Alveolar macrophage depletion is associated with increased surfactant pool sizes in adult rats

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Forbes A, Pickell M, Foroughian M, Yao L-J, Lewis J, Veldhuizen R. Alveolar macrophage depletion is associated with increased surfactant pool sizes in adult rats. J Appl Physiol 103: 637–645, 2007. First published April 19, 2007; doi:10.1152/japplphysiol.00995.2006.—Pulmonary surfactant is a lipid-protein material that is essential for normal lung function. Maintaining normal and consistent alveolar amounts of surfactant is in part dependent on clearance of surfactant by alveolar macrophages (AM). The present study utilized a rat model of AM depletion to determine the impact on surfactant pool sizes and function over time. Male Sprague-Dawley rats were anesthetized and intratracheally instilled with PBS-liposomes (PBS-L) or dichloromethylene diphosphonic acid (DMDP) containing liposomes (DMDP-L) and were killed at various time points up to 21 days for compliance measurements, AM cell counts, and surfactant analysis. AM numbers were significantly decreased 1, 2, and 3 days after instillation in DMDP-L vs. PBS-L, with 72% depletion at 3 days. AM numbers returned to normal levels by 5 days. In DMDP-L rats, there was a rapid increase in surfactant-phospholipid pools, showing a ninefold increase in the amount of surfactant in the lavage 3 days after liposome instillation. Surfactant accumulation progressed up to 7 days, with pools normalizing by 21 days. The increase in surfactant was due to increases in both subfractions of surfactant, the large aggregates (LA) and small aggregates. Surfactant protein A levels, relative to LA phospholipids, were not increased. There was a decreased extent of surfactant conversion in vitro for LA from DMDP-L rats compared with controls. It is concluded that the procedure of AM depletion significantly affects surfactant metabolism. The increased endogenous surfactant must be considered when utilizing the AM depletion model to study the role of these cells during lung insults.

PULMONARY SURFACTANT is a lipid-protein material that is secreted by the alveolar Type II cells of the lung to facilitate normal respiration (15). Surfactant functions to reduce surface tension at the air-liquid interface within the alveoli to maintain alveolar stability (16). In addition, surfactant exerts specific pulmonary host defense roles to protect the lung against the external environment (31). Both the biophysical and host defense functions depend on tight regulation of surfactant composition and metabolism.

Surfactant consists of 90% lipid, the majority of which are phospholipids with a minor proportion of neutral lipids, and 10% surfactant-associated proteins, SP-A, -B, -C, and -D (34, 45). The lipid component, SP-B, and SP-C are largely responsible for the biophysical properties of surfactant to reduce surface tension at the interface (35). SP-A has been implicated in stabilizing the surfactant film and modulating various aspects of the metabolic cycle (6, 29). As well, SP-A and -D have important host defense functions to clear foreign particles from the air space and protect against environmental stressors (8). Maintaining adequate surfactant pools within the air space is essential for lung function and is dependent on the dynamic cycle of surfactant metabolism.

Surfactant metabolism is a complex process involving synthesis and secretion of lamellar bodies from Type II cells, adsorption of functional large aggregates (LA) to the interface, and LA conversion into nonfunctional by-products, the small aggregates (SA) (46). SA are cleared from the air space by Type II cells for recycling or by phagocytic alveolar macrophages (AM) for degradation (20, 33, 37). There is strong evidence that alterations to surfactant metabolism contribute to the lung dysfunction associated with various pulmonary diseases. More specifically, altered surfactant clearance by the AM has been implicated in the surfactant alterations associated with acute respiratory distress syndrome (ARDS) and pulmonary alveolar proteinosis (PAP) (21, 28, 49, 50).

The contribution of AM to surfactant clearance has been investigated using in vitro and in vivo models, although results vary among studies. Poelma et al. (33) suggested that in vitro AM internalize the same amount of liposomes as Type II cells, but in vivo internalize three times the amount of surfactant as Type II cells. When these observations on a per cell basis were interpreted in the context of the proposed number of macrophages and Type II cells in the lung, the calculated contribution of uptake by AM was 30-40% (33). In rabbits, AM cleared 10–20% of an administered di-ether analog of dipalmitoyl phosphatidylcholine over 24 h (37). Instillation of an exogenous surfactant, Curosurf, in newborn and adult rabbits showed negligible uptake by AM after 3 h, although AM degraded Curosurf in vitro (1). In part, the variability between studies may be due to experimental conditions and the lipid composition of the material (32), suggesting that the internalization of exogenous preparations, either in vitro or in vivo, may not accurately reflect the role of AM in clearing endogenous surfactant.

The present study has utilized an in vivo rat model of AM depletion to investigate the role of the AM in endogenous surfactant clearance. This model utilizes dichloromethylene diphosphonic acid (DMDP)-encapsulated liposomes to selectively target AM and initiate apoptosis, and has been employed in a wide range of studies (2, 4, 39, 42, 51). However, the impact of macrophage depletion on the surfactant system has been studied in a wide range of studies (32, 43) but results have been difficult to extrapolate to the in vivo situation. This study utilized a rat model of AM depletion and compared the responses to pulmonary surfactant (Curosurf) and exogenous surfactant preparations, either in vitro or in vivo, in order to investigate the role of AM in clearing the endogenous surfactant.

References


not yet been considered. Therefore, this study has investigated the temporal relationship between AM cell numbers and surfactant pool sizes. We hypothesized that AM depletion would cause an increase in surfactant aggregate pool sizes, which would begin to decrease after repletion of AM numbers.

MATERIALS AND METHODS

Materials. DMDP and egg phosphatidylcholine type X-E were purchased from Sigma-Aldrich (St. Louis, MO). Cholesterol D-150 was obtained from Doosan Serdary Research Laboratories. For immunohistochemistry, the primary antibody and goat serum were purchased from Serotec (Raleigh, NC), and the secondary antibody was obtained from Invitrogen (Burlington, ON, Canada). The SP-A Western blotting primary antibody was a gift from Dr. Henk Haagsman (Univ. of Utrecht, Utrecht, The Netherlands), and the secondary antibody was purchased from Amersham Biosciences (Baied’Urfe, QC, Canada). The molecular weight ladder was a Kaleidoscope Prestained Standard (Bio-Rad, Mississauga, ON, Canada).

Liposome preparation. Liposomes containing either PBS (0.001 M KH2PO4, 0.155 M NaCl, 0.0056 M Na2HPO4, pH 7.4) or DMDP (PBS-L and DMDP-L, respectively) were prepared with sterile techniques according to Van Rooijen and Sanders (42). Briefly, 86 mg of phosphatidylcholine and 8 mg cholesterol were dissolved in 10 ml 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 around 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 liposome 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 4°C, 10,000 g, for 15 min to remove free DMDP. The pellet was washed twice with 7 ml of PBS and centrifuged at 4°C, 16,000 g, for 30 min after each wash. The final pellet was resuspended in 8 ml PBS and stored in aliquots at 4°C under nitrogen gas to be used within 2 wk of preparation.

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

The body weight was recorded, and animals were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5 mg/kg). Sterile technique was used during the instillation surgery. After appropriate anesthesia was confirmed, the trachea was surgically isolated using blunt dissection, and a 14-gauge angiocatheter was inserted. Four-hundred microliters of PBS, PBS-L, or DMDP-L was instilled in 100-μl boluses, each followed by an equal air bolus. The instillation procedure took approximately 5-7 min, after which the incision was closed with subcutaneous sutures. Following instillation, rats were given a 0.1 mg/kg subcutaneous injection of buprenorphine and were monitored until full recovery. After recovery, all rats were housed in the animal quarters with free access to water and chow, and health monitoring was recorded daily until they were utilized for surfactant analysis.

Fig. 1. Pulmonary compliance as measured by pressure-volume curves in the adult rat lung. Static compliance was assessed by stepwise inflation and deflation of the lung. There was no difference in the volume of air required to inflate the lungs to a maximum pressure of 26 cmH2O between PBS (open circles), PBS-liposomes (PBS-L; gray circles), and dichloromethylene diphosphonic acid containing liposomes (DMDP-L; black circles) groups at all time points. Data are expressed as means ± SE; n = 7–10 per group.
Experimental groups. To determine the effect of AM depletion on the surfactant system, two separate experiments were conducted. In experiment 1, rats were intratracheally instilled with PBS, PBS-L, or DMDP-L. Rats were killed either 1, 2, 3, or 7 days after instillation to create a time line of AM depletion and surfactant alterations. This included one cohort to characterize macrophage numbers through immunofluorescence of histological preparations (n = 3 per group), and a second cohort to characterize surfactant alterations (n = 4–7 per group). In experiment 2, rats were intratracheally instilled with PBS-L or DMDP-L and killed 3, 5, 7, 10, or 21 days later. The lungs were lavaged for AM cell number and surfactant analysis (n = 4–7 per group).

Animal euthanasia. At the prespecified time points, rats were killed with an overdose of euthanyle (pentobarbitonal sodium) and exsanguinated by transection of the descending aorta. A midline sternotomy was performed, and the trachea was isolated and catheterized with a 24-gauge angiocatheter. A static pressure-volume curve was generated in experiment 1 to assess lung compliance up to a maximum pressure of 26 cmH2O. Lungs were inflated twice to the maximum pressure followed by passive deflation. Subsequently, the volume instilled was measured at 2-cmH2O pressure intervals, and the lungs were deflated in the same stepwise manner. The animals were then separated into two cohorts, either being formalin-fixed for immunofluorescence or lavaged for surfactant and cell analysis.

Immunofluorescence. In the immunofluorescence cohort, the lungs were inflated to a pressure of 15 cmH2O and immersed in 10% neutral buffered formalin for 24 h. Each lobe was paraffin embedded, and 5-μm-thick slices were cut for fluorescence staining of macrophages. The protocol was obtained through Serotec Workshops 7 and 10 (www.serotec.com). Briefly, the sliced sections were incubated on slides at 50°C overnight before treatment with 1% H2O2 to remove endogenous peroxidase activity. The sections were deparafinized with xylene and rehydrated with ethanol treatments. The sections were washed twice in PBS and blocked with 10% goat serum for 30 min at room temperature. The primary antibody mouse anti-CFCD68 (Serotec, Raleigh, NC) was applied at a concentration of 15 μg/ml in 1% BSA, and the sections were incubated in a humidified chamber at 4°C overnight. The next day, after washing three times in PBS, the secondary antibody Alexafluor 555 goat anti-mouse IgG (Invitrogen, Burlington, ON, Canada) was applied in a 1:500 dilution in 1% BSA and incubated for 1 h at room temperature in the dark. The sections were washed three times in PBS and mounted with aqueous/dry mounting gel. Digital images were taken of six randomly chosen fields of each tissue section, and the number of fluorescing macrophages was determined using the Northern Eclipse Image Analysis System (EMPIX Imaging, Mississauga, ON, Canada) and microscope (Zeiss, Toronto, ON, Canada).

Surfactant and cell analysis. In the lavage cohorts, the lungs were instilled with 5 × 10 ml aliquots of 0.15 M NaCl, with each aliquot being instilled and withdrawn three times (27). One milliliter of the first lavage was aliquoted for cytokine analysis; and the remaining lavages were combined, the volume recorded, and centrifuged at 4°C, 150 g, for 10 min to separate the cellular components from the surfactant. The supernatant represented the total surfactant (TS), and the resulting pellet contained the lavaged cells. The cell pellet was resuspended in 2 ml plasmaute, and the total number of viable alveolar lavage fluid cells (not including erythrocytes) was counted using a hemocytometer. The percentage of AMs was determined through differential cell counts of centrifuged cell preparations stained with a Hemacolor kit (EM Science, Gibbstown, NJ).

For phospholipid analysis, 4.5 ml of the TS was saved and frozen at −20°C. The remaining TS was centrifuged at 4°C, 40,000 g, for 15 min to separate the SA supernatant from the LA pellet, which was then resuspended in 2 ml of 0.15 M NaCl (27). Phospholipid analysis was performed using the Bligh and Dyer (5) lipid extraction and Duck-Chong (12) phosphorous assay on aliquots from the TS, LA, and SA fractions. Briefly, samples were analyzed in triplicate for each surfactant fraction. Samples were added to 1:2 methanol:chloroform in glass test tubes, and the lipids were extracted in the chloroform phase after centrifugation. Magnesium nitrate (10% wt/vol in methanol) was added, and the chloroform phase was evaporated under air in a heated water bath. The dried samples were ashed on an electric heating rack and allowed to cool. One milliliter of 1 M HCl was added to each tube, covered with marbles, and heated for 15 min at 95°C on a heating block. Ninety-six-well plates were loaded with 66 μl of standard and each sample tube in triplicate before 134 μl of dye [3:1 of 4.2% molybdate in 4.5 M HCl:0.3% (wt/vol) malachite green in water] was added. The absorbance was read at a wavelength of 660 nm on the microplate reader (Bio-Rad) and compared with phosphate standards.

Total protein in the lavage was determined using the Micro-BCA protein assay kit on the TS samples, following the manufacturer’s instructions (Pierce Biotechnologies, Rockford, IL).

Phospholipid composition analysis. Phospholipid composition was determined by separating the lipids by thin-layer chromatography (TLC) followed by phosphorus measurements. Aliquots of 350–400 μg SA were extracted (5) and were separated on Whatman K66D TLC plates using chloroform:ethanol:triethylamine:water (30:34:35:8) as a solvent system (40, 44). The plates were developed twice in the same direction. Phospholipid standards were visualized with a Dittmer-Lester (10) phosphorous spray, and the corresponding spot in the sample lanes was scraped off the TLC plate and analyzed for phospholipid phosphorous as described above.

SP-A Western blotting. The LA subfraction of surfactant was analyzed for SP-A using 10% SDS-PAGE and immunoblotting. To determine relative SP-A abundance, samples of 10 μg phospholipid LA were run through the gel and transferred to a nitrocellulose membrane for immunoblotting. The membrane was blocked in 4% milk in PBS.

Table 1. Concentrations of inflammatory mediators and total proteins in the lung lavage

<table>
<thead>
<tr>
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<th>1 Day</th>
<th>2 Days</th>
<th>3 Days</th>
<th>7 Days</th>
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<tr>
<td><strong>IL-6, pg/ml</strong></td>
<td></td>
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<td>PBS</td>
<td>47.5±7.5</td>
<td>ND</td>
<td>ND</td>
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<td>PBS-L</td>
<td>ND</td>
<td>ND</td>
<td>31.3±3.1</td>
<td>ND</td>
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<td>DMDP-L</td>
<td>ND</td>
<td>38.0±3.0</td>
<td>151.9±135.1</td>
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<tr>
<td><strong>IL-4, pg/ml</strong></td>
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<tr>
<td>PBS</td>
<td>2.7±1.3</td>
<td>2.2±1.5</td>
<td>ND</td>
<td>2.1±0.9</td>
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<td>PBS-L</td>
<td>0.8±0.7</td>
<td>ND</td>
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<tr>
<td>DMDP-L</td>
<td>1.4±0.9</td>
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<td>1.1±0.5</td>
<td>10.4±7.4</td>
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<td><strong>Protein, mg/kg body wt</strong></td>
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<tr>
<td>PBS</td>
<td>22.5±4.2</td>
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<td>22.23±2.7</td>
<td>18.76±3.5</td>
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<td>22.0±3.3</td>
<td>26.5±5.3</td>
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<tr>
<td>DMDP-L</td>
<td>26.1±5.7</td>
<td>29.7±4.9†</td>
<td>29.92±3.4†</td>
<td>21.4±2.2†</td>
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<td><strong>MIP-2, pg/ml</strong></td>
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<td></td>
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<tr>
<td>PBS</td>
<td>245.4±27.1</td>
<td>248.5±72.0</td>
<td>182.3±25.1</td>
<td>204.5±34.3</td>
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<tr>
<td>PBS-L</td>
<td>267.5±45.1</td>
<td>206.9±19.1</td>
<td>214.3±21.5</td>
<td>139.4±23.1†</td>
</tr>
<tr>
<td>DMDP-L</td>
<td>384.0±49.0</td>
<td>323.7±89.5</td>
<td>247.8±34.9</td>
<td>194.9±31.3†</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 4–6. Instillation groups: PBS, PBS-liposomes (PBS-L), or dichloromethane diphosphonic acid (DMDP) containing liposomes (DMDP-L). There was no difference in the IL-6 or IL-4 concentrations between any of the groups. There was significantly increased protein in the lavage of DMDP-L groups vs. PBS groups. There was no significant difference in macrophage inflammatory protein-2 (MIP-2) concentrations between the instillation groups. MIP-2 concentration was significantly different in DMDP-L and PBS-L groups at 7 days compared with 1 day, ND, not detectable. *7 days vs. 1 day, P < 0.05. †DMDP-L vs. PBS, P < 0.05.
powdered milk in PBS at 4°C overnight, after which the primary antibody rabbit anti-rat (1:1,000 dilution) was applied and incubated for 2 h at room temperature. The membrane was washed in PBS + 0.1% Tween-20, and the secondary antibody donkey anti-rabbit horseradish peroxidase (1:1,000 dilution) was applied and incubated for 1 h at room temperature, followed by the same washing procedure as above. SP-A was detected with the ECL Western blot analysis system (Amersham Biosciences, Buckinghamshire, UK) and subsequent exposure to x-ray film (Eastman Kodak, Rochester, NY). Densitometry analysis was performed using the gel docking station and Alphalmager 2200 computer software (Alpha Innotech, San Leandro, CA). The densitometry values were calculated as percentage of control (PBS group for experiment 1 and PBS-L group for experiment 2).

Surface area cycling. Aliquots of the LA samples from the lavage of 3- and 7-day PBS, PBS-L, and DMDP-L rats were resuspended in buffer (0.15 M NaCl, 10 mM Tris, 1 mM CaCl2, 1 mM MgCl2, 0.1 mM EDTA, pH 7.4) to a concentration of 0.1 mg phospholipid/ml. A 2-ml aliquot was cycled in a capped plastic tube and rotated (Roto-torque rotator; Cole-Parmer Instruments, Laval, PQ, Canada) at 40 revolutions/min at 37°C for 3 h (17). Controls were represented by identical samples that were not cycled but were kept at 37°C for 3 h. Afterward, the LA and SA fractions were isolated from the cycled and noncycled samples by centrifugation at 40,000 g for 15 min. The surfactant phosphorous analysis was performed on the supernatant (SA) and pellet (LA) as described above.

Cytokine measurements. The 1-ml aliquot saved from the first lavage was centrifuged at 4°C, 200 g, for 10 min. The supernatant was separated into 250-µl aliquots and snap-frozen in liquid nitrogen before being stored at −80°C until cytokine analysis. IL-6 and IL-4 were measured using opti-EIA ELISA kits following the manufacturer’s specifications (Pharmingen, San Diego, CA). Macrophage inflammatory protein-2 (MIP-2) was measured using an ELISA kit purchased from Biosource (Camarillo, CA) and following the manufacturer’s instructions.

Statistical analysis. All data are expressed as means ± SE. A two-way ANOVA was performed to determine interactions of instillation and time. If a significant interaction was found, pairwise comparisons between groups at each time point were performed using independent t-tests. To determine significant differences over time when a significant interaction was found, a one-way ANOVA with a Tukey’s post hoc test was performed on each instillation group. All tests were performed using the SPSS software package for Windows.

Fig. 2. Representative immunofluorescence staining of macrophages in rat lung tissue sections. A: 3 days after instillation, there was a decrease in macrophages in DMDP-L groups vs. PBS and PBS-L groups, with no differences between the controls. B: 7 days after instillation, there were similar macrophage numbers in PBS-L and DMDP-L groups. All images are at the same magnification; scale bar, 250 µm.
macrophages in the lung tissue of PBS groups at 3 days, and PBS-L and DMDP-L groups at 3 days and 7 days, are shown in Fig. 2, A and B, respectively, and the results from the quantification for all groups are shown in Fig. 3. There was no significant difference in the macrophage numbers between the PBS and PBS-L groups. DMDP-L-instilled rats had significantly decreased macrophage numbers at 1, 2, and 3 days after instillation compared with both control groups. Macrophages were approximately 72% depleted 3 days after instillation. Comparison between PBS-L and DMDP-L at 7 days showed no significant differences in macrophage numbers, with 16.0 ± 1.3 and 18.8 ± 1.8 macrophages per field, respectively. There was no change in macrophage number in PBS and PBS-L groups over time. Within the DMDP-L groups, there was a significant effect of time, showing a decrease in macrophages at 1, 2, and 3 days compared with 7 days after instillation.

Figure 4 shows the TS phospholipid levels measured in the lung lavage. Statistical comparisons among groups revealed that there was no significant difference in the TS pool sizes between PBS and PBS-L groups at any time point. TS pools were significantly increased in the DMDP-L compared with PBS and PBS-L groups at 1, 2, 3, and 7 days after instillation. Over time, TS pools did not differ in PBS and PBS-L groups. The DMDP-L groups showed a significant difference in TS phospholipid levels over the time course of 7 days. Comparing 1 day and 7 days after DMDP-L instillation, TS increased from 10.6 ± 1.7 to 29.6 ± 6.4 mg/kg.

TS consists of SA and LA subfractions, which are shown in Fig. 5. There was no difference in SA pools between PBS and PBS-L groups. SA pool sizes in DMDP-L groups were significantly increased compared with both control groups at 1, 2, 3, and 7 days. Three days after instillation, SA phospholipid levels are approximately sixfold higher in the DMDP-L vs. PBS group. Over time, there was no difference in SA pools in PBS and PBS-L groups. In DMDP-L-instilled rats, SA pools were significantly different over the time course of 7 days, with pool sizes increasing from 4.9 ± 0.8 to 19.0 ± 4.1 mg/kg between 1 day and 7 days after instillation. Analysis of the LA
pool sizes revealed no significant differences between PBS and PBS-L groups. Statistical comparison of DMDP-L to the PBS and PBS-L groups revealed a significant increase in LA pools at all time points. In DMDP-L-instilled rats, LA pool sizes were approximately fourfold larger compared with PBS-instilled rats at 3 days. LA phospholipid pools did not significantly change over time in PBS, PBS-L, and DMDP-L groups.

Phospholipid composition of the SA obtained 3 days after instillation of either PBS, PBS-L, or DMDP-L is shown in Fig. 6. There were no significant differences in phospholipid composition between the three groups, with each of them showing the characteristic profile of pulmonary surfactant phospholipid composition. Similar results were obtained with samples obtained 7 days after instillation (results not shown).

Aliquots of LA were analyzed for SP-A by Western blot and densitometry and are expressed as a percentage of the PBS group for the 3- and 7-day time points. There was no significant difference in the relative levels of SP-A per microgram of phospholipid in the LA from the PBS, PBS-L, and DMDP-L groups. Over time, the relative amount of SP-A did not change in any of the groups (Table 2).

Surface area cycling experiments were performed on LA from PBS-L and DMDP-L groups obtained at day 3 and day 7 after instillation. The percent recovery of phospholipid was >90% in all groups and did not differ among the experimental groups. The percent SA (%SA) was calculated for all non-cycled and cycled samples to reflect the percent in vitro conversion of LA to SA (Table 2). The %SA for noncycled samples was not significantly different between PBS-L or DMDP-L groups and was not different between 3 and 7 days. Comparing the %SA after 3 h of cycling, there was a significantly less %SA in DMDP-L groups compared with PBS-L groups overall. As well, comparison between 3 and 7 days showed a decrease in %SA at 7 days overall. In separate cycling experiments, LA from 3 and 7 day PBS animals were analyzed. The SA formation after 3 h of cycling for these samples was 66 ± 3.6%.

A second experiment was performed to further investigate the change in surfactant pool sizes over time. Since there were no main differences between PBS and PBS-L groups in experiment 1, only PBS-L and DMDP-L groups were investigated at 3, 5, 7, 10, and 21 days after instillation.

AM numbers were determined through total and differential cell counts of the lung lavage fluid (Table 3). There was a significant decrease in AM numbers in DMDP-L vs. PBS-L groups 3 days after instillation. AM were ~62% depleted at 3 days, with no significant differences at 5, 7, 10, and 21 days after instillation between the PBS-L and DMDP-L groups. Over time, AM numbers did not significantly change in PBS-L groups. There was a significant decrease in number of AM in DMDP-L-instilled groups at 3 days compared with all other time points.

TS phospholipid levels and percent LA (%LA) are also shown in Table 3. TS pools were significantly increased in DMDP-L vs. PBS-L groups at the time points up to 10 days. At day 21 the amount of TS was not significantly different from
that of the PBS-L controls. TS pool sizes in the DMDP-L group did not significantly change over the time course of 3 to 10 days but were significantly decreased at 21 days.

The %LA was significantly decreased in DMDP-L groups compared with PBS-L groups at all time points. Over time, there was no difference in %LA within the PBS-L- or DMDP-L-instilled groups.

**DISCUSSION**

The objective of this study was to determine the effect of DMDP-liposome instillation on the surfactant system in adult rats. The instillation of DMDP-liposomes has been reported to selectively eliminate macrophages via the uptake of the liposomes and the subsequent phospholipase degradation of the liposome bilayer, resulting in the release of DMDP in the alveolar cells. Subsequent killing of the AMs occurs through apoptotic pathways, as demonstrated with isolated cells in vitro. We hypothesized that this procedure would cause an increase in surfactant pool sizes, which would begin to decrease after AM repletion. The results showed that there was a very rapid and large increase in surfactant pool sizes that was maintained for 10 days after DMDP-liposome administration, with pools returning to normal levels by 21 days. Surfactant pool sizes were analyzed via phospholipid analysis and were confirmed to be surfactant on the basis of the consistent level of SP-A in the LA subfraction and the characteristic surfactant phospholipid profile of the SA subfraction. The fast time course and magnitude of surfactant accumulation after DMDP-liposome administration demonstrates that this procedure has a significant impact on endogenous surfactant metabolism. Furthermore, this model has been reported as selective for AM depletion and has therefore been extensively used to investigate the role of AM in response to lung insults; however, the present study showed that this experimental model disrupts the endogenous surfactant system, which may influence the responses to lung stress.

The simplest explanation for the fast and substantial increase in surfactant in the AM depletion model is decreased surfactant clearance by the AM. The in vitro and in vivo evidence of surfactant clearance by the AM, the progressive increase in surfactant with decreasing AM number, and a greater increase in SA compared with LA pools would certainly support a large contribution of this mechanism to the increased pool sizes. However, the possibility that other mechanisms contribute to the altered surfactant metabolism has to be considered. Specifically, the role of the Type II cell in this response should be considered. For example, uptake of DMDP-liposomes by the alveolar Type II cells may have affected these cells' metabolic activity, resulting in increased surfactant secretion or decreased surfactant uptake. The DMDP-liposomes may also have provided substrate for surfactant production, resulting in increased surfactant, although it should be noted that the control liposomes did not result in significant surfactant accumulation. Regardless, the role of the Type II cell in the observed accumulation of surfactant in the AM depletion model cannot be excluded on the basis of the present set of experiments and should be investigated in future experiments.

Disruption of surfactant clearance leading to increased surfactant pool sizes is not unique to the AM depletion model. For example, total lung saturated phosphatidylcholine (sat PC) and protein levels progressively increased in granulocyte-macrophage colony-stimulating factor (GM-CSF) (-/-) mice from...
birth to adulthood, with a 10-fold increase in alveolar surfactant PC compared with controls by 56 days of age (11, 36). The ratio of SP-A, SP-B, and SP-C to phospholipid was maintained, while SP-D increased fivefold. The lipid accumulation was suggested to be due to decreased clearance since AM from GM-CSF (−/−) mice had decreased surfactant catabolic activity in both in vitro uptake and in vivo metabolic experiments (22, 49). In the present study, there was an almost sevenfold increase in TS phospholipid pools only 3 days after AM depletion, with a normal SP-A-to-phospholipid ratio. Although both models showed impaired AM activity associated with increased surfactant pool sizes, the extent of the changes and the faster time course in the AM depletion model imply a different or a more marked mechanism for surfactant accumulation.

Interestingly, accumulation of surfactant in the AM depletion model consisted of increases in both SA and LA. Decreased clearance of SA in this model is in agreement with the suggested life cycle of surfactant and with the in vitro and in vivo evidence that AM engulf and degrade surfactant liposome forms (24, 26, 33, 47). However, the role of AM in LA clearance in vivo is still undefined. One possible contributing factor is a decreased conversion of LA to SA within the air space, as suggested by the in vitro surface area cycling results. LA conversion was similarly decreased in 7- to 9-wk-old GM-CSF knockout mice, suggesting that the AM may be an important regulator of the surfactant turnover within the air space, which may involve the reported enzymatic activity required for this conversion (18, 22). The role of the AM in this aspect of the metabolic cycle is an interesting concept that requires further investigation.

The accumulation of surfactant was progressive with decreasing AM number; however, there was a discrepancy between the timing of AM repopulation and the restoration of surfactant pools toward normal levels. Evidence from Takashashi et al. (38) suggests that after AM depletion, repopulation was dependent on increased precursor cells in the tissue during the period of depletion and on the specific tissue microenvironment and growth factors, such as macrophage-CSF and GM-CSF. Therefore, it is possible that the returning AM were functionally immature, with decreased activity slowing the restoration of surfactant pools. It is important to note, however, that at day 21 after depletion, surfactant levels were returned to normal; thus macrophage depletion did not lead to a new steady-state level in the alveolar space.

One limitation of the present study was that AM were only 72% depleted. The effect of partial AM depletion on surfactant clearance was so extensive that the remaining 28% of AM could not compensate. We suggest that the remaining AM were less functional and therefore contributed to the surfactant accumulation. Berg et al. (3) showed that with 70% AM depletion, the remaining AM were large, foamy, and functionally abnormal in vitro uptake studies. We noticed a similar AM phenotype in depleted rats, although this finding was not quantified.

Our second conclusion was that surfactant accumulation may have important implications in other studies using this model (7, 13, 14, 25). Our data of lung compliance suggest that lung function is not altered in the DMDP-liposome instillation groups. Although total protein in the lavage was significantly higher in rats receiving DMDP-liposomes compared with PBS animals at day 2 and day 3, these values were not significantly higher than the values in the PBS liposomes group. Furthermore, these protein values were substantially lower than those observed in models of severe lung dysfunction (9, 19). To examine if DMDP liposome administration had an effect on inflammatory mediators, we measured IL-6 as a general marker of inflammation, MIP-2 on the basis of previous studies utilizing the AM depletion model (30), and IL-4 since it has been reported that its overexpression leads to increased surfactant pool sizes (23). Although some IL-6 was detected in a couple of the animals receiving DMDP-liposomes at day 3 and day 7 after administration, overall the results showed no statistically significant increase in the concentrations of these inflammatory mediators in animals administered DMDP-liposome compared with the two control groups. Taken together, these data support previous publications that the AM depletion model does not markedly interfere with these other pulmonary processes (41, 42). However, our study is the first to also measure pulmonary surfactant pool sizes in this model and demonstrates that AM depletion not only decreases AM number but also disrupts surfactant metabolism, resulting in a substantial surfactant accumulation.

Assuming that this increase in surfactant also occurred in other studies, it may have contributed to the outcome of those studies. For example, previous studies have shown that AM depletion protected against pulmonary tuberculosis (25), decreased endotoxin-induced neutrophil recruitment, and cytokine release (3), protected against ventilator-induced lung injury (14), and prevented pulmonary protein leak (13). On the basis of the known biophysical and host defense functions of surfactant (15, 31), the increased surfactant due to the depletion process may have contributed to some of these responses. Therefore, it may be important to consider the potential impact of surfactant accumulation in future studies employing this model.

In conclusion, this study demonstrates that AM depletion causes a severalfold increase in surfactant pool sizes, suggesting that AM are essential for surfactant homeostasis in a healthy lung. The magnitude to which the AM depletion model disrupted the surfactant system may have implications for other studies utilizing this technique and also strengthens the concept that AM have a role in the alterations of surfactant associated with ARDS and PAP. Understanding how the AM mediates surfactant metabolism in the normal healthy lung is an important step to determining its contribution to surfactant alterations in disease states.

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GRANTS

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