CRUCIAL BIOPHARMACEUTICAL ISSUES FACING MACROMOLECULAR CANDIDATES FOR INHALATION: THE ROLE OF MACROPHAGES IN PULMONARY PROTEIN CLEARANCE

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SUMMARY

Our laboratory recently demonstrated that alveolar macrophages (AM) comprised a major barrier to the transport of two large proteins, IgG and human chorionic gonadotropin, from the airways into the bloodstream, while they had no impact on the peptide insulin. This study was aimed at assessing the role of AM in the alveolar clearance of human growth hormone (hGH), a smaller therapeutic protein being investigated as an inhalation aerosol in clinical trials. Using intratracheal instillation of liposome-encapsulated clodronate to deplete AM of rat lungs, we studied the pulmonary absorption of hGH in AM-depleted versus normal animals. The systemic absorption of hGH, following pulmonary administration, did not show significant alterations following AM depletion; absolute bioavailabilities reached 11.4%, 10.7% and 9.0% in clodronate liposome-, PBS liposome- and PBS-treated rats. Absorption from the lungs was rapid in all tested conditions, indicating that hGH crossed the alveolar epithelium quickly, presumably preventing major uptake and degradation by AM. AM uptake of proteins appears to be a significant local elimination mechanism for proteins above a certain size which implies a residence time within the alveolar spaces long enough for significant AM endocytosis.

INTRODUCTION

Inhalation aerosols are proving to be a promising alternative to injection for the systemic administration of proteins (1). Efficient absorption from the lungs results from significant improvement in inhalation systems (2) as well as from the anatomical features of the alveoli that provide a large absorptive surface area (~100 m^2), an extensive vasculature and blood flow (5 L/min) and a

short diffusion path to the bloodstream (0.5 μm). In spite of these advantages, bioavailabilities of inhaled therapeutics remain limited. Uncontrolled biological losses in the respiratory tissue diminish molecular transfer to the bloodstream. Among the barriers to absorption, the alveolar epithelium and local proteases were often thought to be significant (1,3). Recently, our laboratory (4) demonstrated that alveolar macrophages (AM) were also a major barrier to the systemic absorption of certain macromolecules from the airways. Depletion of AM by liposome-encapsulated clodronate induced several fold enhancements in the systemic absorption of IgG (150 kDa) and human chorionic gonadotropin (39.5 kDa) following their pulmonary delivery in rats. In contrast to large proteins, no increase in pulmonary absorption of the peptide insulin (5.8 kDa) was associated with AM depletion. These results suggested that AM clearance is a significant barrier to the pulmonary absorption of large macromolecules.

Recombinant human growth hormone (hGH) is a 22 kDa protein drug that is currently delivered by subcutaneous injection to children with short stature linked to growth hormone deficiency, Turner's syndrome, or chronic renal failure. In contrast to the nasal and the oral routes, which allow low systemic delivery of hGH (< 1%) (5), a bioavailability ranging between 4 and 10% was obtained in humans following inhalation of hGH powder particles (6). Confocal microscopy studies (5) showed that AM took up FITC-hGH that deposited in the deep lungs and that an important accumulation in AM cytoplasm became apparent 1h post-delivery. This result suggested that AM may also limit the pulmonary absorption of hGH even though hGH has a lower molecular weight than proteins like chorionic gonadotropin. Accordingly, this paper describes a study designed to quantify the role of AM in the pulmonary fate of hGH *in vivo*. The results are discussed in the light of data on macrophage clearance of other proteins from the airways.

MATERIALS AND METHODS

Depletion of Alveolar Macrophages

Male Wistar rats (10 to 14 week-old; 320 to 400 g; UCL breeding, Brussels, Belgium) were anesthetized with ketamine (Ketalar®, Pfizer, Brussels, Belgium) / xylazine (Sigma, Bornem, Belgium) (97, 5/2, 5 mg/kg) by intraperitoneal injection. Three hundred microliters of PBS, liposomal-clodronate suspension in PBS or liposome suspension in PBS were instilled in the trachea of rats using a syringe inserted *via* the mouth. Liposomes were prepared as previously described (4). Alveolar macrophages depletion resulting from clodronate (dichloromethylene diphosphonate or Cl_2MDP, Sigma, Bornem, Belgium) was assessed by analysing cellular components in broncho-alveolar lavage fluid (BAL) 48 h after liposome or PBS instillation (4).

Pharmacokinetic Studies

Catheters (polyurethane tubings, 3 French 0.6 mm ID x 0.9 mm OD, Access Technologies, Skokie, IL, USA) for blood sampling and intravenous injection (IV) were implanted in the jugular veins of rats 24 h before the pharmacokinetic study. The day of the study, the animals were anesthetized with intraperitoneal ketamine/xylazine (97, 5/2, 5 mg/kg) prior to hGH (Lilly; Indianapolis, USA) delivery by intravenous injection (IV, 100 μg/mL) or intratracheal instillation (IT, 600 μg/mL) using a 100 μL Hamilton syringe inserted *via* the mouth (Hamilton Bonaduz, Bona-

duz, Switzerland). All hGH solutions were prepared in phosphate buffer at pH 7.4. A total of 12 blood samples (200 μL) were collected from the jugular vein over 4 h (IV) or 7 h (IT) and replaced by an equivalent volume of sodium chloride 0.9% w/v to compensate for the decrease in blood volume. Plasma was separated by centrifugation and stored at −20°C until analysis. The hGH plasma concentrations were measured using an Enzyme Linked ImmunoSorbent Assay (ELISA, DSLabs, Texas, USA). The detection limit was 0.03 ng/mL and the inter-assay coefficient of variation was 6.5%. Plasma samples were assayed in duplicate at proper dilutions. Areas under the plasma concentration-time curve (AUC) were calculated using the linear trapezoidal rule from time 0 to 420 min. The maximal plasma concentration (Cmax) and the time to peak (tmax) were obtained from the individual concentration-time curves. Absolute bioavailabilities (Fabs) were calculated as $[(AUC_{IT} \times Dose_{IV}) / (AUC_{IV} \times Dose_{IT})] \times 100$ and the plasma elimination half-life (t1/2) after IV injection from ln2/k where k was estimated by linear regression of the last points of the log concentration versus time curve. Apparent clearances (CL) were calculated from [dose x Fabs] / AUC and the volume of distribution (Vd) from CL/k. These last two parameters were normalized to rat weight. All experimental protocols in rats were approved by UCL's Ethical Committee for Animal Care and Use.

Statistics

All results are expressed as mean ± standard error of the mean (SEM). The one-way analysis of variance (ANOVA) test and Tukey test were performed to demonstrate statistical differences ($P < 0.05$), using the software GraphPad Prism 4 for Windows.

RESULTS

Depletion of AM

In order to evaluate the role of AM in the absorption of hGH, we depleted AM from rat lungs by IT instillation of liposome-encapsulated Cl_2MDP. Selective depletion of macrophages from tissues *in vivo* has largely been used to study the involvement of macrophages in physiology, pathology or immunity (7). In the lungs of rats, it has been shown that AM numbers are significantly decreased within the first 24 h of liposome-encapsulated Cl_2MDP delivery and that AM number in BAL remained low for the next 36 hours (4). Repopulation of the lungs with AM occurred 5 days after delivery of the liposomes.

In our experiments, AM depletion was verified 48 h after intratracheal instillation of liposome-encapsulated Cl_2MDP, when AM number was at its lowest. A single IT instillation decreased AM number in BAL from 2.38 ± 0.13 million to 0.46 ± 0.08 million 48 h after delivery ($P < 0.001$; Figure 1A). As shown in Figure 2D, Cl_2MDP affected cell metabolism and induced apoptosis of cells (8). In contrast, animals treated with PBS or liposomes prepared with PBS showed normal populations and morphologies of AM 48 h after delivery (Figures 1A and 2A-C).

Intratracheal instillation of liposomes prepared with PBS or clodronate both caused a slight yet similar neutrophil influx into the airspaces ($P > 0.05$; Figure 1B). These data indicated that clodronate liposomes can be used to selectively and efficiently eliminate macrophages from the lungs, without causing significant inflammation.

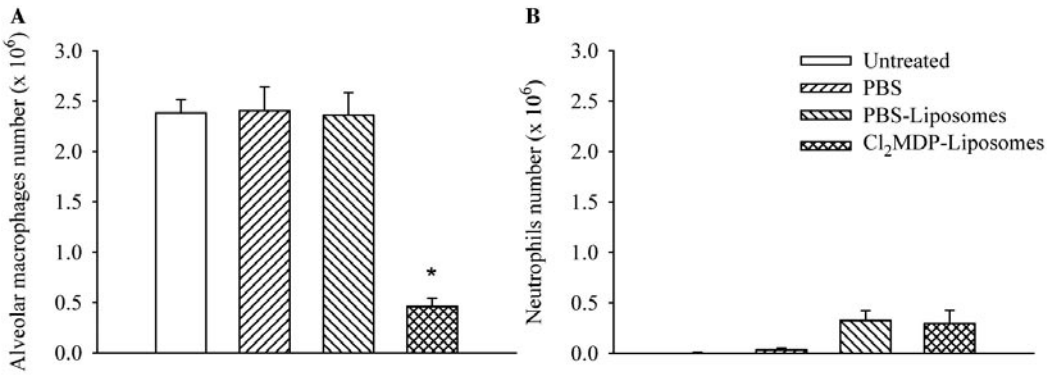

Figure 1. **Impact of the intratracheal instillation of liposome-encapsulated Cl_2MDP on (A) alveolar macrophage and (B) neutrophil numbers (n = 5) following BAL with 50 mL of Hank's Balanced Salt Solution. *Indicates a significant difference in AM number compared to the other groups (Tukey test, P < 0.001).**

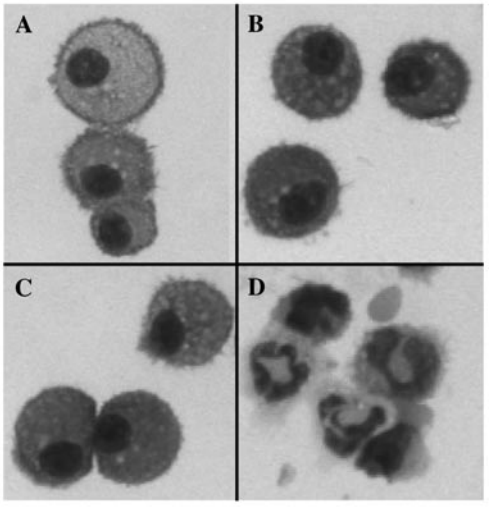

Figure 2. **Alveolar macrophages in bronchoalveolar lavage from (A) untreated (B) PBS-treated, (C) PBS liposome-treated and (D) Cl_2MDP liposome-treated rats (x 400).**

Impact of AM on hGH Pulmonary Bioavailability

The pulmonary absorption of hGH was evaluated following IT hGH administration to rats whose AM had been depleted by instillation of liposome-encapsulated clodronate 48 h earlier. The systemic absorption of hGH failed to show any significant alteration due to AM depletion (Figure 3 and Table 1). The absolute pulmonary bioavailabilities reached 11.4 ± 2.6%, 10.7 ± 2.0% and 9.0 ± 1.3% in Cl_2MDP liposome-, PBS liposome- and PBS-treated rats (Figure 3 and Table 1; P > 0.05). Plasma hGH concentrations peaked at 56 min when alveolar macrophages were depleted and at 106 min and 118 min when rat lungs were instilled with PBS-liposomes and PBS, respectively (tmax, Table 1; P > 0.05). Time to peak plasma concentration and bioavailability values suggest that hGH quickly crossed the alveolar epithelium and that depletion of AM using Cl_2MDP did not affect its pulmonary absorption. Moreover, these pharmacokinetic values are quite similar

in both liposomal treated-conditions, which suggests that intratracheal instillation of liposomes did not cause long-term permeability change to the epithelium.

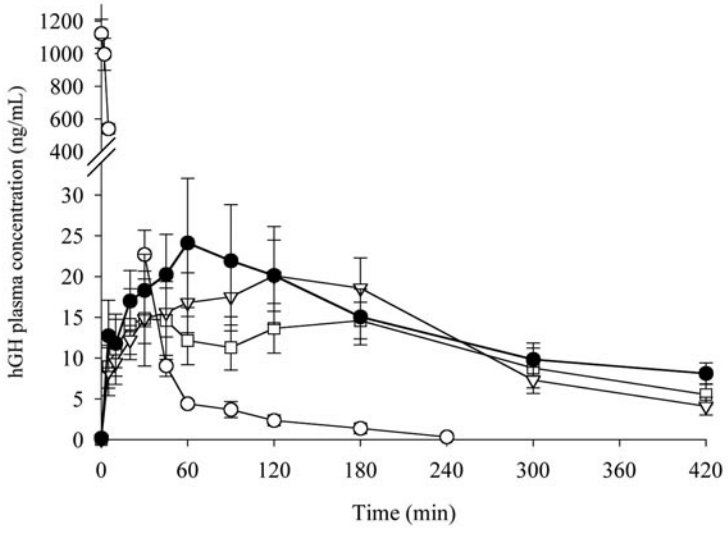

Figure 3. hGH plasma concentration-time curves following : intravenous injection of 100 µg/kg hGH (○, n = 7) or intratracheal instillation in rats pre-treated with Cl_2MDP-liposomes (●, n = 5), PBS-liposomes (▽, n = 5) or PBS (□, n = 5).

Table 1

Pharmacokinetic parameters.

Pre-treatment	-	PBS	PBS-liposomes	Cl_2MDP-liposomes
Delivery of hGH	IV[1]	IT[2]	IT	IT
Number of rats	7	5	5	5
Rat weight (g)	374 ± 8	353 ± 10	346 ± 2	369 ± 12
Dose/kg (µg/kg)	100	600	600	600
Dose/rat (µg)	37.4 ± 1.0	211.8 ± 6.0	207.6 ± 1.1	221.4 ± 7.0
C_0 or C_{max} (ng/mL)[3]	1122 ± 88	20 ± 5	22 ± 4	25 ± 3
t_{max} (min)[4]		118 ± 38	106 ± 35	56 ± 21
AUC $_{0 \rightarrow 420}$ (ng min/mL)[5]	8887 ± 513	4543 ± 708	5252 ± 981	5981 ± 794
CL (mL/min/kg)[6]	11.5 ± 0.7	12.0 ± 0.3	12.2 ± 0.1	11.5 ± 0.4
Vd (mL/kg)[7]	726 ± 97			
$t_{1/2}$ (min)[8]	43 ± 4			
F_{abs} (%)[9]		9.0 ± 1.3	10.7 ± 2.0	11.4 ± 2.6

[1]Intravenous injection. [2]Intratracheal instillation of a hGH solution. [3]Maximal plasma concentration. [4]Time to peak. [5]Area under the plasma concentration-time curve. [6]Clearance. [7]Volume of distribution. [8]Elimination half-life. [9]Absolute bioavailability. Vd and $t_{1/2}$ were not determined in IT groups because the elimination phase was not completely reached at the last sampling times.

DISCUSSION

AM Role in the Clearance of hGH Delivered to the Lungs

In this study, we showed that AM were not involved in a significant way in the clearance of hGH delivered to the lungs. We lowered the number of AM using liposome-encapsulated clodronate before pulmonary delivery of hGH *in vivo* in rats. Serum hGH levels were not affected by macrophage number and the absolute bioavailabilities reached approximately 10% in rats treated with clodronate or not (Figure 3 and Table 1). Plasma hGH concentrations peaked approximately 2 hours following the instillation of hGH. Because hGH is a small protein in comparison with hCG and IgG, it is more likely to be absorbed faster through the alveolar epithelium, perhaps preventing major uptake and degradation by AM.

An absolute bioavailability of 10% for the intratracheal instillation of hGH in rats means that only 10% of the dose of hGH deposited in the lungs reached the systemic circulation and that 90% of it was lost in the airway lumen or lung parenchyma. Alternative causes of protein losses after deposition in the lungs include enzymatic degradation, mucociliary clearance, aggregation with surfactant and/or binding to lung components (9, 10). The involvement of each of these mechanisms in the fate of pulmonary hGH remains to be quantified.

Mechanisms of Macromolecular Endocytosis by AM

Large proteins are endocytosed by macrophages in two general ways : phagocytosis (or "cell eating"), in which large particles (> 0.5 μm) are internalized, and pinocytosis (or "cell drinking"), which refers to the uptake of soluble macromolecules (< 0.5 μm) and fluids. Pinocytosis can occur from the fluid-phase (without binding to the plasma membrane), by non-specific adsorptive pinocytosis (through cell membrane binding, but with no specific receptors) and through recognition of specific sites on the plasma membrane (receptor-mediated endocytosis). When large inhaled macromolecules settle on the alveolar epithelium, their interaction with lung surfactant may lead to their agglomeration and opsonization (coating) by special proteins in the lung lining fluids which then mark the aggregated proteins for phagocytosis by AM (1, 9). However, Lombry et al. have shown that IgG and human chorionic gonadotropin delivered in solution to rat lungs remained principally in soluble form in the alveolar space, implying that these foreign proteins were endocytosed by AM by pinocytosis (4). Moreover, the process was unlikely to be receptor-mediated because these phagocytes have no receptor for human chorionic gonadotropin (4). Because of these observations, we hypothesise that AM engulf proteins by fluid-phase or adsorptive pinocytosis (11).

Relationship Between Protein Molecular Weight and Clearance by AM

We have previously reported a 2.3- and a 3.4-fold increase in pulmonary absorption of IgG and human chorionic gonadotropin, respectively, due to AM depletion in rats (4). However, similar to hGH, AM depletion failed to affect the pulmonary absorption of insulin (4). This suggests that AM hinder the transport of large proteins from the airway lumen into the bloodstream but have no impact on pulmonary absorption of small proteins and peptides. The dependence of AM

clearance on macromolecular weight might be explained quite simply by the relationship between the residence time in the airways and molecular weight. The time it takes peptides and proteins to peak in the blood after pulmonary delivery is molecular weight-dependent; the larger the size, the slower the absorption (1). Small peptides, such as insulin, which are quickly absorbed through the epithelium, are unlikely degraded by AM because of their short residence time within the alveoli. In general, paracellular diffusion across respiratory epithelia regulates the rate of transport into the bloodstream for molecules < 40 kDa and the rate of diffusion between epithelial cells decreases with increasing molecular size (12). Consequently, the higher the molecular weight of the macromolecule, the longer its time of residence and the longer its contact with, and uptake by, AM. Confocal laser scanning microscopy has in fact shown that FITC-IgG and FITC-human chorionic gonadotropin remained visible in the airspaces of rat lungs for at least 24 hours and 6 hours, respectively (4), after intratracheal instillation; while FITC-hGH and FITC-insulin disappeared from the alveolar spaces within 1 hour and 30 minutes postdelivery, respectively (4, 5). It should be noted that a large molecular weight does not systematically involve a long residence time and consequently a degradation by AM. Other mechanisms can affect the relative rate of transport of proteins through the epithelium. For instance, there is evidence that for certain endogenous molecules that normally occur in lung lining fluids, e.g. albumin, immunoglobulins and transferrin, there are specific receptor-mediated transport mechanisms on the alveolar epithelial cell that enable these proteins to be absorbed at higher rates than expected (1).

Other protein properties than molecular weight can affect their uptake by AM. For instance, some proteins are known to be receptor-mediated taken up by macrophages and thus are more likely to be degraded by AM than any proteins of the same molecular weight. Moreover, the physico-chemical properties of therapeutic proteins delivered to the lungs can affect their rate of endocytosis by AM. For instance, a global cationic charge of the protein will positively affect adsorptive endocytosis. These mechanisms could thus explain that a molecule of 50 kDa might be more significantly taken up by AM than a 100 kDa-one. Of note, all the proteins and peptides that we investigated are negatively charged at physiologic pH.

Protein Clearance by AM in Rats versus Humans

It is difficult to predict AM clearance of proteins in humans based on the data obtained in rats. Times to peak plasma concentrations of proteins following inhalation in humans are larger than in rats due in part to lower pulmonary blood flow in humans (7 mL/100 g body wt min^{-1} in humans versus 24 mL/100 g body wt min^{-1} in rats (13)). However, this does not necessarily mean that proteins that are insignificantly taken up by AM in rats (such as hGH), escape human macrophage endocytosis, because rates can be different between species (14).

Methods to Assess AM Clearance of Proteins Delivered to the Lungs

Macromolecules with different sizes have been delivered to the lungs of various animal species, including the monkey, and observed by microscopy to be internalized by AM (Table 2). Both confocal microscopy and electron microscopy have been used.

Table 2

Visualization of macromolecular uptake by AM in different animal species. From (6).

	MW	Animal species	Microscopy
Human growth hormone	22,000 Da	Rat	Confocal
Superoxides dismutases	33,000 Da	Baboon	Electron
Human chorionic gonadotropin	39,500 Da	Rat	Confocal
Ovalbumin	45,000 Da	Mouse	Confocal
Albumin	65,000 Da	Rabbit	Electron
Albumin	65,000 Da	Sheep	Electron
Albumin	65,000 Da	Rat	Confocal
Dextran	70,000 Da	Rat	Electron
Immunoglobulin	150,000 Da	Rat	Confocal
Ferritin	600,000 Da	Rat	Electron

Unfortunately, microscopy provides only a qualitative evaluation of AM uptake with no quantitative assessment of its likely impact on systemic absorption. In this regard, the data obtained with hGH are interesting. Confocal imaging revealed an intense uptake of FITC-hGH by rat alveolar macrophages within 1 hour of pulmonary delivery (5). Yet, the pharmacokinetic study presented in this article indicated that this had no significant impact on pulmonary absorption of hGH (Figure 3 and Table 1).

Measurement of proteins internalized in AM after BAL has been used as a way to assess AM involvement in alveolar protein clearance. Berthiaume et al. (15) instilled 100 mL of autologous serum labelled with ^{125}I-albumin into sheep lungs and measured the quantity of ^{125}I-albumin present in phagocytic cells of the airspaces up to 6 days postdelivery. The amount of ^{125}I-albumin present in AM was less than 1% of the instilled ^{125}I-albumin at all time periods and these authors concluded that AM played a minor role in alveolar protein clearance. However, it was shown later that BAL recovered only 15% of the AM population in rats (16). Therefore, measurement of AM uptake in cells recovered by BAL largely underestimates actual uptake. In addition, Berthiaume et al. (15) instilled large amounts of liquids in the lungs, which probably exceeded the endocytosis capacity of AM.

The use of AM-depleted animals allows the quantitative assessment of their role in the pulmonary clearance of proteins ((4); Figure 3 and Table 1). This method involves depletion by IT administration of liposome-encapsulated clodronate and a comparison of systemic absorption using AM-depleted and normal animals. Both clodronate, which has a short half-life in the circulation, and liposomes are considered to be effectively non-toxic in animals. Free clodronate does not easily cross either the phospholipid bilayers of liposomes or cell membranes in the body. However, liposomes and liposomes containing clodronate are eagerly swallowed by macrophages whose lysosomal enzymes release the drug (Figure 4). Once inside the macrophage, clodronate is trapped and metabolized into a toxic ATP analog which induces the apoptosis (Figure 2) (8). Free clodronate released from apoptotic macrophages is unable to penetrate other cells in amounts sufficient to disturb their metabolism.

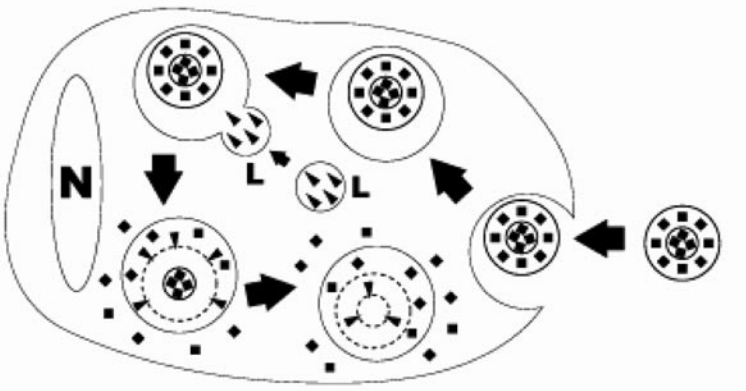

Figure 4. Macrophage "suicide". Liposomes, encapsulating Cl_2MDP molecules (squares), are ingested by macrophages *via* phagocytosis. After fusion with lysosomes (L), liposomal phospholipid bilayers are disrupted and Cl_2MDP is released within the cell (7).

An alternate approach to the use of AM-depleted animals can also be carried out, where surplus AM are intratracheally instilled in rats before a pharmacokinetic study (4). This approach can be used to assess the importance of increased local AM populations upon pulmonary macromolecular bioavailability.

Strategies for Enhancing Pulmonary Protein Bioavailabilities

AM depletion, for example, using liposomal clodronate, is not feasible in humans because the absence of AM would predispose individuals to infections and disrupt pulmonary physiology (17). Therefore, new strategies to prevent protein uptake by AM must be investigated. This represents a particularly challenging task because the methods should not compromise macrophage functions. A first strategy should probably entail protecting proteins from AM uptake. PEGylating peptides and proteins is well-known as a method of protecting proteins from enzymes and phagocytes, to prolong their residence in the body (18). An alternate approach may involve co-administration of ligands to compete with proteins for binding to the AM plasma membrane (11). Increasing the rate of protein transport across the alveolar epithelium is a second strategy. Methods that accelerate systemic absorption of macromolecules from the lungs using membrane permeation enhancers may have limited use due to safety concerns. Alternatives to chemical enhancers might exist that utilize specific active transport pathways in the lungs; for instance, fusion of proteins to the Fc domain of IgG has been claimed to enable efficient transport of the proteins across the airway epithelium via neonatal Fc receptor-mediated transcytosis (19).

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