Protective effects of elevated endogenous surfactant pools to injurious mechanical ventilation

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Yamashita C, Forbes A, Tessolini JM, Yao L-J, Lewis JF, Veldhuizen RA. Protective effects of elevated endogenous surfactant pools to injurious mechanical ventilation. Am J Physiol Lung Cell Mol Physiol 294: L724–L732, 2008. First published January 25, 2008; doi:10.1152/ajplung.00389.2007.—Depletion of alveolar macrophages (AM) leads to an increase in endogenous surfactant that lasts several days beyond the repletion of AM. Furthermore, impairment to the endogenous pulmonary surfactant system contributes to ventilation-induced lung injury. The objective of the current study was to determine whether increased endogenous surfactant pools induced via AM depletion was protective against ventilation-induced lung injury. Adult rats were intratracheally instilled with either control or dichloromethylene diphosphonic acid (DMDP) containing liposomes to deplete AMs and thereby increase endogenous surfactant pools. Either 3 or 7 days following instillation, rats were exposed to 2 h of injurious ventilation using either an ex vivo or in vivo ventilation protocol and were compared with nonventilated controls. The measured outcomes were oxygenation, lung compliance, lavage protein, and inflammatory cytokine concentrations. Compared with controls, the DMDP-treated animals had significantly reduced AM numbers and increased surfactant pools 3 days after instillation. Seven days after instillation, AM numbers had returned to normal, but surfactant pools were still elevated. DMDP-treated animals at both time points exhibited protection against ventilation-induced lung injury, which included superior physiological parameters, lower protein leakage, and lower inflammatory mediator release into the air space, compared with animals not receiving DMDP. It is concluded that DMDP-liposome administration protects against ventilation-induced lung injury. This effect appears to be due to the presence of elevated endogenous surfactant pools.

alveolar macrophages; inflammatory mediators

PULMONARY SURFACTANT is a lipoprotein complex that lines the alveolar air spaces and serves to maintain normal lung function (14). Surfactant is secreted by type II pneumocytes and adsorbs to the air-liquid interface where it serves to maintain alveolar stability and facilitate arterial oxygenation. The freshly secreted and surface layer forms of surfactant are called large aggregates (LA) and represent the functional component of alveolar surfactant (15, 21, 36). Subsequent expansion and compression of the alveolar surface observed during normal respiration results in conversion of LA into nonfunctional, small aggregate (SA) forms, which are either degraded or recycled by resident alveolar macrophages (AM) and type II pneumocytes, respectively (35).

Recently, our laboratory has demonstrated that AM depletion via intratracheal delivery of dichloromethylene diphosphonic acid (DMDP)-encapsulated liposomes resulted in a ninefold increase in the total amount of endogenous surfactant by day 3 postinstillation, likely due to a reduction in clearance of surfactant from the air spaces (10). This accumulation of surfactant included a fourfold increase in the active subtraction of surfactant, the LA, as well as an accumulation of SA. Moreover, these increased surfactant pools remained elevated for 7 days after DMDP-liposome administration, despite re-population of AM by that time point. Of note, these changes were not associated with abnormal lung physiology or evidence of alveolar inflammation. Based on these observations, we hypothesized that the elevated endogenous LA pools acquired through DMDP-liposome administration would be protective in clinical settings in which surfactant dysfunction has been shown to contribute to disease pathophysiology.

One such clinical insult is the lung injury caused by mechanical ventilation (MV) (7). In experimental studies, MV utilizing high tidal volumes and zero end-expiratory pressure levels led to progressive impairments in lung compliance and arterial oxygenation and was associated with alterations of the endogenous surfactant system (7, 28, 32). This injury has been termed ventilator-induced lung injury (VILI). In a broader context, this type of lung injury due to MV is thought to significantly contribute to the pathogenesis of acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) (4). For example, institution of MV in animals or patients with preexisting lung injury has been demonstrated to propagate epithelial damage leading to further lung dysfunction (4, 19). The heterogeneity of lung injury in ALI/ARDS leads to overstretching and collapse of lung units, something that can be mimicked in normal animals by utilizing high tidal volumes and zero end-expiratory pressure (30, 31). More specifically, the cyclic alveolar stretch associated with high tidal volume ventilation has been shown to enhance LA conversion to nonfunctional SA forms, a process felt to represent a key mechanism in the pathogenesis of ALI (29). Furthermore, an intense inflammatory response, known as biotrauma, occurs in parallel to these changes. In this situation, mechanical stretch is translated into biomolecular signals leading to the release of inflammatory mediators such as IL-6 into the air space and potentially into the circulation (6). A greater understanding of the contribution of MV to the development of ALI and the specific role of the surfactant system in this...
process would lead to more effective treatments aimed at preventing this disorder.

The objective of the current study was to employ two separate models of VILI to determine the protective effect of elevated endogenous surfactant pools on the progression of lung injury associated with high tidal volume MV. An ex vivo model was chosen to observe the direct pulmonary effects of injurious MV in an isolated, nonperfused rat model, whereas an in vivo rat model was utilized to study the effects of elevated surfactant pools in a perfused systemic model.

**MATERIALS AND METHODS**

**Liposome preparation.** Liposomes containing either PBS (0.001 M KH₂PO₄, 0.155 M NaCl, 0.0056 M Na₂HPO₄, pH 7.4) or DMDP (Sigma-Aldrich, St. Louis, MO) were prepared using sterile techniques according to Van Rooijen (10, 26). Briefly, 86 mg of phosphatidylcholine and 8 mg of cholesterol were dissolved in 10 ml of chloroform in a round bottom flask. The chloroform phase was removed by low-vacuum rotary evaporation in a 37°C water bath, leaving a thin lipid film within the flask. The lipid film was dispersed in 10 ml of either PBS or 0.6 M DMDP in PBS for 15 min at room temperature. The suspension was placed under nitrogen gas, incubated at room temperature for 2 h, followed by a 3-min water bath sonication and another 2-h incubation at room temperature. The liposomes were then centrifuged at 10,000 g, 4°C for 15 min to remove free DMDP. The pellet was washed twice with 7 ml of PBS and centrifuged at 16,000 g, 4°C for 30 min after each wash. The final pellet was resuspended in 8 ml of PBS and stored in aliquots at 4°C under nitrogen gas to be used within 2 wk of preparation.

**Liposome instillation.** Male Sprague-Dawley rats (Charles River, St. Constant, PQ, Canada) weighing between 340 and 450 g were used for these experiments. All animal procedures were approved by the Animal Use Subcommittee at the University of Western Ontario abiding by the guidelines outlined by the Canadian Council of Animal Care. Rats were allowed 72 h of acclimatization in the Animal Care Facility before experimentation and were allowed free access to water and standard chow.

Body weights were recorded and animals were anesthetized with an intraperitoneal (IP) injection of ketamine (75 mg/kg) and xylazine (5 mg/kg). Sterile technique was employed during the instillation surgery with the use of iodine and alcohol scrubs. After adequate anesthesia was achieved, rats were placed dorsally on an angled stage, the trachea was surgically isolated using blunt dissection, and a 14-gauge angiocatheter and secured with 2–0 surgical silk. The heart and lungs were removed en bloc by blunt dissection. The lungs were inflated once with 20 ml/kg air and held at this static volume for 10 s to exclude iatrogenic air leaks, and, subsequently, the exterior surface of the lungs was washed with 5 ml of 0.9% sterile saline at room temperature to remove residual blood clots. Rat lungs randomized to ex vivo MV were deflated and immediately connected to a volume-cycled rodent ventilator (Ugo Basile; Biological Research Apparatus, Varese, Italy). During ventilation, the lungs were housed in a humidified chamber held at a constant temperature of 37–39°C using a heated water bath. MV occurred with room air and ventilated with a tidal volume of 20 ml/kg, zero end-expiratory pressure (ZEEP), at a rate of 40 breaths/min. An airway pressure monitor was connected in parallel with the ventilator circuit to monitor peak inspiratory pressure (PIP) and was measured at 10-min intervals for a total of 2 h. Following ventilation, the lungs were disconnected from the ventilator and a static pressure-volume (PV) curve was generated to assess lung compliance as previously described (28).

The lungs were then lavaged with 5 × 10 ml aliquots of 0.9% normal saline, with each aliquot instilled and withdrawn three times. A 1-ml aliquot taken from the first lavage sample was frozen at −80°C for subsequent cytokine analysis. The remaining lavage sample was collected and analyzed as described below. Rats randomized to the non-ventilation cohort were euthanized at 3 and 7 days postinstillation and underwent en bloc resection of heart and lungs, immediately followed by PV curve generation and lavage as described above.

Overall, a total of 32 rats were used in this experiment. Twenty rats (n = 5/group) were randomized to receive PBS-L or DMDP-L and then ventilated at either day 3 or 7 after liposome administration. The four ex vivo ventilation groups were designated as: PBS 3(EV), DMDP 3(EV), PBS 7(EV), and DMDP 7(EV). Twelve rats were randomized to the non-ventilation groups at day 3 or 7 after liposome administration (n = 3/group) as described above and were designated as PBS 3(NV), DMDP 3(NV), PBS 7(NV), and DMDP 7(NV).

**Experiment 2: in vivo ventilation.** Rats were randomized to instillation with PBS-L or DMDP-L as described in experiment 1 using the same 3- or 7-day protocols. At the specified time points, rats were normally anesthetized with an IP injection of ketamine (75 mg/kg) and xylazine (5 mg/kg). Once adequate anesthesia was achieved, rats received a subcutaneous injection of buprenorphine (0.05–0.1 mg/kg) for analgesia before surgery. The right and left jugular veins and right carotid artery were isolated and cannulated with PE-50 tubing. The carotid artery line was connected to a pressure transducer for continuous heart rate and blood pressure measurements and was used to access blood samples for analysis throughout ventilation. Propofol (0.5 mg·kg⁻¹·min⁻¹) was administered in one venous line, with dosing adjustments given in response to changes in mean arterial blood pressure (MAP), while a second venous line was used for fluid administration (sterile 0.15 M saline with 1,000 IU heparin/l) at a rate of 1 ml·100 g⁻¹·h⁻¹ via infusion pump. After vascular access was completed, the trachea was isolated and intubated with a 14-gauge endotracheal tube and secured with 2–0 surgical silk.

Immediately following intubation, intravenous pancuronium bromide (2 mg/kg) was administered to induce paralysis and apnea before ventilation. The endotracheal tube was connected to a volume-cycle rodent ventilator (Harvard Instruments, St. Laurent, PQ, Canada) calibrated to a tidal volume of 30 ml/kg, ZEEP, 18 breaths/min, and 100% oxygen. An airway pressure monitor was connected in parallel with the ventilator circuit to monitor PIP throughout the duration of ventilation.

Throughout the 2-h in vivo MV protocol, physiological parameters were recorded every 15 min, whereas arterial blood gas measurements were taken at prespecified time intervals. A repeat dose of IV pancuronium bromide (2 mg/kg) was administered 60 min after the start of ventilation to maintain neuromuscular paralysis. At the end of MV, rats were euthanized with an overdose of IV sodium thiopental.
A

Table 1. Characteristics of DMDP-liposome model at day 3 and day 7

<table>
<thead>
<tr>
<th></th>
<th>PBS 3NV (n = 7)</th>
<th>PBS 7NV (n = 6)</th>
<th>DMDP 3NV (n = 8)</th>
<th>DMDP 7NV (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage count (10⁵)</td>
<td>11.7±2.3</td>
<td>6.23±2.91</td>
<td>4.99±0.81</td>
<td>9.44±2.1</td>
</tr>
<tr>
<td>Large aggregate pool size, mg/kg</td>
<td>1.15±0.20</td>
<td>1.41±0.14</td>
<td>6.11±0.65*</td>
<td>6.78±1.00*</td>
</tr>
<tr>
<td>Minimum surface tension</td>
<td>2.59±0.54</td>
<td>5.93±0.44</td>
<td>2.08±0.43</td>
<td>1.16±0.20*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *P < 0.05 vs. PBS group at the same day. DMDP, dichloromethylene diphosphonic acid.

(60 mg/kg) and exsanguinated by transection of the descending aorta. The protocols prespecified that animals would be euthanized if significant physiological stress was observed. This was defined by a MAP below 30 mmHg or arterial blood oxygenation levels below 60 mmHg at any point during the course of MV. At the end of the ventilation protocol, a midline sternotomy was performed and a static PV curve was generated. lungs were subsequently lavaged for collection of samples to analyze surfactant and cytokines as described above. Rats randomized to the non-ventilation group in experiment 2 were killed and intubated as described above, after which a PV curve and lung lavage was immediately performed.

Overall, a total of 36 rats were used for the in vivo MV experiments. Twenty-four rats (n = 6/group) were randomized to PBS-L or DMDP-L administration and ventilated for 2 h. The four in vivo ventilation groups were designated as either day 3 or 7 for a total of four groups: PBS 3(IV), DMDP 3(IV), PBS 7(IV), and DMDP 7(IV). The remaining 12 rats were randomized to non-ventilation groups at day 3 or 7 after liposome administration (n = 3/group) similar to experiment 1: PBS 3(NV), DMDP 3(NV), PBS 7(NV), and DMDP 7(NV).

Surfactant and cell analysis. Immediately following both ventilation protocols described above, the lavage sample was centrifuged at 150 g for 10 min (4°C) to separate the pellet (cellular components) from the supernatant (total surfactant) (10, 28). Four and a half milliliters of the supernatant was collected for analysis of total surfactant pool sizes, and the remaining supernatant was centrifuged at 40,000 g for 15 min (4°C) to separate the SA supernatant from the LA pellet. The latter was then resuspended in 2 ml of 0.15 M NaCl, and samples of LA, SA, and total surfactant (TS) were frozen and stored at −20°C for subsequent phospholipid analysis.

Phospholipid and total protein analysis. Phospholipid-phosphorous analysis of TS, LA, and SA was performed by chloroform extraction of the lipids followed by phosphorous analysis as described previously by Bligh and Dyer (3) and Duck-Chong (8). Total protein in the lavage was determined using the MicroBCA protein assay kit on the TS samples, following the manufacturer’s instructions (Pierce Biotechnologies, Rockford, IL).

LA functional analysis. LA samples were pooled from experimental groups to assess surface tension in vitro (23). Briefly, LA were resuspended in a buffered solution (0.15 mM NaCl, 2 mM Tris-HCl, 1.5 mM CaCl₂, pH 7.4) to a phospholipid concentration of 300 μg/ml and incubated at 37°C for 1 h. Briefly, using a custom built Captive Bubble Surfactometer, samples were introduced into an air-tight chamber held at a constant temperature of 37°C. Thereafter, an 8-mm bubble was introduced into the sample and allowed to reach an equilibrium surface tension. The chamber was subsequently sealed, and one quasistatic compression cycle was performed to establish the

Fig. 1. LPS induced release of IL-6 (A) and TNF-α (B) by alveolar macrophages (AM). AM were obtained from control rats as well as rats administered PBS-L (PBS-liposome) or dichloromethylene diphosphonic acid (DMDP)-L 7 days after instillation. AM were exposed for 18 h to media containing either 0 μg/ml or 1 μg/ml LPS after which IL-6 and TNF-α were measured in the culture media. *P < 0.05 vs. control and PBS.

Fig. 2. Peak inspiratory pressure (PIP) for ex vivo ventilated rat lungs. Isolated lungs from rats administered PBS or DMDP-L were ventilated either 3 or 7 days after liposome instillation. There were no significant differences among groups in the initial PIP. The lungs obtained from animals that received DMDP compared with PBS at day 3 [DMDP 3(EV) vs. PBS 3(EV)] were significantly different from time 20–100 min. DMDP 7(EV) vs. PBS 7(EV) were significantly different from 10 min onwards.
surface area reduction required to achieve the minimum surface tension before dynamic cycling. Upon initiation of dynamic cycling, a calculation was used to determine the minimum surface tension achieved on the 20th cycle of dynamic compression and was expressed as mN/m.

Cytokine analysis. The 1-ml aliquot obtained from the first lavage sample was centrifuged at 200 g, 4°C for 10 min. The supernatant was separated into 250-μl aliquots and snap frozen in liquid nitrogen before being stored at −80°C. IL-6, macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor-α (TNF-α) were measured using opti-EIA ELISA kits following the manufacturer’s specifications (Pharmingen, San Diego, CA).

Macrophage stimulation. A separate cohort of animals was used to characterize the function of AM recovered from DMDP-instilled rats at day 7 postliposome administration. AM recovered at day 7 from rats instilled with PBS liposomes and a group of rats that did not receive liposomes were used for control experiments. Bronchoalveolar lavage (BAL) was performed as described above and was initially centrifuged at 150 g, 4°C for 10 min to isolate the cellular components of the lavage. The cell pellet was resuspended in 1-ml of RPMI 1600 with 10% FBS culture medium. Cells were immediately aliquoted into a 24-well plate at a concentration of 5 × 10³ cells/ml. LPS derived from Escherichia coli was added to each well at a concentration of either 0 mg/ml or 1 mg/ml. Cell cultures were incubated at 37°C with 5% CO₂ for 18 h. Samples were centrifuged after incubation at 200 g (4°C) for 10 min. Two-hundred fifty microliters of supernatant was collected and stored at −80°C for cytokine analysis as described above.

Statistics. All data are expressed as means ± SE. A two-way ANOVA was performed to determine main effects and interactions of instillation and ventilation, followed by a one-way ANOVA with post hoc analysis to determine differences between individual groups. For data with a homogenous variability among the groups, a Tukey’s post hoc test was performed. For the statistical analysis of cytokine concentrations and lavage protein values, which had heterogeneity in the variance among the groups, a Waller-Duncan post hoc test was performed. This latter statistical analysis was performed for the cytokine concentrations. A Chi-Square analysis was performed to compare the observed and expected frequencies of mortality between ventilated groups. All differences were considered significant at a P value <0.05. All tests were performed using the SPSS software package for Windows, version 11.5 (Chicago, IL).

RESULTS

Baseline characteristics. Table 1 shows the results of lavage analysis in non-ventilated animals including AM counts, LA pool sizes, and functional assessment of the LA fraction. Data are shown for groups of non-ventilated animals instilled with PBS-L and DMDP-L at 3 and 7 days postliposome administration: PBS 3(NV), DMDP 3(NV), PBS 7(NV), DMDP 7(NV). At day 3, there was an ~60% reduction in total AM recovered in lavage fluid from DMDP 3(NV) vs. PBS 3(NV).
groups in response to MV (expected frequency of mortality in DMDP groups vs. PBS mmHg. Overall, there was a significantly lower surface tension at day 7 in the DMDP group compared with the PBS control.

The ability of the repleted AM isolated from animals at day 7 post-DMDP and of AMs from PBS and control animals to secrete inflammatory mediators in response to LPS is shown in Fig. 1. Exposure of AM to 0 μg/ml of E. coli LPS demonstrated minimal secretion of either IL-6 (Fig. 1A) or TNF-α (Fig. 1B) into the culture media of the PBS and control groups. AMs obtained from the DMDP group had higher concentrations of IL-6 and TNF-α in the culture media compared with the other two groups, even when not exposed to LPS. This difference reached statistical significance for TNF-α. When cultured with 1 μg/ml of E. coli LPS for 18 h, the media from all groups had higher concentrations of IL-6 and TNF-α compared with their respective 0 μg/ml groups. In addition, the culture media from the DMDP group exposed to 1 μg/ml LPS had higher concentrations of IL-6 and TNF-α in the culture media compared with the other two groups.

Together, these initial experiments confirm previous experiments that DMDP-liposome administration leads to increased LA pool sizes (10), these LA are surface active, and repleted AMs are functional with respect to cytokine secretion.

Experiment 1 ex vivo ventilation. Immediately after rats were connected to the ventilator, there were no significant differences in the initial PIP between PBS and DMDP groups at either 3 or 7 days (Fig. 2, time 0 values). Over the ensuing 120 min of MV there was a more prominent rise in PIP values for both PBS groups vs. both DMDP groups. This resulted in statistically significant lower PIP values in the DMDP 7(EV) group at all time points after 10 min and in the DMDP 3(EV) group from 20–100 min, compared with their respective PBS groups.

Figure 3 shows the PV curves performed after 120 min of ex vivo MV for PBS and DMDP groups at day 3 (Fig. 3A) and day 7 (Fig. 3B). Corresponding day 3 and day 7 non-ventilated control group values are also included for comparison. There was no difference in PV curve values between PBS (NV) and DMDP (NV) at day 3 or 7. Figure 3A shows lower values at each pressure throughout the PV curve maneuver for the PBS 3(EV) group compared with the PBS 3(NV) group, consistent with a reduction in static pulmonary compliance after 120 min of MV. This is compared with DMDP 3(EV) and DMDP 3(NV), where a reduction in compliance after ventilation was not observed.

Similar findings were observed for the groups at day 7 (Fig. 3B), where PV curve values for PBS 7(EV) were significantly lower than PBS 7(NV). Although the DMDP 7(EV) group had lower PV curve values compared with both NV groups, values in this group were still significantly higher than the PBS 7(EV) group.

Analysis of the surfactant pool sizes after the ventilation period revealed significantly increased TS in the DMDP groups compared with the PBS groups (PBS vs. DMDP: 6.9 ± 1.0 mg/kg body weight (BW) vs. 18.6 ± 0.4 mg/kg BW on day 3, and 6.7 ± 0.8 mg/kg BW vs. 26.2 ± 4.0 mg/kg BW, P < 0.05). Analysis of the LA subfractions revealed a similar pattern with values of 3.7 ± 0.3 mg/kg BW vs. 8.8 ± 0.3 mg/kg BW for PBS 3(EV) vs. DMDP 3(EV) (P < 0.05) and 3.4 ± 0.4 mg/kg BW vs. 7.11 ± 0.7 mg/kg BW for PBS 7(EV) vs. DMDP 7(EV) (P < 0.05). There were no statistical differences between these values when comparing day 3 and day 7.

Figure 4 shows lavage cytokine concentrations for IL-6 and TNF-α in samples recovered after 120 min of ex vivo MV for PBS and DMDP groups at both day 3 and day 7. Corresponding values of non-ventilated groups are included for comparison and demonstrated very low levels of IL-6 and TNF-α at both day 3 and 7, with no significant differences among the latter groups. Ex vivo MV resulted in a significant increase in lavage IL-6 and TNF-α concentrations in PBS (EV) groups compared with PBS (NV) groups at both day 3 and 7. Comparison of the DMDP (EV) vs. PBS (EV) groups revealed
significantly lower IL-6 and TNF-α concentrations in the DMDP (EV) groups at both day 3 and 7.

Experiment 2 in vivo ventilation. Figure 5 demonstrates changes in oxygenation values over the 120 min of in vivo MV for PBS 3(IV), PBS 7(IV), DMDP 3(IV), and DMDP 7(IV) groups. Of note, three of six animals (50%) in the PBS 3(IV) and four of six animals (66.6%) in the PBS 7(IV) group (Fig. 5, A and B) were killed at a mean time of 81.4 (±8.6) min after onset of MV due to a significant drop in oxygenation as prespecified according to the in vivo MV protocol. Conversely, all rats randomized to DMDP 3(IV) and DMDP 7(IV) completed the 120 min of ventilation, with consistently normal oxygenation values over the entire ventilation period and no requirements for euthanasia (Fig. 5, C and D). Statistical analysis of these data revealed that there was a significantly lower than expected frequency of mortality in DMDP groups in response to MV, compared with the PBS groups.

Measurements of PIP during ventilation revealed similar patterns to those obtained for the oxygenation data (data not shown). Specifically, PIP was not significantly different among experimental groups at the start of ventilation and averaged 28.5 ± 0.8 cmH2O for all animals. In 50% of the PBS 3(IV) and 7(IV) instilled rats, an increase in PIP values above 37 cmH2O was observed, corresponding with the decreased oxygenation values and were therefore killed before 120 min. The average PIP for DMDP 3 and 7(IV) groups at 120 min were 27.4 ± 4.0 and 26.3 ± 2.3 cmH2O, respectively.

Similar to the surfactant after ex vivo ventilation, analysis of the surfactant pool sizes after the ventilation period revealed that the TS system had remained elevated during the ventilation period (data not shown). Analysis of the LA subfractions revealed values of 1.6 ± 0.6 mg/kg BW vs. 5.1 ± 1.9 mg/kg BW for PBS 3(IV) vs. DMDP 3(IV) (P < 0.05) and 1.5 ± 0.6 mg/kg BW vs. 8.5 ± 0.9 mg/kg BW for PBS 7(IV) vs. DMDP 7(IV) (P < 0.05).

Figure 6, A and B, shows the PV curves from animals in the day 3 and day 7 cohorts measured after 120 min of ventilation, or immediately after death in the animals that were euthanized at earlier time points. PBS (IV) rats demonstrated a significant reduction in volume in given pressures post-ventilation at both day 3 and day 7. In contrast, both DMDP 3 and 7(IV) rats were not significantly different compared with non-ventilated animals.

Figure 7 shows mean total protein levels measured in lavage fluid after death for the various groups compared with non-ventilated controls at days 3 and 7. There was no significant difference in lavage protein levels between the non-ventilated groups [PBS (NV) and DMDP (NV)]. Both PBS (IV) groups had significantly higher protein values compared with their respective controls. In addition, PBS 7(IV) had significantly higher protein values compared with the DMDP 7(IV) group.

IL-6 and MIP-2 values in the lavage samples are shown in Table 2. In the lung lavage, IL-6 and MIP-2 values were significantly higher in ventilated vs. non-ventilated groups, with no significant effect of the DMDP instillation.

DISCUSSION

Based on previous studies in our laboratory that showed an increase in LA pool sizes in lungs of adult rats at both 3 and 7 days post-DMDP administration (10), the current study inves-

tigated the effect of injurious mechanical ventilation on these lungs. The DMDP-treated animals were protected against VILI, which included superior physiological parameters, lower protein leakage, and lower inflammatory mediator release into the air space, compared with animals not receiving DMDP. Moreover, these protective effects were observed at both time points after DMDP administration, initially at day 3 when AM were depleted, and at day 7 after AM repletion. Based on these findings, we conclude that DMDP-liposome administration protects against VILI and that this effect is primarily due to the presence of elevated endogenous surfactant pools rather than the absence of AMs in these models.

In two previous studies, animals were subjected to an in vivo MV protocol utilizing DMDP administration to lower AM numbers (9, 11). Animals receiving DMDP in those studies were also protected against VILI as reflected by superior outcomes such as blood oxygenation, capillary permeability, wet-to-dry weight ratio, lung compliance, and plasma levels of CXCL1; however, the contribution of elevated surfactant pools was not considered in these previous studies. There are several lines of evidence that would support surfactant as a key mediator in this protective effect. First, our data demonstrated protection against VILI 7 days following DMDP-liposome
administration, a time point at which functional AMs are repleted and surfactant pools are still elevated. Second, there have been several studies demonstrating alteration of surfactant metabolism and function due to high tidal volume ventilation, suggesting an important pathophysiological role for surfactant in this setting (28, 30, 31). Third, increasing surfactant pools by exogenous surfactant administration has been shown to be protective against VILI (27, 33, 34). Together, these observations support the conclusion that elevated LA pools secondary to DMDP-liposome instillation contributed to the protection against VILI in our studies. It should be noted that although this conclusion implies a role for surfactant in the progression of VILI, it does not exclude a role for the AM in the pathophysiological processes associated with this type of lung injury.

As noted above, exogenous surfactant administration has been shown to mitigate the effects of VILI, although to our knowledge, this is the first study to demonstrate that elevated endogenous surfactant pools could have the same effect. Ikegami et al. (16) demonstrated that GM-CSF knockout mice, a model that phenotypically lacks mature macrophages, develop elevated endogenous surfactant pools via a similar mechanism. They showed that the function of the LA pools recovered from these mice had the ability to rescue lung function in preterm, surfactant-deficient rabbits, although specific protection against VILI was not demonstrated. Nevertheless, these results, as well as those presented in the current study, confirm the significance of the AM in surfactant homeostasis, and, in particular, the important role of maintaining LA pool sizes for optimal lung function. Whether using either of these approaches to increase endogenous pools has any feasible relevance in the clinical setting is a different issue. Although depletion of AM appears to be a useful research tool, it may not have clinical utility given the many other important roles of this cell type within the air space, such as host defense functions in the setting of infectious insults. Future studies investigating the specific mechanisms by which AMs take up surfactant, with the aim of interfering with these processes, may prove to be useful.

Although speculative at this point in time, increasing endogenous surfactant pools to protect against lung injury may have certain advantages compared with administration of exogenous surfactant. For example, endogenous pools contain all the components of surfactant, whereas the currently available exogenous surfactant preparations lack several components, most notably surfactant protein A (SP-A) (18). This protein has important host defense functions and protects surfactant against inhibition by serum proteins (5, 20); SP-A may therefore play an important role in the prevention of VILI and other lung injuries. Increasing endogenous pools may also provide a more uniform distribution of newly secreted surfactant, something that is difficult to accomplish via instillation of exogenous surfactant preparations given the current methods of administration (12, 13). Finally, based on our studies, interference with the clearance of endogenous surfactant may result in a more prolonged elevation of surfactant pools, whereas exogenously administered surfactant may be rapidly cleared from the air space. For example, Spragg et al. (25) analyzed BAL fluid of patients with ARDS at 48 and 120 h after exogenous surfactant treatment. These results demonstrated an approximate two- to threefold increase in surfactant concentrations at 48 h posttreatment, but at 120 h, these levels had returned to baseline values. In contrast, the DMDP-liposome administration in the present study demonstrated elevated surfactant pools over a 7-day period, and, in a previous study, up to 10 days (10).

In addition to the physiological outcomes, our study also measured several inflammatory mediators in the lung lavage. Recent literature has suggested that the release of inflammatory mediators due to MV, and the subsequent translocation of these pulmonary-derived mediators into the circulation, may represent a key step in the progression of peripheral organ dysfunction associated with acute lung injury (6, 22, 24). In our ex vivo experiment, we demonstrated a significant reduction in lavage cytokine levels (IL-6 and TNF-α) in DMDP-instilled rats at both day 3 and 7 postventilation compared with the PBS groups. This effect could be a direct anti-inflammatory property of surfactant, or it may reflect a greater maintenance of alveolar stability during injurious MV indirectly leading to reduced inflammatory cytokine secretion. Interestingly, in a previous study utilizing the identical ex vivo ventilated lung

Table 2. IL-6 and MIP-2 concentrations in the lavage of in vivo ventilated animals

<table>
<thead>
<tr>
<th>Instillation</th>
<th>IL-6, pg/ml</th>
<th>MIP-2, pg/ml</th>
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<tr>
<td></td>
<td>3 Days</td>
<td>7 Days</td>
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<td>Non-ventilated</td>
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<tr>
<td>PBS</td>
<td>52 ± 4</td>
<td>58 ± 3</td>
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<tr>
<td>DMDP</td>
<td>147 ± 29</td>
<td>109 ± 25</td>
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<tr>
<td>Ventilated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>579 ± 123*</td>
<td>403 ± 187*</td>
</tr>
<tr>
<td>DMDP</td>
<td>495 ± 104*</td>
<td>988 ± 350*</td>
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Data are expressed as means ± SE. *Ventilated vs. nonventilated, P < 0.05.
model, prophylactic exogenous surfactant administration was protective against compliance changes due to high tidal volume MV, but did not reduce the concentrations of inflammatory mediators in the lavage (34). Thus increasing the endogenous surfactant pools via DMDP-liposome instillation appeared to provide an additional anti-inflammatory effect beyond the physiological protection observed with exogenous surfactant. These observations require further studies that involve more direct inflammatory insults to the lung.

It should also be noted that the in vivo experiments did not confirm the ex vivo results with respect to the cytokine data. This apparent discrepancy illustrates the different advantages and disadvantages of these two distinct models of VILI. The ex vivo MV setup represents a simple and reproducible model that has the advantage of measuring direct changes in local pulmonary physiology and inflammatory mediator release without the confounding factors from the pulmonary circulation. On the other hand, this model fails to account for complex in vivo interactions that occur during MV such as the continuous vascular perfusion of peripheral organ systems, the contribution of chest wall dynamics, and the recirculation of inflammatory mediators through the pulmonary vasculature. Because of these limitations, the in vivo experiments were performed. As was confirmed in these studies, however, the complexities of the whole animal experiments often result in larger variability, not only in physiological parameters but also more definitive outcomes such as mortality. In our study, this variability was most notable in the PBS control groups, as several of the PBS animals were euthanized and lavaged before the end of the study protocol. As a result, the inflammatory cytokine concentrations in these samples were obtained after a shorter ventilation period than those in the DMDP-liposome groups and may have accounted for the lack of differences between these groups in the in vivo experiment. Nevertheless, overall, the results from the two models do emphasize the significant impact of DMDP administration in the setting of VILI in terms of physiological, and, potentially, anti-inflammatory effects.

There are some limitations to this study. Previously, this method of AM depletion has been utilized to elucidate the role of AM in the host response to a variety of lung insults including infection and inflammation (1, 2, 17). Although this method has been generally described as selective depletion, our data demonstrating increased surfactant pools underscores the complex interactions within the alveolar environment and more importantly emphasizes the need to cautiously interpret the findings observed, not only in this, but in other studies as well. For example, we are suggesting that the elevated surfactant pools provided the primary protective effect against VILI; however, we have not investigated the effect of DMDP encapsulated liposomes on other cell types or the direct effect of DMDP on surfactant metabolism independent of the AM. Consequently, we fail to account for the possibility of effects of other pulmonary perturbations in this setting of widespread AM apoptosis. In addition, like many other studies on VILI, we employed a severe ventilation strategy for a relatively short time period. The role of other mechanisms of injury, such as neutrophil infiltration and activation, would involve studies utilizing more prolonged ventilation periods.

To attribute the protective effect of elevated surfactant levels in the setting of VILI, it was important to assess macrophage function at the time of the repopulation of macrophages. We had previously demonstrated normal macrophage numbers at day 7 (10), which was confirmed in the current study. To assess function of the macrophages, we examined IL-6 and TNF-α release after LPS stimulation. Interestingly, the repopulated macrophages demonstrated a slightly higher cytokine release compared with control macrophages, indicating the repopulated macrophages were functional but may respond slightly different than the normal population. In addition, other features of innate macrophage function such as phagocytic activity and oxygen radical release were not assessed. Consequently, it is possible that the protective effect demonstrated at both day 3 and day 7 after DMDP-L administration may have different physiological bases, one attributable to elevated surfactant pools, and the other related to aberrant macrophage function, or possibly a combination of both.

In conclusion, we have demonstrated that selective AM apoptosis with DMDP-L confers a protective effect against the physiological and inflammatory changes observed in two animal models of VILI. This benefit appears to be a reflection of a rise in elevated endogenous surfactant pools associated with macrophage depletion. Future studies should further probe the mechanisms responsible for these effects, since this may represent an effective therapeutic approach for a very common and serious disorder.

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GRANTS

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