Fate of aerosolized recombinant DNA-produced α₁-antitrypsin: Use of the epithelial surface of the lower respiratory tract to administer proteins of therapeutic importance

R. C. Hubbard*, M. A. Casolare, M. Mitchell†, S. E. Sellers*, F. Arabi‡, M. A. Matthey‡, and R. G. Crystal*§

*Pulmonary Branch and †Cardiac Surgery Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; and ‡Cardiovascular Research Institute, Department of Medicine and Physiology, University of California, San Francisco, CA 94143

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ABSTRACT To evaluate the possibility of administering therapeutic proteins via the respiratory route, we administered an aerosol of recombinant DNA-produced human α₁-antitrypsin (rAAT) to anesthetized sheep and measured levels of the protein in epithelial lining fluid (ELF), lung lymph, blood, and urine. Using a nebulizer that generated aerosol droplets with a mass median aerodynamic diameter of 2.7 μm (55% of droplets were <3 μm, a particle size optimal for deposition on the alveolar epithelium), in vitro studies demonstrated that the aerosolized rAAT molecule remained intact and fully functional as an inhibitor of neutrophil elastase. When aerosolized to sheep, the 45-kDa rAAT molecule diffused across the alveolar epithelium, as evidenced by its presence in lung lymph and in blood. Comparison of ELF, lymph, blood, and urine rAAT levels demonstrated that the process was concentration dependent, with highest levels in ELF and in descending concentrations with a 10-fold concentration differences in each consecutive compartment, respectively. Importantly, evaluation with aerosolized 125I-labeled rAAT demonstrated that the rAAT molecules that reached the lung lymph and the systemic circulation remained intact as a 45-kDa protein. These results demonstrate the feasibility of using aerosolization to the pulmonary epithelial surface to administer sizeable proteins of therapeutic interest, thus circumventing the necessity of the traditional parenteral modes of administration of such molecules.

The development of recombinant DNA technologies has made available a variety of human proteins that have potential as therapeutic agents in the treatment of human disorders (1–8). However, in contrast to the relative ease of production of recombinant molecules, one serious obstacle to their use is that as proteins they are susceptible to proteolysis in the gastrointestinal tract and are not absorbed through the skin, thus necessitating that they be administered by a subcutaneous, intramuscular, or intravenous route (1–8). This is not a problem for proteins used therapeutically only in acute situations such as tissue plasminogen activator (8), but it markedly complicates the use of recombinant proteins in the repetitive treatment of chronic disease. For example, recombinant growth hormone and insulin are routinely administered by the subcutaneous route (6, 7), and studies with recombinant interferon-γ, interferon-α, and granulocyte-macrophage colony-stimulating factor, erythropoietin, and interleukin 2 have necessitated subcutaneous, intramuscular, or intravenous routes of administration (1–5, 9, 10).

As an alternative approach to administration of recombinant proteins (and proteins in general, independent of the source), the present study was designed to capitalize on the enormous surface area of the lung as a potential absorptive surface through which proteins of therapeutic importance could gain access to the interstitium of the lung and plasma while remaining intact. This concept is based on the knowledge that (i) droplets of 3-μm diameter inhaled as an aerosol have the potential to reach the alveolar surface (11–14); (ii) the epithelial surface of the human lower respiratory tract is very large, typically 140 m² in adults (15); (iii) plasma proteins as large as 100 kDa are found in the epithelial lining fluid surface of the lung, suggesting that proteins can diffuse across the capillary endothelium, the alveolar interstitium, and the alveolar epithelium (16–19); and (iv) although there are no quantitative data available relating to the measurement of autologous natural or recombinant proteins from lower respiratory tract epithelial lining fluid to plasma, it is known that when solutions of heterologous proteins are instilled in the trachea of experimental animals, the protein can be subsequently detected in the pulmonary interstitium and blood (20–22).

As a model to evaluate the possibility of using the lower respiratory tract to administer proteins of therapeutic interest, we have examined the pulmonary absorption of an aerosol of yeast-produced human recombinant α₁-antitrypsin (rAAT), a 45-kDa single-chain polypeptide that functions as an inhibitor of neutrophil elastase (23). To evaluate the absorption of the molecule, we have used sheep, an experimental animal in which the pulmonary lymphatics can be sampled, thus permitting quantification of protein levels in the fluid of the alveolar interstitium, the first compartment that would be encountered by a protein moving from the epithelial lining fluid across the alveolar epithelial surface (20, 21). Using an aerosol generator capable of developing aerosol droplets of <3 μm, the data clearly demonstrate that aerosolized rAAT deposited on the epithelial surface diffuses across the alveolar epithelial surface, reaches lung lymph and eventually the blood, and remains intact in the process.

METHODS

rAAT Preparation. The rAAT used in these studies was produced by yeast transformed with an expressing plasmid containing a human cDNA encoding the mature normal M1 (Val213) human α₁-antitrypsin protein (23–25). The expressing plasmid pFATPOT was transformed into a Saccharomyces cerevisiae strain to yield zymosan-3, a diploid yeast strain containing the pFATPOT construct (Cooper Laboratories) (23). The rAAT was recovered from lysates of zymosan-3 and purified by sequential ion-exchange, affinity, and size-exclusion chromatography. The final preparation was >99% pure as assessed by SDS/polyacrylamide gel electrophoresis and

Abbreviations: rAAT, recombinant DNA-produced α₁-antitrypsin; 125I-rAAT, 125I-labeled rAAT; ELF, epithelial lining fluid.

†To whom reprint requests should be addressed: Pulmonary Branch, Building 10, Room 6D03, National Institutes of Health, Bethesda, MD 20892.
high-pressure liquid chromatography. The resulting rAAT had a molecular mass of 45 kDa, rather than the normal 52 kDa of human plasma-purified α1-antitrypsin, because of the lack of carbohydrates on the rAAT. The rAAT preparation functions normally as an inhibitor of neutrophil elastase, with an association rate constant (K_a) of 6.8 ± 0.8 M^{-1}sec^{-1} (12).

**Aerosol-Generating System.** To optimize the delivery of aerosolized rAAT to the lower respiratory tract, a nebulizer was used that maximized the generation of aerosol droplets in the <3-μm aerodynamic diameter range, a particle size appropriate for deposition in the alveolar regions (11–14). To accomplish this, 4 ml of a solution of rAAT at 25 mg/ml in physiologic saline (final osmolarity, 286 mosM) was placed in the reservoir of an Ultravent nebulizer (Mallinckrodt), which was driven at 40 psi (1 psi = 6.9 kPa) with compressed air. Evaluation of the aerosol by laser particle analyses carried out by S. P. Newman, P. G. D. Pellow, and S. W. Clarke (Royal Free Hospital, London) and by J. A. Williams and R. M. Platz (Stanford Research Institute) gave identical results. The aerosol generated was heterodisperse but with a narrow size distribution (SD, 1.3 μm), with 90% of the droplets between 0.5 and 5 μm. The droplet mass median aerodynamic diameter (MMAD) was 2.7 ± 1.3 μm. The aerosol was generated at a rate of 10 liters/min and contained ~240 μg of rAAT per liter of air in droplets of <2 μm MMAD (62% of the total mass of rAAT aerosolized) and 380 μg of rAAT per liter of air in droplets <5 μm MMAD (97% of the total mass of rAAT aerosolized).

To evaluate the possible effects of aerosolization on the overall structure and function of rAAT, the rAAT aerosol was collected by bubbling the nebulizer output through phosphate-buffered saline (pH 7.4), and the resultant fluid was concentrated by pressure filtration (UM10 membrane, Amicon). To evaluate the effect of aerosol on the structure of rAAT, pre- and postaerosol samples were applied to a 10% SDS/polyacrylamide gel, and the protein bands were stained with Coomassie blue. To evaluate the effect of aerosol on the function of rAAT, the K_a for human neutrophil elastase of pre- and postaerosol rAAT was measured by the method of Beatty et al. (26) with minor modifications of Ogushi et al. (27).

**Experimental Model.** Female mixed breed sheep (n = 15; 36 ± 3 kg) were used to quantitate the levels of rAAT in the lung interstitium, lung epithelial lining fluid (ELF), blood, and urine following aerosolization of the rAAT. The sheep were anesthetized with intravenous sodium thiopental, and the trachea was intubated with a cuffed endotracheal tube attached to a positive pressure ventilator. Animals were ventilated with a tidal volume of 12 ml/kg with 5 cm of H2O positive end expiratory pressure at a rate of 16 per min. Anesthesia was maintained with 1.0–1.5% fluorothane, 50% nitrous oxide, and 50% oxygen. Lung lymph was collected from the caudal efferent lymphatic duct cannulated with a heparinized silastic catheter by the technique of Staub et al. (28). Bronchoalveolar lavage fluid was obtained by lavage through a fiberoptic bronchoscope (Olympus) using a single 50-ml aliquot of normal saline. Blood samples were obtained from an indwelling catheter in the carotid artery, and urine samples were obtained from an indwelling bladder catheter.

All studies with the rAAT aerosol were performed with the closed chest animal in the prone position. After baseline bronchoalveolar lavage fluid, blood, lung lymph, and urine samples were obtained, 100 mg of rAAT was administrated by aerosol into the inspiratory limb of the ventilation circuit over a 25-min period. Bronchoalveolar lavage fluid, blood, lymph, and urine samples were then obtained at various intervals over a 48-hr period.

**Evaluation of the Amount and Form of rAAT in Vivo.** The levels of rAAT in sheep biologic fluids were quantified by a human α1-antitrypsin specific ELISA using an anti-human α1-antitrypsin antibody (Sigma) as described (23). All measurements were carried out in triplicate. The presence of sheep urine, plasma, lymph, or ELF did not alter the specificity of the ELISA for rAAT. Lymph, blood, and urine volumes are referenced to the volumes of the fluid analyzed, and the lower respiratory tract epithelial lining fluid levels are referenced to the volume of ELF recovered by bronchoalveolar lavage as assessed by the urea method (29). Aerosolization of rAAT did not result in significant inflammation or leakiness of the lower respiratory tract, as determined by differential cell counts and measurements of ELF volumes performed on pre- and postaerosol bronchial lavage fluids (data not shown).

The form of the rAAT in vivo was evaluated by using 125I-labeled rAAT (125I-rAAT), labeled by the chloramine-T method (final specific activity, 1 mCi/mg; 1 CI = 37 GBq; >93% of 125I incorporated in rAAT). The 125I-rAAT (1 mg total) was mixed with 100 mg of unlabeled rAAT, aerosolized as described above, and samples of sheep lymph and blood were obtained 4 hr later. As a control, 125I-rAAT was mixed with sheep lymph and plasma in vitro. All samples were then evaluated by 10% SDS/polyacrylamide gel electrophoresis and autoradiography.

**RESULTS**

**Effect of Aerosolization on the Form and Function of rAAT.** The creation of the rAAT aerosol with the compressed air-driven nebulizer did not alter the structure of rAAT or its ability to inhibit human neutrophil elastase (Fig. 1). After aerosolization, the rAAT remained as an intact 45-kDa protein (Fig. 1A). Importantly, the aerosolization process had no effect on the K_a of rAAT for human neutrophil elastase (P > 0.2, pre- vs. postaerosol) (Fig. 1B).

**Pharmacokinetics of rAAT Following Aerosolization in Sheep.** rAAT was readily measurable in ELF, lung lymph, and blood after aerosolization (Fig. 2). Peak ELF rAAT levels were observed at the first time point (0.5 hr) evaluated after aerosolization. The ELF rAAT level gradually declined over the following 48 hr, and no rAAT was measurable in the alveolar spaces at 48 hr.

### FIG. 1. Characterization of rAAT before and after aerosolization.

(A) SDS/polyacrylamide gel evaluation of rAAT before (lane 1) and after (lane 2) aerosolization. The size of the rAAT is indicated. (B) Association rate constant (K_a) of rAAT for human neutrophil elastase before and after aerosolization.

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*All data are presented as mean ± SEM and all statistical comparisons were made by the two-tailed Student's t test.*
Lung lymph rAAT concentrations lagged significantly behind ELF concentrations; the peak lymph concentration occurred 12 hr after aerosolization. After 2 hr, lung lymph rAAT concentrations were ~10 times less than the corresponding ELF rAAT concentration. By 48 hr postaerosol, small amounts of rAAT were still detectable in lung lymph even though no rAAT was found in ELF. Summation over the 48-hr period of the total rAAT recovered in lung lymph demonstrated 0.17% ± 0.02% of the total aerosolized dose.

Blood plasma levels of rAAT followed a time course similar to the lung lymph levels. On average, the plasma rAAT concentrations were ~10% of that of the lymph levels. Assuming a sheep total plasma volume of 3.6% body weight (30), at 12 hr (the time of maximal plasma concentration), the total rAAT in plasma was ~0.25% of the total aerosolized dose. In urine, rAAT was just at the detection limits of the assay (>1 ng/ml) at 12 and 24 hr; no rAAT was measured in the urine at any other time. Together, these data indicate that a portion of the aerosolized rAAT molecules must have been deposited in the lower respiratory tract, then passed across the alveolar epithelium into the alveolar interstitium and entered lung lymph, and ultimately reached the systemic circulation.

Form of rAAT in Lymph and Blood. After aerosol administration, not only did some of the rAAT molecules move across the epithelium to lymph and blood, but the molecules remained intact in the process (Fig. 3). In this regard, aerosolized 125I-rAAT was found in lymph and blood as an intact 45-kDa molecule, identical to 125I-rAAT prior to aerosolization and to 125I-rAAT combined with sheep lymph in vitro.

**DISCUSSION**

For the epithelial surface of the lower respiratory tract to serve as a potentially useful site for the therapeutic administration of proteins, it is necessary to demonstrate that the protein administered can actually be delivered in a manner feasible for administration to humans, that the protein remains intact and functionally active, and that once deposited on the epithelial surface the protein is absorbed through the epithelium and enters internal body compartments without undergoing alteration. As a model for this concept, we evaluated the pulmonary absorption of human α1-antitrypsin cDNA-directed yeast-produced rAAT administered by aerosol to sheep. The data demonstrate that it is feasible to generate an aerosol that delivers significant quantities of intact functional rAAT to the alveolar surface, and that, once deposited there, at least some of the protein diffuses from the alveolar surface to the lung interstitium and ultimately to the systemic circulation. Importantly, the aerosol process did not alter the form and function of the rAAT molecules, and the rAAT molecules did not undergo degradation during the process of deposition onto the alveolar epithelium and passage into the lung interstitium and the circulation. In this regard, these observations are significant for potential use of the aerosol route to administer proteins of therapeutic importance outside of the lung (e.g., insulin, growth hormone, hematologic growth factors, clotting factors) and for the therapy of pulmonary disorders, such as the potential use of rAAT for the specific therapy of the human hereditary disorder α1-antitrypsin deficiency.

The Lower Respiratory Tract as a Site for Administration of Therapeutic Proteins. Delivery of proteins to the alveolar surface requires that several physiologic obstacles be overcome, including the tortuosity of the upper air passages and the arborization of the bronchial tree, features that lead to substantial deposition of inhaled substances in the pharynx and proximal large airways, rather than in the alveoli (11–14). To maximize alveolar deposition, aerosol particles of 5–3 μm or less must be inhaled; particles of this size are sufficiently small to avoid inertial impaction at airway branch points and do not undergo significant gravitational settling (11–13). However, not all sizes below 3 μm can be used; while particles 0.5 to 3 μm are generally retained in the lung, particles <0.5 μm may escape pulmonary deposition entirely and leave the lung in expired air (12, 13).

We circumvented the natural physiologic obstacles against lower respiratory tract deposition by using a nebulizer capable of transforming a solution of rAAT into aerosol droplets, 55% of which were <3 μm, and because of the narrow droplet size distribution, most droplets were between 0.5 and 3 μm. In addition, use of an endotracheal tube for airway control and ventilation of the anesthetized animal likely contributed to the delivery of aerosolized rAAT to the lower respiratory tract. Importantly, the rAAT remained intact and completely functional after aerosolization, despite
the fact that the active inhibitor site of α₁-antitrypsin is easily oxidizable, and, when oxidized, the function of the α₁-
antitrypsin as an inhibitor of neutrophil elastase is reduced by a factor of 2000 (26). Furthermore, the mass of rAAT that can be aerosolized in this fashion is considerable. In this regard, 100 mg of rAAT was aerosolized within 25 min, a total amount that is likely within the range necessary for potential therapeutic applications and in a time reasonable for chronic administration on a daily basis.

Once delivered to the epithelial surface, the protein must gain access to the interstitium of the alveoli (for proteins destined for local therapy) and the blood (for proteins to act in the blood or in distant organs). The present study clearly demonstrates rAAT passes from the alveolar ELF to lymph and to blood, entering the bloodstream either directly via diffusion of rAAT into alveolar capillaries or by drainage of lung lymph into the venous system. Thus, a relatively large plasma protein can move in a direction opposite that thought to be its normal physiologic path, and the molecule remains intact in the process. Our data do not give insight into how this occurs, but it could occur with endocytic and/or cell junction permeability processes (20, 21, 31–34) and could be related, in part, to the fact that the rAAT is not glycosylated, in contrast to plasma α₁-antitrypsin that contains three asparaginyl-linked complex carbohydrates (23, 24). Consistent with our observations, there are several lines of evidence that the pulmonary “alveolar–capillary” barrier is permeable for proteins of considerable size.

First, morphologic and bronchoalveolar lavage studies have demonstrated intact plasma proteins in the ELF of the lower respiratory tract of experimental animals and humans (16–19, 31). The relative amount of these proteins in ELF in relation to their concentrations in plasma is dependent primarily on the molecular mass of the protein. In this regard, proteins in the 50- to 70-kDa range (such as albumin and α₁-antitrypsin) have an ELF concentration ≈10% that in plasma, while proteins in the 80- to 230-kDa range (such as hemopexin and ceruloplasmin) have concentrations in ELF 5–10% of their plasma concentration, thus indicating some sieving by the lung epithelial barrier in this molecular mass range. Consistent with this concept, very large proteins, such as α₂-macroglobulin (820 kDa) and IgM (900 kDa), are present in ELF in very low concentrations (16–19, 35).

Second, when proteins are infused intravenously to experimental animals, they are readily detected in lung lymph and ELF (20, 21, 28, 30, 36). Furthermore, when α₁-antitrypsin is infused intravenously to humans, the α₁-antitrypsin is readily detectable in the lower respiratory tract ELF at levels ≈15% that achieved in plasma (37). Likewise, when rAAT is infused into primates, the rAAT is readily detectable in lung ELF (23).

Third, when solutions of heterologous proteins are directly instilled into the tracheobronchial tree of experimental animals, some of these proteins can be detected in lung interstitial fluid and in plasma (20–22, 38). In addition, an aerosol of a nonautologous insulin to diabetic individuals resulted in lowering of blood glucose (39), suggesting that at least some protein with a molecular mass of 5 kDa can pass from the alveolar epithelium to blood and remain functionally intact in the process.

There are clear differences between the alveolar epithelial and endothelial “barriers” in the lung, with the epithelial barrier being much the tighter. It is estimated that the overall air-to-blood permeability of the lower respiratory tract to albumin, a protein with the same mass range as rAAT, is \( \approx 2 \times 10^{-9} \text{ cm/sec} \), with ≈90% of the total resistance to flow due to the epithelial barrier (21, 30). In accordance with this restricted permeability, ≈95% of radiolabeled albumin instilled directly into localized areas of the tracheobronchial tree in sheep remains in the lung at 4 hr and is slowly cleared into lymph (21). These observations are consistent with those of the current study, in which rAAT aerosolized into the entire sheep lower respiratory tract also leaves the alveolar surface gradually, and with studies of aerosolized human plasma α₁-antitrypsin to individuals with α₁-antitrypsin deficiency, demonstrating that twice daily administration of 100 mg of α₁-antitrypsin leads to increasing levels of α₁-antitrypsin in lower respiratory tract ELF (40). Thus, the epithelial surface of the lower respiratory tract is permeable to proteins like rAAT, but slowly. In this regard, the epithelial surface of the lower respiratory tract may serve as a “depot” that functions to slowly release proteins deposited by aerosol into the body—i.e., this natural epithelial “barrier” may serve as the equivalent of sustained slow release of the therapeutic protein to the body.

When compared to other epithelial surfaces, the lower respiratory tract has several characteristics that may be advantageous for drug administration. It has a vastly larger surface area (and thus a much larger potential absorptive surface) compared to the nasal, buccal, or rectal surfaces, sites that have been evaluated for administration of proteins (41, 42). In this regard, the use of the lower respiratory tract may obviate the need to augment absorption per unit area, as has been suggested for the nasal epithelium using absorption enhancers such as bile salts, surfactants, or acrylic resins (43–45). Furthermore, unlike the gastrointestinal tract, the lower respiratory tract surface is not a hostile environment to proteins. The lung does not normally contain large quantities of proteolytic enzymes and normally has an adequate antiprotease defensive screen (46), so that most administered proteins should be able to survive in the lower respiratory tract intact with their bioactivity maintained.

**Aerosolization of rAAT for the Therapy of α₁-Antitrypsin Deficiency.** α₁-Antitrypsin deficiency is a hereditary disorder associated with low serum and lung levels of α₁-antitrypsin, a molecule that normally protects the lower respiratory tract from neutrophil elastase, an omnivorous protease capable of degrading the matrix proteins comprising the alveolar interstitium (37, 47, 48). As a consequence of this deficiency state, the lung has an insufficient protective screen against neutrophil elastase, leading to gradual relentless destruction of lung. Although the deficiency of α₁-antitrypsin is systemic, since the lungs are the major site of the pathologic consequences of this hereditary disease in adults, direct targeting of α₁-antitrypsin to the lung is a rational form of treatment. In this regard, while weekly intravenous augmentation therapy for α₁-antitrypsin deficiency with human plasma-purified α₁-antitrypsin is biochemically efficacious (i.e., it augments lung α₁-antitrypsin levels adequate to protect the lung from accelerated destruction), it is inefficient because only ≈2% of the total administered dose actually reaches the lungs (37).

Although rAAT functions as well as plasma-derived α₁-antitrypsin as an inhibitor of neutrophil elastase, the rAAT has no carbohydrate side chains (23). As a result, rAAT undergoes rapid renal clearance when administered intravenously and, consequently, has a very short serum half-life, thus obviating the intravenous route for the chronic administration of rAAT for α₁-antitrypsin deficiency (23). In contrast, aerosol administration of rAAT would permit direct targeting of the rAAT to the site where it is needed and in doing so would avoid the problem of rapid renal excretion and would be highly efficient, with less waste of the administered protein. In this regard, the demonstration that rAAT administered by aerosol penetrates the lung epithelial surface and enters lung lymph supports this concept—i.e., the rAAT would be available to inhibit neutrophil elastase within the lung interstitium, thereby protecting alveolar interstitial connective tissue proteins from neutrophil elastase-mediated degradation.
In the context of these considerations, aerosol administration of α1-antitrypsin may represent an ideal approach to therapy of α1-antitrypsin deficiency. However, to achieve this goal, it will be necessary to demonstrate that enough αAAT can be delivered to the lower respiratory tract such that sufficient αAAT reaches the alveolar interstitium to adequately raise the anti-neutrophil elastase defenses to protect the lung from chronic proteolytic attack. In the present study, ≈1/500th of the administered dose was recoverable in the lung lymph, a value likely too low to provide adequate protection for α1-antitrypsin deficiency. However, this is likely a significant underestimate of the amount that would be delivered to the alveolar interstitium during actual aerosol therapy since (i) only a portion of the total lung lymph flow was recovered; (ii) at least as much αAAT was already present in plasma by 12 hr; (iii) only a single dose was given, in contrast to the chronic aerosol administration that would actually be used for therapy; and (iv) the actual amount of αAAT delivered to the lower respiratory tract is less than the administered dose, due to unavoidable loss of some aerosolized αAAT on various surfaces of the ventilatory apparatus. Unfortunately, for a number of technical considerations and because the human αAAT is viewed as antigenic by experimental animals (49), chronic experimental aerosol studies are not possible. In this regard, evaluation of the feasibility of achieving the necessary therapeutic lung levels of anti-neutrophil elastase protection will have to await human studies with αAAT aerosol therapy.

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