Inactivation of Pulmonary Surfactant Due to Serum-Inhibited Adsorption and Reversal by Hydrophilic Polymers: Experimental

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

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

ABSTRACT The rate of change of surface pressure, π, in a Langmuir trough following the deposition of surfactant suspensions on subphases containing serum, with or without polymers, is used to model a likely cause of surfactant inactivation in vivo: inhibition of surfactant adsorption due to competitive adsorption of surface active serum proteins. Aqueous suspensions of native porcine surfactant, organic extracts of native surfactant, and the clinical surfactants Curosurf, Infasurf, and Survanta spread on buffered subphases increase the surface pressure, π, to ~40 mN/m within 2 min. The variation with concentration, temperature, and mode of spreading confirmed Brewster angle microscopy observations that subphase to surface adsorption of surfactant is the dominant form of surfactant transport to the interface. However (with the exception of native porcine surfactant), similar rapid increases in π did not occur when surfactants were applied to subphases containing serum. Components of serum are surface active and adsorb reversibly to the interface increasing π up to a concentration-dependent saturation value, π_{max}. When surfactants were applied to subphases containing serum, the increase in π was significantly slowed or eliminated. Therefore, serum at the interface presents a barrier to surfactant adsorption. Addition of either hyaluronan (normally found in alveolar fluid) or polyethylene glycol to subphases containing serum reversed inhibition by restoring the rate of surfactant adsorption to that of the clean interface, thereby allowing surfactant to overcome the serum-induced barrier to adsorption.

INTRODUCTION

Lung surfactant is a mixture of lipids (primarily dipalmitoylphosphatidylcholine) and four lung surfactant specific proteins (SP-A, B, C, and D) that line the interior of the lung alveoli. Lung surfactant lowers the interfacial tension in the lungs, thereby insuring minimized work of breathing and uniform lung inflation (1). The absence of lung surfactant due to prematurity leads to neonatal respiratory distress syndrome (NRDS). In NRDS, the lack of functional surfactant results in a progressive failure of the lungs, which is manifested clinically by atelectasis (collapsed alveoli), decreased lung compliance (stiff lungs), decreased functional residual capacity (a measure of the amount of air left in the lungs after exhalation), systemic hypoxia (oxygen starvation), and lung edema (protein rich fluid in the lungs) (1–3). Treating NRDS with currently available replacement surfactants has significantly reduced neonatal mortality in developed countries (1,3). However, there are certain cases, meconium aspiration syndrome being one example, in which surfactant therapy is less effective because surfactant loses the ability to reduce surface tension and is said to be ''inactivated'' (3–6).

Surfactant inactivation is likely one cause of acute respiratory distress syndrome (ARDS), which affects both adults and children. ARDS has an incidence of 150,000 cases per year (United States) and a mortality rate of ~30% (3,7,8). The pathophysiology of ARDS involves injury to the alveolar-capillary barrier, lung inflammation, atelectasis, surfactant dysfunction, and intrapulmonary shunting. The disorder typically appears rapidly within 12–24 h of an identifiable clinical event and may be due to direct lung injury, such as gastric content aspiration, pneumonia, near-drowning, toxic gas inhalation, or chest/lung trauma. In addition, ARDS may be associated with systemic processes such as sepsis, nonthoracic trauma, acute pancreatitis, major surgery, multiple blood transfusions, fat embolism, or shock. No specific therapy for ARDS currently exists. Although ARDS has a more complicated pathology than the simple absence of surfactant, ARDS shares many NRDS symptoms such as diminished lung compliance, marked restriction of lung volumes, and profound hypoxemia. Hence, it was hoped ARDS might respond favorably to surfactant replacement therapy. However, clinical trials with the most effective formulations used in NRDS yield gains in ARDS patients that are both modest and transient (3,7,9–12), suggesting that ARDS involves not only a lack of functional surfactant, but an inactivation of the endogenous or exogenous surfactant present.

There are many ways surfactant can be inactivated at various points in the surfactant life cycle; from transcription and protein translation, during multivesicular and lamellar body formation in the type II cell (13,14), secretion into the hypophase liquid layer, transformation from lamellar bodies to tubular myelein to membrane vesicles (3), transport through subphase to the alveolar interface, reuptake by type II cells or macrophages, or losses due to transport out of the alveoli to the airways (3). Inactivation can be slow (enhanced

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Address reprint requests to Dr. H. William Taeusch, Dept. of Pediatrics, SFGH, 1001 Potrero Ave., San Francisco, CA 94110. Tel.: 415 206 3681; Fax: 415-206-3686; E-mail: btaeusch@sfghpeds.ucsf.edu.

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degradation from increased volume changes), permanent (from lipase or protease activity), or rapid and reversible. In ARDS and other acute lung injuries, the inactivation is rapid and likely reversible and may explain why exogenous surfactant does not have the dramatic immediate effect when treating adult lung injuries seen when treating premature newborns with surfactant deficiency (NRDS) (8,11,15–22). In ARDS, increased concentrations of serum proteins in the alveolar hypophase are a likely cause of rapid inactivation; albumin concentrations in ARDS alveolar fluid may reach 100 mg/ml, with an average concentration reported by Ishizaka and co-workers of 25 mg/ml (23). Surfactants may be made resistant to serum and other inactivating substances in the alveolar spaces during acute lung injury (24–28); a common finding is that increased concentrations of surfactant and increased fractions of surfactant specific proteins reduce inactivation by serum (20,29–34). A more recent and surprising finding is that hydrophilic, nonadsorbing polymers added to aqueous mixtures of organic surfactant extracts (which contain SP-B and SP-C, but not SP-A) reduces rapid inactivation by serum, meconium, albumin, and other substances both in vitro and in vivo (4–6,35–42). The added polymers allow these surfactants to better mimic fully constituted native mammalian surfactant, which appears to be more resistant to inactivation than clinical organic solvent-extracted surfactants.

Diemel and co-workers have recently shown the utility of studying spreading rates of various surfactant preparations applied to the surface of a buffered subphase in a Langmuir trough to examine surfactant inactivation (43). The change of surface pressure, π, as a function of time after surfactant deposition gave an indication of the degree of surfactant inhibition after addition of albumin (used as an inactivating agent) to the subphase. The method models tracheal instillation of surfactants for treatment of neonatal or adult respiratory distress syndrome. Using this technique, we have studied a variety of surfactants applied to subphases that include serum and/or polyethylene glycol ((PEG) 10 kDa) or hyaluronic acid ((HA) 1240 kDa). Brewster angle microscopy (BAM) was used to visualize the surfactant film during adsorption and spreading.

When clinical surfactants that are approved for use in the United States for treatment of neonatal respiratory distress syndrome (Infasurf, Survanta, or Curosurf) are spread as aqueous dispersions, adsorption from the subphase is the major route for surfactant accumulation at the interface. Clinical surfactants adsorb quickly ($<$100 s) to a clean interface, in a concentration and temperature-dependent manner up to the equilibrium spreading pressure, π_e, ($\sim$40–45 mN/m), in a very similar fashion to native porcine surfactant. Adding serum to the subphase before surfactant deposition caused serum components to reversibly adsorb to the interface, leading to a serum concentration-dependent surface pressure that saturates at $\pi_{max} \sim 20$ mN/m (19,44). Surfactant adsorption was significantly slowed for all the clinical surfactants at surface pressures below π_{max}. Above π_{max}, surfactant adsorbed at roughly the same rate as to a clean interface. From these experiments, the reduced rate of surfactant adsorption caused by the need to displace the serum proteins from the interface is one origin of rapid surfactant inactivation. As shown in the companion article (45), the surface-active proteins in serum create a steric and/or electrostatic barrier (46,47) to surfactant adsorption below π_{max}. The surfactant aggregates remain in suspension; the surfactant simply cannot reach the interface due to the serum protein barrier. Above π_{max}, the serum proteins are displaced from the interface by surfactant, and there is no longer a significant resistance to surfactant adsorption from the subphase. Hence, to reverse inactivation, it is necessary to either remove sufficient serum from the subphase or to enhance surfactant adsorption to displace the surface-active serum components from the interface.

Adding 5 wt% PEG or 0.125 wt% HA to the subphase restored the rate of clinical surfactant adsorption with serum in the subphase to that of the clean subphase, effectively reversing this form of surfactant inactivation. Native porcine surfactant containing SP-A was not inactivated by serum; sufficient SP-A may have the same effect as adding polymer to the subphase. The enhanced adsorption of surfactant in the presence of hydrophilic polymers can be explained using a simple ''depletion attraction'' (42,48–52) model described in the companion article. 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. The model also explains why high molecular weight, anionic polymers like HA can reverse inhibition at lower weight fractions than neutral, lower molecular weight polymers like PEG. Lower concentrations of higher molecular weight polymers would be beneficial in vivo to minimize edema due to osmotic imbalance in the lungs (42,53,54). The model also suggests that the SP-A and hyaluronan normally present in the alveolar hypophase may act to accelerate surfactant adsorption under normal conditions and may allow lower surfactant concentrations to be used in treatments.

METHODS

Materials

Infasurf (Forest Pharmaceuticals, St. Louis, MO) was purchased from the hospital pharmacy at University of California, San Francisco. Survanta (Abbott Laboratories, Columbus, OH) was obtained from the San Francisco General Hospital nursery. Curosurf was purchased from Dey Laboratories (Napa, CA). Serum was obtained from healthy laboratory volunteers and refrigerated until use. Differences in inactivation were not seen with sera from different volunteers. Protein content of sera was 6.8 g/dl. Polyethylene glycol (10 kDa), hyaluronic acid (1240 kDa), and bovine serum albumin were obtained from Sigma (St. Louis, MO). HA (250 and 100 kDa) was a gift from GlycoMed Research (New York, NY).

Native surfactant from slaughtered adult pigs (obtained from Matadero Municipal, Pozuelo de Alarcon, Madrid, Spain) was prepared by lavaging the lungs with ice-cold 0.9% NaCl buffer. Saline (2.5 liters) was introduced in aliquots into the trachea and withdrawn. The recovered lavage fluid was centrifuged at 1000 × *g* for 5 min to remove debris, then again in 0.9% (w/v) NaCl, at 105,000 × *g* for 1 h. The supernatant was discarded and the pellets were combined and homogenized in 24 ml 16% NaBr and 0.9% NaCl. To form discontinuous density gradients, 4 ml of the homogenate was put into each of six tubes, and then 6 ml of NaBr (13% in 0.9% saline) was layered into each tube, and a final layer of 2.5 ml of 0.9% NaCl was added. These tubes were centrifuged in a swinging bucket rotor at 120,000 × *g* for 2 h at 4°C. The lipid bands were removed, rehomogenized in 0.9% saline, and centrifuged (105,000 × *g* for 1 h). Pellets were placed in buffer or distilled water and centrifuged a second time. Lipid phosphorus (55) was measured and the pellets were stored at −70°C until further use. From one adult pig, ~200 mg of surfactant was recovered. Native surfactant extract was prepared by adding 1 ml of surfactant (~90 mg phospholipid) in saline to a mixture of 2 ml methanol and 1 ml chloroform; the surfactant lipids and hydrophobic proteins were extracted into the organic phase (56).

Methods

Surfactant spreading was done either at 24–25°C or 34–37°C using a temperature-controlled trough with a surface area of 228 cm^2 (Kibron, Helsinki, Finland) and a depth of ~1 mm. The surface pressure was measured by the pull of the film on a metal wire contacting the interface. The distance between the point of application of surfactant and the pressure sensor was 27 cm. The subphase volume was ~30 ml. Three or more measurements using at least two different vials or batches of each surfactant were carried out and compared, with standard deviations of ~5%, although the figures show representative results from single experiments. The standard buffer was either 2.5 mM HEPES or 2.5 mM Tris base, with 0.9% NaCl and 2.5 mM $CaCl_2$ adjusted to a pH of 7.0. The as-received surfactants were diluted with the same buffer to 15 mg/ml and mixed by vortex immediately before use (5 s × 6 over 1 min). Unless otherwise indicated, the surfactant suspensions were applied by micropipette in one drop to the surface (total volume 7 μl; 15 mg/ml; 105 μg total surfactant). The volume of applied material was fixed at 7 μl and the surfactant was applied to the interface at 25 s (on the horizontal axis) for all experiments, unless otherwise specified. Plotting in this way shows that the subphases with different additives had different initial surface pressures.

For experiments using polymers, the appropriate amount of PEG or HA were mixed in the same buffer until the solution was clear. HA in buffer was sometimes heated to 50°C for 1 h for complete solubilization. The standard concentration of PEG was 5% w/v, and HA was 0.125% w/v, based on prior in vitro and in vivo work (4,36,37,57). Human serum from laboratory volunteers, diluted to 1–10 μl/ml in buffer, was used as a nonspecific inhibitor. These concentrations of serum are similar to those found to reduce surface activity of surfactants in a pulsating bubble surfactometer at 37°C (4,36,37,57). Albumin concentrations in ARDS alveolar fluid may reach 100 mg/ml, with an average concentration reported by Ishizaka and coworkers of 25 mg/ml (23). Hence, the concentrations used here are significantly lower than typically found in ARDS patients. In certain experiments, a fixed amount of serum was added to the subphase as described. In other experiments, sufficient serum was added to the subphase to obtain a surface pressure from 10–20 mN/m after 60 s. The subphase temperature was measured with a Barnant thermocouple (Barrington, IL) and maintained with a temperature-controlled water circulator.

Brewster angle microscopy of the surface was carried out on a custom, temperature-controlled Langmuir-Wilhelmy balance (58–60). A *p*-polarized He-Ne laser is the light source, which was directed at the surface at an angle of 53.1° relative to vertical (the Brewster angle for water). At the Brewster angle, water (buffer) does not reflect any light; all of the incident light is transmitted into the water. However, if the interface is covered by any material with a refractive index different from water (or buffer), light is reflected and detected by a CCD camera. The intensity of reflected light depends on thickness of surfactant material and the variation in refractive index. Sequential images (30/s) were collected for 2 min from the time surfactant was applied to the surface. Images were collected in mpeg format for analysis by VirtualDub 1.5 (www.virtualdub.org) and PhotoShop 6.0. Magnification was constant with a field of view of 872 × 946 μm.

RESULTS

Fig. 1 *a* shows the effect of spreading different amounts of native porcine surfactant on a buffered subphase at 24°C. The rate of surface pressure increased roughly exponentially with time up to a limiting value that depends on surfactant concentration. Increasing the surfactant concentration increased the maximum surface pressure from <5 mN/m (35 μg) to 25 mN/m (70 μg) to 40 mN/m (140 μg). The maximum surface pressure saturated at ~40 mN/m for surfactant concentrations ≧100 μg, which is consistent with the equilibrium spreading pressure, π_e, measured by other techniques (3). The concentration dependence and the amount of time (~100 s) required to reach π_e (compare to Fig. 4 *b*), suggests that the primary mode of surfactant accumulation at the interface is by adsorption of surfactant aggregates from the subphase. Surfactant spread as a molecular solution from organic solvent reached π_e in seconds and the rate of surface tension reduction was relatively independent of the surfactant concentration. The spreading solvent keeps the surfactant at the interface so that there is nothing to adsorb from the subphase.

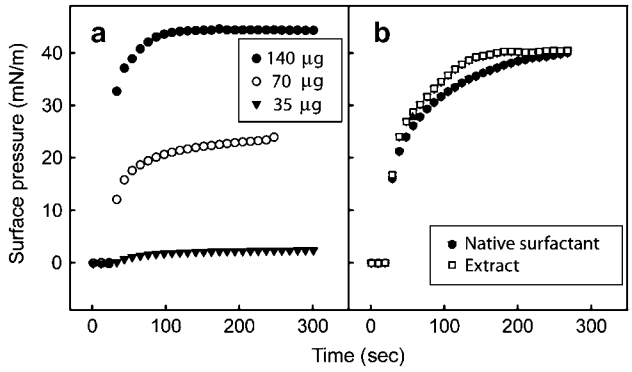

FIGURE 1 (*a*) Whole porcine surfactant (7 μl aqueous suspensions; different concentrations) applied to a clean subphase (24°C) at 25 s (● 140 μg; ○ 70 μg ; ▼ 35 μg total surfactant). Increasing the amount of surfactant increased the maximum surface pressure up to a limiting concentration of ~100 μg, at which the maximum surface pressure saturated at the equilibrium spreading pressure of the surfactant, ~40 mN/m (3). No differences in the maximum surface pressure were found between 105 μg (the amount used in most of the following results) and the 140 μg shown here. (*b*) Equal amounts and volumes (15 mg/ml; 7 μl; 105 μg total surfactant) of native whole porcine surfactant (●) and an aqueous suspension of organic extract of whole porcine surfactant (lyophilized and taken up in standard buffer) (□) applied to clean subphase (24°C) at 25 s. Minimal differences are seen between the two preparations. This suggests that SP-A and SP-D, the main hydrophilic proteins in lung surfactant that are removed by solvent extraction, are not essential to surfactant adsorption to a clean interface.

Fig. 1 *b* compares the same amounts of native porcine surfactant in aqueous buffer versus a lyophilized chloroform/methanol organic extract of porcine surfactant resuspended in aqueous buffer. Similar results were obtained; both samples caused rapid increases in surface pressure to $\pi_e \sim 40$ mN/m. The hydrophilic proteins SP-A and SP-D are not extracted into the chloroform-methanol (61). The close correspondence between the spreading rates and maximum surface pressure shown in Fig. 1 *b* shows that SP-A (or SP-D) is not essential to the rate of spreading or net amount of material adsorbed on a clean subphase.

Fig. 2 *a* shows a comparison at 37°C between the adsorption rates of the three commonly used clinical surfactants: Curosurf (porcine lung mince extract), Survanta (bovine lung mince extract), and Infasurf (calf lavage extract). As for the native surfactant, the surface pressure rose exponentially to the equilibrium spreading pressure, π_e. Curosurf consistently increased the surface pressure faster and to a higher π_e than Infasurf or Survanta when applied in this fashion. The clinical surfactants adsorb similarly to both native and extracted native surfactant on a clean subphase (Fig. 1).

Fig. 2 *b* shows the effect of subphase temperature on the spreading of Curosurf. The spreading rate increased with increasing temperature, but the maximum spreading pressure was the same for all temperatures. The viscosity of the Curosurf suspensions, ~1.3 centipoise, is similar from 15–37°C (62), and does not differ significantly from that of saline, hence the diffusivity of the Curosurf aggregates should also be similar at all temperatures. However, the surface viscosity of the Curosurf film decreases with increasing temperature, so it is likely that the greater interfacial fluidity of the lipids at higher temperature (63–66) is responsible for the increase in the spreading rate with temperature. However, π_e does not appear to change much with temperature.

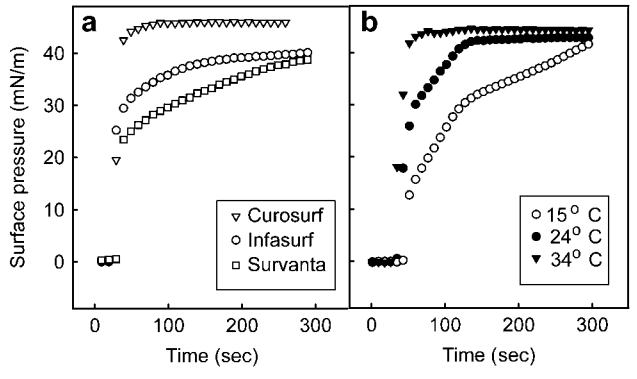

FIGURE 2 (*A*) Curosurf (▽), Infasurf (○), and Survanta (□) (15 mg/ml; 7 μl; 105 μg total surfactant) spread on a clean subphase at 37°C at 25 s. Curosurf consistently increased the surface pressure faster and to a higher value than Infasurf or Survanta when applied in this fashion. Infasurf was intermediate in both spreading rate and maximum surface pressure. (*B*) Effect of subphase temperature on the spreading of Curosurf (15 mg/ml; 7 μl; 105 μg total surfactant) on a clean subphase. (▼ 34°C; ● 24°C; ○ 15°C). Subphase temperature increased the rate of surfactant spreading, but not the equilibrium surface pressure.

Fig. 3 shows the progressive decrease of the rate of adsorption of Curosurf with increasing concentrations of subphase serum. Serum contains a number of hydrophilic, water soluble proteins that are surface active (19,44). Even before surfactant is added, increasing the serum concentration causes an increase in the surface pressure to ~10 mN/m for 1.7 μl/ml serum. Like many soluble, surface-active amphiphiles such as detergents and lysolipids (19,67), serum has a subphase concentration-dependent surface pressure that saturates at a maximum value, π_{max}. Increasing the serum concentration above ~3 μl/ml serum does not increase the surface pressure above ~20 mN/m. Krishnan and co-workers (44) have measured π_{max} for human serum and many of the proteins present in serum, such as albumin, hemoglobin, thrombin, IgG, IgM, etc. and have found a rather narrow range for the maximum surface pressure, π_{max}, of 20–25 mN/m. Most of the proteins in serum reduce the surface tension by ~20 mN/m at the saturation concentration, independent of the details of the protein structure, molecular weight, or function (44).

The increase in surface pressure on addition of Curosurf to the subphases containing serum was significantly slower at surface pressures below π_{max} than for a serum-free interface (Fig. 3). The time required for Curosurf to reach π_{max} increased with serum concentration. However once π_{max} was

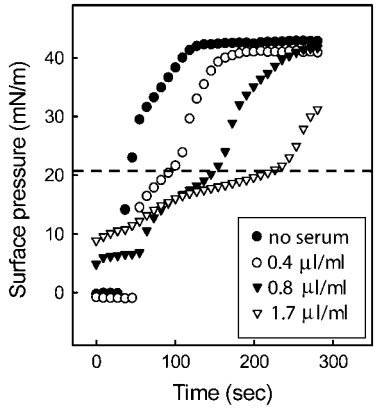

FIGURE 3 Curosurf (15 mg/ml; 7 μl; 105 μg total surfactant) applied to buffers (24°C) with increasing concentrations of serum. (● no serum; ○ 0.4 μl serum/mL buffer; ▼ 0.8 μl serum/mL buffer; ▽ 1.7 μl serum/mL buffer.) Increasing the serum concentration increased the buffer surface pressure (data before 25 s). The increase in surface pressure after addition of Curosurf at 25 s was slow below ~20 mN/m (*dotted line*), and was proportional to the serum concentration. Above ~20 mN/m, the increase in surface pressure was similar to that of the serum-free surfaces (see Figs 1 and 2). The critical surface pressure at which the rates change is roughly equal to the maximum surface pressure, $\pi_{max,}$ of a subphase containing a saturation concentration of serum, ~20 mN/m (19,44). The data suggest that as the surfactant adsorbs, the surfactant compresses the serum components at the interface up to π_{max}, at which the serum components are squeezed out from the interface back into the subphase. At higher serum concentrations in the subphase, more serum is adsorbed to the interface (44), and it takes longer for surfactant to adsorb and raise the surface pressure to π_{max}.

reached, the increase in surface pressure was similar to that of the serum-free surfaces and was independent of the serum concentration (see Fig. 2). The data suggest that as the surfactant adsorbs, it first concentrates the serum proteins so that the surface pressure increases to π_{max}. Further surfactant adsorption then starts to displace the serum proteins from the interface; as the surface pressure increases, the equilibrium partition of serum proteins is altered so that the proteins return to the subphase. At higher serum concentrations in the subphase, more serum is adsorbed to the interface up to saturation (44), and it takes longer for surfactant to adsorb and raise the surface pressure to π_{max}. Above π_{max}, the adsorption rate is similar to the rates shown in Figs. 1 and 2; the surfactant adsorbs at a similar rate as to a ''clean'' serum-free, surfactant-coated interface. For sufficiently high serum subphase concentrations, the clinical surfactants by themselves never increase the surface pressure above π_{max} (see Figs. 4–7).

When aqueous Curosurf was applied on buffer containing serum (see Fig. 4 *a*), only minimal increases in surface pressure were observed. Before application of the surfactant, the serum-containing buffer had a surface pressure of ~12 mN/m. As in the higher serum concentrations in Fig. 4, application of Curosurf did not change the surface pressure significantly, suggesting that a sufficiently high density of serum proteins at the interface prevents adsorption of Curosurf. However, when native porcine surfactant containing the hydrophilic proteins SP-A and SP-D was applied at 200 s after Curosurf had been applied to the buffer with serum, a rapid increase in surface pressure to ~40 mN/m occurred. The rate of increase was similar to that of whole surfactant on a clean interface (Fig. 1 *b*). If additional aqueous Curosurf was added at 200 s instead of native porcine surfactant, no increase in surface pressure was seen (data not shown).

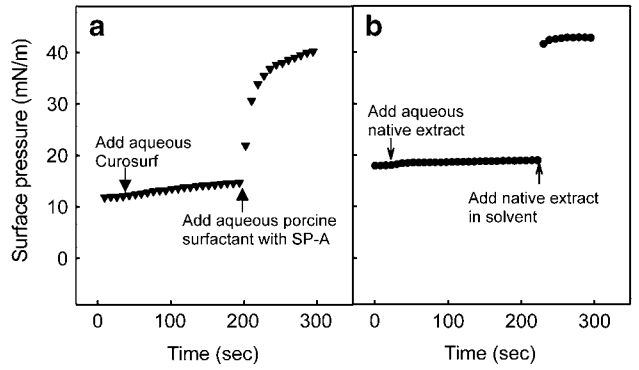

FIGURE 4 (*a*) Aqueous Curosurf (15 mg/ml; 7 μl; 105 μg total surfactant) deposited at 25 s on a subphase containing serum (▼). The initial surface pressure due to the serum was ~12 mN/m before adding Curosurf. There was a very slow and gradual increase in surface pressure on addition of the surfactant. At 200 s (*right arrow*), whole aqueous porcine surfactant (15 mg/ml; 7 μl; 105 μg total surfactant) was applied to the surface and a rapid rise in surface pressure to ~40 mN/m was seen. The rate of change of the surface pressure was similar to that on a clean interface (Fig. 1 *b*). If a second application of Curosurf was added at 200 s instead of whole porcine surfactant, no increase of surface pressure occurred (data not shown). (*b*) Whole porcine surfactant solvent extract, lyophilized and resuspended in aqueous buffer (15 mg/ml; 7 μl; 105 μg total surfactant) was applied at 25 s (*left arrow*) on serum (●) containing buffer (initial surface pressure, 18 mN /m). No change in surface pressure was observed. At 240 s, the same surfactant extract in organic solvent (15 mg/ml; 7 μl; 105 μg total surfactant) was applied (*right arrow*), leading to an almost instantaneous rise in surface pressure to ~40 mN/m. Applying the surfactant from solvent bypasses the serum proteins at the interface, allowing for fast decrease in surface pressure. This also confirms that the serum proteins primarily inhibit the adsorption of surfactant from the subphase, rather than interacting specifically with the surfactant.

However, Fig. 4 *b* shows that aqueous suspensions of organic extracts of porcine surfactant added to the subphase (at 25 s) do not cause a similar rise in surface pressure on a serum-containing subphase. The surface pressure does not rise above π_{max}, suggesting that the extracted surfactant cannot displace the serum proteins from the interface. The main difference between the composition of organic extract and that of porcine surfactant is the presence of the hydrophilic proteins SP-A and SP-D, which apparently assist in surfactant adsorption from the subphase. In addition to the presence of these proteins, surfactant adsorption could also be much more efficient via the formation of different bilayer aggregate structures such as lamellar bodies and tubular myelin surfactant formed by native surfactant (see Fig. 7), which are likely different than the structures formed by the reconstituted organic extracts (62).

However, spreading the porcine organic extract surfactant directly at the interface in chloroform/methanol solution (at 200 s) causes the surface pressure to rise immediately to the equilibrium spreading pressure. This confirms that surfactant inactivation is primarily caused by a decrease in the rate of surfactant adsorption from the subphase due to the presence of the surface-active components of serum. If surfactant can reach the interface, either due to the particular structure of the native complexes, including the presence of SP-A in the subphase (Fig. 4 *a*), or by directly applying the surfactant to the interface via an organic solvent (Fig. 4 *b*), serum has little effect on the rate of change of surface pressure or the equilibrium spreading pressure.

Serum inactivation occurred for all aqueous surfactants (with the exception of whole native porcine surfactant) including suspensions of Infasurf, Curosurf, Survanta, and organic extract of porcine surfactant at both 24° and 34–37°C, although greater amounts of serum were required at 34–37°C to induce inactivation (700 vs. 210 μg serum proteins per milliliter of buffer). The amount of serum needed for inactivation for the different surfactants varied as Survanta < Curosurf < Infasurf < surfactant extract < whole surfactant. In other words, whole surfactant was the least susceptible to serum inactivation. On the other hand, any of the surfactants that were lyophilized and resuspended in 2:1 chloroform methanol and deposited from organic solvent caused a rapid rise in surface pressure to ~40 mN/m, even if the subphase contained serum. This confirms that this form of surfactant inactivation is caused by a decrease in

the rate of surfactant adsorption from the subphase due to the presence of surface-active serum components at the air-water interface.

Previous work has shown that hydrophilic polymers can also reverse inactivation both in vitro and in vivo (4–6, 35–42). Fig. 5 *a* (Infasurf) and Fig. 5 *b* (Curosurf) show that when either HA or PEG was added to the serum-containing subphase, a rapid increase in surface pressure to ~40 mN/m occurred after addition of the aqueous suspensions of surfactant. The standard concentrations of 5% w/v 10 kDa PEG and 0.125% w/v of 1240 kDa HA were based on prior in vitro and in vivo work (4,36,37,57).

Fig. 5 shows that when either aqueous Infasurf (Fig. 5 *a*) or Curosurf (Fig. 5 *b*) was added to serum-containing subphases, little to no increase in surface pressure occurred and the maximum surface pressure was set by the serum at $\sim\pi_{max}$. HA by itself does not affect the surface pressure of the subphase without serum (the HA curve begins from a surface pressure of nearly zero), or the subphases with serum. However, HA does accelerate the adsorption of Infasurf for both serum-containing and serum-free subphases (compare to Fig. 2). For Infasurf, both HA and PEG lead to similar increases in the rate of surfactant adsorption. For Curosurf (Fig. 5 *b*) on subphases that also contained HA or PEG, the surface pressure increased to ~40 mN/m, either very quickly for PEG, or after a short induction time for HA. This difference in initial adsorption rate may be related to the higher initial serum surface pressure of ~15 mN/m for HA compared to ~12 mN/m for the PEG experiments. As in Fig. 4, once the surface pressure exceeded π_{max} of the serum, the rate of change of surface pressure increased dramatically and was similar to that for a clean interface (Figs. 1 and 2). The equilibrium surface pressure of Curosurf was not significantly changed by the presence of PEG or HA. Porcine surfactant extract (data not shown) and Survanta (data not shown) also show an increase in the rate of adsorption by both PEG and HA. Reduction of serum inactivation in the presence of subphase polymers was found both at 24°C and at 35–37°C. Again, the equilibrium spreading pressure of Curosurf and Infasurf were not affected by HA or PEG. It appears that the polymers do not interact specifically with either the serum or particular surfactants and alter the surface pressure by themselves. Instead, the polymers decrease the barrier to adsorption and thereby accelerate the transport of whatever surfactant is available in the subphase to the interface.

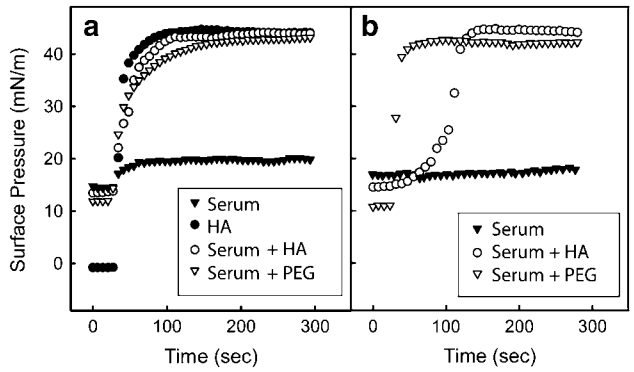

FIGURE 5 (*a*) Infasurf (15 mg/ml; 7 μl; 105 μg total surfactant) applied to different subphase mixtures at 24°C at 25 s: ● HA-buffer; ○ serum + HA (0.125%); ▽ serum + PEG (5%); ▼ serum alone. HA by itself does not raise the surface pressure of the subphase; however, serum alone or serum with HA or PEG raises the subphase surface pressure to ~12 mN/m at these concentrations. HA and PEG do not alter the serum surface pressure in the absence of surfactant. However, HA in the subphase accelerates the adsorption of Infasurf both with and without serum (compare to Fig. 2) in the subphase. With serum in the subphase, both HA and PEG prevent inhibition. (*b*) Curosurf (15 mg/ml; 7 μl; 105 μg total surfactant) applied at 25 s to subphase containing serum alone (▼), or serum + HA (0.125%) (○), or serum + PEG (5%) (▽), at 24°C. The rate of adsorption to the equilibrium spreading pressure is increased with both PEG and HA. PEG induced a rapid adsorption whereas HA showed a slow adsorption below π_{max} of the serum followed by a rapid adsorption for surface pressures above π_{max}.

Brewster angle microscopy

BAM images showed rapid lateral motion immediately following application of Curosurf to the subphase (Fig. 6, *top row*). BAM reveals differences in the local refractive index at the interface due to variations in monolayer density, orientation, structure, etc. Larger objects floating at or near the interface that scatter or reflect light are also visible (60). The rate of lateral spread slowed as surface pressure increased, consistent with a simple surface tension driven flow (68). A rough estimate of the flow velocity was made by measuring the speed of various structures as they moved across the field of view (~1000 μm or 1 mm). For example, the rate of lateral spreading of an individual white domain observed after spreading Curosurf was 7 cm/s at a surface pressure of 4 mN/m (~5 s after applying surfactant to the surface); 0.7 cm/s at a pressure of 18 mN/m (30 s after applying surfactant); and, 0.3 cm/s at a pressure of 27 mN/m (60 s after applying surfactant). Fig. 6 *a* shows a variety of bright and dark spots in the image corresponding to Curosurf aggregates trapped at or near the interface (58,60). Such structures were not evident on serum-containing subphases (Fig. 6 *b*) in which the surface pressure remained low after Curosurf addition. No lateral spreading of Curosurf was evident with serum in the subphase; it is likely that the serum proteins occupying the surface prevented the adsorption of surfactant. In addition, adsorption of serum over the entire interface lowered the surface pressure (to $\sim\pi_{max}$) and reduced the surface tension gradient driving the spreading of surfactant. However, when serum and PEG or HA were both added to the subphase, lateral spreading rates and the number of surface structures were similar to those obtained on buffer without serum (Fig. 6 *c*). Results were similar for other clinical surfactants on buffer and when HA was present in the buffer with serum (42). Native porcine surfactant, which was not inactivated by serum, showed a high density of surfactant aggregates near the interface for both clean subphases and serum-containing subphases (Fig. 6, *d* and *e*).

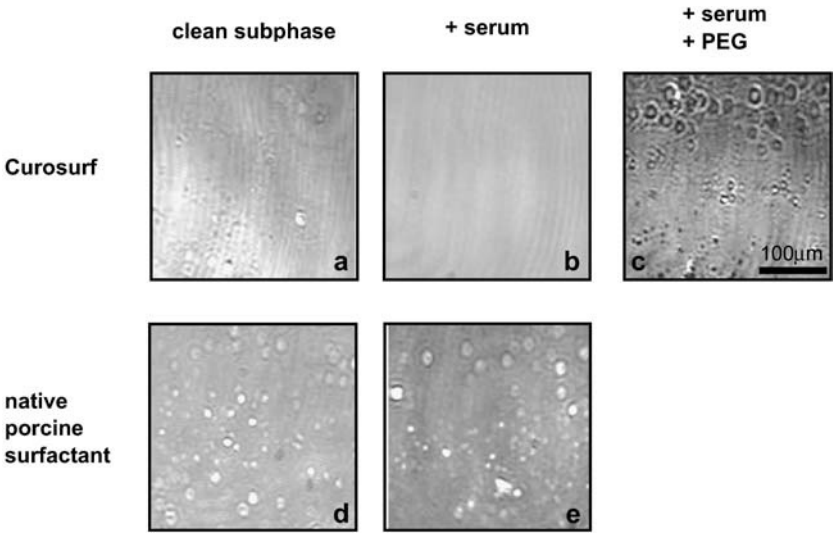

FIGURE 6 Brewster angle microscopy of the air-liquid interface ~2 min after application of surfactant. (*a*) Curosurf on clean buffer. (*b*) Curosurf on buffer with serum. (*c*) Curosurf on buffer with serum and PEG. With either clean buffer or buffer containing serum and PEG, diverse surface structures are seen and surface pressures are >35 mN/m. With serum alone in buffer, the surface is featureless, and surface pressure remains low. (*d*) Native porcine surfactant with SP-A on clean buffer. The surface pressure is ~40 mN/m. (*e*) Native porcine surfactant with SP-A on a serum containing buffer (up to 10 μl/ml at 24°C). Surface pressure is ~40 mN/m. There is no apparent difference between panels *d* and *e* suggesting that the SP-A in native surfactant can help overcome serum inactivation. A higher density of bright structures is noted in the experiments with native surfactant compared with those with surfactant extracts or commercial surfactants, suggesting better adsorption of native surfactant regardless of conditions.

DISCUSSION

In normal lungs, after secretion of surfactant in the form of multilamellar bodies from alveolar type II cells (13,69,70), surfactant must unpack, move across the alveolar hypophase, adsorb to the air-water interface, and then transform from bilayer to monolayer and spread over the interface (Fig. 7 *a*) (71). The primary function of lung surfactant is to vary the surface tension at the alveolar surface with changes in lung volume (hence alveolar surface area), the effects of which are to minimize the work of breathing, maintain a reservoir of air at end-expiration, prevent pulmonary edema, and allow equal inflation and deflation of different size alveoli. Similarly, aqueous mixtures of surfactant, introduced into the airway of a patient with lung disease, must travel to the periphery of the lung, adsorb, and spread to cover the air-liquid interface, despite the presence of alveolar inhibiting substances (Fig. 7 *b*). For both normal and exogenous surfactant, adsorption through the liquid subphase is the primary route of surfactant accumulation at the interface.

The spreading assay described here is a simple and reproducible method of studying surface activities of surfactants under a variety of conditions. Our experiments show that for surfactants deposited as aqueous suspensions, significant accumulation and exchange of surfactant occurs by adsorption of surfactant aggregates from the subphase. BAM

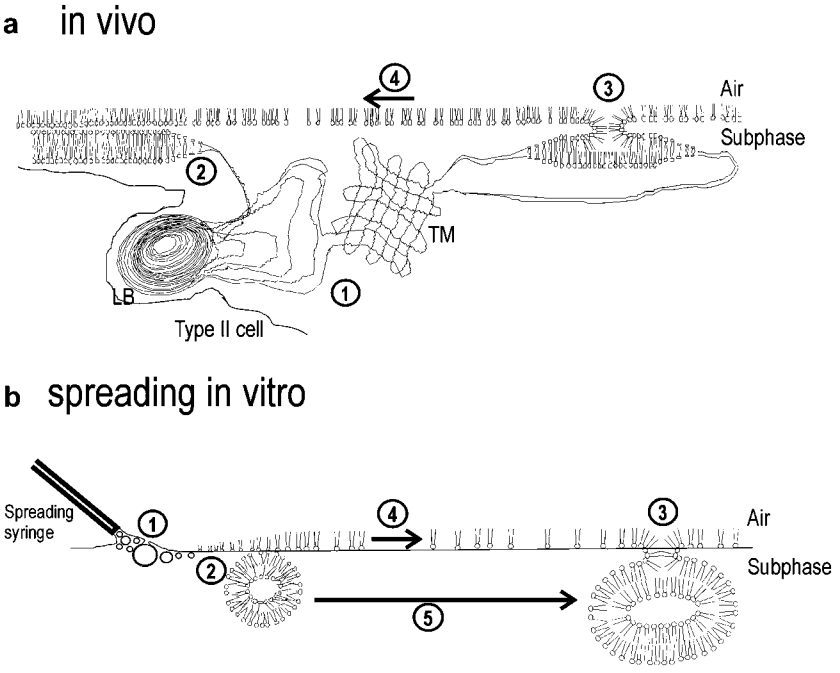

FIGURE 7 A model for surfactant spreading and adsorption of surfactant. Panel *b* illustrates surfactant secreted from the alveolar type II and the steps necessary for it to form a surface layer on alveolar subphase (in vivo): 1), secretion of multilamellar bodies from the Type II cell is generally followed by formation of tubular myelin surfactant; 2), the tubular myelin surfactant spreads as individual bilayers; 3), fusion and conversion of bilayers to monolayers; and 4), lateral spreading of surfactant monolayers. Panel *b* summarizes processes that may occur when aqueous surfactant is spread on the subphase surface (spreading in vitro): 1), deposition as aqueous suspension from syringe; 2), surfactant aggregates suspended in the subphase; 3), fusion and conversion of bilayers to monolayers; 4), spreading on monolayers at the air-liquid interface; and 5), transport of surfactant aggregates in the subphase.

images show a surface ''twinkling'' that looks like tiny raindrops hitting the surface of a lake as the aggregates approach the interface. This twinkling has been described by Winsel and co-workers in less phenomenological (and less poetic) terms. They studied whole human surfactant obtained by lavage from patients with sarcoidosis (72). After placing surfactant in subphase buffer, they aspirated the surface and observed by BAM the reaccumulation of surface material for times up to 1 h with surface pressures increasing from 20 mN/m to 44 mN. Complex surface structures were seen as surface pressures rose, with overall surface reflectivity increasing, indicating formation of structures on and below the surface. They state, ''Frequently one can observe a 'particle' approaching the surface from the subphase. In the moment of appearance of the particle at the interface, a spherical domain of film is forming in a very rapid way.''

Inactivation of surfactant by capillary to alveolar leak of serum is probably relevant to acute lung injuries. Albumin concentrations in ARDS alveolar fluid may reach 100 mg/ml, with an average concentration of 25 mg/ml (23). In excised rat lungs or in living rats, introduction of serum proteins into the trachea causes changes in mechanical behavior of the lungs consistent with surfactant inactivation at even lower concentrations (18,73). In vitro studies show that addition of serum to the subphase, or mixing serum with surfactant reduces the surface activity of surfactants (3).

Serum, like lung surfactant, is surface active (44). However, unlike surfactant, the serum proteins likely responsible for its surface activity (albumin, IgG, IgM (44)) are soluble in the subphase and are in rapid exchange equilibrium between the subphase and the interface. The surface activity of serum proteins is more similar to simple, water-soluble amphiphiles such as detergents and lysolipids (19,44,67) than the water-insoluble lipids in lung surfactant. Increasing the subphase serum concentration causes an increase in the surface pressure proportional to the logarithm of the concentration (19,44) up to a maximum value, π_{max}. Krishnan and co-workers (44) have measured π_{max} for human serum and many of the proteins present in serum, such as albumin, hemoglobin, thrombin, IgG, IgM, etc., and have found a rather narrow range for π_{max}. The surface-active proteins in serum adsorbed to the air-water interface create a steric and/or electrostatic barrier (46,47) to surfactant adsorption. Slowly compressing a serum-covered interface does not lead to a significant increase in the surface pressure; instead serum at the interface equilibrates with serum in the subphase to keep the serum surface concentration and surface pressure roughly constant at π_{max} (19,44), although protein unfolding or denaturation at the interface can lead to some hysteresis in the surface pressure.

Our data suggest that as surfactant adsorbs, it compresses the serum proteins at the interface up to π_{max}, at which point the serum proteins likely leave the interface and are solubilized in the subphase. At higher serum concentrations in the subphase, more serum is adsorbed to the interface that must eventually be displaced (44), and it takes longer for surfactant to adsorb and raise the surface pressure to π_{max}. Above π_{max}, the surfactant adsorbs to an effectively surfactant-coated interface, and the rate of adsorption above π_{max} is similar to the rates shown in Figs. 1 and 2 for serum free interfaces. However, to raise π significantly above π_{max}, the serum at the interface must be replaced by surfactant, a process that is slower the higher the concentration of serum in the subphase.

For sufficiently high serum subphase concentrations, the clinical surfactants never increase the surface pressure above π_{max} (see Figs. 3–6) within the timescale of these experiments. The slow surfactant adsorption in the presence of serum suggests that the serum imposes an electrostatic and/or steric barrier to surfactant adsorption from the subphase. We cannot distinguish if the barrier to adsorption comes from serum adsorbed to the interface or to the bilayer aggregate in solution. However, when the surfactant is applied directly to the interface by an organic solvent, thereby bypassing the serum barrier to adsorption, the change in surface pressure is instantaneous, regardless if serum is present in the subphase (Fig. 4 *b*). Adding PEG or HA largely reverses this inactivation by enhancing surfactant adsorption below $\pi_{max.}$ (35,40,74). Adding synthetic polymers to surfactants improves responses to treatment in animal models of acute lung injury (42,53,54).

The natural polymer, HA, and other glycosaminoglycans, are secreted by alveolar epithelial cells (75,76). These molecules may interact physically with surfactant under normal and abnormal conditions in the alveoli (13,74,77–82). Like PEG, HA is hydrophilic and not particularly surface active and does not appear to adsorb to surfactant bilayers or monolayers. The unifying features of the polymers that reverse inactivation are: 1), they do not specifically adsorb to surfactant aggregates; 2), they are small compared to surfactant aggregates (nanometers versus microns); 3), inhibition reversal occurs for all surfactant and polymer mixtures tried so far (4,35,36,40,42,57,74,83). This suggests that a generic interaction, the so-called ''depletion interaction'' as discussed in the companion article (45), leads to inactivation reversal, rather than a specific interaction between particular surfactants and polymers or between serum and polymer. The depletion interaction results from the increased free volume available to the polymer as the surfactant aggregates flocculate or adsorb to the interface (49–51).

CONCLUSIONS

Our spreading experiments show that serum adsorbs to the air-water interface, which reduces the rate at which surfactants adsorb due to a combination of steric effects and likely, electrostatic repulsion. Less surfactant adsorption means a slower increase in the surface pressure, and the need for greater compression to reach the nearly zero surface tensions

required for proper lung function. This is similar to what was observed in our earlier work that showed respreading of collapsed monolayers was inhibited by serum proteins (19) or electrostatic interactions between charged lipids (47). At the much more rapid rates at which surfactant must adsorb in the alveoli during respiration, this can lead to much higher surface tensions than can be accommodated by normal lung function. Hydrophilic polymers, and the hydrophilic surfactant protein SP-A, accelerate the adsorption of surfactant, likely via a nonspecific depletion attraction as discussed in the companion article (45). If lowering the energy barrier to adsorption via the depletion attraction can increase the rate of surfactant adsorption, sufficient surfactant will adsorb to quickly reach its equilibrium spreading pressure, π_e. As this is greater than the equilibrium spreading pressure of the serum, π_{max} (and most other proteins present in the alveoli), the surfactant should be able to displace the serum from the interface and eliminate inactivation.

The depletion model may also help explain certain in vivo structures as well. Whole, native porcine surfactant with SP-A is less inhibited by serum than surfactants from which SP-A has been extracted (surfactant extract, Survanta, Curosurf, and Infasurf). SP-A is the most abundant protein in lung surfactant and is hydrophilic and soluble, and may provide a depletion force, similar to the hydrophilic polymers. In vivo, surfactant is released from the type II cell as micron-sized lung multilamellar bodies (3,13,14). On entering the liquid layer lining the alveolus, multilamellar bodies transform into tubular myelin, a structure that has a regular rectangular lattice of bilayers with water layers ~50–500-nm thick (3,69). It is generally believed that this transition requires SP-A, SP-B, and calcium (3,84–86). SP-A, as well as the other natural macromolecules in the alveolar fluid (which include HA) may induce depletion forces that increase surfactant adsorption. The polymers added to replacement surfactants may replicate or enhance this function of SP-A.

Native surfactant with SP-A remains the gold standard that has not yet been duplicated by commercial or extracted surfactants. Results indicate that current surfactants used in therapy can be improved by addition of hydrophilic polymers that promote adsorption of the surfactant to the interface via a depletion force. We speculate that one of the reasons that surfactant replacement has not worked well in ARDS may be that serum, and other surface-active materials not normally present in the alveolar fluids, adsorb to the interface and act as a barrier to surfactant adsorption. Insufficient surfactant adsorption leads to higher surface tension in the lungs, and subsequent difficulties in respiration. Addition of hydrophilic polymers that accelerate the adsorption of surfactant to the interface, especially in the presence of serum, may be useful to include with surfactant treatment.

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