Early surfactant administration protects against lung dysfunction in a mouse model of ARDS

Vijay P. A. Rasaiah, Jaret L. Malloy, James F. Lewis, and Ruud A. W. Veldhuizen

Departments of Physiology and Pharmacology and Medicine, Lawson Health Research Institute, University of Western Ontario, London, Ontario N6A 4V2, Canada

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ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) is defined by severe lung dysfunction involving decreased lung compliance and hypoxemia (1). The pathophysiology of ARDS is complex and evolves after a variety of possible initiating events including direct insults to the lung, such as acid aspiration and pneumonia, and indirect insults, such as sepsis and pancreatitis. Because not all patients with these conditions develop ARDS, it has been proposed that this syndrome develops because of a number of additional or subsequent insults that may occur after the initiating event (17). The end result is an overwhelming inflammatory response within the host targeting a number of organs, including the lung.

One such example is ARDS induced by sepsis from the gut (3). Lung involvement occurs secondary to the systemic inflammatory response induced by peritonitis. If the illness is severe enough to result in deteriorating respiratory function, mechanical ventilation may be required. Unfortunately, this intervention itself may damage the lung, thereby contributing to the pathophysiology of ARDS as a “second insult” (25). Extensive research has focused on this latter issue, and recent studies have shown that more protective ventilatory strategies may improve the outcome of these patients (6). Despite these promising results, however, the morbidity and mortality of patients with ARDS remain high, particularly when associated with sepsis.

Several studies have shown that one of the mechanisms by which both sepsis and mechanical ventilation may contribute to progressive lung dysfunction is via alterations in the pulmonary surfactant system (19, 23, 29). The main function of surfactant is to lower surface tension and maintain alveolar stability, although recent studies have suggested that surfactant may also play an important role in host defense (11, 24). Surfactant is composed of ~90% lipids and 10% surfactant-associated proteins (11). Within the air space, the functional forms of surfactant are called large aggregates (LA), which during respiration are converted into smaller, nonfunctional forms called small aggregates (SA) (5, 33). In patients with established ARDS as well as in patients at risk for ARDS, the composition of surfactant is altered, resulting in impaired biophysical function (10, 12, 13, 28). Studies involving animal models of ARDS have demonstrated that these alterations of surfactant contribute directly to the severe lung dysfunction associated with this syndrome (10, 16).

Far less is known about the role of the surfactant system during the development of ARDS. In an animal model of sepsis with relatively mild lung dysfunction, alveolar surfactant pool sizes were altered, but surfactants’ biophysical function were not affected (19, 21). We hypothesized that the surfactant changes during sepsis may have influenced the host’s pulmonary inflammatory response, thereby increasing the susceptibility of the lung to a subsequent insult such as mechanical ventilation. To address this hypothesis, we used a mouse model of sepsis induced by cecal ligation and perforation (CLP) followed 18 h later by ex vivo mechanical ventilation. The specific objectives of the current study were to examine the effects of adminis-
tering exogenous surfactant at the time of CLP on lung compliance and the inflammatory cytokine concentrations in the lung and to determine the fate of the exogenous surfactant at a remote time point after administration.

MATERIALS AND METHODS

CLP/sham surgery and experimental groups. Sepsis was induced in mice via CLP as previously described (21). Briefly, male BALB/c mice weighing between 20 and 25 g (7–9 wk old) were anesthetized with an intraperitoneal injection of ketamine/xylazine (0.25 and 0.025 mg/kg, respectively). A laparotomy was performed to expose the entire cecum, which was then ligated distally to the ileocecal valve and punctured twice by an 18-gauge needle. The cecum was gently manipulated to extrude a small amount of fecal material and placed back into the abdomen. The abdominal incision was then closed with 4-0 silk suture. Sham-operated, control groups consisted of animals undergoing identical anesthetic and laparotomy procedures, but with no manipulation of the cecum. Of note, the sham/CLP surgeries were performed with the surgeon blinded to the subsequent treatment/ventilation protocol.

Surfactant treatment. While the animals were still under general anesthetic from the CLP/sham procedure, a small incision was made on the ventral region of the neck, and the trachea was carefully exposed. Animals were then placed supine on a slight incline, and a 25-gauge needle was used to puncture the trachea. Either bovine lipid extract surfactant (BLES, 27 mg/ml, 100 mg lipid/kg body wt; BLES Biochemical, London, Ontario, Canada) or air (non-Rx) was instilled to puncture the trachea. Either bovine lipid extract surfactant (BLES, 27 mg/ml, 100 mg lipid/kg body wt; BLES Biochemical, London, Ontario, Canada) or air (non-Rx) was instilled through the needle into the lungs followed by 50 µl of air. Animals used for analyzing recovery of the exogenous surfactant after death were given BLES radiolabeled with [3H]diplamitoylphosphatidylcholine ([3H]DPPC, NEN, Boston, MA) as previously described (15). A continuous flow of oxygen (2 l/min, FIO2 = 100%) was administered to animals during these procedures and shortly thereafter until normal spontaneous breathing was apparent. The neck incision was then closed with 4-0 silk sutures, and animals were given an injection of buprenorphine (0.04 mg/kg sc) in 1.5 ml of saline to maintain adequate analgesia and blood pressure.

Ex vivo mechanical ventilation and lung compliance measurements. Eighteen hours after CLP/sham surgery, all animals were killed by injection with ketamine/xylazine (0.25 and 0.025 mg/kg, respectively). A midline sternotomy was performed, and blood was sampled via cardiac puncture by a 22-gauge needle. The descending aorta was then transected and blood was withdrawn twice more before recovery (20, 30). The three recovered lavages were combined, and the total volume was recorded. The lavage was then centrifuged at 150 × g for 10 min to remove cellular material. The 150-g supernatant was utilized for measurement of total surfactant. In addition, 1-ml aliquots of the 150-g supernatant were used to separate the LA and SA fractions, as well as an aliquot of the LA fraction was resuspended in a humidiﬁed chamber (37°C) and mechanically ventilated to a pressure of 25 cmH2O, lungs were inflated with air in a stepwise fashion with the corresponding pressure recorded at each volume. The first five inflations consisted of 20-µl volumes each, followed by inflations of 50-µl volumes until a pressure of 25 cmH2O was reached. The lungs were then subsequently deflated in a similar fashion while pressures were recorded at each level.

Experimental groups. The above procedures resulted in a total of eight experimental groups into which the mice were randomized: 1) a nonsurfactant-treated, sham group (non-Rx sham), 2) a surfactant-treated, sham group (Surf-Rx sham), 3) a nonsurfactant-treated, sham group with lungs removed at 18 h to undergo ex vivo mechanical ventilation (non-Rx sham Vent), 4) a surfactant-treated, sham group with lungs removed at 18 h to undergo ex vivo mechanical ventilation (Surf-Rx sham Vent), 5) a nonsurfactant-treated, CLP group (non-Rx CLP), 6) a surfactant-treated, CLP group (Surf-Rx CLP), 7) a nonsurfactant-treated, CLP group with lungs removed at 18 h to undergo ex vivo mechanical ventilation (non-Rx CLP Vent), and 8) a surfactant-treated, CLP group with lungs removed at 18 h to undergo ex vivo mechanical ventilation (Surf-Rx CLP Vent).

All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the University Animal Care Subcommittee.

Lung lavage analyses. After death and pressure-volume curve measurements, lungs were lavaged three times using three aliquots of 1 ml 0.15 M NaCl. Each lavage consisted of instilling and withdrawing the saline with reinstillation and withdrawal twice more before recovery (20, 30). The three recovered lavages were combined, and the total volume was recorded. The lavage was then centrifuged at 150 × g for 10 min to remove cellular material. The 150-g supernatant was utilized for measurement of total surfactant. In addition, 1-ml aliquots of the 150-g supernatant were used to separate the LA from the SA via centrifugation at 40,000 × g for 15 min. The pellet of the 40,000-g spin (the LA) fraction was resuspended in 300 µl of 0.15 M saline and frozen at −20°C until further use. The SA fraction was also stored at −20°C.

The total quantity of surfactant phospholipids was determined by phospholipid-phosphorus measurements using aliquots from the 150-g supernatant (total surfactant), 40,000-g pellet (LA), and 40,000-g supernatant (SA). Samples were extracted using the method of Bligh and Dyer (2), and phospholipid-phosphorus levels were determined by a modification of the Duck-Chong method (7, 21). Total protein was measured in the 150-g supernatant by the method of Lowry and colleagues (18) with bovine serum albumin as the standard.

Radiolabel recovery was assessed by measuring [3H]DPPC in both lung tissue and lung lavage fractions of the animals that were given the radiolabeled surfactant. The lung tissue of these animals was homogenized after the lavage procedure. Aliquots of the lung homogenate, the 150-g supernatant, the LA and SA fractions, as well as an aliquot of the administered radiolabeled BLES were extracted by the method of Bligh and Dyer (2). Radioactivity was determined via scintillation counting.

Cytokine analysis. A separate cohort of animals (n = 6/group) was used to measure the concentrations of the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the lavage. Previous studies using the CLP and ex vivo ventilation models have consistently demonstrated that these particular cytokines are elevated in the lungs of these animals (30). Identical experimental procedures were performed on these animals, although nonradio-
labeled BLES was instilled. In addition, the lungs from these animals were lavaged only with two 1-ml aliquots of 0.15 M saline for cytokine measurements. This lavage was then centrifuged at 200 g for 10 min at 4°C, and the supernatant was aliquotted into 350-μl amounts, flash frozen with liquid nitrogen, and stored at −70°C. Subsequently, samples were thawed at room temperature, and enzyme-linked immunosorbent assays (ELISA; BD Pharmingen, Mississauga, Canada) were performed to measure concentrations of these cytokines.

Inhibition of BLES activity by serum. To assess the potential of serum obtained from both CLP and sham animals to inhibit the function of a known concentration of BLES, we performed in vitro analyses of surface activity using a pulsating bubble surfactometer (Electronetics, Buffalo, NY) (9). Blood was obtained via cardiac puncture from both CLP and sham animals at death and was then centrifuged at 500 g (4°C) for 5 min. The serum was carefully removed, flash frozen using liquid nitrogen, and stored at −70°C for further analysis. Samples were subsequently thawed, and total protein recovery was determined by the Lowry method with bovine serum albumin as a standard (18). Varying concentrations of the serum samples ranging from 0 to 0.1 mg protein/ml were added to 2 mg/ml BLES suspended in 0.15 M NaCl and 1.5 mM CaCl2. After incubation for at least 90 min at 37°C, samples were then pulsed for 5 min at 20 pulsations/min on the pulsating bubble surfactometer (9). The surface tension at minimum bubble size after 100 pulsations was recorded.

Statistics. Data are expressed as means ± SE. Statistical analysis was performed using the SPSS statistical software package for Windows, version 9.0.0 (SPSS, Chicago, IL). Values among groups were compared using a three-way analysis of variance. A least significant difference post hoc test was used for multiple comparisons. A probability level of \( P < 0.05 \) was considered statistically significant.

RESULTS

A total of 116 animals were used in this study, with no significant differences in mean body weights of the animals between groups. Although not statistically significant, there were differences in mortality observed among the groups. Although there were no deaths in either of the sham groups, whether they were given exogenous surfactant or not, 21% (7/34) of the CLP animals not given surfactant died compared with only 7% (2/32) of those animals given BLES at the time of CLP. Although the specific cause of death is unknown, all deaths occurred at least 6 h after surgery and anesthetic, suggesting that these animals developed overwhelming sepsis with multiple organ failure.

Figure 1A shows the peak inspiratory pressure (PIP) values (reflecting dynamic lung compliance) both at the start (time 0) and after 2 h of mechanical ventilation in lungs that were removed from animals at the 18-h time point for ex vivo ventilation. Figure 1B shows volumes measured at maximum pressure (Vmax) obtained from the pressure-volume curves (reflecting static lung compliance), which were performed either immediately after death at 18 h (non-Vent) or after 2 h of ex vivo mechanical ventilation (Vent). The CLP procedure had no significant effect on either dynamic (Fig. 1A, time 0) or static (Fig. 1B, non-Vent) lung compliance compared with the sham procedure. Surfactant administration also had no effect on nonventilated sham and CLP lungs compared with their respective nonsurfactant-treated control groups. After 2 h of mechanical ventilation, however, significant increases in PIP and decreases in Vmax were observed in both sham and CLP groups not given exogenous surfactant (Fig. 1, A and B). Interestingly, although surfactant administration had no significant effect on the decrease in compliance noted in the sham lungs after mechanical ventilation, BLES did mitigate the changes in both PIP (Fig. 1A) and Vmax (Fig. 1B) induced by the ventilation in the CLP animals. As a result, lung compliance values were significantly higher in ventilated CLP lungs that had received exogenous surfactant compared with ventilated, CLP lungs that were not given surfactant.

Lung lavage analyses. Figure 2 shows the total amounts of alveolar surfactant recovered from these animals’ lungs at death (Fig. 2A) and the proportion of this surfactant existing as LA (Fig. 2B) and SA (Fig. 2C) forms. Compared with their respective nonsurfactant-treated control groups, surfactant treatment significantly increased total surfactant and LA pools in nonventilated sham lungs and significantly increased
the LA fraction in the nonventilated CLP lungs. Two hours of mechanical ventilation increased SA pools in both nontreated sham and CLP lungs compared with nonventilated lungs. Administration of exogenous surfactant at the time of sham/CLP surgery followed by ventilation resulted in a further increase in alveolar surfactant pool sizes compared with the surfactant-treated groups that were not ventilated. This increase in surfactant was due to significant increases in both LA and SA in these surfactant-treated lungs and was not significantly different between sham and CLP lungs.

Table 1 shows the amount of total protein recovered in the lung lavage of the various groups. In the non-surfactant-treated groups, the CLP procedure did not result in an increase in alveolar protein compared with the sham procedure. In addition, the administration of exogenous surfactant did not result in an increase in lavage protein values in the nonventilated, sham, or CLP lungs. Two hours of mechanical ventilation re-

![Fig. 2. Surfactant pool sizes measured by phospholipid-phosphorus analysis from lung lavage from the 8 experimental groups outlined in MATERIALS AND METHODS. A: total exogenous surfactant; B: large aggregates; C: small aggregates. Values are means ± SE, n = 6/group. Statistical significance (P < 0.05) * vs. Non-Rx, # vs. non-ventilated.](image1)

![Fig. 3. Exogenous surfactant pool sizes measured by [3H]dipalmitylphosphatidylcholine-phosphorus amounts from lung lavage from the 4 experimental groups receiving radioactive exogenous surfactant. A: total exogenous surfactant, B: large aggregates, and C: small aggregates. Values are means ± SE, n = 6/group. Statistical significance (P < 0.05) # vs. nonventilated, $ vs. sham.](image2)
resulted in a significant increase in the protein values in both CLP and sham lungs compared with the nonventilated control groups. Surfactant administration did not affect this ventilation-induced increase in protein levels in the lavage in either CLP or sham lungs.

On the basis of the differences in compliance, it was speculated that the serum proteins leaking into the air space in the CLP animals may have been less inhibitory to the exogenous surfactant than the protein leaking into the sham, ventilated lungs. In vitro experiments were therefore performed to determine whether serum obtained from either sham or CLP animals immediately before death had different effects on the surface activity of the exogenous surfactant preparation, BLES. Figure 4 shows that increasing amounts of serum protein mixed with 2 mg phospholipid/ml BLES resulted in significant increases in surface tension for both sham and CLP serum samples. There were no significant differences between the sham and CLP serum with respect to their inhibitory effects on the BLES.

Figure 5 shows the concentrations of both TNF-α (Fig. 5A) and IL-6 (Fig. 5B) in the alveolar lavage of the various groups. The CLP procedure resulted in an increase in IL-6 concentration compared with the sham procedure, although this did not reach statistical significance. No significant differences in TNF-α concentrations were observed. Surfactant administration did not affect either the TNF-α or IL-6 concentrations in the nonventilated sham or CLP lungs compared with their respective nonsurfactant-treated groups. Two hours of ex vivo mechanical ventilation significantly increased both TNF-α and IL-6 concentrations in both sham and CLP lungs compared with their respective nonventilated groups. Furthermore, the CLP lungs that were mechanically ventilated had significantly higher TNF-α and IL-6 concentrations than the sham lungs undergoing mechanical ventilation. Surfactant administration had no effect on the cytokine concentrations in the ventilated sham lungs compared with nontreated sham lungs that were ventilated. In contrast, surfactant-treated, ventilated CLP lungs had lower cytokine concentrations than the nonsurfactant-treated, ventilated CLP lungs, and this difference was statistically significant for TNF-α.

**DISCUSSION**

The development of ARDS usually involves an initial insult, such as sepsis, that may subsequently predis-

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**Table 1. Total protein (mg/kg) in lung lavage**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Non-Rx</th>
<th>Surfactant-Rx</th>
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<tbody>
<tr>
<td>Sham</td>
<td>21.2 ± 2.1</td>
<td>20.9 ± 6.0</td>
</tr>
<tr>
<td>Sham + ventilation</td>
<td>78.2 ± 10.7*</td>
<td>90.0 ± 15.1*</td>
</tr>
<tr>
<td>CLP</td>
<td>25.7 ± 5.7</td>
<td>32.7 ± 6.5</td>
</tr>
<tr>
<td>CLP + ventilation</td>
<td>64.5 ± 11.6*</td>
<td>89.0 ± 27.8*</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 6/group. CLP, cecal ligation and perforation; Non-Rx, nonsurfactant treated; Surfactant-Rx, surfactant treated. *Statistical significance (P < 0.05) vs. nonventilated.
pose the lung to further insults such as mechanical ventilation (17, 23). We studied the effects of administering exogenous surfactant at the time of the CLP procedure on the subsequent development of sepsis over 18 h and on the response of these lungs to 2 h of ex vivo mechanical ventilation at this time point. Our results demonstrated that, during the 18 h after CLP, exogenous surfactant had no impact on sepsis-induced IL-6 concentrations in the lungs and that the administered surfactant was metabolized similarly to endogenous surfactant over this time period. Interestingly, the administered surfactant did have a significant protective effect on the lung dysfunction induced by mechanical ventilation, but only in the CLP lungs.

To reflect the paradigm of ARDS resulting from multiple insults rather than one massive initiating event, this experimental study used the combination of systemic sepsis induced by CLP followed by a mode of mechanical ventilation that was previously shown to induce lung dysfunction (30). Although the CLP model clearly has clinical relevance, the ex vivo ventilation model has some limitations. However, as pointed out in previous reports, the specific ventilatory strategy used in this study reflects the damaging effects of overdistension as well as the repeated collapse and reopening of alveolar units that occur within regions of the lung in mechanically ventilated patients (22, 27, 30). In addition, a significant advantage of this model is the relatively short period of time during which the injury occurs, making it conducive to experimental study. To investigate the role of the surfactant system in the processes leading to lung dysfunction in this paradigm, we administered a relatively large dose, 100 mg/kg, of a BLES at the time of the CLP procedure. This surfactant was chosen since it is used clinically to treat neonates with RDS and has been used in a clinical trial for the treatment of ARDS (8, 14).

Consistent with previous studies using the CLP model, our results showed that this procedure did not result in significant lung injury, since lung compliance measurements and bronchoalveolar lavage (BAL) protein values were not significantly different from sham animals (21). Also consistent with previous studies were the alterations in the surfactant system and the increase in IL-6 concentrations in lung lavage observed in CLP animals compared with the sham procedure. Administering exogenous surfactant at the time of surgery did not affect lung compliance in either the CLP or sham groups at 18 h, nor did it affect IL-6 concentrations in the lavage of the CLP lungs. It is interesting to note, however, that CLP animals given surfactant had a lower mortality than their nonsurfactant-treated counterparts. Although the number of animals used in this study was not sufficient for this result to reach statistical significance, it is possible that surfactant administration may indeed impact the outcome of an animal’s response to CLP. Further studies, powered to evaluate additional outcome parameters such as mortality, are warranted.

Analyses of the fate of the exogenous surfactant 18 h after administration revealed significant differences in the recovery of this material in the nonventilated, sham, and CLP groups (Figs. 2 and 3). In general, it appeared that the metabolism of the exogenous surfactant was similar to the endogenous surfactant system. For example, the total recovery of the radiolabeled surfactant in the alveolar lavage of CLP animals was lower than the sham group. In previous studies, this decrease in total alveolar surfactant and the SA fractions in CLP lungs was attributed either to decreased synthesis and/or secretion or to an increased uptake of SA from the air space (19, 21). Because our exogenous surfactant obviously does not require the same synthetic/secretory pathways as endogenous material, we conclude that the decreased pools in the CLP lungs were likely due to increased surfactant uptake. Furthermore, measurements of the radioactivity from exogenous surfactant subsequent to ventilation revealed significant increases compared with the nonventilated lungs (Fig. 3). This implies that at least part of the exogenous surfactant that was taken up during the 18 h subsequent to treatment was available for resecretion into the alveolar space.

Overall, mechanical ventilation, the second insult in our experimental paradigm, was shown to significantly decrease lung compliance, increase SA surfactant pool sizes, increase inflammatory mediators within the lungs, and increase BAL protein concentrations, compared with nonventilated lungs. However, possibly the most striking observation in the current study was the effects of the exogenous surfactant on lung compliance measurements in the ventilated, CLP lungs. These lungs had compliance values superior to those of both the nonsurfactant-treated CLP lungs and the surfactant-treated sham lungs after ventilation. Interestingly, the ventilated sham lungs given surfactant had no such benefit (protection) in response to the surfactant.

The specific mechanism(s) responsible for the protective effect of surfactant in the CLP lungs, but not the sham lungs, is unknown. Simply increasing alveolar surfactant pool sizes was not responsible, as similar changes in the total, LA, and SA pools were observed in both the sham and CLP lungs given surfactant (Fig. 2). In addition, the increase in protein levels within the air space induced by the ventilation was not affected by the administered surfactant. Because previous studies have shown that exogenous surfactant administered immediately before the onset of mechanical ventilation mitigates the lung dysfunction (31, 32), the absence of such a response observed in the sham lungs in the present study is likely due to the remote time point before mechanical ventilation when the surfactant was administered. We then speculated that the protein leaking into the air space in the CLP animals may have been less inhibitory to the exogenous surfactant than the protein leaking into the sham, ventilated lungs. Although there was a trend toward less inhibition of the serum from CLP animals on BLES, there were no significant differences observed between the groups, indicating that this mechanism was not responsible for
the different effects of the administered surfactant in the CLP and sham lungs.

It is also possible that the surfactant may have had an impact on pulmonary IL-6 and TNF-α concentrations during mechanical ventilation, as previous studies have shown that surfactant can influence cytokine release (4, 26). In our study, CLP alone resulted in an increase in IL-6, whereas ventilation alone caused an increase in both IL-6 and TNF-α. The combination of CLP and ventilation resulted in a synergistic increase in the concentrations of both cytokines within the air space. These specific changes in inflammatory mediators may represent one of the mechanisms by which ventilation contributes to the progression of injury in lungs with pre-existing damage (25). Although the administration of surfactant did significantly decrease the concentration of TNF-α in the lung lavage of the ventilated, CLP lungs compared with the nonsurfactant-treated, ventilated, CLP lungs, it is unlikely that this relative difference in TNF-α concentration was responsible for the marked differences in compliance noted between these two groups. This is supported by the observation that the sham ventilated group had even lower TNF-α concentrations than the CLP groups, but worse compliance. It is more likely that the converse was true. The superior compliance of the surfactant-treated, ventilated, CLP lungs may have been indirectly responsible for the lower TNF-α concentrations compared with the nonsurfactant-treated group. Cytokine release through ventilation is thought to occur from overstretching and collapse/reopening of lung units (27), and the exogenous surfactant may have limited some of these processes, resulting in less cytokine release.

In conclusion, we administered a large dose of surfactant at an early time point to evaluate the role of the surfactant system both on the inflammatory response induced by the CLP procedure and the lung dysfunction created by the addition of mechanical ventilation. Although the exogenous surfactant had no effect on the pulmonary cytokine concentrations or metabolism of surfactant over the course of sepsis, the administered BLES did protect the CLP lungs from the deleterious effects of mechanical ventilation. The mechanism(s) responsible for this protection are currently unknown but are unlikely to involve the increased surfactant alveolar pool sizes, inflammatory cytokine concentrations, or serum protein inhibition. We speculate that the surfactant within the CLP lungs may be modified, for example, due to the incorporation of SP-A, to a greater extent than in the sham lungs, rendering these treated CLP lungs more resistant to the damaging effects of ventilation. Studies evaluating such mechanisms are currently underway.

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Current address for J. L. Malloy: Box 3709, Dept. of Cell Biology, Duke University Medical Center, Durham, NC 27710.

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