Mechanisms of alveolar protein clearance in the intact lung

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Mechanisms of alveolar protein clearance in the intact lung. Am J Physiol Lung Cell Mol Physiol 286: L679–L689, 2004; 10.1152/ajplung.00205.2003.—Transport of protein across the alveolar epithelial barrier is a critical process in recovery from pulmonary edema and is also important in maintaining the alveolar milieu in the normal healthy lung. Various mechanisms have been proposed for clearing alveolar protein, including transport by the mucociliary escalator, intra-alveolar degradation, or phagocytosis by macrophages. However, the most likely processes are endocytosis across the alveolar epithelium, known as transcytosis, or paracellular diffusion through the epithelial barrier. This article focuses on protein transport studies that evaluate these two potential mechanisms in whole lung or animal preparations. When protein concentrations in the air spaces are low, e.g., albumin concentrations <0.5 g/100 ml, protein transport demonstrates saturation kinetics, temperature dependence indicating high energy requirements, and sensitivity to pharmacological agents that affect endocytosis. At higher concentrations, the protein clearance rate is proportional to protein concentration without signs of saturation, inversely related to protein size, and insensitive to endocytosis inhibition. Temperature dependence suggests a passive process. Based on these findings, alveolar albumin clearance occurs by receptor-mediated transcytosis at low protein concentrations but proceeds by passive paracellular mechanisms at higher concentrations. Because protein concentrations in pulmonary edema fluid are high, albumin concentrations of 5 g/100 ml or more, clearance of alveolar protein occurs by paracellular pathways in the setting of pulmonary edema. Transcytosis may be important in regulating the alveolar milieu under nonpathological circumstances. Alveolar degradation may become important in long-term protein clearance, clearance of insoluble proteins, or under pathological conditions such as immune reactions or acute lung injury.

acute respiratory distress syndrome; endocytosis; diffusion; protein transport pulmonary edema

CLEARANCE OF SERUM PROTEINS from the alveolar space is an important process in recovery from pulmonary edema. Albumin and immunoglobulin G (IgG) are present in pulmonary edema fluid in concentrations that are 40–65% of plasma levels in hydrostatic pulmonary edema and 75–95% in lung injury pulmonary edema. Concentrations of albumin, for example, may be 5 g/100 ml or more. Protein concentrations rise even higher during the recovery phase from alveolar edema because the salt and water fraction of edema fluid is cleared much faster than albumin and IgG (6, 37, 58, 59, 72). High protein concentrations are significant because increased protein osmotic pressures slow alveolar fluid clearance. For example, soluble protein concentrations in alveolar liquid rose from 5.9 ± 0.4 g/100 to 10.2 ± 1.2 g/100 ml over 12 h after instillation of autologous serum into the distal spaces of sheep lungs, and calculated protein osmotic pressure in alveolar liquid increased from 40 to 53 cmH2O (57). Alveolar liquid clearance slowed from 8%/h in the first 4 h to 3%/h at the end of this period. In addition to limiting liquid clearance, highly concentrated alveolar protein may precipitate, necessitating clearance of insoluble as well as soluble protein. Inability to clear alveolar protein may play a role in poor outcomes after pulmonary edema. Patients dying with acute lung injury and the acute respiratory distress syndrome (ARDS) have large quantities of insoluble protein in their air spaces, and nonsurvivors of lung injury after ARDS have three times as much protein in their alveoli as survivors (1, 13). Accordingly, the mechanism of alveolar protein clearance is a physiologically and clinically significant problem.

Several mechanisms have been proposed for the removal of protein from the alveoli, including clearance by the mucociliary escalator, phagocytosis by macrophages, intra-alveolar catabolism, passive diffusion between cells in the epithelial barrier, and endocytic transport across the epithelial cells in vesicles, a process known as transcytosis. Wang et al. (83) recently showed that liquid microinjected into small groups of surface alveoli of gas-inflated lungs redistributed within seconds to adjacent alveoli. This movement appeared to be convective flow because it was unaffected by inhibitors of active fluid transport. Convection from alveoli to distal airways could
occur during initial alveolar flooding but would not necessarily contribute to clearance from the lung (65). It has yet to be studied as a means for alveolar protein clearance to our knowledge. In contrast, the other mechanisms have received considerable attention. For example, the rate of alveolar protein clearance is unchanged by the presence or absence of a cuffed endotracheal tube, indicating that the mucociliary escalator is an insignificant route for escape of protein from the lung (57, 59). Only small amounts of alveolar tracer protein are found in macrophages, and most alveolar protein reaches the bloodstream intact in the first few days after instillation of solutions of protein in the air spaces (5, 33, 57), suggesting minimal impact for these processes. The importance of the first three mechanisms has been discounted in acute alveolar protein clearance, as discussed in detail in previous review articles (25, 38, 56, 67). Thus paracellular diffusion and transcytosis across the distal lung epithelium remain the two most likely mechanisms for clearance of soluble protein from alveolar edema fluid in normal lungs and will be the focus of this review. Net diffusion of protein from the air spaces to the interstitium would be supported by the positive protein concentration gradient between alveolus and plasma, established by more rapid clearance of alveolar liquid than alveolar protein during the resolution of pulmonary edema. Macrophages and catabolism could play a role in long-term clearance of precipitated protein, which will be discussed in a later section.

A recent article by Kim and Malik (48) provided an excellent review of how transcytosis might apply to alveolar protein clearance. The article focused primarily on studies that have utilized monolayers of alveolar epithelial cells grown on permeable supports. After primary culture, alveolar type II epithelial cells form confluent monolayers that are joined by tight junctions and exhibit apical-basal polarity similar to the epithelium in vivo. The cells differentiate into type I-like cells within days and form a high resistance (>2,000 ohm-cm²) monolayer, providing a reasonable in vitro model for transport across the tight alveolar epithelium. Drs. Kim and Malik discuss evidence in favor of receptor-mediated transcytosis of albumin and IgG by rat alveolar epithelial monolayers, including asymmetric apical-basolateral vs. basolateral-apical transport, saturation, competition between labeled and unlabeled protein, and temperature dependence (49, 53, 54). Transport of albumin appeared to be mediated by gp60, the albumin glycoprotein binding protein (46), whereas FcRn, the IgG binding receptor, could carry IgG (22). Alveolar epithelial monolayers are advantageous for studying transcellular transport because of their high resistance. However, they comprised only one of the 40 or more cell types present in the distal lung, and they model only the type II cells of alveolar epithelial barrier with no components representing the endothelium or the bronchial epithelium. Consequently, data from monolayer studies may not present a complete picture of transport mechanisms active in the lung in vivo.

This review will focus on studies of alveolar protein clearance in whole animal or whole lung models under predominantly normal conditions. We are most interested in how clearance applies to the setting of resolution of alveolar edema. The main question is: are plasma proteins cleared in vivo predominantly by transcytosis or by paracellular pathways? Evidence will be presented to demonstrate that both processes operate. The problem is to determine which pathway is more important and their relative quantitative contributions under different conditions. The article will examine several characteristics that may differentiate the two mechanisms: size dependence, saturation kinetics, temperature dependence, and sensitivity to agents that manipulate the rate of endocytosis. The review will also briefly consider cell mechanisms of clearance of insoluble protein from the distal air spaces of the lung in vivo.

**VESICLES VS. CHANNELS**

Bignon and colleagues (7, 8) were the first of several investigators (17, 46) to report that alveolar type I and type II cells contained endogenous albumin and immunoglobulins in intracellular vesicles. Micrographs of protein in vesicles demonstrate that the alveolar epithelium is capable of endocytosis of serum protein but do not establish whether the protein is transported across the epithelium, indicate the direction of transport, or quantify how much protein is transported. Vesicles do not necessarily transport their contents across a barrier. Protein in vesicles could be returned to the alveolar space with no net transport across the epithelium, akin to type II cell recycling of surfactant phospholipid between intracellular and extracellular compartments (34). Williams (87) found that type II cells transported cationic ferritin from alveolar liquid to organelles of the secretory pathway, supporting the operation of recycling routes in the lung. Microscopy studies also provide no information about how the protein entered the alveolar vesicles. More recent work suggests that albumin may undergo receptor-mediated uptake through binding to gp60 on alveolar epithelial cells. John and colleagues (46) have shown that cultured pneumocytes internalize fluorescent albumin into plasmaemmal vesicles together with gp60 and that albumin and gp60 are located in caveolae, vesicles coated with the protein caveolin. Furthermore, cross-linking gp60 with anti-gp60 antibodies and secondary antibodies stimulates endocytosis of gp60 and albumin, whereas prolonged stimulation with the cross-linking antibody depletes the type II cell surface of gp60 and abolishes endocytic albumin uptake. Albumin undergoes receptor-mediated transcytosis bound to gp60 in pulmonary capillary endothelium (71). Thus gp60 could play a role in alveolar albumin clearance. The evidence concerning this hypothesis is discussed in the section PHARMACOLOGICAL MANIPULATION OF ENDOCYTOSIS to follow.

The alveolar epithelium is a tight epithelium and is the primary barrier restricting passage of solutes and water into or out of the alveolar space. Consequently, some authorities have questioned whether molecules as large as serum proteins can pass through the epithelium through paracellular channels (77). Direct microscopic observation of proteins passing through paracellular passages in the epithelium has not been reported, but the slow rate of transalveolar protein flux connotes that channels of sufficient size to transmit large macromolecules would be rare and exceedingly unlikely to be encountered by electron microscopy. We can demonstrate this point with a few simple calculations, using results from a study that modeled large pore frequency in the lung. Conhaim and coworkers (17) made theoretical calculations of the number and size of pores necessary to support the observed transport rates of various-sized hetastarch molecules across the alveolar epithelial barrier in rat lungs (Table 1). On the basis of their modeling work, we
estimate that one of these large pores would be found in the alveolar epithelium on average in a volume of $3.5 \times 10^{-10}$ ml (per pore). For comparison, we next calculate the volume of tissue that can be examined by electron microscopy. We use the micrographs by Conhaim’s team to examine transport of colloidal gold-labeled molecules in the lung epithelium as our example. Based on section thickness and micrograph cross-sectional area, each micrograph samples a tissue volume of $4.5 \times 10^{-13}$ ml. The volume corresponding to each micrograph is nearly a million times greater than the volume of one pore, so pore volume has little or no impact on how well the pores are assessed by electron microscopy. To conclude the problem, we note that the volume of alveolar epithelium containing one pore is nearly 100,000 times greater than the volume examined in one micrograph, $3.7 \times 10^{-8}$ vs. $4.5 \times 10^{-13}$ ml. Thus surveying a region of the epithelium that contained one of these pores might require taking tens of thousands of micrographs. Even then, the pore might not be readily apparent in the micrograph or might not be observed passing a labeled protein molecule due to the orientation of the pore. We conclude that electron microscopy has insufficient sensitivity to discover or rule out paracellular transport of macromolecules in the alveolar epithelium.

Most of the evidence in favor of paracellular alveolar protein clearance comes from physiological studies on factors related to diffusive or endocytic transport that will be described in later sections of this review. However, a few tracer studies also provide evidence in favor of passive diffusive transport. Conhaim and coworkers (15) filled isolated dog lobes with solutions containing a variety of tracers ranging in size from 1.2 to 405 nm in radius and measured concentrations in interstitial liquid. The 405-nm fluorescent polystyrene particles were not found in the interstitium, but 85-nm-radius latex beads and 95 nm-radius India ink particles crossed the epithelium. This model could be subject to the criticism that the stress imposed on lungs inflated to near total capacity with liquid could make the epithelial barrier leaky. However, the investigators had previously shown no difference in alveolar epithelial albumin permeability in lungs inflated with low volumes compared with lungs inflated to total lung capacity (16). Transcytosis of particles 85–95 nm in radius is unlikely because they are so large compared with the dimensions of endocytic vesicles. Vesicles typically have radii on the order of 25–75 nm (75). Williams (87) found some vesicles in the alveolar epithelium with radii as large as 130 nm but reported that the radius of the large majority of vesicles was <90 nm. Hastings and coworkers (39) reported that alveolar epithelial vesicles averaged 35 ± 2 nm in radii. Thus Conhaim argued that large pores must be present to provide a passage for their large tracers. These pores were localized to the distal airway epithelium in the region of the alveolar ducts, respiratory bronchioles, or their associated microalveoli (14). The presence of pores of adequate size shows that paracellular passage of alveolar protein is possible but says nothing about the contribution of this mechanism to alveolar clearance of serum proteins. Physiological studies described below help to delineate the relative importance of diffusion versus transcytosis.

**SIZE DEPENDENCE**

The rate of absorption from the alveoli in vivo is size dependent for most proteins. Effros and Mason (21) assembled measurements of alveolar epithelial protein permeability from many sources. The combined data demonstrated a consistent inverse relationship between permeabilities and the corresponding molecular weights (MW) (Fig. 1). Theodore and colleagues (79) reported that the alveolar epithelial permeabilities of sucrose, inulin, and dextran (60–90 kDa) in saline-filled dog lungs varied inversely with molecular size and directly with the free diffusion coefficient in water. Wagensteen and Yankovich (84) found a larger permeability-surface area (PS) product for sucrose than for albumin in rabbit lungs. Folkesson

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**Table 1. Theoretical volume occupied by one large pore in the alveolar epithelium vs. the volume examined by electron microscopy**

<table>
<thead>
<tr>
<th>Theoretical Number of Large Alveolar Epithelial Pores</th>
<th>f = 1 pore/7 alveoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of pores/alveoli*</td>
<td>$A = 3.0 \times 10^7$</td>
</tr>
<tr>
<td>Alveoli/lung†</td>
<td>$n_p = A \times f = 4.3 \times 10^5$</td>
</tr>
<tr>
<td>Alveolar Volume Containing One Pore</td>
<td>$V_a = 0.161$ ml</td>
</tr>
<tr>
<td>Alveolar epithelial volume (per one lung)‡</td>
<td>$V_a/pore = V_a/n_p = 3.7 \times 10^{-8}$ ml</td>
</tr>
<tr>
<td>Volume/pore</td>
<td>$T = 100$ nm = $1 \times 10^{-5}$ cm</td>
</tr>
<tr>
<td>Tissue Volume Sampled by Electron Microscopy</td>
<td>$M = \pi \times 100,000$</td>
</tr>
<tr>
<td>Section thickness*</td>
<td>Area_{tiss} = 23.5 cm x 19 cm</td>
</tr>
<tr>
<td>Magnification (1 cm = 0.1 μm)*</td>
<td>$= 2.35 \times 10^{-4}$ cm x $1.9 \times 10^{-4}$ cm</td>
</tr>
<tr>
<td>Micrograph area</td>
<td>$= 4.5 \times 10^{-8}$ cm²</td>
</tr>
<tr>
<td>Tissue Area*</td>
<td>$V_{tiss} = T \times \text{Area}_{tiss} = 4.5 \times 10^{-13}$ ml</td>
</tr>
<tr>
<td>Tissue volume examined</td>
<td>$r = 17$ nm = $1.7 \times 10^{-6}$ cm</td>
</tr>
<tr>
<td>Large Pore Volume</td>
<td>$L = 0.7$ nm = $7 \times 10^{-8}$ cm</td>
</tr>
<tr>
<td>Pore radius*</td>
<td>$V_p = \pi \times r^2 \times L = 6.6 \times 10^{-19}$ ml</td>
</tr>
<tr>
<td>Pore length*</td>
<td>Ratio</td>
</tr>
<tr>
<td>Epithelial Volume Containing One Pore versus Volume Examined by Microscopy</td>
<td>$3.7 \times 10^{-8}$ ml vs. $4.3 \times 10^{-13}$ ml</td>
</tr>
<tr>
<td>V_a per pore vs. V_{epith}</td>
<td>82,000:1</td>
</tr>
</tbody>
</table>

Table 1. Theoretical volume occupied by one large pore in the alveolar epithelium vs. the volume examined by electron microscopy. From Conhaim et al. (17); †From Tenney and Remmers (78); ‡From Harris et al. (35).
and colleagues (27, 28, 30) found that the absorption of intratracheally instilled proteins and peptides in unanesthetized rats and pigs varied inversely with MW. In a study of pulmonary absorption of different-sized fluorescein isothiocyanate (FITC)-labeled dextrans, Ohtani and colleagues (66) found that rate of absorption decreased with increasing molecular size. Conhaim and colleagues (17) found that transport of different-sized hetastarch molecules out of the alveoli could be modeled by a system of epithelial pores of at least two sizes, 5 and 17 nm in radius, allowing size-dependent variation in the rate of passage. Hastings and colleagues (37) reported an inverse relationship between the Stokes radius and the amount of passage across upper airway epithelium, saturation kinetics do not necessarily be size dependent. Fluid-phase endocytosis begins with vesicles formed from caveolae, clathrin-coated pits that close around fluid adjacent to the cell. The protein concentration in the vesicle would be expected to match the concentration of the outside fluid regardless of size, unless other factors such as unstirred layer effects or steric hindrance to caveolar access imparted sieving properties. Experimental studies support independence of size for fluid phase endocytosis in the distal lung epithelium. Matsukawa and colleagues (52) reported that 70-kDa dextran and 150-kDa dextran particles undergo pinocytosis across alveolar epithelial monolayers at the same rate, whereas Williams (87, 88) observed that vesicles in alveolar epithelial cells in vivo took up similar amounts of 70-kDa dextran and two lectins with limited epithelial binding, concanavalin A (108 kDa) and Wisteria floribunda agglutinin (58 kDa).

There are reasons to expect that the rate of receptor-mediated endocytosis should not depend on size either. Receptor binding can concentrate proteins in the vesicle above concentrations in the extracellular fluid, so the transport rate may be greater than expected for ligand size, as is the case for hGH (24, 68, 69) and other proteins. For example, vasoactive intestinal peptide (VIP, MW 3,450), which undergoes receptor-mediated endocytosis, appears to escape the lungs with a half-time of 19 min, nearly as fast as pertechnetate (MW 163) at 10 min and almost 10 times faster than diethylene triamine pentaaetacetate (MW 492), a much smaller molecule (3). The calculated kinetic constant for VIP is $3.7 \times 10^{-2}$ min$^{-1}$, nearly an order of magnitude greater than would be predicted for its size (see Fig. 1). Thus the observation that albumin and IgG permeate the alveolar epithelial barrier at a rate expected for their size is consistent with a paracellular mechanism but would not necessarily be anticipated for clearance by transcytosis.

**SATURATION KINETICS**

When receptors become fully occupied by ligand, increases in concentration do not increase the rate at which ligand is taken up by endocytosis. Thus lung protein clearance by receptor-mediated endocytosis or transcytosis should exhibit saturation kinetics. hGH clearance demonstrates this relationship (24). The appearance of hGH in the blood circulation after lung instillation increases nonlinearly with instilled dose (Fig. 2A) (24), consistent with clearance, at least in part, by a receptor-mediated mechanism. Albumin transport across the upper airway epithelium follows saturation kinetics also and may therefore depend on an endocytic transport process (47). The half-maximal flux across bronchial epithelium occurs at $\sim 1$ g/100 ml albumin. However, unlike the situation for passage across upper airway epithelium, saturation kinetics do...
Temperature, generally a Q10. On the other hand, endocytic transport requires energy by the Q10, the ratio of the rates of a biological process with a constant. Temperature dependence is described conveniently the dimensions of the channel and the molecule remained 40% for a fall in temperature from 37 to 17°C.

The rate of diffusion would decrease by a factor of 10 for a 10°C change in temperature. The Q10 for diffusion would be 100. Temperature dependence at higher concentrations was consistent with paracellular transport.

TEMPERATURE DEPENDENCE

The magnitude and pattern of temperature dependence distinguish between endocytic and diffusive transport. Diffusion through aqueous channels slows as temperature decreases due to increases in the viscosity of water and a decrease in the diffusion coefficient. The rate of diffusion would decrease by ~40% for a fall in temperature from 37 to 17°C, assuming that the dimensions of the channel and the molecule remained constant. Temperature dependence is described conveniently by the Q10, the ratio of the rates of a biological process with a 10°C change in temperature. The Q10 for diffusion would be ~1.6. On the other hand, endocytic transport requires energy and should demonstrate a much greater dependence on temperature, generally a Q10 >2. Furthermore, endocytic uptake is abolished at a temperature between 10 and 20°C (63). Thus a plot of the rate of transcytosis as a function of temperature should show an inflection point somewhere in that range. In contrast, diffusive transport should demonstrate a monotonic relationship with temperature.

Hostetter and colleagues (41) aerosolized 125I-albumin, total albumin concentration 0.02 g/100 ml, into isolated rabbit lungs and compared clearance at two temperatures, 37 and 12°C. Clearance decreased 89% between the two temperatures (Q10 = 2.4), much more than the 40–50% decrease expected for diffusion above. Thus these data suggested that transport of low concentrations of albumin out of the alveolar space occurred by transcytosis.

John et al. (46) examined the effects of concentration and temperature on tracer albumin clearance. The investigators studied the effects of temperature on clearance of 0.05 g/100 ml albumin, representing saturable transport, and 5 g/100 ml albumin, the nonsaturable component. At 0.05 g/100 ml albumin, the PS product for 125I-albumin decreased slightly over 50% when temperature was reduced from 37 to 27°C, a Q10 of 2.1. At 5 g/100 ml, the PS product fell by ~40%, a Q10 of 1.6. Thus temperature sensitivity was greater at the lower albumin concentration, corresponding to saturable transport (see previous section SATURATION TRANSPORT), than at the higher concentration corresponding to nonsaturable albumin clearance. In the opinion of the authors, the temperature dependence at higher concentrations and the absence of saturation were both consistent with albumin transport by a paracellular route. In summary, the Hostetter study and the John study both found temperature sensitivity consistent with energy-dependent transport, such as transcytosis, at low albumin concentrations, 0.02–0.05 g/100 ml. Temperature dependence at higher concentrations was consistent with a passive process, such as paracellular diffusion.

PHARMACOLOGICAL MANIPULATION OF ENDOCYTOSIS

Effects of monensin and nocodazole on albumin transport. Several investigators have evaluated the role of transcytosis in alveolar protein clearance by attempting to increase or decrease the rate of endocytosis in vivo with pharmacological agents.
Hastings and colleagues (39) studied the effects of monensin and nocodazole on alveolar clearance of albumin and IgG in anesthetized rabbits. The rationale for this study was that inhibiting endocytosis should block alveolar protein clearance if endocytosis is the major pathway. Monensin is an ionophore that interferes with vesicular Na+/H+ exchange. It blocks acidification of endocytic compartments and disrupts recycling of membrane components to the plasma membrane, thus inhibiting endocytosis (61, 76). Nocodazole reversibly disrupts microtubules, which are necessary for vesicle translocation across the cell. It inhibits transcytosis in Madin-Darby canine kidney epithelial cells, Caco-2 intestinal epithelial cells, and tracheal epithelial cells (12, 19, 55).

The investigators performed four control experiments to demonstrate that monensin and nocodazole were active in whole lung and that they effectively reduced endocytosis: 1) electron microscopy with morphometric techniques demonstrated that the inhibitors increased the numerical density of vesicles in the alveolar epithelium and capillary endothelium. Thus the two drugs increased the size of internal membrane compartments as expected if membrane traffic were disrupted. 2) The inhibitors were studied for effects on clearance of surfactant apoprotein A (SP-A) from the alveolar space. SP-A is a positive control for endocytosis inhibition because type II cells remove SP-A from the air spaces by receptor-mediated endocytosis. SP-A binds selectively to the apical surface of type II cells in a dose-dependent and saturable fashion (70, 89). After internalization, the protein dissociates from its receptor and progresses through vesicles, endosomes, multivesicular bodies, and lamellar bodies (70, 90). Eventually, SP-A is recycled back to the alveolar space (73, 90). Monensin increased the quantity of SP-A associated with the lung tissue after 2 h (Fig. 3). Thus monensin inhibited recycling of SP-A from the epithelial cells to the air spaces by either blocking dissociation from the receptor or interfering with intracellular sorting. In either case, the action was consistent with the pattern expected for a compound that inhibited endocytosis (3).

Endocytosis of FITC-labeled albumin uptake was studied in cultured alveolar epithelial cell monolayers. Monensin and nocodazole reduced uptake by 20–50% over 4- and 8-h experiments, verifying that they were active against alveolar epithelial cells (4). Immunohistochemical techniques were used to evaluate the effect of inhibitors on parenchymal uptake of 5 g/100 ml human albumin from the air space. Albumin immunoreactivity appeared in the epithelium in the absence of inhibitors but was not found in alveolar epithelium treated with monensin or nocodazole (Fig. 4). Because the inhibitors would not block paracellular transport, monensin and nocodazole must have blocked endocytic uptake of albumin. In summary, the control experiments demonstrate that the two drugs blocked transport by cultured alveolar epithelial cells and disrupted alveolar membrane traffic in whole lung. Furthermore, monensin and nocodazole reduced transport of SP-A and albumin, indicating that they inhibited endocytosis of protein by the alveolar epithelium in vivo.

The studies were completed by investigating the effects of the endocytosis inhibition on alveolar clearance of 125I-IgG and 131I-albumin. The tracers were instilled into the lungs in autologous plasma. Thus concentrations of unlabeled protein were high, approximately equivalent to concentrations in lung injury pulmonary edema fluid. There were no significant differences in the quantities of the two tracer proteins recovered in lung or appearing in the plasma among control animals and animals receiving monensin or nocodazole (Fig. 5). In other words, alveolar clearance of albumin and IgG occurred at the
same rate whether or not alveolar endocytosis was inhibited. Therefore, a pathway other than transcytosis, possibly paracellular transport, must be important for clearing of serum protein in high concentrations from the alveolar space in normal lungs. A possible location for this pathway was suggested by the micrograph from a lung treated with nocodazole in Fig. 4B. The nocodazole had inhibited albumin uptake by the parenchyma, but albumin immunoreactivity was still present in the interstitium of a small airway. Nocodazole is known to inhibit albumin endocytosis by airway epithelium (19). Thus the albumin must have reached the airway interstitium by crossing a paracellular channel in the airway epithelium proximal to the alveolar barrier. Furthermore, no such channel was apparent at the alveolar level. This observation is consistent with the report of Conhaim et al. (15) that proximal airway epithelium contains pores large enough for passage of particles up to 94 nm in radius.

Effects on transport of riboflavin-albumin vs. albumin. Wangelsteen and colleagues (85) examined the effect of monensin and nocodazole on alveolar transport of bovine serum albumin (BSA), riboflavin-conjugated albumin (riboflavin-albumin), and sucrose. Riboflavin-albumin was tested because many cells possess riboflavin receptors (40), and the authors believed that the conjugate might undergo receptor-mediated transcytosis utilizing this receptor. In fact, tracer [3H]riboflavin-albumin was cleared twice as fast as [3H]albumin when instilled into the lungs of anesthetized rats in 0.5% unlabeled protein, indicating that it was transported differently from unconjugated albumin. In isolated perfused rabbit lungs, nocodazole and monensin decreased the PS product of riboflavin-albumin by >50%, consistent with absorption by transcytosis mediated by riboflavin receptors. The PS product of sucrose, a marker for paracellular transport, and the PS product for albumin were unaffected by the endocytosis inhibitors. Thus transcytosis was quantitatively important for riboflavin-albumin but did not play an important role in alveolar clearance of 0.5% albumin. The conclusion of this study was similar to the one reached by Hastings et al. (39).

Stimulation and inhibition of gp-60-mediated albumin uptake. John et al. (46) used pharmacological interventions to investigate the role of gp60 in alveolar clearance of albumin in isolated perfused rat lungs. They incorporated the effects of concentration in their experiments because they had demonstrated the presence of saturable and nonsaturable albumin transport pathways as discussed earlier. The experiments employed interventions both to stimulate and to inhibit gp60-mediated endocytosis.

The intervention to stimulate endocytosis was to instill gp60 primary antibodies and secondary antibodies into the lung with tracer 125I-albumin plus low and high concentrations of unlabeled albumin. This treatment crosslinks gp-60 and stimulates endocytosis of albumin in cultured endothelial cells (60) and cultured type II cells (46). At low unlabeled albumin concentrations, 0.05 g/100 ml, gp60 cross-linking stimulated 125I-albumin PS product by 58%. At high concentrations, 5 g/100 ml, cross-linking had no effect. The intervention to inhibit endocytosis was to instill intratracheal filipin, a lipophilic polyene antibiotic that binds to cholesterol. Filipin prevents gp60-activated vesicle formation in endothelial cells (60). In cultured type II cells, filipin depletes caveolae, the vesicles that carry gp60 and albumin, within minutes of treatment (46). In the isolated lung experiments, filipin reduced the PS product for 125I-albumin by 40% when the air space levels of unlabeled albumin were low. The antibiotic did not affect clearance of tracer albumin at high levels of unlabeled carrier, nor did it alter the PS product for sucrose, a marker for paracellular transport. Thus stimulation of endocytosis augmented alveolar albumin clearance, and endocytosis inhibition reduced clearance at low albumin concentrations, conditions in which saturable transport predominates. Transport at high concentrations was unaffected by manipulating the rate of endocytosis. On the basis of the saturation kinetics, the temperature dependence described earlier, and the effects of pharmacologically manipulating endocytosis, the authors proposed that alveolar albumin permeation occurred by gp60-mediated transcytosis of albumin at nonsaturating albumin concentrations. At higher concentrations, they suggested that the transport mechanism was a paracellular pathway. The three studies, by John et al. (46), Hastings and coworkers (37), and Wangelsteen and colleagues (85), are consistent with each other. All three found no effect of endocytosis inhibitors on alveolar albumin clearance with high concentrations of alveolar albumin. John and coworkers provided data that allow calculation of the approximate capacity of the transcytosis mechanism relative to the paracellular route. They measured clearance of 125I-albumin at low and high carrier albumin concentrations.
Approximately 35% of the tracer albumin was cleared from the lung in 1 h when the carrier albumin concentration was 0.05 g/100 ml, but the investigators estimated that only 25% was cleared by the saturable route. Thus the capacity of the transcytosis pathway to clear albumin in 1 h was \(-0.5 \text{ mg} \times 0.25 = 0.125 \text{ mg}\). At 5 g/100 ml albumin, conditions saturating the receptor-mediated process, 90% remained at the end of the hour. In this case 50 mg \times 0.1 = 5 \text{ mg} was cleared from the air spaces. Thus when high concentrations of albumin are present in the alveolar space, as with pulmonary edema, transcytosis accounts for 2.5% of the total albumin clearance and paracellular pathways for the other 97.5%.

**ALVEOLAR DEGRADATION**

*Alveolar macrophages.* Alveolar macrophages are well known for their capacity to endocytose and degrade exogenous protein and peptides (74). Their endocytic capacity is greater than that of type II alveolar epithelial cells (36). However, alveolar macrophages do not appear to play a major role in clearing protein over 24–48 h from the alveoli of the normal lung. For example, the number of alveolar macrophages increased by 10-fold over 24 h after autologous serum was instilled into sheep lungs, but the rate of protein clearance remained constant during this period (5, 57). Less than 10% of the instilled \(^{125}\text{I}\)-albumin was associated with alveolar macrophages 24 and 48 h after the instillation (5). Alveolar macrophages may become important in alveolar protein clearance after 48 h. Berthiaume and colleagues (5) followed alveolar protein clearance in awake sheep for 6 days after instilling \(^{125}\text{I}\)-albumin into the lungs. The influx of macrophages into the air spaces increased substantially between the second and sixth days. Increased quantities of protein tracer were associated with macrophages by the fourth day, and phagolysosomes with staining characteristics similar to that of the instilled serum were present in the macrophage cytoplasm. On the sixth day, the quantities of protein-free \(^{125}\text{I}\) in lung lavage, urine, and feces increased four- to fivefold compared with the quantity at 48 h. Thus as time progressed, resident cells in the air spaces (and/or alveolar epithelial cells) appeared to catabolize more proteins. Alveolar macrophages have been demonstrated to be important for the clearance of SP-A in rabbit lung (81). In summary, macrophages may not play a major role in the short-term removal of excess protein from the intact lung, but they may be more important for long-term protein clearance or for clearance of surfactant apoproteins. The role of alveolar macrophages in the injured lung has not been studied yet.

*Peptidases and proteases.* Catabolism by the airway epithelial cells with absorption of smaller protein fragments was first proposed as a mechanism for alveolar protein clearance by Drinker and Hardenbergh (20). Various peptidases, such as neutral endopeptidase and cathepsin H, are present on the apical surface of the airway and alveolar epithelium and may be important for clearing small peptides. (45, 64). Morimoto and colleagues (62) suggested that aminopeptidases in the apical cell plasma membranes of the alveolar epithelial cells cultured on porous substrates could degrade a model dipeptide, glycyl-L-phenylalanine. Protein might also be degraded after endocytosis and transport to lysosomes in the epithelial cells. Intra-alveolar degradation is not a major clearance mechanism in the uninjured lung for most proteins, including serum proteins. The vast majority of studies indicate that \(>95\%\) of the protein instilled into the distal air spaces of the normal lung reaches the blood circulation intact (4, 5, 27, 28, 31, 33, 57). However, peptidases and lysosomes could participate in metabolism of specific peptides or larger proteins, such as VIP, gastrin, or insulin. VIP is cleared from the air spaces more rapidly than expected for its size and is completely degraded during transit across the pulmonary epithelium into the vascular spaces (3). Gastrin is also degraded in the passage from the air spaces into blood (R. H. Hastings, unpublished data). Although cultured pneumocytes degrade substantial quantities of insulin (50), insulin is cleared intact at a rate consistent with its size relative to larger proteins in rabbit lungs (37).

**IMMUNITY**

If proteins are not handled appropriately at the membrane, they may become antigenic and trigger local and systemic immune and inflammatory responses. Folkesson and colleagues (29) investigated whether a prior immunization with BSA altered the alveolar epithelial permeability of a closely related protein, human serum albumin (HSA). The investigators picked HSA as the bystander protein for estimating permeability because it was similar in most characteristics to BSA, the antigen. Alveolar epithelial HSA permeability increased when animals with anti-BSA IgG in their air spaces were challenged with BSA. Thus high levels of circulating specific antibodies and antigen may influence alveolar epithelial permeability to protein in general. Other investigators have suggested that a preceding immunization may reduce or even totally inhibit the passage of the specific antigen from the alveolar space to the systemic circulation (9–11, 32, 42, 80). These effects may be attributed to increased degradation of the antigen and/or reduced penetration across the epithelial barrier due to the increased size of immune complexes compared with antigen alone. Significant levels of protein are present in bronchoalveolar lavage fluid after sensitive asthmatics are exposed to allergen (23). One implication of increased nonspecific protein permeability after allergen challenge is that it may expose sensitive asthmatics to other potential antigens, resulting in development of additional allergies unrelated to the original disease.

**INSOLUBLE PROTEIN CLEARANCE**

This review has focused on the mechanisms for removal of soluble protein from the distal air spaces of the lung. From a clinical perspective, hydrostatic pulmonary edema and increased permeability pulmonary edema (acute lung injury) represent the most important clinical settings in which large quantities of soluble protein collect in the distal air spaces of the lung. In the presence of severe acute lung injury, some of the protein in the air spaces of the lung precipitates out of solution and forms an insoluble matrix in conjunction with extracellular matrix proteins and fibrin. Microscopically, the insoluble protein is identified pathologically as hyaline membranes, a classic hallmark of diffuse alveolar damage in patients with acute lung injury. Recent work indicates that the mechanism for accumulation of the insoluble protein components in the distal air spaces of the lung is probably a product of both procoagulant and proinflammatory mechanisms that are activated in the presence of acute lung injury (43, 86).
Removal of the insoluble protein fragment will be much slower than the removal of soluble protein components. Alveolar macrophages probably play a primary role in the removal of insoluble proteins, matrix, and cell debris, although there is very little quantitative information regarding this process. Observational studies in experimental conditions, such as bleomycin-induced lung injury (26), indicate that the process probably requires weeks for removal of insoluble protein in the distal air spaces of the lung. The resolution process may also be complicated by the presence of intra-alveolar fibrosis and potentially by a loss of capillary blood flow and destruction of some lung lymphatics.

With the availability of new therapies that reverse the procoagulant environment and potentially stimulate fibrinolysis, it is possible that this clearance process of removal may be accelerated. Conceivably, the use of activated protein C, for example, might reverse the procoagulant environment, limit deposition of insoluble protein in the air space in the lung, and potentially activate fibrinolytic mechanisms that may lead to a more rapid resolution of hyaline membranes and insoluble protein complexes in the distal air spaces of the lung (43, 86). Thus, although we have learned a great deal about the mechanisms for removal of the salt and water fraction of the edema fluid from the distal air spaces of the lung (58), as well as the mechanisms for removal of soluble protein (as discussed in this review), much more information is required to understand the intra-alveolar protein deposition that results in widespread insoluble protein fragments deposited in the distal air spaces of the lung.

**Future directions.** Protein transport has been studied extensively in normal lungs over relatively short periods, but pathological states, clearance of precipitated protein, and therapies have received little attention. Future studies might explore protein elimination in disease, clearance mechanisms for insoluble protein, and the impact of therapies on accelerating liquid and/or protein clearance. For example, the previous section mentions possible benefits of reversing the procoagulant state in the alveolar space. Other potential therapies include proteins and peptides that open tight junctions for small solutes and macromolecules (18, 44, 51). Such agents could be investigated for their ability to speed alveolar protein clearance, although opening tight junctions could slow liquid clearance by allowing back flow into the air spaces. The impact of beta agonists and other agents that speed liquid removal on protein clearance is also an interesting question. Theoretically, increased protein concentration could speed protein clearance by augmenting the diffusion gradient or slow it by hastening protein precipitation.

The site of paracellular protein passage along the bronchoalveolar tree under normal and pathological conditions is another important area for exploration. The discovery that alveolar fluid can undergo convective transport to distal upper airways (83) lends credence to the hypothesis that tight junctions at the bronchoalveolar interface could be involved in alveolar protein clearance (see Fig. 4). Recent work has suggested that the permeability of tight junctions is subject to physiological regulation by factors such as peptides, secondary messengers, kinases, and toxins (67). Thus paracellular protein clearance could be a dynamic process that could respond to different pathological situations and stimuli in an injury-specific manner. Types of channels other than junctions between two cells have been considered lately as possible routes of paracellular transport. For example, edges of three epithelial cells frequently meet at one spot, a structure known as trijunctional complex (67). Trijunctional complexes leave a gap that contains glycocalyx but is still considerably larger than the opening of a tight junction (82). These gaps could serve to pass protein. Defects left by removal of apoptotic cells have also been recognized as potential though temporary pores for paracellular transport of macromolecules such as horseradish peroxidase (2).

Finally, studies are indicated to evaluate the role of receptor-mediated transcytosis of small quantities of protein in alveolar physiology under normal conditions. Receptor-mediated removal of serum proteins could serve to maintain low protein concentrations in the epithelial lining fluid under normal conditions, thus preserving low surface tension and optimal surfactant function (46). A carrier-mediated process could also be involved in moving proteins, such as IgA, into the alveolar space.

**SUMMARY**

The removal of protein from the distal air spaces of the lung is an essential step in the recovery from pulmonary edema. A variety of mechanisms have been studied for the removal of alveolar protein, including transport by the mucociliary escalator, as well as alveolar degradation by macrophages. There is some evidence for endocytosis across the alveolar epithelium, a process known as transcytosis. There is also evidence for paracellular diffusion through the distal epithelial barrier. When protein concentrations in the distal air spaces are low (all edema concentrations <0.5 g/100 ml), protein transport demonstrates saturation kinetics, temperature dependence, and sensitivity to pharmacological agents that can stimulate or inhibit endocytosis. In the presence of higher concentrations of alveolar protein, the rate of protein clearance appears to be proportional to concentration with no saturation kinetics. Protein clearance is also inversely related to protein size and insensitive to endocytosis inhibition under these conditions. On the basis of these findings, alveolar protein clearance can occur by a receptor-mediated transcytosis mechanism at low protein concentrations but probably proceeds by passive paracellular mechanisms at higher concentrations, once the receptors are saturated. Because protein concentrations in pulmonary edema fluid are relatively high (>3 g/100 ml) (58), clearance of alveolar protein probably occurs primarily by paracellular pathways during the resolution of clinical pulmonary edema. Transcytosis may be an important mechanism in regulating the alveolar environment under nonpathological circumstances. Also, alveolar degradation of protein may be an important mechanism in long-term protein clearance, as well as in the clearance of insoluble protein under pathological conditions such as acute lung injury.

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