JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 57–66

Received December 14, 1999
Accepted August 21, 2000
ISSN 0022-3573

Microparticulate uptake mechanisms of in-vitro cell culture models of the respiratory epithelium

Kimberly A. Foster, Mehran Yazdanian and Kenneth L. Audus

Abstract

The objective of this study was to examine the uptake mechanisms of fluorescent polystyrene microspheres of various diameters and surface chemistry by two human cell lines derived from the respiratory epithelium, A549 and Calu-3.

Briefly, A549 and Calu-3 cells were grown to confluence in 12-well cluster plates and the uptake of fluorescent microspheres by the cells was determined at various time points. The amount of microspheres internalized by the cells was determined by correcting for non-specific binding to the cell surface. The data showed that A549 cells appeared to have more phagocytic activity than Calu-3 cells. Albumin-coated microspheres as large as 3 μm diameter can be internalized by A549 cells. The amount of internalization by A549 cells observed for 0.5-μm diameter albumin-coated microspheres was approximately 10-times greater than that observed for 1-μm diameter spheres and approximately 100-times greater than values observed for 2- and 3-μm diameter beads. Transmission electron micrographs confirmed that the microspheres were internalized by the cells. Uptake experiments conducted with Calu-3 cells indicated that albumin-coated microspheres were neither bound nor internalized by the cells. The effect of microsphere surface chemistry on the uptake mechanism indicated that amidine microspheres were internalized more rapidly and to a greater extent by both A549 and Calu-3 cells than carboxylate microspheres and non-coated microspheres. This phenomenon is thought to be attributed to masking of the negative polystyrene core by the positive amidine functional group; this effect was less marked for the carboxylate microspheres.

These results suggest that A549 and Calu-3 cells can internalize microspheres and that size and effective charge played an important role in the uptake process.

Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66047, USA

Kimberly A. Foster, Kenneth L. Audus

Boehringer Ingelheim Pharmaceuticals Incorporated, Ridgefield, CT 06877–0368, USA

Mehran Yazdanian

G. D. Searle, Pharmaceutical Development, Skokie, IL 60070, USA

Kimberly A. Foster

Correspondence: K. L. Audus, 236B Simons, 2095 Constant Avenue, Lawrence, KS 66047–3729, USA.

Acknowledgements: This work was supported by Boehringer Ingelheim and an NIGMS predoctoral training programme in biotechnology (KAF). The authors gratefully acknowledge Dr Bruce Cutler for his skillful assistance in obtaining the TEM and the support of Corning Costar for the Cellular and Molecular Biopharmaceutics Handling Laboratory at the University of Kansas.

Introduction

In recent years, the pharmaceutical industry has witnessed increasing interest in pulmonary drug delivery. The possibility of delivering drugs and gene fragments via the pulmonary route offers several attractive features to the pharmaceutical scientist. These include a large absorptive area, extensive vasculature, and low extracellular and intracellular enzymatic activity (Liu et al 1993). Pulmonary delivery of pharmaceuticals also allows for an essentially instantaneous absorption of drug into the bloodstream, as well as local application of drugs in the case of pulmonary disease. Recent reports have shown that the pulmonary route is promising for the delivery of peptides such as leuprolide acetate (Adjei & Garren 1990) and proteins such as insulin (Edwards et al 1997).

One major challenge of pulmonary drug delivery is that the epithelial cell uptake and transport mechanisms of the pulmonary barrier are not well understood. This

Table 1 Lectin binding profile of A549 and Calu-3 cell lines.

Lectin	Blocking Sugar	A549	Calu-3
Con-A	Mannose	++	++
DBA	*N*-Acetylgalactose	+	+++
PNA	Galactose	+++	+
RCA	Galactose	++	+++
SBA	*N*-Acetylgalactose	+++	++
UEA-1	Fucose	+++	++
WGA	*N*-Acetylglucose	++	+

Staining patterns were defined as follows: +, slight intensity; ++, moderate intensity; and +++, strong intensity.

has prompted the need for cell culture models of the respiratory epithelium in order to understand and elucidate the mechanisms of pulmonary drug delivery at a cellular level. The human lung is a very complex organ which is composed of over 40 different cell types. However, the cells comprising the epithelial lining of the lung are believed to be the major barrier to pulmonary delivery (Taylor 1990). Two human cell lines derived from the respiratory epithelium, A549 and Calu-3, were used in this study. The A549 cell line possesses properties characteristic of Type II pulmonary epithelial cells (Foster et al 1998). The Calu-3 cell line has recently been shown to have properties consistent with submucosal gland serous cells (Shen et al 1994; Illek et al 1997).

Dry powder inhaler (DPI) formulations of proteins typically have been used to produce particles ranging in size from 1 to 5 μm (Lucas et al 1998). It is believed that this size range will deposit in the tracheobronchial and pulmonary regions in the lung (Hickey 1993).

This work examined the uptake of polystyrene microspheres ranging in size from 0.5 to 5.0 μm by A549 and Calu-3 cell lines. The goal was to determine the effect of size and effective charge on the uptake mechanisms of the cell models to evaluate the usefulness in exploring particulate interactions with the pulmonary epithelium.

Materials and Methods

Cell subcultures and plating

A549 cells were obtained from the American Type Culture Collection (ATCC) and maintained in Ham's F12-K medium (Sigma), with 10% fetal bovine serum (Atlanta Biologicals) and supplemented with 100 μg mL^{-1} penicillin G (Sigma) and 100 μg mL^{-1} streptomycin sulfate (Sigma). The cells were passaged when the cell monolayer was near confluence in a 175-cm^2 cell culture flask using a 0.1% trypsin solution containing 0.04% EDTA (Sigma). The cells were split 1:5 during each passage. The passages used for the following experiments were 78 to 99.

Calu-3 cells were obtained from the ATCC and maintained in a 1:1 mixture of Ham's F-12 (Life Technologies) and DMEM (Sigma) with 10% fetal bovine serum and supplemented with 100 μg mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin sulfate. The cells were passaged when the cell monolayer was near confluence in a 150-cm^2 culture flask using a 0.25% trypsin solution containing 0.1% EDTA. The cells were split 1:2 during each passage. The passages used for the following experiments were 19 to 27.

Lectin binding studies

The lectin binding pattern of both cell lines was examined using a biotinylated lectin kit from Vector Laboratories (Burlingame, CA) containing diaminobenzidine and the biotinylated peroxidase complex. The biotinylated lectins Con-A, RCA, UEA-1, SBA, DBA, PNA, and WGA were also purchased from Vector Laboratories. The solubilized stock solutions of lectins contained 1 mg mL^{-1} lectin, 10 mM HEPES, 0.15 M NaCl, 0.1 mM Ca^{2+}, and 0.04% NaN_3. The blocking sugars mannose, fucose, *N*-acetylgalactose, *N*-acetylglucose, and galactose were purchased from Sigma (St Louis, MO).

The lectins were stained according to the protocol provided by the Vector Laboratories. Briefly, A549 cells and Calu-3 cells were plated at 10^6 cells cm^{-2} and 5×10^5 cells cm^{-2}, respectively, on Lab-Tek chamber slides (Nunc). After the cells became confluent, the cells were washed twice with PBS and fixed with 0.4% formaldehyde for 20 min at room temperature. The cells were washed twice with PBS and incubated with 0.3% H_2O_2 in methanol for 10 min to block endogenous cellular peroxidases. The cells were then washed three times with 0.05 M Tris-buffered saline (TBS), pH 7.6. Side-by-side experiments were conducted in which a test monolayer was stained with a specific lectin and a control monolayer which had been pretreated with the specific blocking sugar was also stained with the lectin. Stock solutions of the lectins were made by adding 10 μg lectin to 1 mL TBS. Control lectin samples were made by adding 0.1 M blocking sugars to each specific lectin (Table 1) and allowed to equilibrate with shaking for 30 min before being added to the cells. Control cell monolayers were also pretreated with 0.1 M of the blocking sugar for 30 min before the addition of the lectin solution containing the blocking sugar. The lectin stock solutions were added to the cells for 45 min at 37°C in

an incubator with 95% relative humidity and 95% O_2/5% CO_2. The cells were washed three times with TBS and incubated for 30 min at 37°C with avidin-biotinylated peroxidase complex. The cells were washed three times with TBS and treated with diaminobenzidine for approximately 2 min. After visible staining could be detected under a light microscope, the cells were washed with distilled water for 5 min. The cells were then allowed to air-dry and were photographed using a Nikon camera attached to a light microscope.

Preparation of albumin-coated microspheres

Fluorescent polystyrene microspheres were obtained from Duke Scientific (Palo Alto, CA). Samples (0.5 mL) of stock solution were taken from the 1% vendor stock and washed three times with borate buffer (pH 8.5). A 1% solution of human serum albumin (Sigma) was added to the microspheres and the suspension was vortexed. The albumin was allowed to adsorb to the beads overnight. The next day, the microsphere suspension was centrifuged and the supernatant was saved for protein analysis. The beads were washed three times with PBS (pH 7.4) and resuspended in 1 mL PBS. Stock suspensions were stored at 4°C until used in an experiment. Before each experiment, an experimental stock was prepared by diluting the stock suspension 1:4 with PBS.

Carbodiimide-coupling of polyamines to carboxylate microspheres

Polyamine microspheres were prepared according to a protocol provided by Molecular Probes (Eugene, OR), by coupling the polyamines to carboxylate-modified microspheres using carbodiimide (Sigma, St Louis, MO). The polyamines, spermine (MW 202, three positive charges), spermidine (MW 145, two positive charges), and putrescine (MW 88, one positive charge), were obtained from Sigma (St Louis, MO). Carboxylate-modified microspheres (0.5 μm) were obtained from Molecular Probes (Eugene, OR). Polyamine stock solutions (2–5 mg mL^{-1}) were made by dissolving each of the polyamines in morpholenoethane sulfonic acid (MES), pH 6. Carboxylate-modified microspheres (5 mL, 2% (w/v) suspension) were added to each polyamine stock solution. The polyamine microsphere suspension was incubated at room temperature for 1 h. After the incubation period, 40 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) was added to each suspension. The resulting suspension was vortexed and the pH was adjusted to 6.5 ± 0.2 with dilute NaOH. The reaction mixture was placed on an orbital shaker for 2 h at room temperature. Glycine (100 mM) was then added to the suspension and the microspheres were incubated for 30 min at room temperature. The unreacted polyamine was removed from the suspension by centrifugation at 10 000 rev min^{-1} removing the supernatant, and resuspending the microspheres in 100 mM PBS. The washing procedure was repeated three times. The microsphere suspensions were stored in the refrigerator until used in experiments.

Qualitative analysis

Fluorescence microscopy was used to qualitatively determine microsphere binding and uptake into the cells. Internalized microspheres were detected in cross sections of A549 cells using transmission electron microscopy. Briefly, A549 monolayers grown on Snapwell polycarbonate filters were washed three times for 10 min with 100 mM phosphate-buffered saline supplemented with 0.63 mM $CaCl_2$, 0.74 mM $MgSO_4$, 5.3 mM dextrose, and 0.1 mM ascorbic acid, pH 7.4 (PBSA), and fixed with a 2.5% glutaraldehyde solution in PBSA for 4 h at 4°C. After fixing, the membranes were washed three times for 10 min with PBSA, the samples were post-fixed in 1% OsO_4, dehydrated in a series of ethanol and acetone solutions, and embedded in EmBed 812. The sample was sectioned at 80 nm on Sorvall Mt 500 Ultratome with a diamond knife. The sections were stained for 10 min each with uranyl acetate and lead citrate and examined under a Jeol 1200 EX II transmission electron microscope.

Effect of size on microparticulate uptake

A549 cells were plated at a density of 1.6×10^5 cells cm^{-2} in 12-well cluster plates. The culture medium was changed every two days. Uptake experiments were conducted once the cells had formed confluent monolayers as determined by light microscopy (approx. 3 days after seeding). All experiments were conducted in culture medium supplemented with 1.3 mM $MgSO_4$ and 1.3 mM $CaCl_2$. Before each experiment, fresh culture medium was added to the wells and allowed to equilibrate at the desired temperature (37 or 4°C). During the experiments, the plates were placed on an orbital shaker so that the microsphere suspensions were well mixed during the experiments. At various time points, a sample of fluorescent microspheres was added to each well. At the end of the experiment the medium was removed from the cells, and the monolayers were washed three times with cold PBS. The monolayers were then digested with 0.2 M NaOH containing 10% Triton X-100. The amount of microspheres taken up was determined by fluores-

cence spectroscopy as a percentage of control. Control suspensions of microspheres were made in 0.2 M NaOH containing 10% Triton X-100 supplemented with 1% BSA. The experimental samples were normalized spectrophotometrically (Shimadzu UV160U) for protein content using a BCA Protein Assay Reagent Kit (Pierce). The internalization of the fluorescent microspheres was determined by subtracting the number of microspheres bound to the cell surface at 4°C from the number of microspheres associated with the cells at 37°C (Steinkamp et al 1982). All time point values were done in triplicate.

Effect of surface group on microparticulate uptake

The 0.5-μm carboxylate-modified and amidine microspheres were obtained from Molecular Probes. The 0.5-μm polystyrene microspheres were obtained from Duke Scientific. Charged microsphere stock suspensions were made by taking samples of the vendor stock suspension and washing with PBS. The microsphere suspensions were then centrifuged at 10000 rev min^{-1} for 10 min. The supernatant was discarded and the microspheres were washed again with PBS. The washing procedure was repeated three times. The final microsphere suspension was resuspended in PBS. It should be noted that the amidine microsphere stock suspensions contained 0.01% Tween-20 because the microspheres adhered to plastic. The final concentration of Tween-20 in the cell wells was 0.001% and did not alter cell viability as determined by trypan blue exclusion (data not shown).

A549 cells were plated at a density of 1.6×10^5 cells cm^{-2} and Calu-3 cells were plated at a density of 5×10^{-5} cells cm^{-2} in 12-well cluster plates. The culture medium was changed every two days. Experiments were conducted when the cells had formed confluent monolayers as determined by light microscopy. Uptake experiments were conducted in serum-free culture medium to prevent masking the microsphere functional group. Binding experiments were conducted in serum-free culture medium containing 1% NaN_3 (Steinman et al 1983). Before each experiment, fresh serum-free medium (with or without NaN_3) was added to the wells and allowed to equilibrate for 1 h. During the uptake experiments, the plates were placed in a thermal box set at 37°C on an orbital shaker so that the microsphere suspensions were well mixed during the experiment. At various time points, a sample of charged fluorescent microspheres was added to each well. At the end of the experiment, the medium was removed from the cells and the monolayers were washed three times with cold PBS. The monolayers were then digested with 500 μL 0.2 M NaOH containing 0.5% Triton X-100. A 10-μL fraction of each sample was removed for protein determination. Ethanol (95%; 500 μL) was added to the cell lysate and the sample was transferred to a cuvette. The amount of microspheres taken up was determined by fluorescence spectroscopy (SLM fluorometer) as a percentage of control. Control samples were prepared in cell lysates of known protein levels and corrected for background cellular fluorescence. The samples were normalized spectrophotometrically (Shimadzu UV160U) for protein content using a BCA Protein Assay Reagent Kit (Pierce). The internalization of the fluorescent microspheres was determined by subtracting the binding of microspheres to the cell surface (microspheres/cell) at 37°C + 1% NaN_3 from the values at 37°C. All time point values were done in triplicate.

Zeta potential measurements

Microsphere bead suspensions were prepared in 1 mM KCl solution and the zeta potential of the microspheres was measured using a Nicomp 380/ZLS zeta potential analyser (Particle Sizing Systems, Santa Barbara, CA). The measurements were done under the following conditions: frequency range 500 Hz; temperature 23°C; fluid refractive index 1.33; antipoise viscocity 0.933; dielectric constant 78.0; electric field strength 10 V cm^{-1}; and sample run times were 1 to 5 min. The values reported represent the average of five determinations and standard deviations of less than 1 mV.

Statistical analysis

Internalization data were defined as the residual values between means of the uptake and binding experiments for each time point. The propagation of error was determined from the standard deviation of the means from the uptake and binding experiments. Standard errors of the means for each time point were calculated by dividing the propagated error by the square root of the sample size. Polyamine data were analysed with the Minitab statistical software package using a Tukey's test in a one-way analysis of variance ($\alpha = 0.05$). Each data point represents the mean $\pm$ s.e.m. of at least three values.

Results and Discussion

Lectin binding studies

The A549 cell line has been described as a model for Type II pulmonary epithelium (Kobayashi et al 1995;

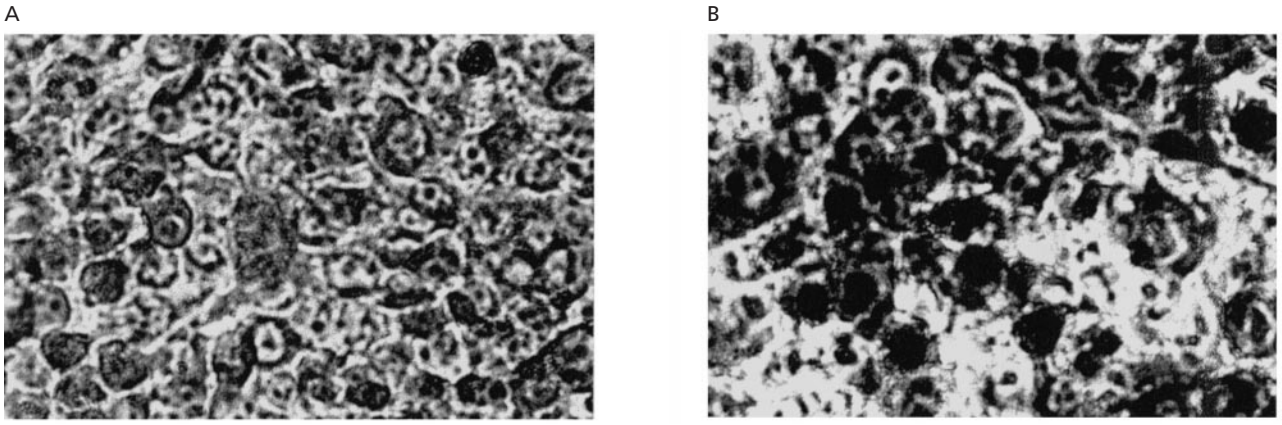

Figure 1 Light micrograph of A549 monolayer stained with UEA-1 lectin (400 ×). The control monolayer (A) was pre-incubated for 30 min with 0.1 M fucose. The dark patches on the test monolayer (B) indicate UEA-1 lectin binding to the surface of A549 cells.

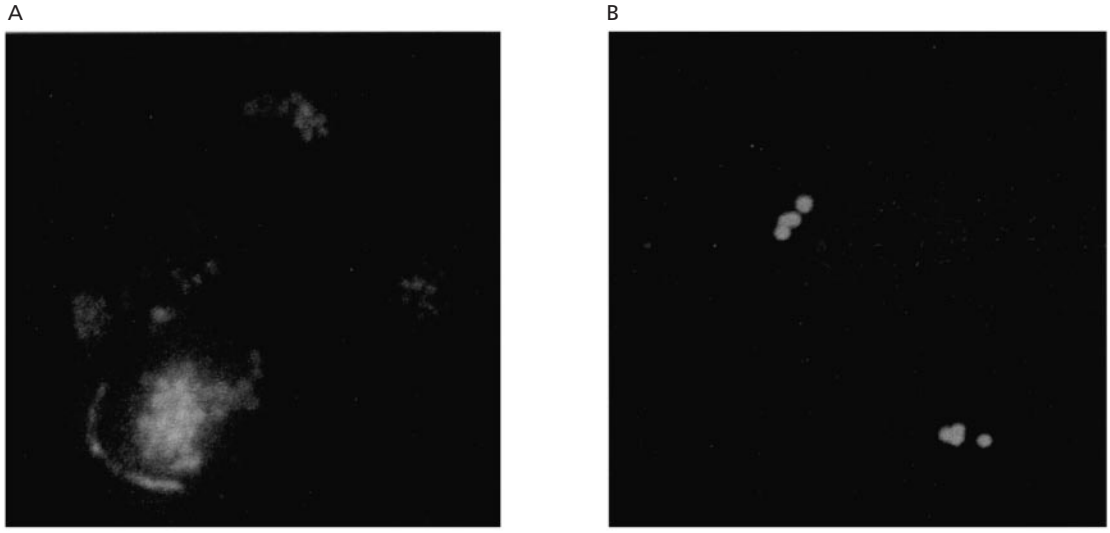

Figure 2 Fluorescent micrograph of A549 cells exposed to fluorescent polystyrene microspheres. These micrographs indicate that the microspheres are associated (bound to the cell surface or internalized) with the cells and that smaller 0.5-μm microspheres (A) are associated to a greater extent to A549 cells than larger 1.96-μm microspheres (B).

Foster et al 1998). The major limitation of this cell line is that it does not possess tight junctions apparently characteristic of the pulmonary epithelium in-vivo. The A549 cell line has been used previously to examine how the pulmonary epithelium interacts with environmental particulates such as titanium dioxide, iron oxide, α-quartz, and concentrated air particulates (Stringer & Imrich 1996), as well as asbestos (Leser et al 1992; Rosenthal et al 1994). The Calu-3 cell line has been used as a transport model for insulin and other proteins (O'Shaughnessy & Prosser 1996) but has not been examined for particulate uptake.

The respiratory epithelial cell lines chosen for this study were compared on the basis of lectin binding pattern. Light microscopy was used to characterize the lectin binding patterns of the A549 and Calu-3 cell lines (Table 1). A representative micrograph of UEA-1 staining of an A549 monolayer is given in Figure 1. There were some qualitative differences between our lectin staining results and lectin binding results for normal pulmonary tissue (Alvarez-Fernandez & Carretero-Albinana 1990). The A549 model binds the lectins SBA and UEA-1 to a greater extent than normal tissue. The Calu-3 model also showed positive staining for the lectins DBA, SBA and UEA-1 which is in contrast with data obtained for normal human serous cells. The Calu-3 cell line also apparently binds WGA to a lesser extent than normal tissue. Anomalous lectin binding patterns for lung adenocarcinomas as compared with normal tissue have been reported by Alvarez-Fernandez & Carretero-Albinana (1990). Therefore, with the exception of normal tissue failing to bind SBA, the cell lines,

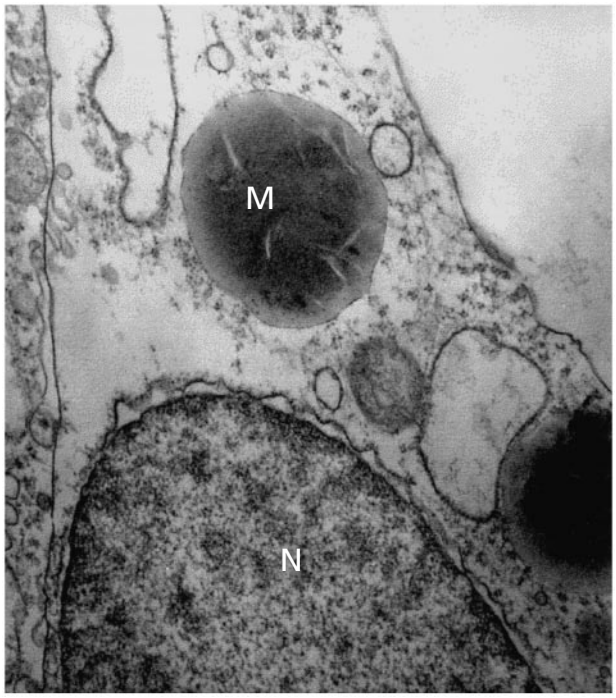

Figure 3 Electron micrograph of cross section of an A549 cell grown in Transwell. The cells in the Transwell were exposed to a stock suspension of 0.5-μm albumin-coated microspheres for 1 h at 37°C. After the incubation period, the cells were washed and fixed for electron microscopy. The micrograph indicated that the microspheres were internalized by the cells. The microspheres (M) and cell nucleus (N) are labelled.

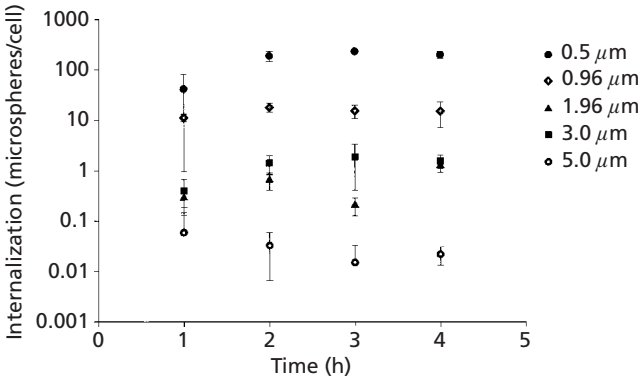

Figure 4 Effect of size on the internalization of albumin-coated microspheres by A549 cells in the presence of fetal bovine serum. The cells were grown to confluence in 12-well Transwells and incubated with microsphere stock suspensions in culture medium with serum at either 37°C or 4°C with 0.5% NaN_3. The data show the number of microspheres taken up by the cells and are corrected for non-specific binding to the cell surface.

normal and adenocarcinoma cells were all bound to some degree with the lectins. Differences in surface biochemistry as reflected by lectin binding could suggest that interactions of particulates with the different cell types should not be identical.

Qualitative analysis

Fluorescence and transmission electron microscopy were used to qualitatively assess particulate uptake by the cells. Figure 2 shows typical micrographs obtained when monolayers exposed to fluorescent microspheres were examined under an inverted fluorescent microscope. These experiments allowed us to establish that the microspheres were in fact associated with the cells. However, internalization could not be determined from these studies. Electron micrographs of cross sections of A549 cells exposed to 0.5-μm albumin-coated microspheres confirmed that the microspheres were internalized by the A549 cell line (Figure 3).

Effect of size on microparticulate uptake

Size-effect experiments were conducted with albumin-coated microspheres. It was necessary to coat the polystyrene microspheres with albumin to keep the microspheres from aggregating during the experiments. Experiments conducted with non-coated polystyrene microspheres were problematic. Aggregation of albumin-coated microspheres was encountered during the analysis procedure of early experiments. Therefore, the concentration of the detergent in the lysing buffer, Triton X-100, was increased from 0.5 to 10% in later experiments.

Due to the size of the microspheres used in this study, the microsphere internalization mechanism was believed to be phagocytosis, an energy-dependent process. Internalization was determined by conducting side-by-side uptake experiments at 37°C and experiments at 4°C with 0.05% NaN_3 (conditions in which the energy-dependent mechanisms of the cells are impeded). The average number of microspheres associated with each cell was determined for both experiments with internalization being defined as the difference between data from the two temperatures.

The results indicated that albumin-coated microspheres as large as 3 μm in diameter were internalized by the A549 cells (Figure 4). The amount of internalization observed for 0.5-μm diameter albumin-coated microspheres by A549 cells was approximately 10-times greater than that observed for 1-μm diameter spheres, and approximately 100-times greater than values observed for 2- and 3-μm diameter beads. Initial experiments were conducted over 4 h. Later experiments revealed that this internalization mechanism was a very rapid process, and thus the time frame for subsequent experiments was reduced to 15 min (data not shown).

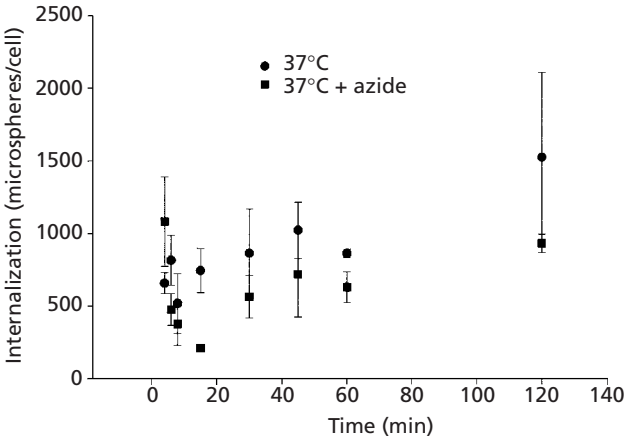

Figure 5 Uptake and binding results of A549 monolayers treated with a 0.5-μm albumin-coated microsphere suspension in culture medium without serum at 37°C or 37°C+azide. The data indicate that there is no significant internalization of albumin-coated microspheres by A549 cells under these conditions.

Table 2 Zeta potential measurements of representative microsphere suspensions.

Microsphere	Zeta potential (mV)
Amidine	−29.5
Carboxylate	−39.5
Non-coated	−49
Putrescine	−31
Spermidine	−30
Spermine	−30
Albumin 0.5 μm	−34
0.96 μm	−39
1.96 μm	−39
3 μm	−44
5 μm	−37.5

Microsphere bead suspensions were prepared in 1 mM KCl solution and the zeta potential of the microspheres measured using a zeta potential analyser. The values represent the average of five determinations and standard deviations of less than 1 mV.

Experiments conducted with the Calu-3 model revealed that those cells did not bind or internalize albumin-coated microspheres in the presence of serum within the 15-min time frame. The uptake data for albumin-coated microspheres > 0.5 μm in Calu-3 cells also indicated that fluorescent readings were due to background cellular fluorescence only (data not shown).

The internalization of albumin-coated microspheres by A549 cells was in contrast with that reported for uptake of albumin-coated microspheres by other cell types, including lung and peritoneal macrophages, neutrophils and monocytes (Ayhan et al 1995). It is believed that macrophages do not readily take up albumin-coated microspheres because they are not recognized by the reticuloendothelial system (RES) as foreign particulates (Ayhan et al 1995), and because of their hydrophilic surface. However, opsonization of microspheres by serum proteins has been shown to increase their uptake by the RES (Strand et al 1988; Armstrong et al 1997). Moreover, the phagocytic activity of Kupffer cells from normal rat liver has been reported to increase 17-fold in the presence of normal rat serum and heparin as compared with buffer conditions without serum (DiLuzio et al 1972).

Our size-effect experiments were conducted in culture medium containing fetal bovine serum. Later uptake experiments conducted with albumin-coated microspheres in serum-free culture medium showed no statistical difference between 37°C and 37°C+1% NaN_3 (Figure 5). These experiments were conducted with different microsphere stock suspensions and human serum albumin. The reason for this discrepancy is unclear; however, it appears to indicate that some component of FBS was important in the uptake of albumin-coated microspheres.

Effect of surface group on microparticulate uptake

Experiments were conducted to further explore the internalization mechanism using amidine microspheres and carboxylate microspheres possessing positively-charged and negatively-charged surface functional groups, respectively. The zeta potential for non-coated polystyrene microspheres reflected the large negative nature of polystyrene. Zeta potential measurements were conducted on representative microsphere stock suspensions to confirm masking of the negative charge on the polystyrene microspheres (Table 2). This negative charge appears to be masked by the addition of functional groups onto the polystyrene surface.

The results showed that internalization by both cell types of 0.5-μm amidine microspheres was approximately 10-fold greater (Figure 6) than that of 0.5-μm carboxylate microspheres (Figure 7). Previous groups have shown that uncharged molecules are taken up by pulmonary epithelial cells to a lesser extent than cationized particles, and anionic molecules appear to be largely excluded by Type II pulmonary epithelial cells (Farquhar 1978). This trend has been shown with macromolecules such as anionic and cationic ferritin (radius approx. 10 nm) and is presumably due to the net negative charge on the apical membrane. This mechanism may involve a non-specific ionic interaction or a specific

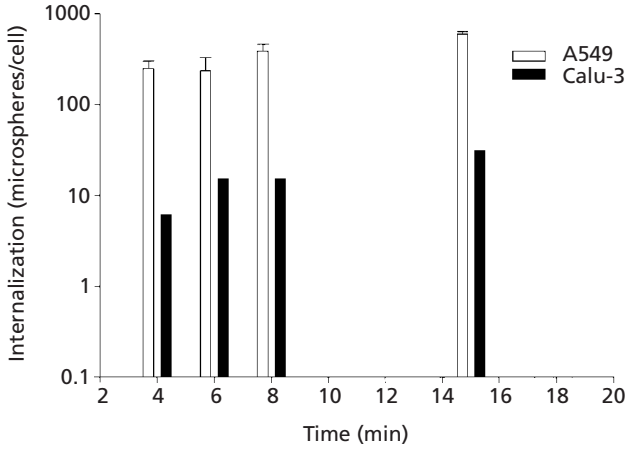

Figure 6 Internalization data from uptake experiments conducted with positively-charged amidine microspheres with A549 and Calu-3 cells in serum-free medium. The data are corrected for non-specific binding to the cell surface. The results indicate that both cell types internalize the positively-charged microspheres, and that A549 cells have more phagocytic capacity than Calu-3 cells.

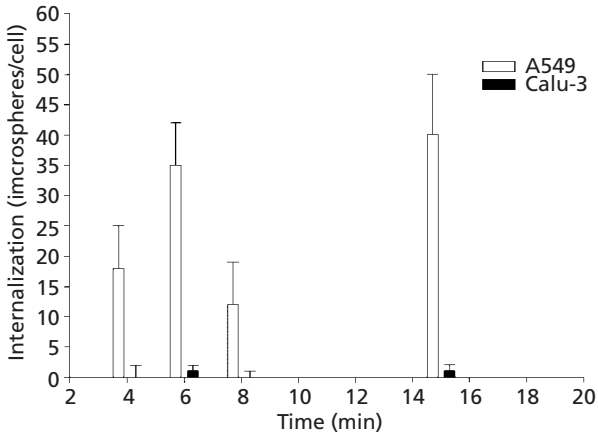

Figure 7 Internalization data from uptake experiments conducted with negatively-charged carboxylate microspheres with A549 and Calu-3 cells in serum-free medium. The data are corrected for non-specific binding to the cell surface. The results indicate that phagocytosis was reduced compared with data for internalization of positively-charged microspheres as shown in Figure 6. The results also show that A549 cells internalize negatively-charged microspheres to a greater extent than Calu-3 cells.

interaction with a membrane glycoprotein (Williams 1984).

Polyamine experiments

The results that amidine microspheres are internalized preferentially by both cell types prompted consideration of various polyamines, and whether the presence of increased positive charges alter uptake of the microspheres by A549 cells. These experiments were done using the A549 model because of its greater phagocytic ability compared with the Calu-3 model, and lectin-binding properties which suggested a better correlation with normal pulmonary epithelium.

The results of the polyamine uptake experiments revealed that conjugating polyamines onto carboxylate-modified microspheres did not enhance the internalization of the microspheres (Figure 8A–C). There was no statistical difference between 37°C and 37°C + 1% NaN_3 uptake data for spermine- and spermidine-coated microspheres (Figure 8A and B, respectively) at all time points. Data for putrescine microspheres (Figure 8C) appeared to indicate that fluorescence readings were due to background cellular fluorescence only; however, the 8- and 15-min time points showed binding of the putrescine to the cell membrane, possibly indicating that the duration of the experiment was not long enough. These results might also imply that charge localization plays a key role in the internalization process, or that the efficiency of polyamine binding onto the microspheres is low. However, zeta potential measurements confirmed that suspensions of polyamine microspheres were overall less negative than carboxylate microspheres (Table 2). The zeta potential reflects the entire suspension and the surface of the carboxylate-modified microsphere may not have been entirely covered by the polyamines.

Summary and conclusions

This study examined the phagocytic properties of the A549 cells, a Type II epithelial cell line, and the Calu-3 cell line, a sub-bronchial cell line. These models could be potentially useful for examining particulate interactions with the respiratory epithelium. One concern in using these cell lines was their tumour origin. Lectin binding confirmed that the A549 had a similar lectin binding pattern as normal pulmonary tissue. However, Calu-3 deviates somewhat from normal cells with respect to lectin binding.

We examined the internalization of microspheres of varying diameters and surface chemistry by the cell models. The fate of the microspheres once internalized by the cells was not examined. Polystyrene microspheres were used in these studies because of their commercial availability and convenient fluorescence properties which allowed qualitative and quantitative examination of their uptake by cells. Pharmaceutical microsphere carriers should, of course, be composed of biodegradable polymers such as polylactic acid and polylactic-co-glycolic acid, and possibly also possess a non-spherical geometry (Gonda 1992; Lacasse et al 1998). Future work should address these issues.

Our results confirm the findings of Griese & Reinhardt (1998) who showed that rat alveolar type II pneumocytes

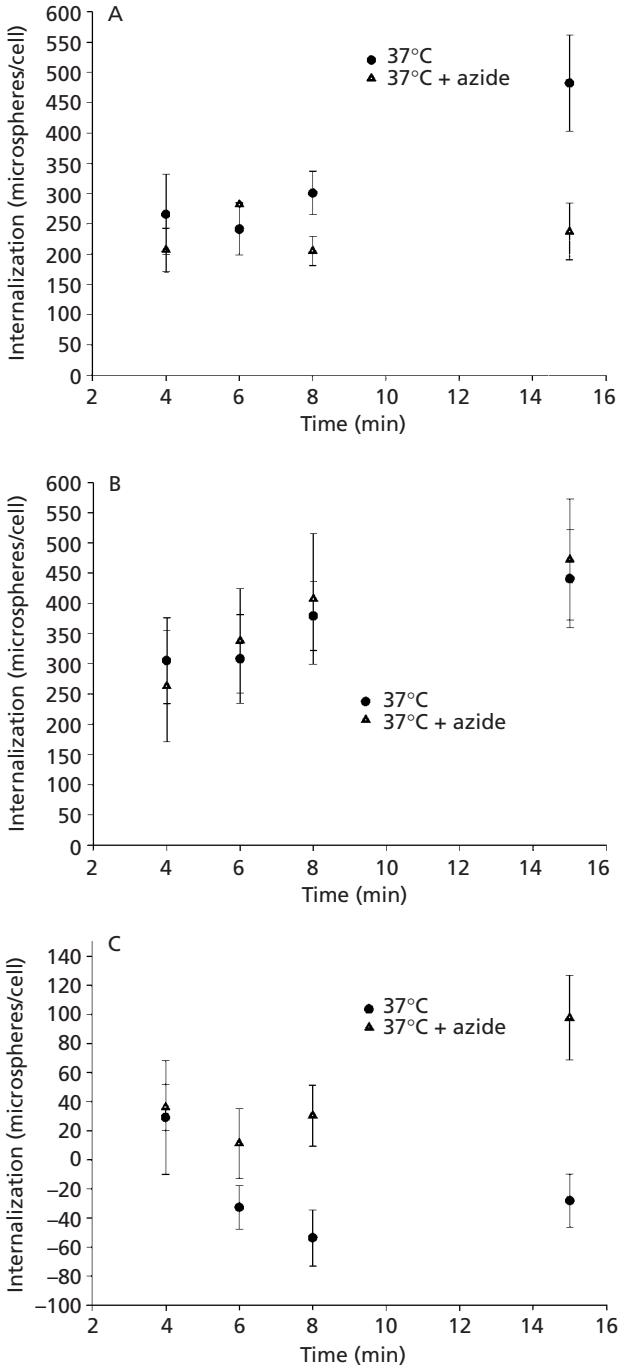

Figure 8 A. Uptake and binding results of A549 monolayers treated with a 0.5-μm spermine-coated microsphere suspension in culture medium without serum. The data indicate that there was no significant internalization of spermine-coated microspheres by A549 cells. B. Uptake and binding results of A549 monolayers treated with 0.5-μm spermidine-coated microsphere suspension in culture medium without serum. The data indicate that there was no significant internalization of spermidine-coated microspheres by A549 cells. C. Uptake and binding results of A549 monolayers treated with 0.5-μm putrescine-coated microsphere suspension in culture medium without serum. The data indicate that the fluorescent readings from the early time points were due mainly to background cellular fluorescence. Binding of putrescine-coated microspheres to A549 cells appeared to take place after 8 min. No internalization of putrescine-coated microspheres was observed during the course of the experiment. In all cases the cells were incubated at 37°C or at 37°C + 1% NaN_3.

preferentially took up smaller 15-nm beads as opposed to larger 1-μm beads; however, their findings showed no size-dependency on uptake from 200 to 1000 nm. The particles chosen for the present study covered a broader size range (500–5000 nm); however, it is still unclear whether particle size alone or the number of particles, or both factors, contribute to increases in uptake.

Our finding that uptake of microparticulates is inversely related to the size of the particle is not new (Carr et al 1996). Our in-vitro data showed that smaller microspheres were internalized to a greater extent than larger microspheres. However, in-vivo data indicate that larger particles are better delivery devices because they are less readily cleared from the lung than smaller particles (Edwards et al 1997). This has also been demonstrated in data from gastrointestinal tract experiments; the smallest size is not always the optimum size for bioavailability. In fact, a 6-μm diameter appears to be the optimum size for particles delivered to the gastrointestinal tract. Studies using 100-nm microspheres showed that those particles were not taken up by rat peritoneal macrophages (Pratten & Lloyd 1986). It was concluded that this size of particle was too large for uptake by pinocytosis, but too small to initiate the phagocytic mechanism (Pratten & Lloyd 1986).

Since A549 cells were shown to take up microspheres as large as 3 μm, one could propose that this cell line might be a good candidate to investigate particulate–cell interactions for formulations in practical size-ranges for delivery to the alveolar region (1–3 μm) (Gonda 1992). Their usefulness in the study of clearance of environmental particulates has already been established (Stringer & Imrich 1996).

Reports have shown that the geometric shape of the particle has an effect on internalization by pulmonary epithelium (Gonda 1992). Other studies have indicated that the bioavailability of insulin and testosterone was increased when delivered to subjects using a large nonporous biodegradable polymer vehicle (Edwards et al 1997). These results were believed to be due to the larger size yielding a favourable deposition in the lung and an increased residence time in the lung due to lack of phagocytosis (i.e., clearance) of the larger nonporous spheres. Future work may be carried out using various shapes of microspheres, both porous and nonporous.

To conclude, we have shown that the A549 and Calu-3 cell lines internalize microspheres, and that charge and size play roles in the mechanism of internalization. These cell lines, and particularly the A549 cell line, may be useful for examining the mechanisms of macromolecule drug delivery from drug delivery vehicles such as biodegradable microspheres to the respiratory epithelium.

References

Adjei, A., Garren, J. (1990) Pulmonary delivery of peptide drugs: effect of particle size on bioavailability of leuprolide acetate in healthy male volunteers. *Pharm. Res.* **7**: 565–569

Alvarez-Fernandez, E., Carretero-Albinana, L. (1990) Lectin histochemistry of normal bronchopulmonary tissues and common forms of bronchogenic carcinoma. *Arch. Pathol. Lab. Med.* **114**: 475–481

Armstrong, T., Davies, M., Illum, L. (1997) Human serum albumin as a probe for protein adsorption to nanoparticles: relevance to biodistribution. *J. Drug. Target.* **4**: 389–398

Ayhan, H., Tuncel, A., Bor, N., Piskin, E. (1995) Phagocytosis of monosize polystyrene-based microspheres having different size and surface properties. *J. Biomater. Sci. Polym. Ed.* **7**: 329–342

Carr, K., Hazzard, R., Reid, S., Hodges, G. (1996) The effect of size and charge on uptake of orally administered latex microparticles in small intestine and transport to mesenteric lymph node. *Pharm. Res.* **13**: 1205–1209

DiLuzio, N., Miller, R., McNamee, R., Pisano, J. (1972) Alterations in plasma recognition factor activity in experimental leukemia, RES. *J. Reticuloendothel. Soc.* **11**: 186

Edwards, D., Hanes, J., Caponetti, G., Hrkach, J., Ben-Jebria, A., Eskew, M., Mintzes, J., Deaver, D., Lotan, N., Langer, B. (1997) Large porous particles for pulmonary drug delivery. *Science* **276**: 1868–1871

Farquhar, M. (1978) Recovery of the surface membrane in anterior pituitary cells. Variations in traffic detected with anionic and cationic ferritin. *J. Cell Biol.* **77**: R35–R42

Foster, K., Oster, C., Mayer, M., Avery, M., Audus, K. (1998) Characterization of the A549 cell line as a Type II pulmonary epithelial cell model for drug metabolism. *Exp. Cell Res.* **243**: 359–366

Gonda, I. (1992) Targeting by deposition. In: *Pharmaceutical Inhalation Aerosol Technology*. Marcel Dekker Inc, New York, pp 61–82

Griese, M., Reinhardt, D. (1998) Smaller sized particles are preferentially taken up by alveoloar type II pneumocytes. *J. Drug Target.* **5**: 471–479

Hickey, A. (1993) Lung deposition and clearance of pharmaceutical aerosols: what can be learned from inhalation toxicology and industrial hygiene? *Aerosol Sci. Tech.* **18**: 290–304

Illek, B., Yankaskas, J., Machen, T. (1997) cAMP and genistein stimulate HCO_3^- conductance through CFTR in human airway epithelia. *Am. J. Physiol.* **272**: L752–L761

Kobayashi, S., Kondo, S., Juni K. (1995) Permeability of peptides and proteins in human cultured alveolar A549 cell monolayer. *Pharm. Res.* **12**: 1115–1119

Lacasse, F., Filion, M., Phillips, N., Escher, E., McMullen, J., Hildgen, P. (1998) Influence of surface properties at biodegradable microsphere surfaces: effects of plasma protein adsorption and phagocytosis. *Pharm. Res.* **15**: 312–317

Leser, O., Melloni, B., Cantin, A. (1992) Silica-exposed lung fluids have proliferative activity for type II epithelial cells: a study on human and sheep alveolar fluids. *Exp. Lung Res.* **18**: 633

Liu, F., Shao, Z., Kildsig, D., Mitra, A. (1993) Pulmonary delivery of free and liposomal insulin. *Pharm. Res.* **10**: 228–232

Lucas, P., Anderson, K., Staniforth, J. (1998) Protein deposition from dry powder inhalers: fine particle multiplets as performance modifiers. *Pharm. Res.* **15**: 562–569

O'Shaughnessy, C., Prosser, E. (1996) Calu-3 in the horizontal diffusion chamber system: a model of pulmonary drug transport. *Pharm. Res.* **13**: S169

Pratten, M., Lloyd, J. (1986) Pinocytosis and phagocytosis: the effect of size of a particulate substrate on its mode of capture by rat peritoneal macrophages cultured in vitro. *Biochim. Biophys. Acta* **881**: 307–313

Rosenthal, G., Germolec, D., Blazka, M., Corsini, E., Simeonova, P., Pollock, P., Kong, L., Kwon, J., Luster, M. (1994) Asbestos stimulates IL-8 production from human lung epithelial cells. *J. Immunol.* **153**: 3237–3244

Shen, B., Finkbeiner, W., Wine, J., Mrsny, R., Widdicombe, J. (1994) Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl^- secretion. *Am. J. Physiol.* **266**: L493–L501

Steinkamp, J., Wilson, J., Saunders, G., Stewart, C. (1982) Phagocytosis: flow cytometric quantitation with fluorescent microspheres. *Science* **215**: 64–66

Steinman, R., Mellman, I., Muller, W., Cohn, Z. (1983) Endocytosis and recycling of the plasma membrane. *J. Cell Biol.* **96**: 1–27

Strand, S., Anersson, L., Bergqvist, L. (1988) Applications and characterization of radiolabeled or magnetizable nano- and microparticles for RES, lymph, and blood flow studies. In: Rembaum, A.; T-k-s, Z. (eds) *Microspheres: Medical and Biological Applications*. CRC Press Inc., Boca Raton, pp 166–187

Stringer, B., Imrich, A. (1996) Lung epithelial cell (A549) interaction with environmental particulates: quantitation of particle binding and IL-8 production. *Exp. Lung Res.* **22**: 495–508

Taylor, G. (1990) The absorption and metabolism of xenobiotics in the lung. *Adv. Drug Deliv. Rev.* **5**: 37–61

Williams, M. C. (1984) Endocytosis in alveolar type II cells: effect of charge and size of tracers. *Proc. Natl Acad. Sci. USA* **81**: 6054–6058