Inhibition of Pulmonary Surfactant Adsorption by Serum and the Mechanisms of Reversal by Hydrophilic Polymers: Theory

Joseph A. Zasadzinski,* T. F. Alig,* Coralie Alonso,* Jorge Bernardino de la Serna,† Jesus Perez-Gil,† and H. William Taeusch‡

*Department of Chemical Engineering, University of California, Santa Barbara, California; †Bioquimica y Biologia Molecular, Universidad Complutense, Madrid, Spain; and ‡Department of Pediatrics, University of California, San Francisco, California.

ABSTRACT A theory based on the Smolukowski analysis of colloid stability shows that the presence of charged, surface-active serum proteins at the alveolar air-liquid interface can severely reduce or eliminate the adsorption of lung surfactant from the subphase to the interface, consistent with the observations reported in the companion article (pages 1769–1779). Adding nonadsorbing, hydrophilic polymers to the subphase provides a depletion attraction between the surfactant aggregates and the interface, which can overcome the steric and electrostatic resistance to adsorption induced by serum. The depletion force increases with polymer concentration as well as with polymer molecular weight. Increasing the surfactant concentration has a much smaller effect than adding polymer, as is observed. Natural hydrophilic polymers, like the SP-A present in native surfactant, or hyaluronan, normally present in the alveolar fluids, can enhance adsorption in the presence of serum to eliminate inactivation.

INTRODUCTION

Under normal conditions, the mixture of lipids and proteins in lung surfactant quite reliably lowers the interfacial tension in the lungs to nearly zero on exhalation, thereby insuring a negligible work of breathing and uniform lung inflation (1,2). The absence of lung surfactant in premature infants leads to neonatal respiratory distress syndrome (NRDS), which can be treated by delivering replacement surfactants, often derived from animals, to the lungs. Such surfactants often provide immediate relief from symptoms and improved oxygenation and gas exchange (1,2). However, there are certain cases, meconium aspiration syndrome being one example, in which surfactant therapy is less effective because substances not normally present in the alveolar fluid inactivate surfactant, leading to a decreased ability to reduce surface tension to the levels necessary for proper lung function (2–5).

Surfactant inactivation is also thought to be one factor in the development of acute respiratory distress syndrome (ARDS), which affects both adults and children (2,6–8). Extracted bronchial fluid (lavage) from ARDS patients shows elevated levels of serum and inflammatory proteins (2,9,10) and the ratio of soluble protein to lung surfactant in lavage correlates with severity and outcome in ARDS (11). ARDS lavage also has a reduced surface activity both in terms of the speed with which it adsorbs to an exposed air-water interface and the minimum surface tension at a given compression (9). Biophysical studies of lung surfactant mixed with serum proteins show that, at sufficiently high protein concentrations, ARDS-like depression of surfactant surface activity is obtained (2,6,12). Recent clinical reports (13,14) confirm earlier in vitro experiments (6) showing that high, 300–500 mg/kg doses of bovine extract surfactant (relative to the ~100 mg/kg typically given for NRDS treatment) delivered directly to the lung via bronchoscope significantly improved oxygenation in patients with ARDS (14), and decreased expected patient mortality. In a second study, large volumes of surfactant were administered by bronchoscope, followed by removal of at least 50% of the volume instilled. This study found that the fluid removed contained large amounts of inflammatory and serum proteins (15). These studies suggest that increasing the exogenous surfactant pool, while at the same time, lowering the protein load in the alveoli, could improve results for ARDS patients (6).

This recent work contributes to the general consensus that serum proteins, inflammatory agents, and other surface-active species not normally found in the lung can be a significant factor in the inactivation of lung surfactant, and hence, may be involved in the development of ARDS. As serum proteins inactivate both endogenous and replacement lung surfactants in vitro, serum proteins likely also reduce replacement surfactant efficacy in NRDS (6,9,12,16). Unfortunately, the mechanism of lung surfactant inactivation by serum proteins has remained obscure, frustrating efforts to rationally construct surfactant formulations appropriate for ARDS.

In the companion article (pages 1769–1779), we showed that serum proteins in the subphase greatly reduce or even eliminate the normal adsorption of clinical lung surfactants to the air-water interface at low surface pressures. Surfactant spread via organic solution directly onto the air-water interface was not affected by serum. Hence, reduced surfactant adsorption from bilayer aggregates in the subphase to a serum-covered interface is one cause of rapid surfactant inactivation. The diminished quantity of surfactant at the interface means that the surface tension is higher than normal for a given compression of the interface during exhalation.

Submitted March 21, 2005, and accepted for publication June 20, 2005.

Address reprint requests to Joseph A. Zasadzinski, Dept. of Chemical Engineering, University of California, Santa Barbara, CA 93106. Tel.: 805-893-4769; Fax: 805-893-4731; E-mail: gorilla@engineering.ucsb.edu.

0006-3495/05/09/1621/09 $2.00

doi: 10.1529/biophysj.105.062646

Hence, the low surface tensions required for proper lung function may never be reached (12).

As shown in the companion article and elsewhere (3–5, 16–23), ionic or nonionic polymers added to clinical and model surfactants (without SP-A) enhance surface activity and reduce surfactant inactivation in vitro and in vivo. Dextrans, polyethylene glycol (PEG) and natural hyaluronic acid (HA) restore the normal rates of surfactant adsorption in the presence of serum. Neither serum nor polymers in the subphase have an effect on the equilibrium spreading pressure of the surfactant or the collapse pressure (12). Polymer added to surfactants with no serum present also increased the rate of adsorption (17), but not the equilibrium spreading pressure or the collapse pressure of the surfactant. Hence, the origin of surfactant inactivation by serum proteins and reversal by hydrophilic polymers is likely the enhanced adsorption of surfactant due to the polymer induced depletion force (24,25) overcoming the reduction of surfactant adsorption by the electrostatic and steric repulsion from serum proteins at the interface.

To explain the results of the companion article, as well as develop a model for surfactant inactivation in ARDS, we present a quantitative theory of surfactant adsorption based on the classical Smolukowski analysis of colloid stability (26). The result shows that the presence of charged, surface-active serum proteins at the alveolar air-liquid interface, below the maximum surface pressure of the serum proteins, π_{max} (12,27), can induce a repulsive energy barrier to surfactant transport to the interface, severely reducing or even eliminating the amount of lung surfactant at the interface. Similar to the work of Hall and co-workers (28), we have divided the adsorption process into two steps: 1), transport from the subphase to the interface, followed by 2), conversion from bilayer aggregates to interfacial film. This theory examines only the first step in the process: transport. The reduction in adsorption is due to a combination of steric and electrostatic repulsive interactions caused by the presence of serum proteins at the interface (29), consistent with the observations reported in the companion article.

The enhanced adsorption of surfactant in the presence of hydrophilic polymers can be explained within the same theory by adding a simple ''depletion attraction'' term to the interaction potential (22,24,25,30–32). The surfactant aggregates are pushed toward the interface by an osmotic pressure induced by the exclusion of the polymer from the ''excluded volumes'' of the surfactant aggregates and the interface. The depletion attraction is sufficiently strong that it can overcome the electrostatic and steric repulsion imposed by the serum at the interface. The increased surfactant adsorption helps to drive the serum from the interface, resulting in a reversal of the inactivation. The depletion attraction model also explains why high molecular weight, anionic polymers like HA can reverse inactivation at lower weight fractions than neutral, lower molecular weight polymers like PEG, as was shown in the companion article.

The serum protein induced energy barrier to surfactant adsorption

Hall and co-workers (28) have outlined a two-step process for transfer of surfactant lipids from the subphase to the air-liquid interface. The first step is the transport of surfactant aggregates from the bulk solution to the interface. The second step, the conversion from bilayer structures to surface monolayer, is affected by the acyl group composition of the lipids and the surfactant proteins. Monolayer surface spreading does not appear to be rate limiting in Hall's experiments, which do not include inhibition of adsorption or application of surfactants directly onto liquid surfaces (28). From our experiments, the rate of surfactant adsorption is primarily affected below π_{max} of the serum, which suggests that the conversion from bilayer to monolayer is not affected significantly by serum. Rather, it is the transport of surfactant to the interface that is inhibited by serum and reversed by the addition of polymers.

The surface pressure exerted by a surface active, soluble protein (or any other species) is typically a logarithmic function of concentration up to a certain bulk concentration at which the surface becomes saturated (26,27,33–35). The serum protein concentrations used in the companion article exerted surface pressures of 10–20 mN/m; the maximum surface pressure of serum is ~24 mN/m at saturation and higher concentrations (27). This relationship between inhibition and surface activity appears to hold for nonprotein inhibitors as well. Lysophosphatidylcholine is a more potent inhibitor than serum; the surface pressure at the surface saturation concentration is 34 mN/m, which is significantly greater than serum (36). From a variety of experiments, inactivation of surfactant occurs when a surface active species not normally present in the alveoli, such as albumin, serum, lysolipids, etc., compete successfully for the air-water interface with lung surfactant (6,12). Most of these species are water soluble and rapidly adsorb to the air-water interface to produce moderate (20–34 mN/m) maximum surface pressures, π_{max}. However, as the interfacial area is reduced during exhalation, these soluble molecules at the interface can exchange with the subphase much more easily than lung surfactants. Hence, the surface pressure rises much more slowly with compression than for a surfactant-covered interface and surface pressures greater than π_{max} cannot be maintained for long because the serum is sufficiently soluble to leave the interface. A certain hysteresis of the surface pressure on compression of serum or albumin is observed; a fast compression will cause the surface pressure to rise, but it rapidly returns to the saturation surface pressure once the fast compression ends.

The lipids and hydrophobic proteins in lung surfactant are essentially insoluble as molecular species in the subphase; lung surfactants exist at the air-water interface as monolayer or multilayer films, as bilayer aggregates in the subphase, or as some intermediate structure (such as the tubular myelin

surfactant (2) that forms during the conversion of lung multilamellar bodies after secretion from the type II cell into the alveolar hypophase (37)). The energy difference between surfactant in the subphase and at the interface determines the equilibrium spreading pressure, π_e of the surfactant; π_e is directly related to the surface concentration of the surfactant at equilibrium. However, when surfactant films are compressed, the surface pressure increases well above π_e until the film collapses, generally by the generation of folds, cracks, etc., in the film (38–40). The maximum surface pressure of the surfactant film is set by this collapse pressure, and the surface pressure can remain at levels well above π_e even without fast compression. Proper lung function requires that the surface tension in the alveoli drop to near zero as the alveolar surface area decreases, which requires a high collapse pressure and an interface densely packed with surfactant. Mixed surfactant-serum films require greater compression to reach the maximum surface pressure because the serum is constantly being removed from the monolayer at pressures above π_{max}. Hence, the combined film never reaches the low surface tensions necessary for proper lung function, and breathing becomes more difficult (6,41).

The rate of adsorption from subphase to interface determines the relative amount of surfactant versus inhibitor at the interface. As surfactant is in large aggregates that must undergo structural rearrangements during and after adsorption (28,37,42), surfactant adsorption is slow compared to inhibitor adsorption. Hence, if an inhibitor is present in the subphase, the inhibitor will adsorb rapidly up to π_{max} (12) as shown in the companion article; when serum is added to the subphase the minimum surface pressure remains between 10–18 mN/m.

In the companion article we found that serum present at the air-water interface slows or stops adsorption of surfactant below π_{max}, although there is much less effect on adsorption above π_{max}. This suggests that the serum components at the interface must be pushed aside for surfactant to be adsorbed below π_{max} and that the serum proteins are removed from the interface at surface pressures above π_{max}. In addition to this steric barrier to adsorption, serum components and the phosphatidylglycerol, phosphatidylserine, and fatty acid components of lung surfactant are negatively charged. The electrostatic double layer induced by the charges provides an additional longer-ranged potential that repels surfactant aggregates (33). The anionic lipids are usually located in domains within the monolayer along with the cationic surfactant specific proteins SP-B and SP-C (38,43,44). The net interaction of the surfactant monolayer with the surfactant aggregate may be attractive due to the proper mixture of cationic proteins and anionic lipids, and is likely purely repulsive when purely anionic serum proteins occupy the interface due to a less-than-optimal balance of charge (45). Surfactant appears to need to encounter either a free or surfactant-covered interface to optimize surfactant adsorption.

Energy barriers and adsorption

An analysis similar to the classical Smoluchowski model of colloid aggregation can be derived to quantify the effect of the serum proteins at the interface (26,46). Fick's first law of diffusion states that the flux/area, $-J = d\Gamma/dt$, to an interface located at $x = 0$, is proportional to a friction factor, D/k_BT (D is the diffusion constant for the surfactant aggregate, T is the absolute temperature, k_B is Boltzmann's constant, Γ is the surface concentration), the bulk surfactant concentration, C, and the driving force, which in general, is the gradient in chemical potential, $(\partial\mu/\partial x)$ (26):

$$-\frac{d\Gamma}{dt} = J = -\frac{D}{k_BT}C\frac{\partial\mu}{\partial x}. \tag{1}$$

The chemical potential of an ideal solution in an external potential, $V(x)$, where x is the distance from the interface is:

$$\mu = \mu^{\circ} + k_BT\ln C + V(x). \tag{2}$$

$V(x)$ may be due to electrostatic forces, the steric work needed to push aside the serum, the depletion attraction, etc. Combining Eqs. 1 and 2 leads to a general diffusion equation:

$$\frac{-J}{D} = \frac{dC}{dx} + \frac{C}{k_BT}\frac{dV}{dx}. \tag{3}$$

Under steady-state conditions, the flux/area, J, is constant, and Eq. 3 can be integrated by multiplying both sides by $\exp(V/k_BT)$ (26):

$$\frac{-J}{D}\exp\frac{V}{k_BT} = \exp\frac{V}{k_BT}\left[\frac{dC}{dx} + \frac{C}{k_BT}\frac{dV}{dx}\right] = \frac{d}{dx}\left[C\exp\frac{V}{k_BT}\right], \tag{4}$$

which is integrated as follows:

$$\frac{-J}{D}\int_0^{\infty}\exp\frac{V}{k_BT}\,dx = \int_0^{C_B} d\left[C\exp\frac{V}{k_BT}\right]. \tag{5}$$

The limits of integration are taken to be such that the surfactant concentration is zero anytime a surfactant aggregate gets to the interface, ($C = 0$ at $x = 0$, this assumes that conversion from bilayer aggregate to interfacial film is fast compared to the transport to the interface), and the concentration reaches the bulk concentration, $C = C_B$, and far from the interface, the potential, $V = 0$, as $x \to \infty$. This is an idealization of the real situation, and assumes that the slow step in adsorption is transport of surfactant past the barrier, rather than conversion of surfactant from bilayer to monolayer (28). Integrating the right-hand side of Eq. 5 gives:

$$\int_0^{C_B} d\left[C\exp\frac{V}{k_BT}\right] = C_B. \tag{6}$$

The integral on the left-hand side of Eq. 5 is approximated by noting that the exponential term is dominated by the value of V at its maximum, V_{max}, and the potential can be expanded in a Taylor series about the maximum:

$$V \cong V_{max} + \frac{dV_{max}}{dx}(x - x_{mzx}) + \frac{d^2V_{max}}{dx^2}\frac{(x - x_{max})^2}{2} + \ldots, \tag{7}$$

at V_{max}, the first derivative is zero. Combining Eqs. 6–8 gives:

$$C_B = \frac{-J}{D}\exp\frac{V_{max}}{k_BT}\int_0^\infty \exp\frac{\frac{d^2V_{max}}{dx^2}\frac{(x - x_{max})^2}{2}}{k_BT}dx. \tag{8}$$

In Eq. 8, the integrand is simply a Gaussian and gives:

$$C_B = \frac{-J\underline{\pi}^{0.5}}{2Dp}\exp\frac{V_{max}}{k_BT}$$
$$p^2 = \frac{-d^2V_{max}}{dx^2}\Big/2k_BT, \tag{9}$$

where p is roughly constant, and reflects the shape of the potential around the maximum, and has units of length^{-1} (in Eqs. 9–11, $\underline{\pi}$ is 3.1416, not the surface pressure). Rearranging Eq. 9,

$$J = \frac{-2DC_Bp}{\underline{\pi}^{0.5}}\exp\frac{-V_{max}}{k_BT}. \tag{10}$$

Equation 10 shows that the flux is proportional to the bulk concentration and the negative exponential of the potential maximum.

We can approximate $V_{max} \cong (E_1 - E_0) + \pi\Delta A + E_{elect}$ near the maximum of the interaction potential. $E_1 - E_0$ is the energy difference between surfactant in bilayers and in the monolayer, which leads to adsorption onto a clean interface with zero surface pressure. $\pi\Delta A$ is the work required to clear an area of already-occupied interface with surface pressure π to give the surfactant room to adsorb. E_{elect} accounts for the electrostatic repulsion between a charged interface and the anionic surfactants (29,33).

If the concentration near the interface is depleted by adsorption, a boundary layer will form, which causes the effective concentration to be decreased as $C_B = C_o/(Dt)^{0.5}$ as the boundary layer thickness grows. Combining all these terms together, lumping the unknown constants into an effective diffusivity gives a general expression for adsorption to an interface in the presence of an energy barrier.

$$\frac{d\Gamma}{dt} = -J = C_o\left(\frac{D_{eff}}{\underline{\pi}t}\right)^{1/2}\exp[-(E_1 - E_0 + \pi\Delta A + E_{elect})/k_BT]. \tag{11}$$

If there is no surfactant depletion, or if the subphase is well mixed, $D_{eff}/(\underline{\pi}t)^{0.5}$ is replaced by D_{eff}/x, in which x is the diffusion boundary layer thickness (47). The adsorption to the interface depends primarily on the magnitude of the potential maximum due to the exponential. The decrease in the rate of adsorption due to the presence of the potential barrier imposed by the serum is:

$$\frac{J_{serum}}{J} = \exp[-(\pi_{max}\Delta A + E_{elect})/k_BT]. \tag{12}$$

Hence, if an inhibitor has already adsorbed to the interface, there is a minimum surface pressure of π_{max}, the interface is charged, and the rate of surfactant adsorption slows due to the barrier. The energy barrier may be sufficiently large that on experimental timescales, insufficient surfactant adsorbs to the interface to raise the surface pressure above π_{max} to displace the serum (see Figs. 4–6). To stabilize colloidal particles indefinitely against equilibrium aggregation, the energy barrier height need only be ~15 k_BT (26,33). For the relatively fast cycles of expansion and compression under normal breathing, the energy barrier likely does not have to be even that high to effectively prevent sufficient surfactant adsorption for proper lung function. Doubling the surfactant concentration, C_o, in Eq. 11 will not have nearly the effect of reducing the potential barrier by 50%, i.e., reducing $\pi_{max}\Delta A + E_{elect}$ from 10 to 5 k_BT. Equation 11 predicts the increased rate of lipid adsorption and respreading after monolayer collapse that results from decreasing the electrostatic potential, E_{elect} by increasing the salt concentration of the subphase, and hence the Debye length as shown in Alig et al. (29).

Effects of polymers: lowering the energy barrier

Enhancing the rate of surfactant adsorption in the presence of serum or other charged proteins requires that the energy barrier in Eq. 11 be lowered. Although it is possible to lower E_{elect} by increasing the salt concentration in the subphase in vitro (29), this would not be practical in vivo as it could lead to edema. A second possibility would be to reduce the serum concentration at the interface (15); however, due to the logarithmic dependence of surface pressure on the serum concentration, a substantial fraction of the protein would have to be removed to see an effect.

However, adding hydrophilic, nonadsorbing polymers to the subphase creates a force of entropic origin that pushes the surfactant aggregates to the interface and can provide the necessary attractive potential to enhance adsorption. A mixture of two different sizes of noninteracting ''hard spheres'' maximizes its entropy by maximizing the volume accessible per ''sphere'' (24,25,30–32). Here, the small spheres are the polymers with radius of gyration, R_g (typically nm), and the large spheres are surfactant aggregates of radius R (typically microns). The polymer is sterically excluded from the regions where the aggregates come within R_g of each other or the interface (*shaded* regions in Fig. 1). As the large sphere moves toward another large sphere or the wall, the volumes excluded from the small spheres (Fig. 1) overlap, causing the total volume accessible to the small spheres to increase (*small box* in *right corner*) (24,25,30–32). This increases the total entropy of the mixture (decreases the free energy) by an amount proportional to the size of the excluded volume overlap region, multiplied by the osmotic pressure of the small spheres. The depletion potential, $W(l)$, as a function of separation, l, between the large sphere and the rigid wall (32) is:

$$W(l) = -3\phi_pk_BT\frac{R}{R_g}\left(1 - \frac{l}{2R_g}\right)^2, \tag{13}$$

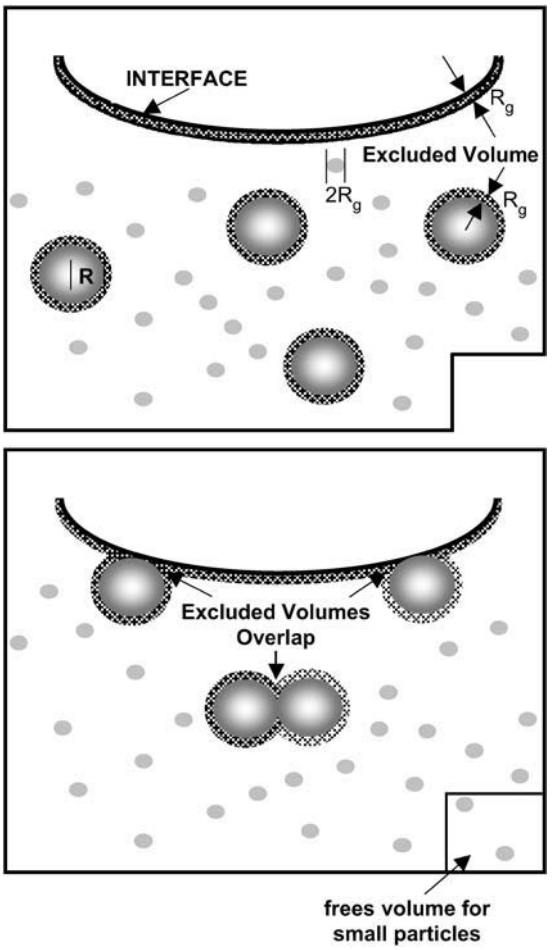

FIGURE 1 Origin of depletion forces in a binary sphere mixture. (*Top*) The centers of the small spheres are excluded from the hatched regions within one small sphere radius (R_g) of the larger spheres (radius R) or the interface. (*Bottom*) When the larger spheres move to the interface or toward each other, the hatched regions overlap, and the total volume accessible to the small spheres increases by this amount (small volume in the bottom right-hand corner). The increase in the volume accessible to the polymer increases the entropy of the system, resulting in a net force pushing the large spheres toward the interface or each other.

for $l < 2R_g \ll R$, and $W(l) = 0$ otherwise; ϕ_p is the volume fraction of polymer, k_B is Boltzmann's constant, and T is the temperature (25). Moving a single large sphere to a rigid surface decreases the mixture's free energy by $3(R/R_g)\phi_p k_B T$. If the surface or the sphere deforms, an even larger excluded volume overlap region can result, with a larger force pushing the large sphere toward the surface (25). The depletion potential is independent of the chemistry of the large and small spheres, as long as the polymer does not adsorb to surfactant or interface. The increase in the flux to the interface is proportional to

$$\frac{J_{\text{serum}+\text{polymer}}}{J_{\text{serum}}} = \exp[-(W(l))/k_B T]. \quad (14)$$

The depletion interaction effectively ''pushes'' the surfactant to the interface (Fig. 1). For the volume fractions ($\phi_p \sim 1$–10%) and aspect ratios ($R/R_g \sim 10$–300) that reduce inhibition, $W(l) \sim 10$–100 k_BT (3–5,16,18,19,22,23,48,49). The range of the depletion force is twice the radius of gyration of the polymer, which is comparable to the range of electrostatic forces in physiological saline, ~1–5 nm (26) for the 10–1250 kDA polymers used in the experiments in the companion article. Adding the polymer lowers V_{max} by an amount proportional to $W(l)$, providing an exponential increase in the rate of surfactant adsorption, thereby reversing the effects of inhibition. Yu et al. (22) have shown that increasing the PEG concentration increases the rate of adsorption of surfactant even in the absence of inhibitors, consistent with the predictions of Eq. 14.

Fig. 2 shows that the depletion forces between surfactant aggregates can push aggregates together against electrostatic repulsion and thermal motion to form large flocs. $W \approx 1.5 (R/R_g)\phi_p k_B T$ between two spheres because of the smaller excluded volume overlap (25), half that between a sphere and a surface. Infasurf, Curosurf, and Survanta form large flocs when 5 wt% 10 kDa PEG is added to the buffer, which confirms that the depletion interaction is large compared to thermal motions (k_BT) and large compared to any electrostatic repulsion between the anionic surfactant particles (26,33). Aggregation of surfactant particles after polymer addition was also observed by Yu et al. (22) and was ascribed to depletion attraction. Hence, it is clear that the depletion attraction is capable of overcoming the electrostatic repulsions and thermal motions in physiological salt conditions and should significantly lower the barrier to surfactant adsorption.

Molecular weight and polymer charge: optimizing surfactant formulations

The magnitude of the depletion interaction, and hence the increase in adsorption, can be controlled by changing the surfactant volume fraction and/or the aspect ratio of the surfactant to the polymer. For a polymer in a good solvent, such as PEG in water, R_g scales as $M^{0.6}$ (33), in which M is the polymer molecular weight. For highly charged polyelectrolytes like hyaluronic acid, R_g can vary from $M^{0.6}$ to $M^{1.0}$ depending on the charge distribution of the polymer and the ionic strength of the solution. Hence, for PEG, the depletion potential can be expressed as: $W \propto RN_pM^{1.2}/V$. N_p is the number of polymer chains in a volume, V. For HA, the scaling with molecular weight is more complex, $W \propto RN_p M^{1.2-2.0}/V$. In most experiments regarding inhibition, the polymer concentration is expressed as a weight of polymer per unit volume, $\rho = N_pM/V$ (3,4,16,20–23,48). The molecular weight dependence of inhibition reversal has not been systematically explored, although this simple argument suggests that higher molecular weight polymers should reduce inhibition at lower weight/volume ratios than low molecular weight polymers. This is consistent with our experiments that show it required 5 wt/v 10 kDa PEG to

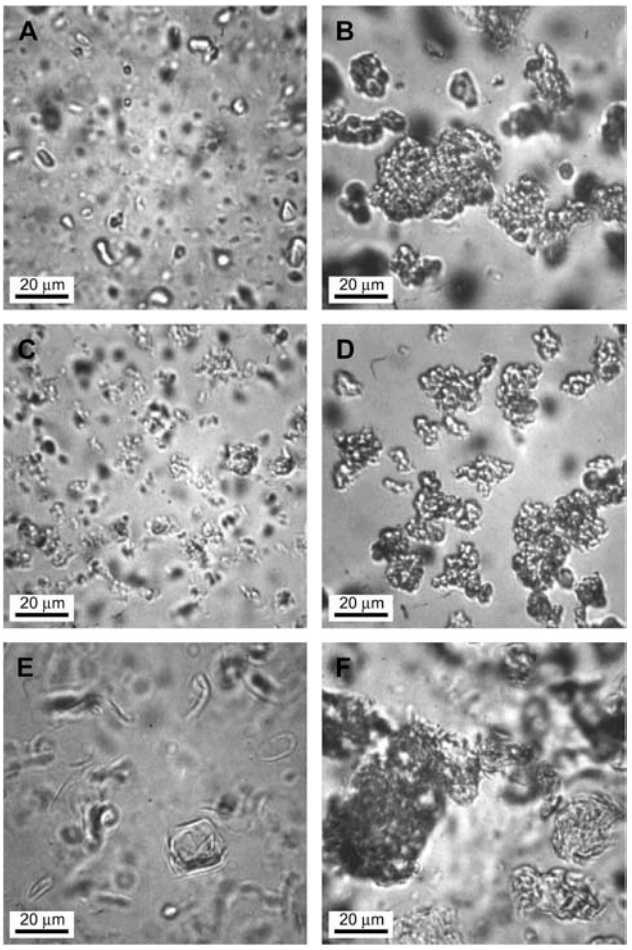

FIGURE 2 Optical microscope images of different clinical surfactants before (*left* column) and after (*right* column) addition of 5 wt% 10 K PEG. (*A*) Curosurf; (*B*) Curosurf with 5 wt% 10 K PEG. Formation of large flocculates is obvious in panel *B* compared to the much smaller particles in panel *A*. The depletion interaction between particles is roughly half that between surfactant particles and the interface, but still sufficient to overcome any thermal or electrostatic repulsion between the surfactant aggregates. (*C*) Infasurf; (*D*) Infasurf with 5 wt% 10 K PEG. Large, irregular flocs also form for Infasurf, suggesting that the interaction between the surfactant and polymer does not depend on the specific composition of the surfactant as suggested by the depletion interaction. (*E*) Survanta. Survanta has larger particles before polymer addition than either Curosurf or Infasurf. (*F*) Survanta with 5 wt% 10 K PEG. Survanta exhibits the greatest amount of flocculation on addition of polymer, consistent with Eq. 13. All the images are consistent with the entropic depletion interaction forcing the large particles together to maximize the accessible volume of the polymers. All the commercial surfactants contain anionic lipids, and hence are negatively charged. The depletion potential is sufficient to overcome the electrostatic repulsion and the thermal motion that would otherwise keep the aggregates apart.

provide similar inhibition reversal as .125 wt/v 1240 kDa hyaluronic acid. From the scaling arguments, to provide the same depletion attraction, $(\rho_{PEG}/\rho_{HA}) = (M_{HA}^{0.2-1.0})/(M_{PEG}^{0.2}) \approx 3 - 750$, which spans the experimental value of ~40. Yu et al. (22) have also shown that adsorption is increased for a given weight/volume of PEG as the molecular weight of the PEG is increased.

The osmotic pressure of the polymer-surfactant solution, which should be minimized to prevent infiltration of liquid into the lung during ARDS treatment, is proportional to N_p/V, so high molecular weight polymers should have a distinct advantage in treatments of ARDS and other lung injuries. For example, for 10 kDa PEG, increased lung water reduced the beneficial effects of the polymer in a rabbit model of acute lung injury (22). The adverse effects were reversed by giving the animals a diuretic and using a hypotonic saline vehicle for administration of the surfactant/PEG mixture (50,51).

The range of the depletion force is $2R_g$ (Eq. 13), which shows that using higher molecular weight polymers will increase the range as well as the magnitude of the depletion interaction. The range of electrostatic interactions are roughly κ^{-1}, the Debye length (26,31,33), which is proportional to the ionic strength of the subphase. $\kappa^{-1} \sim 1$ nm for 150 mM physiological saline (33). The charge density of the serum at the interface likely increases with the surface pressure up to π_{max}, so the magnitude of the electrostatic repulsion should change with surface pressure. It is not known what the range of the steric interactions with serum components might be. PEG (4 kDa) has $R_g = 2.7$ nm, and 6 kDa PEG has $R_g = 3.3$ nm (52), so even relatively small molecular weight polymers should have a sufficient range of depletion forces. Yu et al. found some effect on surfactant adsorption with 3.3 kDa PEG (22). PEG (1 kDa) has $R_g = 1.3$ nm, which may be too small for depletion forces to overcome electrostatic repulsion. Future investigations are needed to determine the molecular weight and concentration to provide the optimal osmotic pressure, depletion attraction, and solution viscosity for clinical formulations.

Larger surfactant aggregates will feel a larger push to the interface, as the depletion interaction is proportional to the aggregate radius, R; larger particles also deliver more surfactant to the interface. Additional considerations in the model include the diffusivity of the surfactant aggregates in the subphase. According to the Stokes-Einstein model for diffusion of a surfactant aggregate, $D = k_B T/6\pi\eta R$; η is the subphase viscosity, which increases with polymer concentration and polymer molecular weight. Hence, there is likely an optimal combination of polymer concentration and molecular weight to maximize the rate of surfactant diffusion to the interface. Yu et al. (22) found that for 300 kDa PEG, surfactant adsorption was inhibited by the high viscosity of the PEG solution. The diffusivity is also inversely proportional to the surfactant aggregate radius, R, so there is likely an upper limit on the optimal surfactant aggregate size. Survanta aggregates are much larger than Curosurf aggregates, and there is a larger fraction of very small aggregates in Infasurf (Fig. 2) (53).

Another interesting aspect of the depletion interaction is the coupling with electrostatic forces. Experimentally, it is found that if both surfactant aggregates and polymer have the same charge, then the depletion layer can extend further than

R_g (31). The electrostatic repulsion between the large and small particles increase the effective radius of both large and small particles by roughly κ^{-1}, the Debye length (31) in Eq. 13, leading to an effective depletion attraction of the form:

$$W = 4\pi N_p (R + \kappa^{-1})(R_g + \kappa^{-1})^2 k_B T/V. \quad (15)$$

In physiological saline, $\kappa^{-1} \sim 1$ nm. Lung surfactant contains a substantial fraction of anionic lipids, so this suggests that an anionic polymer such as HA might provide a larger depletion interaction for a given concentration than a nonionic polymer such as PEG. This may also contribute to the smaller concentration of HA needed to reverse inhibition than the uncharged PEG.

CONCLUSIONS

In ARDS, increased concentrations of serum and inflammatory proteins in the alveolar hypophase are a likely contributing factor to the rapid inactivation of lung surfactant. Albumin concentrations in ARDS alveolar fluids may reach 100 mg/ml, with an average concentration reported by Ishizaka and co-workers of 25 mg/ml (54). Many of the proteins in serum reach their saturation surface pressure, π_{max}, at much lower concentrations (12,27). For both albumin and fibrinogen, π_{max} is achieved for concentrations $>\sim$0.1 mg/ml (12), which means that >99% of the albumin typically found in ARDS alveolar fluid (54) would have to be removed to eliminate the adsorbed albumin and lower the energy barrier to surfactant adsorption. Although Wiswell and co-workers (15) saw improvements in oxygenation after they administered large volumes of surfactant by bronchoscope and removed ~50% of the volume instilled along with large amounts of serum and inflammatory proteins, surfactant inactivation, and the symptoms of ARDS persisted. It is likely to be impractical, if not impossible, to physically remove the inactivating substances in vivo during ARDS therapy.

Reversing surfactant inactivation likely will require restoring the rate of surfactant adsorption to normal levels by lowering the energy barrier as shown in Eq. 11. From the theory presented here, the rate of surfactant adsorption at a given surface pressure should increase linearly with the bulk surfactant concentration (Eq. 11). This is consistent with the in vitro observations reported in Part 1 and also found by others (17,22). In vivo, instilling 3–5 times the concentration of surfactant used to treat NRDS directly by bronchoscope to the lungs of ARDS patients resulted in some improvement (13,14). Increasing the available exogenous surfactant pool, while at the same time, lowering the protein load in the alveoli, could improve results for ARDS patients. However, if sufficient serum protein remains that the electrostatic and steric barrier to adsorption is of order 10–15 k_BT, which is certainly possible given the known magnitude of electrostatic and steric effects for charged proteins at the air-liquid interface (29,33), it would be difficult to increase the concentration sufficiently to overcome inactivation.

According to Eq. 14, a significantly greater increase in the rate of surfactant adsorption can be obtained by lowering the energy barrier to adsorption by adding hydrophilic polymers to the surfactant suspension as shown in the companion article. The added polymers create a depletion attraction potential that can be of the same order of magnitude as the electrostatic and steric repulsive potentials (see Eq. 13) and can easily be adjusted by changing the polymer molecular weight, charge, and concentration. As the energy barrier is lowered, the rate of adsorption should increase exponentially. These results are consistent with the experimental observations in the companion article and in the literature (3,16–22, 50,51) that PEG, dextran, and HA can reverse surfactant inactivation both in vivo and in vitro. The model presented here provides readily testable predictions of the effects of molecular weight, polymer and surfactant concentration, polymer charge, and surfactant aggregate size that are qualitatively consistent with the results in the companion article. High molecular weight, anionic polymers should provide the best results for a given polymer weight fraction, as is observed in the companion article. Low polymer concentrations will help minimize the osmotic stress in vivo to minimize complications of ARDS therapy. A systematic study of surfactant adsorption rates will show that there is an optimal combination of surfactant structure, polymer charge, molecular weight, and solution viscosity that can be guided by the theory presented here to develop new therapies for ARDS treatment.

We thank David Pine for discussions on the depletion forces near interfaces.

Support for this work comes from DGESIC (BIO 2003-9056 to J.P.G. and J.B.D.L.S) and CAN (08.2/0054.1/2001 to J.P.G. and J.B.D.L.S.) in Spain, and the National Institutes of Health (HL-66410 to H.W.T. and HL-51177) and the Tobacco-Related Disease Research Program (11RT-0222 and 14RT- 0077 to J.A.Z., C.A., and T.F.A.) in the United States. International collaboration has been facilitated by a grant from the New Del Amo Program of the University of California and the Universidad Complutense.

REFERENCES

1. Clements, J. A., and M. E. Avery. 1998. Lung surfactant and neonatal respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* 157: 59–66.
2. Notter, R. H. 2000. Lung surfactant: basic science and clinical applications. Marcel Dekker, New York, NY and Basel, Switzerland.
3. Lu, K. W., H. William Taeusch, B. Robertson, J. Goerke, and J. A. Clements. 2000. Polymer-surfactant treatment of meconium-induced acute lung injury. *Am. J. Respir. Crit. Care Med.* 162:623–628.
4. Tashiro, K., T. Kobayashi, and B. Robertson. 2000. Dextran reduces surfactant inhibition by meconium. *Acta Paediatr.* 89:1439–1445.
5. Tashiro, K., X. Cui, T. Kobayashi, T. Curstedt, and B. Robertson. 2003. Modified protocols for surfactant therapy in experimental meconium aspiration syndrome. *Biol. Neonate.* 83:49–56.
6. Holm, B. A., G. Enhorning, and R. H. Notter. 1988. A biophysical mechanism by which plasma proteins inhibit lung surfactant activity. *Chem. Phys. Lipids.* 1988:49–55.

7. Hyers, T. M. 1991. Adult respiratory distress syndrome: definition, risk factors and outcome. *In* Adult Respiratory Distress Syndrome. W. M. Zapol and F. Lemaire, editors. Marcel Dekker, New York, NY. 23–33.
8. Spragg, R. J., and R. M. Smith. 1998. Surfactant replacement in patients with ARDS: result of clinical trials. *In* Acute Respiratory Distress Syndrome: Cellular and Molecular Mechanisms and Clinical Management. S. Matalon, J. I. Sznajder, and North Atlantic Treaty Organization Scientific Division, editors. Plenum Press, New York, NY. 107–116.
9. Spragg, R. G. 1991. Abnormalities of lung surfactant function in patients with acute lung injury. *In* Adult Respiratory Distress Syndrome. W. M. Zapol and F. Lemaire, editors. Marcel Dekker, New York, NY. 381–395.
10. Nakos, G., E. I. Kitsiouli, I. Tsangaris, and M. E. Lekka. 1998. Bronchoalveolar lavage fluid characteristics of early intermediate and late phases of ARDS. Alterations in leukocytes, proteins, PAF and surfactant components. *Intensive Care Med.* 24:296–303.
11. Hallman, M., V. Glumoff, and M. Ramet. 2001. Surfactant in respiratory distress syndrome and lung injury. *Comp. Biochem. Phys. A Mol. Integr. Physiol.* 129:287–294.
12. Warriner, H. E., J. Ding, A. J. Waring, and J. A. Zasadzinski. 2002. A concentration-dependent mechanism by which serum albumin inactivates replacement lung surfactants. *Biophys. J.* 82:835–842.
13. Anzueto, A. 2002. Exogenous surfactant in acute respiratory distress syndrome: more is better. *Eur. Respir. J.* 19:787–789.
14. Guenther, A., M. Schmidt, J. Harodt, T. Schmehl, D. Walmrath, C. Ruppert, F. Grimminger, and W. Seeger. 2002 . Bronchoscopic administration of bovine natural surfactant in ARDS and septic shock: impact on biophysical and biochemical surfactant properties. *Eur. Respir. J.* 19:797–804.
15. Wiswell, T. E., R. M. Smith, L. B. Katz, L. Mastroianni, D. Y. Wong, D. Willms, S. Heard, M. Wilson, R. D. Hite, A. Anzueto, S. D. Revak, and C. G. Cochrane. 1999 . Bronchopulmonary segmental lavage with Surfaxin (KL4-surfactant) for acute respiratory distress syndrome. *Am. J. Respiratory and Critical Care Medicine* 160.
16. Taeusch, H. W., K. W. Lu, J. Goerke, and J. A. Clements. 1999. Hydrophilic polymers added to surfactant enhance treatment of lung injury by reducing surfactant inactivation. *Am. J. Respir. Crit. Care Med.* 159:A595. (Abstr.)
17. Lu, J. J., L. M. Y. Yu, W. W. Y. Cheung, Z. Policova, D. Li, M. L. Hair, and A. W. Neumann. 2003. The effect of concentration on the bulk adsorption of bovine lipid extract surfactant. *Colloids Surf. B.* 29:119–130.
18. Lu, J. J., W. W. Y. Cheung, L. M. Y. Yu, Z. Policova, D. Li, M. L. Hair, and A. W. Neumann. 2002. The effect of dextran to restore the activity of pulmonary surfactant inhibited by albumin. *Respir. Physiol. Neurobiol.* 130:169–179.
19. Lu, K., H. W. Taeusch, B. Robertson, J. Goerke, and J. A. Clements. 2001. Polyethylene glycol/surfactant mixtures improve lung function after HCl and endotoxin lung injuries. *Am. J. Respir. Crit. Care Med.* 164:1531–1536.
20. Lu, K. W., H. W. Taeusch, J. Goerke, and J. A. Clements. 1999. Hydrophilic polymers prevent surfactant inactivation. *J. Invest. Med.* 47:32A. (Abstr.)
21. Taeusch, H. W. 2000. Treatment of acute (adult) respiratory distress syndrome: the holy grail of surfactant therapy. *Biol. Neonate.* 77 (suppl. 1) :2–8.
22. Yu, L. M. Y., J. J. Lu, I. W. Y. Chiu, K. S. Leung, Y. W. Chan, L. Zhang, Z. Policova, M. L. Hair, and A. W. Neumann. 2004. Polyethylene glycol enhances the surface activity of a pulmonary surfactant. *Colloids Surf. B Biointerfaces.* 36:167–176.
23. Kobayashi, T., K. Ohta, K. Tashiro, K. Nishizuka, W.-M. Chen, S. Ohmura, and K. Yamamoto. 1999. Dextran restores albumin-inhibited surface activity of pulmonary surfactant extract. *J. Appl. Physiol.* 86:1778–1784.
24. Dinsmore, A. D., A. G. Yodh, and D. J. Pine. 1996. Entropic control of particle motion using passive surface microstructures. *Nature.* 383: 239–242.
25. Dinsmore, A. D., D. T. Wong, P. Nelson, and A. G. Yodh. 1998. Hard spheres in vesicles: curvature-induced forces and particle induced curvature. *Phys. Rev. Lett.* 80:409–412.
26. Hiemenz, P. C. 1986. Principles of Colloid and Surface Chemistry. Marcel Dekker, New York, NY.
27. Krishnan, A., J. Sturgeon, C. A. Siedlecki, and E. A. Vogler. 2003. Scaled interfacial activity of proteins at the liquid-vapor interface. *J. Biomed. Mater. Res.* 68A:544–557.
28. Walters, R. W., R. R. Jenq, and S. B. Hall. 2000. Distinct steps in the adsorption of pulmonary surfactant to an air-liquid interface. *Biophys. J.* 78:257–266.
29. Alig, T. F., H. E. Warriner, L. Lee, and J. A. Zasadzinski. 2004. Electrostatic barrier to recovery of DPPG monolayers after collapse. *Biophys. J.* 86:897–904.
30. Kaplan, P. D., J. L. Rourke, A. G. Yodh, and D. J. Pine. 1994. Entropically driven surface phase separation in binary colloidal mixtures. *Phys. Rev. Lett.* 72:582–585.
31. Mondain-Monval, O., F. Leal-Calderon, J. Phillip, and J. Bibette. 1995. Depletion forces in the presence of electrostatic double layer repulsion. *Phys. Rev. Lett.* 75:3364–3367.
32. Bickel, T. 2003. Depletion forces near a soft interface. *J. Chem. Phys.* 118:8960–8968.
33. Israelachvili, J. N. 1992. Intermolecular and Surface Forces. Academic Press, London, UK.
34. McClellan, S. J., and E. I. Franses. 2003. Effect of concentration and denaturation on adsorption and surface tension of bovine serum albumin. *Colloids Surf. B.* 28:63–75.
35. Phang, T. L., Y. C. Liao, and E. I. Franses. 2004. Dynamic adsorption and surface tension of aqueous dilauroylphosphatidylcholine dispersions under physiological conditions. *Langmuir.* 20:4004–4010.
36. Holm, B. A., Z. Wang, and R. H. Notter. 1999. Multiple mechanisms of lung surfactant inhibition. *Pediatr. Res.* 46:85–93.
37. Haller, T., P. Dietl, H. Stockner, M. Frick, N. Mair, I. Tinhofer, A. Ritsch, G. Enhorning, and G. Putz. 2004. Tracing surfactant transformation from cellular release to insertion into an air-liquid interface. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286:L1009–L1015.
38. Lipp, M. M., K. Y. C. Lee, D. Y. Takamoto, J. A. Zasadzinski, and A. J. Waring. 1998. Coexistence of buckled and flat monolayers. *Phys. Rev. Lett.* 81:1650–1653.
39. Diamant, H., T. A. Witten, A. Gopal, and K. Y. C. Lee. 2000. Unstable topography of biphasic surfactant monolayers. *Europhys. Lett.* 52:171–177.
40. Lu, W., C. M. Knobler, R. F. Bruinsma, M. Twardos, and M. Dennin. 2002. Folding Langmuir monolayers. *Phys. Rev. Lett.* 89:146107.
41. Mbagwu, N., R. Bruni, J. M. Hernández-Juviel, A. J. Waring, and F. J. Walther. 1999. Sensitivity of synthetic surfactants to albumin inhibition in preterm rabbits. *Mol. Genet. Metab.* 66:40–48.
42. Diemel, R. V., H. P. Haagsman, and G. Putz. 2002. In vitro and in vivo intrapulmonary distribution of fluorescently labelled surfactant. *Crit. Care Med.* 30:1083–1090.
43. Ding, J., I. Doudevski, H. Warriner, T. Alig, J. Zasadzinski, A. J. Waring, and M. A. Sherman. 2003. Nanostructure changes in lung surfactant monolayers induced by interactions between POPG and SP-B. *Langmuir.* 19:1539–1550.
44. Ding, J., D. Y. Takamoto, A. Von Nahmen, M. M. Lipp, K. Y. C. Lee, A. J. Waring, and J. A. Zasadzinski. 2001. Effects of lung surfactant proteins, SP-B and SP-C, and palmitic acid on monolayer stability. *Biophys. J.* 80:2262–2272.
45. Hu, Y., I. Doudevski, D. Wood, M. Moscarello, C. Husted, C. Genain, J. A. Zasadzinski, and J. N. Israelachvili. 2004. Synergistic interactions of lipids and myelin basic proteins. *Proc. Natl. Acad. Sci. USA.* 101:13466–13471.
46. Evans, D. F., and H. Wennerstrom. 1994. The Colloidal Domain. VCH Publishers, New York, NY.

47. Schürch, S., F. H. Y. Green, and H. Bachofen. 1998. Formation and structure of surface films: captive bubble surfactometry. *Biochim. Biophys. Acta.* 1408:180–202.

48. Kobayashi, T., K. Tashiro, X. Cui, T. Konzaki, Y. Xu, C. Kabata, and K. Yamamoto. 2001. Experimental models of acute respiratory distress syndrome: clinical relevance and response to surfactant therapy. *Biol. Neonate.* 80:26–28.

49. Cui, X., K. Tashiro, H. Matsumoto, Y. Tsubokawa, and T. Kobayashi. 2003. Aerosolized surfactant and dextran for experimental acute respiratory distress syndrome caused by acidified milk in rats. *Acta Anaethesiol. Scand.* 47:853–860.

50. Dehority, W., K. W. Lu, J. A. Clements, J. Goerke, and H. W. Taeusch. 2005. Polymer-surfactant therapy for adult rats with acute lung injury induced by saline lavage. *Pediatr. Res.* In press.

51. Dehority, W., K. W. Lu, and H. W. Taeusch. 2001. Increased lung water may affect responses to treatment of acute lung injury in rats with polymer-surfactant mixtures. *Am. J. Respir. Crit. Care Med.* 163:A81.

52. Bhat, R., and S. N. Timasheff. 1992. Steric exclusion is the principle source of the preferential hydration of proteins in the presence of polyethylene glycols. *Protein Sci.* 1:1133–1143.

53. Braun, A. 2004. Microstructure and properties of a lipid-based drug delivery system and lung surfactant suspensions. Masters thesis. Department of Chemical Engineering, University of California, Santa Barbara, CA.

54. Ishizaka, A., T. Matsuda, K. Albertine, H. Koh, S. Tasaka, N. Hasegawa, N. Kohno, T. Kotani, H. Morisakai, J. Takeda, M. Nakamura, X. Fang, et al. 2004. Elevation of KL-6, a lung epithelial cell marker, in plasma and epithelial lining fluid in ARDS. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286:L1088–L1094.