

Both rat and human lung paraffin wax sections (5–10 μm) were mounted on SuperFrost microscope slides (Shanndon). After removal of paraffin from the mounted sections, endogenous peroxidase activity within the rehydrated sections was blocked with 0.6% hydrogen peroxide in methanol for 15 min at room temperature. The slides were then briefly washed in tap water before each section was equilibrated in OptiMax wash buffer (pH 7.4; Menerium Diagnostics, Oxford, UK) at room temperature for an additional 10 min. After draining, the P-gp monoclonal antibody JSB-1 (ID Labs, Inc., Glasgow, UK) was applied to each section at a dilution of 1:10 [diluent was 0.6% bovine serum albumin (BSA) in OptiMax wash buffer] and incubated overnight (15 h) at 4°C within a humidified slide chamber. A parallel set of sections was also stained using another P-gp monoclonal antibody, C494 (ID Labs, Inc.), at dilutions of 1:10 to 1:20. The following day, the slides were washed to remove unbound primary antibody and a goat anti-mouse secondary HRP-conjugated antibody (dilution of 1:100) (Dako, High Wycombe, UK) was then applied for 1 h at room temperature. Immunoreactivity was subsequently detected using the 3,3′-diaminobenzidine system (Sigma Chemical, Poole, Dorset, UK). The sections were counterstained with hematoxylin and mounted. Images were captured using a BX 41 microscope (Olympus, Tokyo, Japan) fitted with a Color View 12 camera and equipped with AnalySIS software (Norfolk Analytical, Downham Market, UK).

Isolation and Culture of Primary Rat AE Cells. Male pathogen-free CD rats (120–180 g b.wt.) were used throughout and were bred and maintained at Cardiff University in controlled temperature and lighting under barrier conditions with access to food and water ad libitum. Animal procedures were conducted in compliance with the Animal (Scientific Procedures) Act 1986. Isolation of AE type II cells was undertaken following previously described approaches (Danto et al., 1992; Campbell et al., 1999). Briefly, lung tissue was enzymatically disaggregated using aqueous porcine elastase (2 units/ml) (Worthington Biochemicals, Freehold, NJ). Purification of AE type II cells was undertaken by density centrifugation (250*g* for 20 min) upon a discontinuous Percoll gradient (1.040 and 1.089 g/ml) (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). AE type II cells enriched in the opaque band located at the interface of the discontinuous gradient were removed and plated onto a Petri dish containing DMEM (Invitrogen, Paisley, UK) for 1 h at 37°C to aid further purification. After differential attachment the cell suspension containing the isolated AE type II cells (purity >95% AE type II cells) was either plated onto tissue culture-treated plastic or semipermeable polycarbonate membranes (Transwells, 0.4-μm pore size; Corning Costar, High Wycombe, UK) at a seeding density of 0.9×10^6 cells/cm^2. Cultures were maintained in a humidified atmosphere (5% CO_2, 95% air) with culture medium comprising DMEM supplemented with 10% fetal bovine serum, the antibiotics

penicillin G (100 units/ml) and gentamicin (50 μg/ml), and with or without the addition of dexamethasone (0.1 μM) as indicated. Culture medium was replenished every 48 h. After seeding the AE type II cells were allowed to adhere and their purity reconfirmed at 40 h by tannic acid stain to detect the presence of lamellar bodies. Beyond this time in culture, the AE type II cells, when grown on a plastic substratum, acquire (over the subsequent 3 to 6 days) a phenotype more characteristic of the AE type I cell (Dobbs et al., 1988; Cheek et al., 1989; Danto et al., 1992; Campbell et al., 1999). However, parallels between in vivo and in vitro AE cell differentiation remain to be fully defined; hence, the term AE type I-like cell is often adopted to represent the in vitro-derived AE type I phenotype.

Cell Lines. Investigations also made use of various cell lines, including the human colon adenocarcinoma cell line Caco-2 (passage 35) and the human lung type II alveolar adenocarcinoma cell line A549 (passage 98–101), both obtained from European Collection of Animal Cell Cultures (Porton Down, UK). The Madin-Darby canine kidney (MDCK) epithelial cell line and its recombinant clone containing the human MDR-1/P-gp gene (MDCK-MDR1) were both kind gifts from Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The human breast carcinoma cell line MCF-7 and the human nasopharyngeal carcinoma cell line KB3-1, both P-gp negative, and their respective P-gp-induced sublines MCF-7/ADR and KBV1 were all obtained as kind gifts from the Imperial Cancer Research Fund (London, UK). All cell lines were routinely cultured in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin G. The culture media for KBV1 and MCF-7/ADR cell lines were supplemented with vinblastine (1 μg/ml) and doxorubicin (0.1 μg/ml), respectively, to maintain the MDR phenotype. However, before experimentation these cells were taken through one round of subculture in the absence of cytotoxic agents.

RT-PCR Analysis for the Detection of mdr-1/mdr-2 P-gp mRNA in Rat Alveolar Epithelial Cells. Total RNA was harvested from freshly isolated rat AE type II cells, denoted as AE(0). Total RNA was also extracted from the isolated AE cells grown in primary culture to 60 h, AE(60); 120 h, AE(120); and 192 h, AE(192) postseeding. By the 120 and 192 h time points postseeding, the cells had adopted a characteristic AE type I-like phenotype. Total RNA was harvested using a commercial kit (Ultraspec RNA reagent; Biogenesis, Dorset, UK) following the manufacturer's protocol. For each variable, three or four separate samples from different isolations were collected. Quantification and purity assessment of the extracted RNA was carried using a GeneQuant *pro* RNA/DNA calculator (Pharmacia Biotech, Cambridge, UK). First-strand cDNA was synthesized using 500 ng of RNA that was initially reverse transcribed using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The reverse transcription reaction consisted of 10 pmol of random hexamers (pdN6; Amersham Biosciences UK, Ltd.), 10 mM dithiothreitol, 1 mM dNTPs, and 1 U/μl RNasin (20-μl total volume). Samples were heat denatured at 80°C for 4 min before the addition of reverse transcriptase, followed further by incubations at 25°C for 10 min, 42°C for 50 min to complete the reverse transcription process, and finally at 99°C for 2 min to inactivate the reverse transcriptase enzyme to terminate the whole reaction. The cDNA reaction product was either held at 4°C for immediate use or stored at −80°C until required.

Aliquots of cDNA equivalent to 500 ng of RNA were subjected to PCR for detection of mdr-1a, mdr-1b, and mdr-2 genes as well as the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Previously published primers were used for these analyses with the primer sequences and expected product sizes are shown in Table 1. All primers were synthesized within the University of Wales College of Medicine (Cardiff, UK) on a Beckman DNA synthesizer with high-performance liquid chromatography high-purity isolation. Separate aliquots of cDNA in a final volume of 50 μl using 2 U of *BioTaq* DNA polymerase and 0.3 μM of each primer was used in each PCR reaction for the detection of mdr-1a, mdr-1b, and mdr-2. The PCR reactions were conducted using an MJC Research PTC-200 thermal cycler (Genetic Research Instrumentation Ltd., Braintree, UK) and consisted of an initial denaturation process at 94°C for 2 min, followed by a total of 28 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s (Fardel et al., 1996). The housekeeping gene GAPDH was amplified separately, however, the same reverse transcription product was used. Thermal cycling parameters adopted for GAPDH were 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s (28 cycles) (Vos et al., 1998). Negative control samples for all primers consisted of omitting the RNA from the reverse transcription reaction and/or the cDNA product from the final PCR.

A sample (10 μl) from each PCR product was subjected to electrophoresis on a 2% agarose gel (0.5× Tris borate-EDTA buffer) and the resolved cDNA stained with ethidium bromide (final concentration 0.5 μg/ml). The gel was then destained and the fluorescent bands imaged under UV light with semiquantitative analysis performed using commercial software (Molecular Analyst; Bio-Rad, Hemel Hempstead, UK). Data obtained were normalized with respect to GAPDH densitometric values. The products were examined with respect to a 1-kilobase DNA ladder (New England Biolabs, Herts, UK).

P-gp Immunoblot Analysis in Rat Alveolar Epithelial Cells. Confluent cell monolayers of AE(192), A549, KBV-1, KB3-1, and MDCK-MDR1 were harvested in lysis buffer containing 50 mM Tris (pH 7.5), 1% Triton, 5 mM EGTA, 150 mM NaCl, and protease/phosphatase inhibitors and incubated on ice for 20 min. After this, the crude cell lysates were centrifuged in a microcentrifuge at 10,000*g* for 15 min and the cell supernatants subjected to immunoblot analysis. Total protein (equivalent to 30 μg for each sample) was loaded and resolved by 7.5% SDS-PAGE and then electroblotted to nitrocellulose (0.2-μm pore size) membrane (Schleicher & Schuell, Dassel, Germany). Rainbow prestained molecular weight markers (Amersham Biosciences UK, Ltd.) were concurrently run. For signal generation the membrane was incubated at room temperature first for 2 h with 5% nonfat-dried milk in Tris-buffered saline with 0.1% Tween 20 (TBST, 0.1 M, pH 7.4) and then for 16 h at 4°C with the monoclonal P-gp C219 antibody (ID Labs, Inc.) diluted 1:100 in TBST. After this, the membrane was washed three times in TBST and further incubated for 1 h at room temperature with HRP-conjugated anti-mouse IgG rabbit antibody (Dako, Cambridge, UK) di-

TABLE 1
RT-PCR conditions and primers used

GENE	Reference	Primer Sequence (5′ to 3′)	Expected Product size
			bp
mdr-1a	Fardel et al. (1996)	GATGGAATTGATAATGTGGAC (FP) TGCTGTTCTGCCGCTGGA (RP)	321
mdr-1b	Fardel et al. (1996)	AGTGACACTGGTGCCTCTGA (FP) CAAACACTGGTTGTATGCAC (RP)	264
mdr-2	Fardel et al. (1996)	AAGGCTGCTGGAGCCGTGGC (FP) CAGGATGATGGAGAATGCCG (RP)	254
GAPDH	Vos et al. (1998)	CCATCACCATCTTCCAGGAG (FP) CCTGCTTCACCACCTTCTTG (RP)	576

FP, forward primer; RP, reverse primer; bp, base pair.

luted 1:6000 in TBST. The membrane was finally washed six times with TBST and the chemiluminescence signal generated (Super Signal Ultra; Pierce, Chester, UK) and recorded onto Hyperfilm ECL (Amersham Biosciences UK, Ltd.). Image acquisition and band quantitation were undertaken on GS-700 densitometer with Molecular Analyst software (Bio-Rad).

Flow Cytometric Immunofluorescence Assay for P-gp Expression in Rat Alveolar Epithelial Cells. Freshly isolated AE(0) cells and confluent monolayers of AE(192) cells and of select continuous cell lines were harvested by trypsin/EDTA disaggregation and washed with phosphate-buffered saline (PBS, 0.1 M, pH 7.4) containing 1% BSA (Sigma-Aldrich, Poole, Dorset, UK).

Approximately, 5×10^5 cells derived from the AE(0), AE(192), and MDCK cultures were permeabilized and fixed using a Fix and Perm kit (Caltag Laboratories, Burlingame, CA) according to the manufacturer's instructions, allowing access of the C219 Pgp antibody to its internal epitope. After the initial fixation, the cells were incubated with the C219 (3.3 μg/ml) antibody for 30 min at room temperature in the presence of permeabilizing reagent. In the case of the other control cell lines expressing human MDR-1/P-gp, a similar number of cells (5×10^5) was incubated with the anti-human monoclonal P-gp antibody MRK-16 (TCS Biologicals Ltd., Oxford, UK) (4 μg/ml) at 4°C for 30 min in the dark. After the primary antibody incubation the cells were briefly washed with PBS and pelleted in a microcentrifuge at 1000*g* for 5 min. Resultant cell pellets were resuspended in PBS/BSA (0.6%) solution containing a 1:25 (for C219 primary) or a 1:50 (for MRK16 primary) dilution of anti-mouse fluorescein isothiocyanate-conjugated $F(ab')_2$ secondary reporter (Dako). Controls consisted of cells incubated in the presence of an inappropriate mouse isotypic IgG antibody subtype (Dako) used at an equivalent protein concentration.

Cell associated immunofluorescence distributions were obtained from 10,000 events per cell sample through a bandpass filter FL1 using a FACScalibar flow cytometer (BD Biosciences, Oxford, UK). The fluorescence of gated cell populations was analyzed using validated analysis software, WinMDI. Each cell culture was probed using P-gp and isotypic control antibodies a total of five times, with statistical analysis performed on the raw (median fluorescence intensity) data.

Retention and Accumulation of Rhodamine-123 in Rat Alveolar Epithelial Cells. To examine the functional expression of P-gp, the intracellular accumulation and efflux of the highly selective fluorescent P-gp substrate, rhodamine-123, were studied using a previously described method (Lee et al., 1994). In this particular assay, the inhibition of P-gp-mediated cell efflux leads to the increased cellular accumulation and retention of rhodamine-123. Briefly, confluent monolayers of AE(192) cells and of select continuous cell lines (harvested at 120–192 h postseeding) were rinsed with Ca^{2+}- and Mg^{2+}-free PBS, and the cells harvested by treatment with 0.05% trypsin and 0.02% EDTA for 2 min at 37°C. Cells were then suspended (5×10^5 cells/ml) in DMEM (minus serum) containing rhodamine-123 (0.2 μg/ml) in the presence or absence of the P-gp competitive inhibitor verapamil (40 M). Cells were then incubated in the dark for 1 h at 37°C. After this, the cells were pelleted (100*g* centrifugation for 5 min), washed in PBS, and 50% of the cells then aliquoted for flow cytometric analysis representative of the accumulation phase of rhodamine-123. For the examination of rhodamine-123 efflux the remaining cells were resuspended in fresh DMEM (minus serum) either with or without verapamil (40 μM) but devoid of rhodamine-123. The cell efflux of the accumulated rhodamine-123 was then conducted over 2 h at 37°C, after which the cells were pelleted, washed in ice-cold PBS, and analyzed by FACScan flow cytometry. The fluorescence of rhodamine-123 was collected in a FL1 band pass filter. Each treatment was represented by at least six replicates, minimum of 4000 events was collected for each sample and each experiment repeated at least twice. Sample analysis gated the cell population to exclude cell debris.

Polarized Transepithelial Transport of Vinblastine across Monolayer Cultures of Rat Alveolar Epithelial Cells. Freshly isolated AE type II cells were seeded upon Transwell polycarbonate inserts (0.9×10^6 cells/cm^2) and cultured to 192 h postseeding AE(192) in the presence of dexamethasone. The MDR-1/P-gp-positive cells, Caco-2, and MDCK-MDR1 were also seeded onto Transwell inserts (both at a seeding density of 0.04×10^6/cm^2) and cultured to days 21 and 4 postseeding, respectively. The formation of restrictive monolayers was monitored for all cell types by microscopical examination and measurement of transepithelial electrical resistance (TEER) using an EVOM epithelial voltammeter (WPI, Sarasota, FL).

The polarized transport of the radiolabeled P-gp substrate [^{3}H]vinblastine sulfate (Amersham Biosciences UK, Ltd.), in the apical-to-basal (A-B) and basal-to-apical (B-A) directions was examined over a period of 90 min across the confluent cell monolayers incubated in DMEM (without serum) and subject to stirring on an orbital shaker (100 rpm). At 30 min before the transport experiment the culture media were replaced with fresh DMEM, and at the end of this preincubation period the TEER was measured and the Transwell inserts were distributed evenly between treatment groups on the basis of the TEER measurements. Transport experiments in the A-B or B-A directions were initiated by adding, respectively, either 250 μl (to the apical chamber) or 1 ml (to the basal chamber) of [^{3}H]vinblastine (radioactive concentration 0.0157 MBq/ml or vinblastine concentration 53.2 nM) dissolved in DMEM. For each of the above-mentioned groups, parallel treatment groups were run in the presence of the P-gp inhibitors 40 μM verapamil or 10 μM cyclosporin, where the inhibitor was present in both apical and basal chambers throughout the experiment, and applied to the cells for an equilibration period of 30 min before the start of the transport study. At predetermined times over the course of the 90-min transport study, 100-μl samples were taken from the respective receiver chamber and replenished with fresh DMEM. Each treatment group comprised $n = 4$ replicates, with each study repeated at least twice.

The cumulative transport of [^{3}H]vinblastine as a function of time in A-B and B-A directions was determined and the apparent permeability coefficients (ρ) ($\times 10^{-6}$ cm/s) calculated according to the equation $dM/dt = \rho \times A \times Co$, where dM/dt is the rate of change in cumulative mass of [^{3}H]vinblastine transferred to the receiver chamber, A represents the surface area of Transwell membrane, and Co represents the initial concentration of radiolabeled substance in the donor chamber assumed to remain essentially constant (i.e., <5% loss) throughout the experiment.

Statistics. Results are presented as mean ± S.D unless otherwise stated. Statistical analysis was undertaken using either nonpaired Student's *t* test or analysis of variance with post hoc analysis by Duncan's multiple range test. Details are provided in figure legends. Statistical significance was at $P < 0.05$ unless otherwise stated.

Results

Immunohistochemical Localization of mdr-1/MDR-1 P-gp within Alveolar Epithelium of Normal Intact Lung Tissue. Paraffin wax sections (5–10 μm) of rat and human normal lung tissue were prepared and immunostained for P-gp using JSB-1 and C494 antibodies, both of which react specifically with the MDR-1 protein and not the MDR-3 protein but also have been shown previously to react with both rat and human species (Jette et al., 1993). Immunocytochemistry with C219 failed to provide any reproducible staining pattern even within positive control tissue.

Figure 1, a–c, show immunohistochemical staining for P-gp within rat lung tissue using the JSB-1 antibody, although an identical pattern of staining was obtained with the C494 antibody. In Fig. 1a, the marked staining can be seen for P-gp across the entire alveolar epithelial surface mem-

brane with staining clearly present at the apical (airspace) surface of the alveolar type I epithelium. Figure 1c is a higher magnification of the boxed region in Fig. 1a and highlights the apical airspace staining within alveolar type I epithelium. There was some evidence of weaker staining for P-gp associated with the alveolar type II epithelium, although this is somewhat equivocal given the considerably reduced proportion of total alveolar epithelial area occupied by this cell. Within certain fields of view smooth muscle tissue was also observed to stain weakly for P-gp, whereas large arterioles, lymph glands, mesothelium, and other supporting tissues were devoid of stain (data not shown). Figure 1b shows bronchial epithelium labeling positive for P-gp and serves as a positive control given the recognized expression of P-gp at this site (Lechapt-Zalcman et al., 1997). Negative control sections were run in parallel consisting of antibody omission and replacement of primary antibody with an isotypic IgG antibody; no staining was observed in any of these control sections.

The immunostaining of human lung tissue showed considerable parallels to that of the rat, with Fig. 1, d–f, showing staining for P-gp within human lung tissue using the JSB-1 antibody. An identical pattern of staining was obtained when the C494 antibody was used instead of JSB-1. Serving as a positive control specific staining for P-gp was observed in the ciliated cells of the lower bronchial epithelium (Fig. 1e). Additionally, the squamous alveolar type I epithelium clearly showed P-gp stain across its apical membrane surfaces (Fig. 1, d and f). Smooth muscle tissue was weakly stained for P-gp, whereas large arterioles, lymph glands, and mesothelium were devoid of stain (data not shown). Appropriate negative control human sections showed an absence of any staining.

mdr-1/P-gp mRNA in Primary Rat Alveolar Epithelial Cell Cultures. Total RNA was isolated from primary cultures of rat alveolar epithelial cells and analyzed by RT-PCR using previously published mdr-1a and mdr-1b and mdr-2 rat gene-specific primers (Table 1). The alveolar cultures were grown from seeding in either the presence or absence of dexamethasone (0.1 μM); the extensive literature base reporting the use of this primary cell system to study alveolar epithelial cell differentiation or alveolar epithelial solute transport varies in either the inclusion or exclusion of the above-mentioned glucocorticoid.

Figure 2 shows a representative agarose gel of the amplification of the mdr1a and mdr1b RNA gene products from the primary rat alveolar epithelial cell cultures. Also shown in Fig. 2 is the amplified transcript for the control "housekeeper" gene GAPDH, which indicates consistency between treatment samples in the level of RT-PCR amplification and allows semiquantitative interpretation of the data. Densitometric quantitation of respective samples from three to four different isolations are also shown. Transcript for mdr2 was not detected at any time throughout the cultures or indeed in the freshly isolated AE type II cells (data not shown). The mdr-1a and mdr-1b products were not detected in freshly isolated rat AE type II cells (lanes 1 and 2) and also were absent in the 60-h cultures grown in the absence of

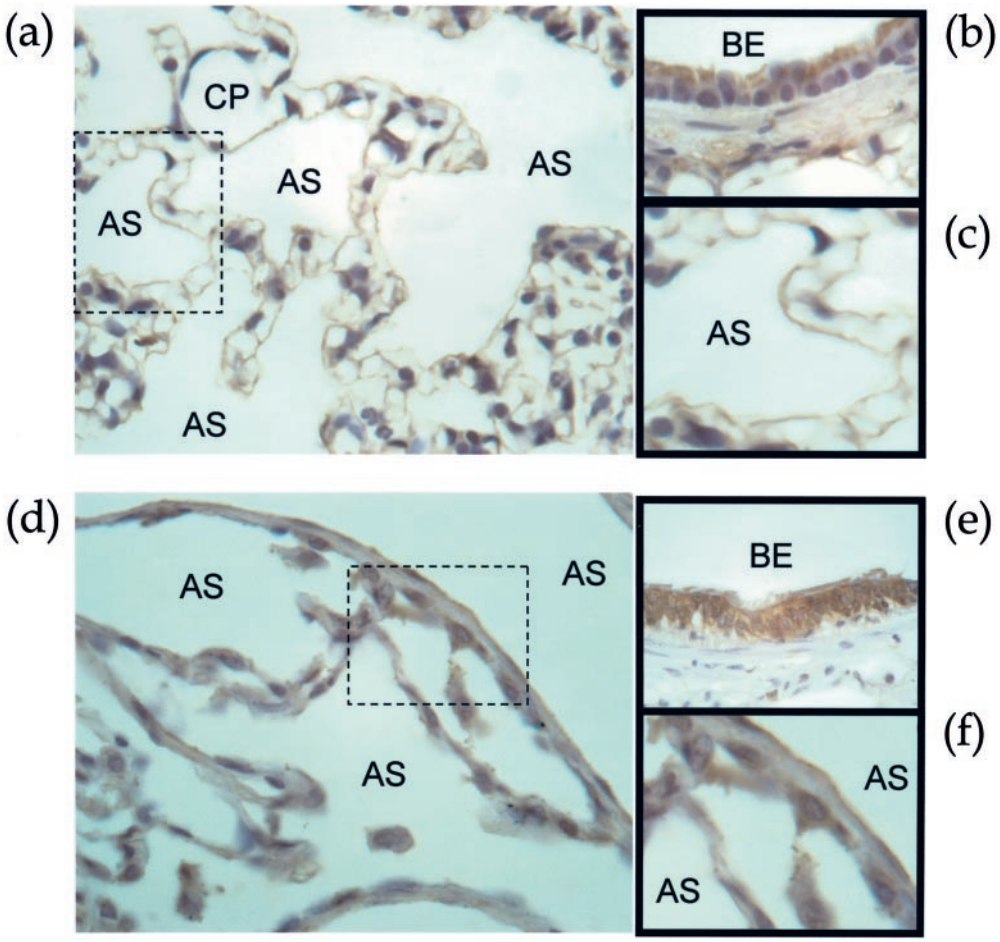


Fig. 1. Immunohistochemical staining for P-gp (JSB-1 antibody) in normal rat (a–c) and human (d and e) lung tissue. Paraffin wax sections (5 μm in thickness) were prepared and labeled with JSB-1 antibody, as described under *Materials and Methods*. In rat tissue, immunostaining for P-gp was observed across what appeared as the entire alveolar epithelial surface membrane, with staining clearly present at the apical surface of the type I alveolar epithelial cell (a, magnification, 67×; c, higher magnification, 100× image of the boxed region in a). b, serves as a positive control showing labeling for P-gp within bronchial epithelium (BE) (magnification, 67×). Similarly, in human tissue the squamous alveolar type I epithelium clearly showed P-gp immunostaining across its apical membrane surfaces (d, magnification, 67×; g and f, a higher magnification (100×) image of the boxed region in d). Specific staining for P-gp was observed in the ciliated cells of the lower bronchial epithelium (e). Negative control sections were run in parallel consisting of antibody omission and replacement of primary antibody with an isotypic IgG2a antibody; no staining was observed in any of these control sections. CP, capillary; AS, alveolar airspace.

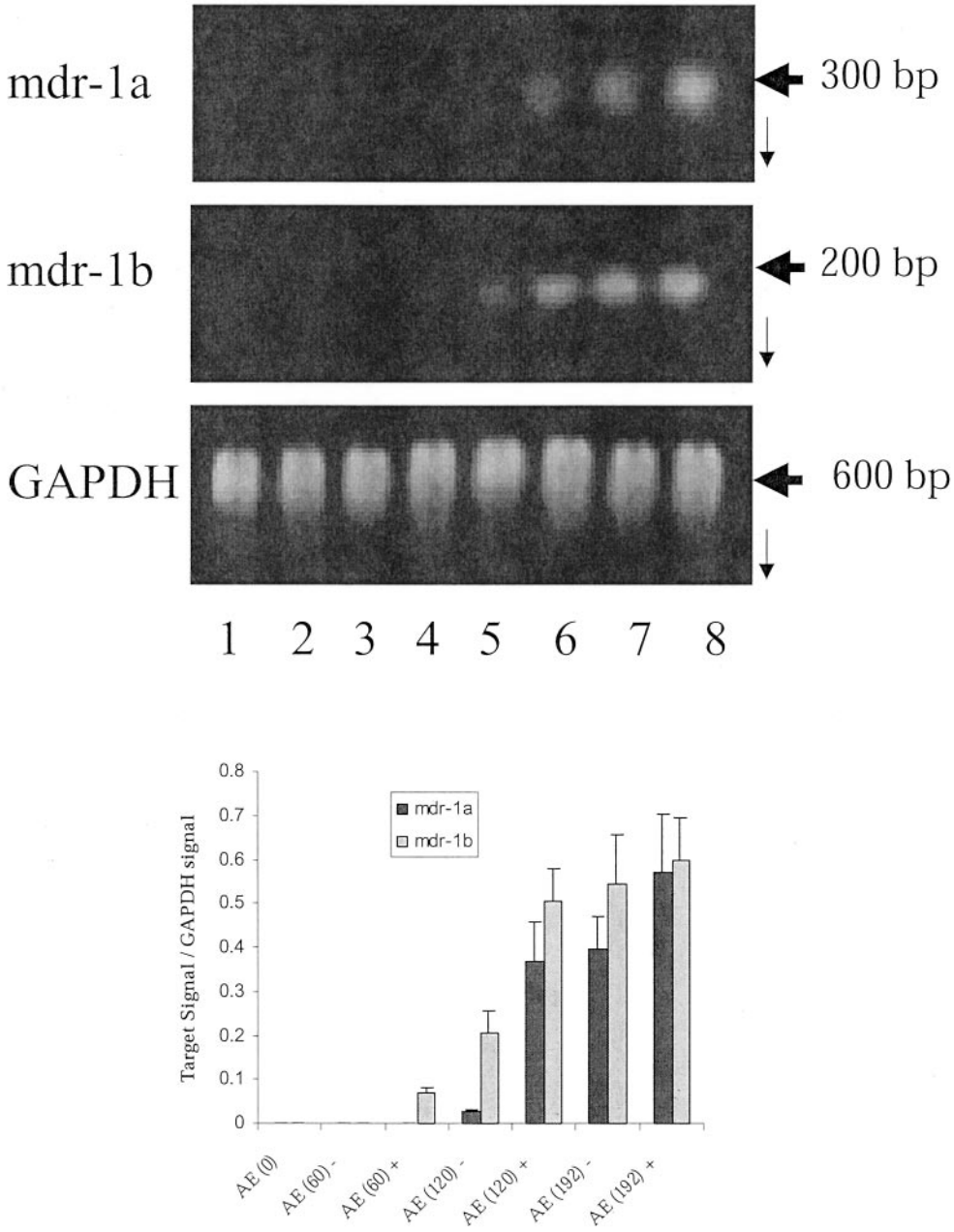


Fig. 2. Agarose gel (loading of cDNA equivalent to 100 ng of RNA) of the RT-PCR analysis of total RNA obtained from primary rat AE cell culture using oligonucleotide primers shown in Table 1 for rat mdr1a and mdr1b and the control housekeeper gene GAPDH. Lanes 1 and 2, freshly isolated rat AE type II cells [AE(0)]. Lanes 3 and 4, AE(60) grown in the absence or presence of glucocorticoid, respectively. Lanes 5 and 6, AE(120) grown in the absence or presence of glucocorticoid, respectively. Lanes 7 and 8, AE(192) grown in the absence or presence of glucocorticoid, respectively. GAPDH levels are consistent throughout the samples. Transcript for mdr-2 was not detected in any of the AE samples. The vertical arrow shows direction of electrophoresis. Histogram shows band quantitation, where for each variable three or four separate samples from different isolations were collected. Statistical analyses by analysis of variance and Duncan's multiple range test, where + represents a statistical difference ($P < 0.05$) from all other mdr-1a signals, and where * represents a statistical difference ($P < 0.05$) from all other mdr-1b signals.

glucocorticoid (lane 3). A very weak signal for mdr-1b was observed at 60 h in the AE cells treated with dexamethasone (lane 4). This is consistent with the data of Lehmann et al. (2001) whose work noted the expression of mdr-1b in 2- to 3-day cultures of primary rat AE epithelial cells that were grown in the presence of glucocorticoid; no details were supplied in the report of Lehmann et al. (2001) about mdr-1a or mdr-1b transcript levels at later time points in culture. In our current work, the mdr-1 transcript levels increased as the cultures progressed toward an AE type-I-"like" phenotype, with signals evident for both mdr-1a and -1b within the 120-h cultures treated with dexamethasone (lane 6). At this time the dexamethasone treatment clearly had an effect upon the level of mdr-1 transcription product because cells grown in the absence of dexamethasone lacked a signal for mdr-1a and the signal for mdr-1b was considerably weaker than that obtained for the equivalent dexamethasone-exposed sample. By 192 h (lanes 7 and 8) the level of mdr-1 transcripts had increased further (compare lane 7 with 5 and lane 8 with 6), with relatively strong signals evident in the AE(192) cells for both mdr-1a and mdr-1b, and little difference observed (with respect to mdr-1b in particular) in signal intensity between dexamethasone-treated or untreated cultures. Collectively, these data indicate that mdr-1a and mdr-1b transcripts are absent from freshly isolated AE type II cells but transcript is increasingly seen at higher levels because the rat primary AE cultures adopt a more AE type I phenotype. Exposure to glucocorticoid may accelerate the earlier appearance of the mdr-1 gene products but by 192 h postseeding there emerged little difference in mdr-1 transcript level between untreated and dexamethasone-treated cells.

P-gp Protein Expression by Western Blot in Primary Rat Alveolar Epithelial Cell Cultures. Immunoblot analysis (Fig. 3) with the antibody C219 was used to confirm Pgp

protein expression in the late AE cultures grown to 192 h postseeding in the absence (lane 1) or presence (lane 2) of 0.1 μM dexamethasone; little morphological difference was observed between cells grown in the presence or absence of the glucocorticoid.

In the AE(192 h) cells, a single band was detected at 170 to 190 kDa (Fig. 3), the appropriate molecular mass range for P-gp. The level of Pgp protein expression seemed independent of treatment with dexamethasone, an observation consistent with RT-PCR analysis of the 192-h cultures. As expected, strong signals for P-gp were detected in the positive control cells comprising the recombinant MDCK-MDR1 cell line (lane 6) and the KBV1 cells, the latter induced to express P-gp by exposure to vinblastine (lane 5); no P-gp was detected in the recognized P-gp-negative parental KB3-1 cells (lane 3). A very weak signal at approximately 180 kDa can be seen in the lane for the A549 lung adenocarcinoma cell line (lane 4), which seemed reflective of signal "bleed" from the adjacent intense band obtained for KBV-1 (lane 5).

P-gp Protein Expression by Immunoflow Cytometry in Primary Rat Alveolar Epithelial Cell Cultures. Further verification of P-gp protein expression in the late (192-h) primary cultures of AE cells was provided by immunoflow cytometry. Figure 4 shows some examples of the fluorescent distributions obtained by immunoflow cytometry for P-gp protein expression in the primary rat alveolar epithelial cells together with other select cell lines. The tabulated data in Fig. 4 show for each cell culture the ratio of the pooled median fluorescence intensities obtained with P-gp antibody compared with that obtained with an isotypic control antibody; ratios >1.0 indicate increasing evidence of P-gp protein expression.

The freshly isolated AE cells [AE(0)] were found to be negative for P-gp expression by this immunoflow cytometry assay, which is consistent with the RT-PCR data. The AE cells at 192 h [AE(192)] in the presence or absence of dexamethasone were positive for P-gp in agreement with both RT-PCR and Western blot data, although the difference between the P-gp and isotypic control fluorescent distributions was not as marked as for the high P-gp expressing KBV1, MCF7/ADR, and MDCK-MDR1 cells or, indeed, compared with the Caco-2 and the wild-type MDCK cells.

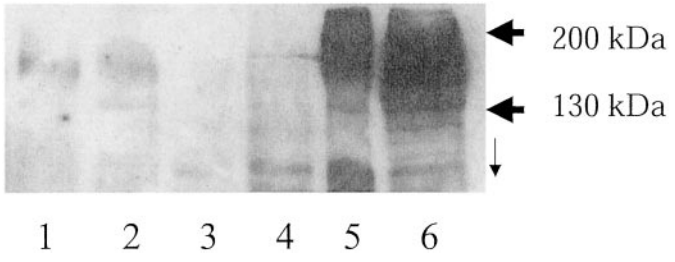
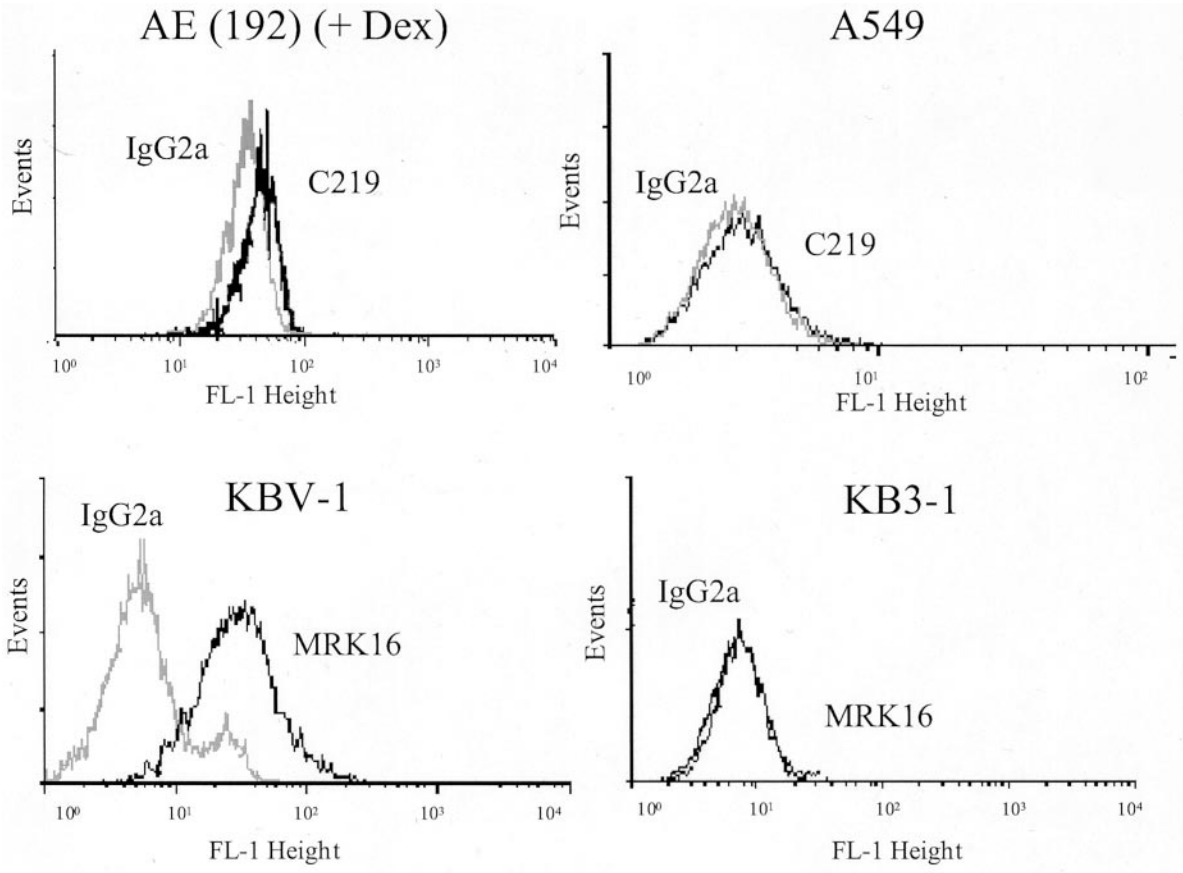


Fig. 3. Western blot showing expression of P-gp in cultured AE(192) cells and control cell lines. Expression of P-gp in the AE(192) cells is evident by a single band at 170 to 190 kDa, the appropriate molecular mass range for P-gp in both AE(192) cells cultured with glucocorticoid (lane 1) or without glucocorticoid (lane 2). Lanes 3 to 6, select cell lines: KB3-1 cells (lane 3); A549 cells (lane 4); KBV-1 cells (lane 5), and MDCK-MDR (lane 6). Each lane was loaded with 25 μg, separated by SDS-polyacrylamide gel electrophoresis (7.5%), blotted onto nitrocellulose membrane, and P-gp detected by C219 monoclonal antibody. Signal bleed is evident in lane 4 arising from the adjacent lane 5. The vertical arrow shows direction of electrophoresis.

Functional Expression of P-gp in Primary Rat Alveolar Epithelial Cell Cultures: Retention and Accumulation Assay of Rhodamine-123. A fluorescent based flow cytometry assay using the P-gp substrate rhodamine-123 was used as one of the techniques to confirm P-gp functionality in the AE cells.

Figure 5, a (low accumulation) and b (high accumulation), shows histograms of rhodamine-123 cellular accumulation (over 1 h) in the AE cells and other select cell lines. Figure 5, c and d, shows corresponding histograms for the retention of rhodamine-123 in the cells at the end of the efflux phase (2-h duration). The results for treatments coincubated with verapamil (40 μM) are expressed as a percentage of the untreated control value. During the accumulation phase, the coincubation with verapamil leads to inhibition in the activity of P-gp and to greater intracellular accumulation of the fluorescent P-gp substrate rhodamine-123. Figure 5, a and b, shows the effects of verapamil (40 μM) coincubation upon the extent of rhodamine-123 accumulation relative to the untreated control cells. Statistically significant ($P < 0.05$) increases in accumulation were observed for the MDCK wild-type cells (MDCK-WT) (a 90% increase over control), the MCF7/ADR cells (238% increase over control), and MDCK-MDR1 cells (an 880% increase over control); all of these cells display high levels of P-gp expression. Although the KBV1 cells also have relatively high P-gp expression, they did not display a statistically significant ($P > 0.05$) increase in accumulation with verapamil treatment, nor did other cells expressing P-gp such as Caco-2 and the AE (192-h) cells. However, the accumulation phase in such studies is recognized to be less sensitive to detecting P-gp functionality compared with the efflux or retention phase of the assay.

The retention or efflux phase of the assay immediately followed the accumulation of rhodamine-123 and was undertaken by incubating the cells in rhodamine-123-free media for 2 h in the presence or absence of verapamil. Here, P-gp functionality is displayed by verapamil inhibition of P-gp, leading to increased retention of rhodamine-123 within the cells at the end of the efflux phase. Figure 5, c and d, shows the effects of verapamil (40 μM) coincubation upon the extent of rhodamine-123 retention at the end of efflux. In this phase, all the highly expressing P-gp cells demonstrated significant ($P < 0.05$) increased retention over control cells. For MDCK-MDR1, MCF7/ADR, MDCK-WT, and KBV1 cell lines the increase in retention over control was 3500, 2444, 900, and 800%, respectively. For Caco-2 cells the increase was 46% over control ($P < 0.05$) and for the AE cells (+ dexamethasone) the increase ($P < 0.05$) was 59%. The cell lines determined to be P-gp negative in this current work and in previous reports (A549; Courage et al., 1997; Trussardi et al., 1998) (MCF-7 and KB3-1; Abulrob and Gumbleton, 1999) showed no significant ($P > 0.05$) increase in rhodamine-123 retention with verapamil treatment compared with control. It must be noted, however, that the absence of P-gp expression in A549 cells is equivocal with some other investigators observing low-to-moderate MDR-1/P-gp expression within A549 cells (Yang et al., 1998; Hamilton et al., 2001) and even expression localized solely to intracellular sites (Meschini et al., 2002). Such divergent reports likely reflect intralaboratory phenotypic shift from the original cell bank deposit. Nevertheless, collectively the retention and accumulation assay of rhodamine-123 clearly demonstrates that the P-gp



Cell	Ratio MFI (P-gp AB : Iso AB)	Statistical Significance
AE(0)	1.03	P > 0.05
AE (192) + Dex	1.73	P < 0.05
AE (192) -Dex	1.71	P < 0.05
KBV1	6.32	P < 0.01
KB3-1	1.00	P > 0.05
MCF7	1.05	P > 0.05
MCF7/ADR	5.88	P < 0.01
CACO-2	3.80	P < 0.01
A549	1.10	P > 0.05
MDCK	5.11	P < 0.01
MDCK-MDR1	9.21	P < 0.01

Fig. 4. Expression of P-glycoprotein by immunoflow cytometry. Figure shows some example fluorescence distributions for AE(192) cells grown in the presence of dexamethasone (+dex), and some other select cell lines. Each fluorescence distribution was obtained from collecting 10,000 events per sample (n = 4 samples for each cell) using a FACScan flow cytometer, and for each cell line compares the P-gp antibody (either C219 or MRK16) to that of an isotypic control antibody. The tabulated data in Fig. 4 shows for each cell culture the ratio of the pooled median fluorescence intensities obtained with P-gp antibody compared with that obtained with an isotypic control antibody; ratios >1.0 indicate increasing evidence of P-gp protein expression. Statistical analysis by nonpaired Student's t test.

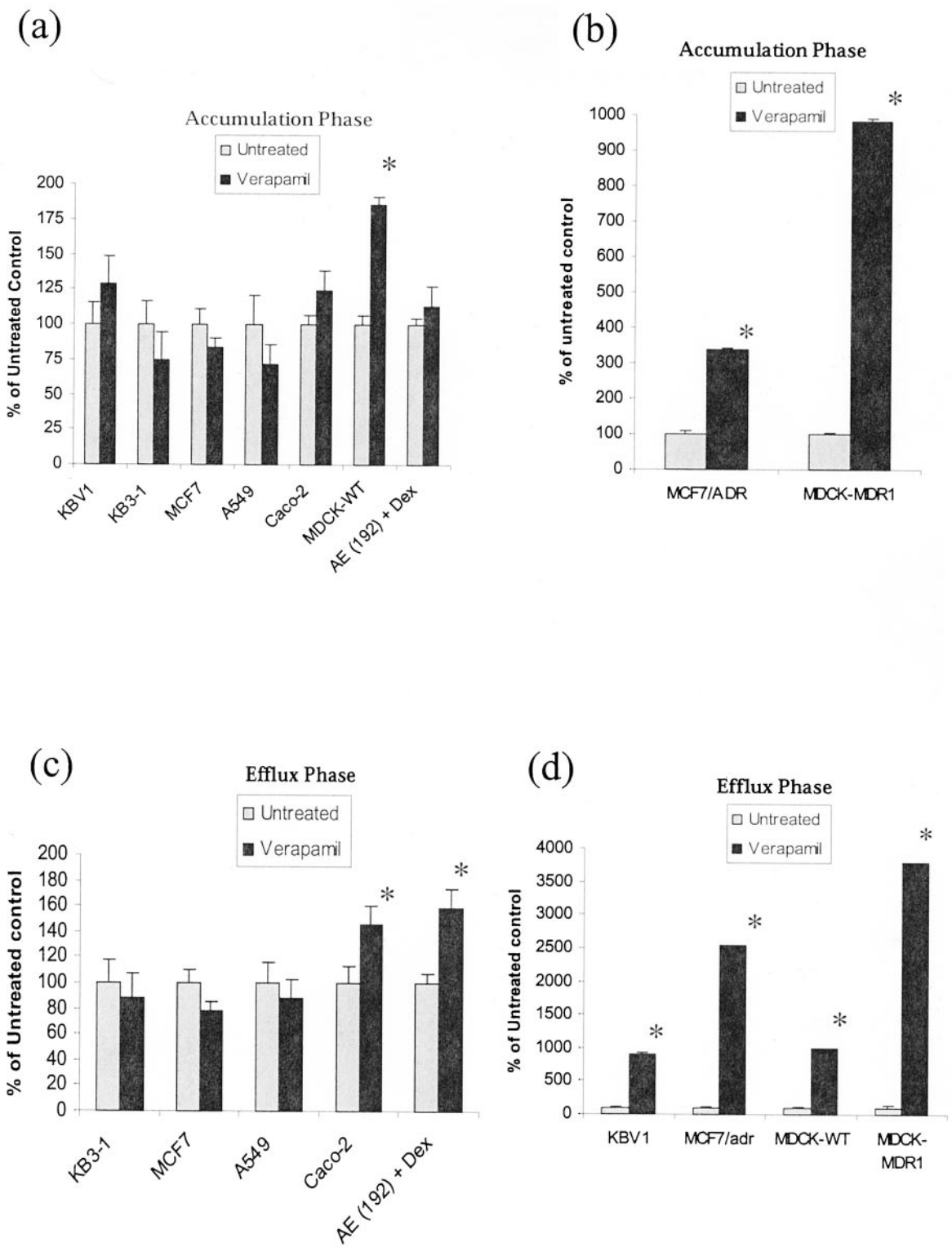


Fig. 5. Intracellular accumulation and retention of rhodamine-123. a and b, accumulation. Cells were incubated for 60 min at 37°C in media containing rhodamine-123 in the absence (untreated control) or presence of P-glycoprotein inhibitor verapamil at 40 μM. c and d, retention. Cells were then washed and rhodamine-123 efflux carried out over 120 min at 37°C in media not containing rhodamine-123 and in the absence (untreated control) or presence of 40 μM verapamil. Data are mean ± S.D. ($n = 6$) and presented as a percentage of untreated control cells. Statistical analysis by nonpaired Student's t test. *, indicates statistical difference ($P < 0.05$) compared with the respective untreated control.

expressed in primary rat AE type I-like cells at 192 h post-seeding is functional as an efflux pump with an activity (assessed by a rhodamine-123 accumulation/efflux assay) comparable with that of a population of Caco-2 cells.

Functional Expression of P-gp in Primary Rat Alveolar Epithelial Cell Cultures: Polarized Transport of Vinblastine. To provide a more ready assessment of the potential transepithelial transport barrier provided by P-gp

within the cultured AE cells we examined the polarized (A-B and B-A) cell monolayer permeability to vinblastine in the presence of P-gp inhibitors verapamil or cyclosporin. For comparative purposes, in addition to monolayers of AE(192) cells we examined the polarized permeability of MDCK-MDR1 and Caco-2 cell monolayers to vinblastine.

Figure 6, a–c, shows the histograms for the determined permeabilities of the respective cell monolayers. The permeability of the AE(192) monolayers (Fig. 6a) to vinblastine was significantly ($P < 0.05$) greater in the B-A (5.71×10^{-6} cm/s) compared with the A-B (1.77×10^{-6} cm/s) direction, with a polarized effect equating to an approximate permeability ratio (B-A/A-B) of 3.2, and one that was abolished by undertaking the transport study in the presence of either verapamil or cyclosporin. The TEER value for the AE monolayers at 192 h postseeding grown in the presence of dexamethasone was $1455 \pm 228\ \Omega \cdot cm^2$. The permeability ($\times 10^{-6}$ cm/s) of the Caco-2 monolayers (Fig. 6b) to vinblastine, although greater in both directions (i.e., A-B = 16 0.28 and B-A = 5.40) compared with the respective values determined for the AE(192) monolayers, also showed a directional effect equating to an approximate permeability ratio (B-A/A-B) of 3.0, and one which was also reduced considerably (verapamil) or abolished (cyclosporin) by the presence of a P-gp inhibitor. The TEER value for the Caco-2 monolayers at 21 days postseeding was $680 \pm 90\ \Omega \cdot cm^2$. The recombinant P-gp expressing cell line MDCK-MDR1 displayed a marked directional effect with regard to the permeability of vinblastine (Fig. 6c) with an approximate permeability ratio (B-A/A-B) of 18.5. The TEER value for the MDCK-MDR-1 monolayers at 4 days postseeding was $200 \pm 38\ \Omega \cdot cm^2$. The magnitude of the directional effect we observed for vinblastine in both Caco-2 and MDCK-MDR monolayers is in agreement with work from other laboratories (Lentz et al., 2000). The polarized transport data for the alveolar epithelial monolayers clearly shows a significant P-gp-mediated activity opposing the apical (~airspace)-to-basal (~pulmonary interstitium) transport of vinblastine in the AE type I-like cell model, an effect comparable with that seen for the Caco-2 cell monolayer.

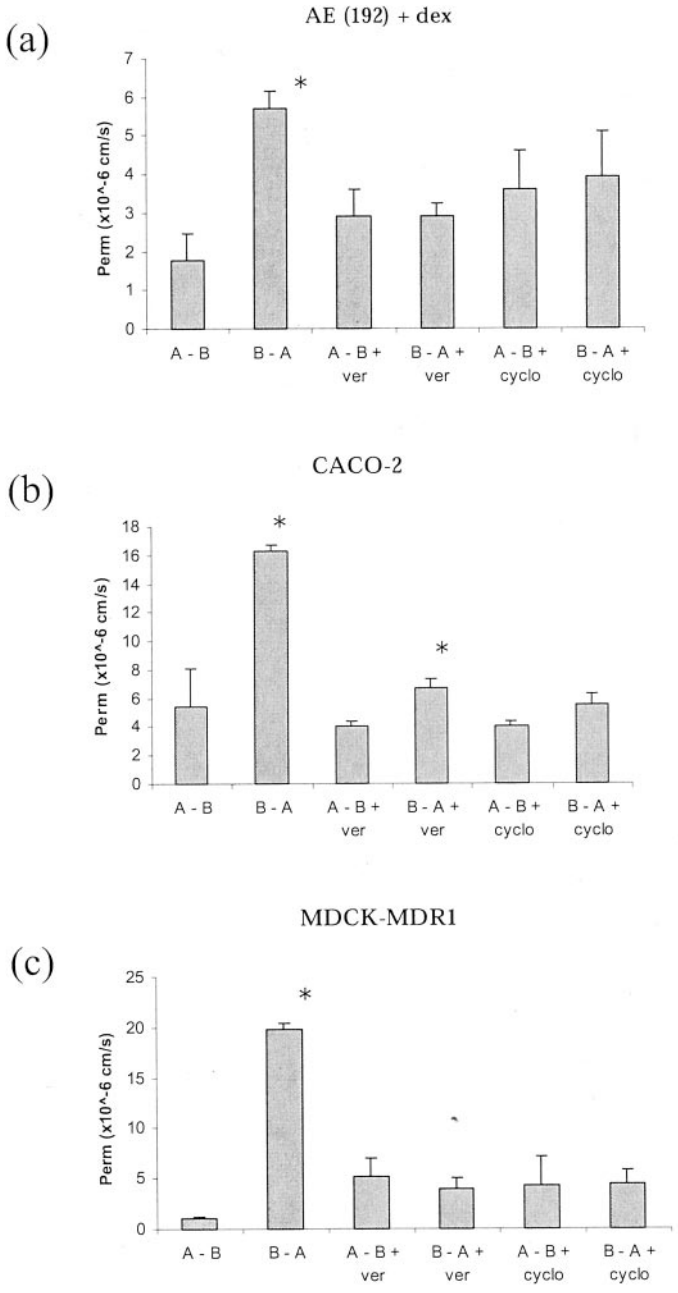


Fig. 6. Polarized transport of vinblastine. Permeability of vinblastine across monolayers of AE(192) + dexamethasone (dex) cells (a), Caco-2 (b), and MDCK-MDR1 (c). Transport studies were undertaken in the A-B and B-A direction in the absence or presence of verapamil (40 μM) or cyclosporin (10 μM) added to both the apical and basal compartments. *, indicates statistical ($P < 0.05$) difference in the B-A direction compared with the respective A-B direction.

Discussion

mdr-1/MDR1 P-gp is constitutively expressed at a number of anatomical barriers separating "environment" from systemic blood, e.g., luminal membranes of intestinal enterocytes or of proximal tubule epithelial cells of the kidney, or systemic blood from certain tissues, e.g., luminal membranes of endothelial cells that form the blood-brain barrier (Schinkel et al., 1994), the blood-retinal barrier (Holash and Stewart 1993), and the blood-testes barrier (Holash et al., 1993). Such a spatial distribution of this efflux transporter has defined it as a functionally important element in reducing the systemic exposure and specific tissue access of potentially harmful xenobiotics (for review, see Schinkel, 1997). The lung is constantly exposed to inhaled xenobiotics from the immediate atmosphere. With expression of P-gp within the epithelium of the lung conducting airways already recognized (Lechapt-Zalcman et al., 1997), we sought in this investigation to use several different but complimentary experimental approaches to examine for functional P-gp expression within normal mammalian alveolar epithelium, which comprises the majority of the total lung epithelial surface area (~120-m^2 surface area for alveolar epithelium versus ~3 m^2 for the conducting airways). In particular, we sought to define whether P-gp expression was evident within the terminally differentiated squamous AE type I cell that constitutes the major part (>95%) of the total alveolar epithelial surface area, and the loss of which to chemical injury would be highly detrimental to the maintenance of the alveolar-capillary blood barrier.

The immunohistochemical studies we present in both rat and human lung tissue show clear staining for mdr-1/MDR-1 P-gp, respectively, along the alveolar type I epithelial mem-

branes lining the alveolar airspace. Previous studies that have commented upon P-gp expression within the alveolar region are limited with a lack of definitive investigations examining the alveolar epithelium specifically. Using an in situ hybridization technique with probes against mdr-1b, Johannesson et al. (1997) noted positive staining in rat lung parenchyma, although they were unable to identify the cell type expressing the mdr-1b transcript. Using RT-PCR analysis of primary cultures of human lung epithelial cells (separated on the basis of size into ciliated epithelial cells >40 μm and alveolar epithelial cells <40 μm), Bagru et al. (1998) reported the presence of P-gp transcript in primary 7 day cultures of the <40-μm cell population. Using immunohistochemistry techniques with C219 antibody (that cross-reacts with both MDR-1 and MDR-3 P-gp), Cordon-Cardo et al. (1990) found P-gp to be undetectable within normal human lung alveolar tissue, while observing luminal staining in the bronchial epithelium, the latter finding in bronchial epithelium in agreement with the subsequent work of Lechapt-Zalcman et al. (1997).

Extending our investigations to examine P-gp functional expression within the cultured alveolar epithelial cell, we exploited a well characterized in vitro model system using rat primary culture of AE cells (Dobbs et al., 1988; Cheek et al., 1989; Danto et al., 1992; Campbell et al., 1999). Paralleling an in vivo process whereby the AE type II cell serves as a progenitor for, and differentiates into, the AE type I cell (for review, see Uhal, 1997), the isolation and primary culture of AE type II cells over a 5- to 8-day period on a substratum of tissue culture-treated plastic leads to the loss of the AE type II phenotype and acquisition of the morphology, and expression of biochemical markers, characteristic of the in vivo AE type I cell phenotype. When grown in the presence of low glucocorticoid concentrations (0.1 μM dexamethasone) such cultures not only adopt with time the well characterized squamous morphology but also generate a highly restrictive solute barrier with TEERs of between 1000 and 2000 $\Omega \cdot cm^2$ (Cheek et al., 1989; Campbell et al., 1999).

With the above-described model system, we confirmed the expression of both mdr-1a and mdr-1b mRNA transcripts in the cultured cells as they develop toward the AE type-I-like phenotype, i.e., transcripts became evident between 60 and 120 h postseeding with an absence of both mdr-1a or -1b in the freshly isolated AE type II cells. The lack of P-gp protein in the freshly isolated AE type II cells was also observed by immunoflow cytometry (C219 antibody) and leads to the tentative conclusion that the absence of mdr-1 transcript or P-gp protein in fresh isolates of pure (>95% AE type II) AE type II cells is a true reflection of the in vivo cell phenotype in this species. Of more note with respect to the aim of the investigation was confirmation of expression of mdr-1 transcript and P-gp protein (immunoflow cytometry and Western blot) within the cultured AE cells as they progress in culture toward an AE type I-like phenotype at 120 to 192 h postseeding. The temporal nature of this change was apparent for both mdr-1a and mdr-1b transcripts and in both the − and + dexamethasone-treated cultures. Beyond this temporal pattern (a function of the differentiation status of the culture) a direct comparison between the − and + dexamethasone-treated cells revealed a glucocorticoid mdr-1-inductive effect (for the mdr-1b notable at 60 h, and for both mdr-1a and mdr-1b notable at 120 h postseeding). This inductive effect was not evident by the time the cells had reached 192 h postseeding. A number of reports exist noting dexamethasone induction of P-gp levels in liver, brain, and intestinal tissue and also in lung tissue (Demeule et al., 1999), an effect which seems to be glucocorticoid concentration-dependent. The above-mentioned RT-PCR data would not be inconsistent with low-level glucocorticoid induction of cellular mdr-1 transcript levels against a background where the changing phenotype of the cell itself is also leading to increased mdr-1 transcript. Nevertheless, whether the alveolar epithelial cells were grown in the presence or absence of glucocorticoid the AE type I-like cells at 120 and 192 h express both mdr-1a and 1b and P-gp protein, whereas the freshly isolated AE type II progenitor cells, and the AE cells in early primary culture at 60 h, lack both the protein and transcript.

The functionality of P-gp within the AE type I-like cells (192) cells was established by a flow cytometric accumulation-retention assay using rhodamine-123 as substrate, and also by the polarized transport of vinblastine across confluent AE monolayers grown on semipermeable supports. The studies confirmed a basal to apical efflux mechanism present within the AE type I-like (192) cells that presents a transport barrier that can be considered, within the limits of this study design, to be comparable with that observed in the well characterized Caco-2 cell model.

The functional studies provide further support for the supposition that P-gp expressed in alveolar type I epithelium can serve as an efflux pump available to extrude potentially harmful inhaled xenobiotics and environmental pollutants from the alveolar epithelial cell back into the alveolar airspace away from the systemic circulation. Beyond this fundamental protective role, however, other speculative physiological functions of P-gp within the alveolar airspace may be considered. For example, MDR-1 or mdr-1a P-gp seems able to positively regulate cell volume-dependent chloride ion channel activity (for review, see Higgins, 1995), although P-gp itself lacks intrinsic chloride ion transport function. This modulatory role could subserve homeostatic volume regulation within the alveolar epithelium and help to maintain efficient respiratory gaseous exchange. Some evidence suggests that phosphatidylcholine, among other phospholipids, behaves as substrate for MDR1/P-gp (Bosch et al., 1997; Abulrob et al., 1999). The alveolar epithelial type I cell through P-gp functionality may fulfill a role in alveolar surfactant phospholipid homeostasis.

From a pharmacological perspective, the presence of P-gp within alveolar epithelium is of note given the current intense interest in exploiting the inhaled pulmonary route of drug delivery to gain increased systemic absorption for a range of therapeutic entities, from proteins and peptides (for review, see Patton, 1998) to, for example, inhaled opiate analgesics (Ward et al., 1997). With anatomical determinants and pharmacokinetic data supporting the view that deep penetration of therapeutic aerosols leads to improved systemic bioavailability, particularly for biotechnology products, it is natural that the alveolar epithelium is considered an appropriate absorption surface to target. In the knowledge that peptides and peptide-like drugs (Sharom et al., 1996), opiates (Wandel et al., 2002), and a wide range of structurally diverse molecules can serve as P-gp substrates, the finding of functional P-gp expression with alveolar epithelium provides a further possible mechanism that may limit the systemic

absorption of inhaled products or indeed lead to nonlinear absorption pharmacokinetics for pulmonary-administered products.

In summary, we have demonstrated expression of P-gp within alveolar type I epithelium from normal human and rat lung tissue. Using a well characterized rat primary alveolar epithelial cell culture model, we observed mdr-1 transcript and P-gp protein expression within the cultured alveolar type I cell phenotype. The P-gp expressed was functional and supports a role for MDR-1/mdr-1 as an element in mediating the reduced alveolar airspace to epithelium, and alveolar airspace-to-blood exposure of potentially harmful xenobiotics. Given the number of possible physiological functions that P-gp may serve within normal alveolar epithelium a critical evaluation of lung physiology and biochemistry within P-gp knockout mice is warranted and would extend to the examination of alveolar fluid composition and determination of possible underlying pathology compared with their wild type counterparts. Such studies would ultimately improve our knowledge of the exact physiological function of P-gp in normal alveolar epithelium and how these may change under pathological conditions.

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