Clearance of surfactant lipids by neutrophils and macrophages isolated from the acutely inflamed lung

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Quintero, Omar A., and Jo Rae Wright. Clearance of surfactant lipids by neutrophils and macrophages isolated from the acutely inflamed lung. Am J Physiol Lung Cell Mol Physiol 282: L330–L339, 2002; 10.1152/ajplung.00190.2001.—Pulmonary surfactant reduces surface tension at the lung air-liquid interface and defends the host against infection. Several lines of evidence show that surfactant levels are altered in animal models and patients with inflammatory or infectious lung diseases. We tested the hypothesis that cells responding to lung injury alter surfactant levels through increased phospholipid clearance. Acute lung injury was induced by intratracheal administration of lipopolysaccharide (LPS; Escherichia coli 026: B6) into rats. LPS exposure resulted in a 12-fold increase in the number of cells isolated by lavage, the majority of which were neutrophils. Isolated macrophages and neutrophils from LPS-treated lungs internalized and degraded lipids in vitro, and LPS injury stimulated uptake by macrophages twofold. We estimate that lipid clearance by lavage cells in LPS-treated lungs could be enhanced 6- to 13-fold with both activated macrophages and increased numbers of neutrophils contributing to the process. These data show that the increased number of cells in the alveolar space after acute lung injury may lead to alterations in surfactant pools via enhanced clearance and degradation of lipids.

Lipopolysaccharide; surfactant protein-A; lipid degradation

PULMONARY SURFACTANT is a mixture of lipids and proteins synthesized and secreted by alveolar epithelial type II cells (20). The surfactant lipids form a monolayer at the air-liquid interface, reducing the surface tension of the fluid lining the alveoli. By weight, surfactant is composed of ~90% lipid. Surfactant lipid composition varies in different animal species, though the major phospholipid component is phosphatidylcholine (PC; 70–80%). Dipalmitoylphosphatidylcholine (DPPC) is the major surface tension-reducing component of surfactant and makes up ~50% of surfactant PC. Surfactant contains variable amounts of phosphatidylinositol (2–4%), phosphatidylserine (0.1–2%), and phosphatidylglycerol (PG; 7.5–18%), the second most abundant surfactant phospholipid in rodents and humans. Cholesterol and free fatty acids are also present in surfactant (reviewed in Ref. 51). The roles of the minor lipid components are not yet clear, but there is evidence that PG aids in the adsorption of DPPC to the air-liquid interface (33).

Surfactant proteins have been shown to function in defending the lung against infection, both in vitro and in vivo. Both surfactant protein-A (SP-A) and surfactant protein-D (SP-D) are members of the collectin superfamily of proteins and bind foreign particles through interactions mediated by the proteins’ carbohydrate recognition domain (13, 38). SP-A has been shown to bind to a variety of pulmonary pathogens (reviewed in Ref. 23). Enhanced clearance of bacteria is mediated by interactions among SP-A, the foreign particle, and the macrophage, in vitro (49). Bacterial killing can also be stimulated by SP-A (32, 39). It has been reported that SP-A deficiency in mice leads to defects in clearance of both Group B streptococci (27) and Pseudomonas aeruginosa (28) in vivo. In addition to a role in clearing infectious organisms, SP-A has also been shown to affect the production of inflammatory mediators such as cytokines in vitro by U-937 cells (43) and by macrophages (30, 42). It has been shown in SP-A-deficient mice that SP-A regulates production of tumor necrosis factor-α, as well as nitric oxide (5).

Although in vitro data suggest that surfactant homeostasis may be mediated by SP-A, the in vivo data to date do not completely support this hypothesis. Purified SP-A binds and aggregates phospholipids in a calcium-dependent manner (16, 21), and SP-A has been shown to be essential for formation of tubular myelin (48). With the use of in vitro assays, SP-A has been shown to stimulate lipid uptake by isolated type II cells (55) and alveolar macrophages (4, 56). SP-A has also been shown to alter other functions related to surfactant lipid metabolism, including inhibition of agonist-stimulated lipid secretion by type II cells (41) and inhibition of phospholipase A2 (PLA2) in lung tissue homogenate (11). SP-A has also been shown to stimulate lipid degradation in intact alveolar macrophages in vitro (40). However, once SP-A knockout mice were generated, it became apparent that SP-A deficiency did not dramatically disrupt surfactant metabolism. SP-A (−/−) mice had a relatively mild phenotype. Alveolar surfactant pool sizes were not altered, and tissue DPPC pools were slightly increased. SP-A

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mice lacked tubular myelin, and surfactant isolated from these mice contained a lower percentage of large aggregate forms (19, 22, 23). To complicate matters further, a deficiency in SP-D, which has not been shown to mediate functions related to lipid clearance in vitro, leads to disruption of surfactant pool sizes in mice (6, 24).

Acute lung injury has been shown to alter the abundance of surfactant components in the alveolar space and to change the populations of cells present in the alveolar space. However, very little is known about the mechanisms responsible for the changes in surfactant pool size in the injured lung and the contributions of the inflammatory cells to the process, which will be impacted by changes in synthesis, secretion, and clearance of both lipid and protein components. It has been shown that incubation of isolated type II cells with lipopolysaccharide (LPS) increased the amount of cholesterol incorporation into PC in vitro (1). LPS exposure in rats has been shown to decrease phospholipid levels in lung lavage as early as 2 h after exposure in ex vivo experiments (10), although studies using rats instilled with LPS showed increased phospholipid in the lungs 4 h after exposure (34). Intratracheal instillation of LPS (1 mg) into rats led to a 50% decrease in intracellular surfactant lipid, a 50% increase in intracellular surfactant pools, a fourfold increase in SP-A found in lung tissue, and a 10-fold increase in the cells found in bronchoalveolar lavage 3 days after LPS exposure (52). In addition to macrophages, the lavage cells after LPS treatment contained ~30% neutrophils. Instillation of 0.5 mg LPS/kg body wt into rats increased SP-A levels in tissue by 6 h and in lavage by 72 h. In this model, lavage phospholipid levels decreased at 72 h (31).

We hypothesize that changes in the alveolar resident cells impact surfactant levels after LPS-induced acute lung injury and that SP-A may be important in regulating surfactant phospholipid levels. Although it is well established that alveolar macrophages degrade surfactant components, the degradative ability of macrophages isolated from the inflamed lung has not been reported. In addition, the ability of neutrophils obtained from the lung to degrade surfactant components has not been evaluated, although it has been reported that neutrophils obtained from peripheral blood degrade SP-A (45), and neutrophils derived from the rabbit peritoneal cavity internalize liposomes (8, 46). Our model system, consisting of isolated cells, purified SP-A, and synthetic surfactant-like liposomes, was used to determine whether alveolar macrophages and neutrophils isolated from LPS-injured lungs are activated for phospholipid uptake in vitro and to determine if these changes could lead to alterations in surfactant phospholipid pool sizes after acute lung injury.

MATERIALS AND METHODS

Materials. DPPC, dipalmitoylphosphatidylglycerol (DPPG), egg PC, and cholesterol were purchased from Avanti Polar Lipids (Birmingham, AL). α-dipalmitoyl[b-3H]-PC (89 Ci/mmol) was obtained from DuPont New England Nuclear (Boston, MA). Phosphate-buffered saline (PBS) was purchased from GibCO. Chloroform (CHCl3) and methanol (MeOH) were from Mallinkrodt. Bovine serum albumin, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), ammonium molybdate, Fiske and Subbarow reducer, Percoll, and ammonium hydroxide (NH4OH) were obtained from Sigma (St. Louis, MO). BCA protein assay reagent was purchased from Pierce (Rockford, IL). Hemacolor differential hematoxylin staining kit was obtained from EM Science (Gibbstown, NJ). The fluorocent lipid 2-(4,4-difluoro-5-(4-phenyl-1,3-buta dienyl)-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-gly ce ro-3-phosphocholine (BODIPY-PC) was purchased from Molecular Probes (Eugene, OR).

Animals. Male Sprague-Dawley rats (200–500 g) were purchased from Charles River (Raleigh, NC).

Purification of alveolar proteinosis SP-A. SP-A was purified as previously described (55). Briefly, SP-A was isolated from the lavage fluid of patients with alveolar proteinosis by butanol extraction of the sedimented lipids. Purified protein was treated with polymyxin to lower endotoxin levels (30).

Purification of liposomes. Small unilamellar liposomes were prepared with a lipid composition similar to that of surfactant: 52% DPPC, 26% egg PC, 15% DPPG, and 7% cholesterol by weight with trace amounts of [3H]DPPC (12 μCi/mg phospholipid) (55). The lipids were resuspended in 0.9% NaCl and extruded from a French pressure cell, resulting in small unilamellar liposomes at a concentration of ~1 mg lipid/ml (14). For confocal studies, liposomes were labeled with 150 μg of BODIPY-PC/mg lipid.

LPS injury model system. Rats were anesthetized with halothane and intubated. A dose of 100 μg of E. coli 026:B6 LPS/kg body wt (31) was delivered in 400 μl of PBS through the endotracheal tube.

Purification of alveolar macrophages and neutrophils. Male Sprague-Dawley rats weighing 200–500 g were anesthetized with pentobarbital sodium and killed by exsanguination. The trachea and lungs were exposed. A cannula was inserted in the trachea, and the lungs were filled with PBS containing 0.2 mM EGTA at 37 °C to total lung capacity for a total of five washes. For control experiments in which the lavage cell population was >93.2 ± 1.5% macrophages, cells were removed from the lavage fluid by centrifugation at 228 g for 10 min using a Beckman GS-6R centrifuge. To separate macrophages and neutrophils after LPS exposure, the lavage fluid of LPS-exposed animals was centrifuged at 228 g, and the cell pellet was separated over a discontinuous Percoll gradient (4 ml PBS: 5 ml 44% Percoll in PBS: 4 ml 52% Percoll in PBS) at 386 × g for 12 min. Macrophages were collected from the interface between the PBS and 44% Percoll, and the interface between the 44% Percoll and 52% Percoll. Neutrophils were collected from the cell pellet. The cells were resuspended in 10 ml of PBS and centrifuged at 228 g for 10 min to remove the Percoll. Purity of the different populations of cells were determined by Hemacolor staining of cytospins that were prepared in a Shandon CytoSpin 2 centrifuge. Macrophages isolated using this method were ~85% pure. The contaminating cells in the macrophage preparations were primarily neutrophils. Neutrophils isolated by this method were ~90% pure; the majority of the contaminating cells were macrophages.

Purification of peripheral blood neutrophils. Peripheral blood neutrophils (PMN) were isolated as previously described with minor modifications (44, 47, 53). Briefly, rats were anesthetized, and blood was collected through the jugular vein by a buffer exchange procedure (6% hetastarch solution containing 100 U/ml heparin) until the rats expired. The collected blood was allowed to settle 30–45 min at room...
temperature, and the red blood cell-depleted fraction was separated on a five-step Percoll gradient with densities of 1.081, 1.085, 1.089, 1.093, and 1.097. The fractions at the interfaces of 1.085–1.089 and 1.089–1.093 density layers were pooled, washed, and resuspended in the appropriate buffer. The neutrophil preparations from the peripheral blood contained ~83% neutrophils and 7% eosinophils, and lymphocytes and monocytes made up the remaining 10%, as determined by Hemacolor staining.

Lipid uptake, lipid binding, and lipid degradation assays in vitro. Isolated cells were resuspended in PBS with 1 mM Ca\(^{2+}\) and 0.1% BSA at a concentration of 2 × 10^6 cells/ml. Radiolabeled liposomes were added to a concentration of 40 nM phospholipid/ml. In some cases, SP-A was also added. Cells were incubated at 37°C (for uptake) or 4°C (for binding) with gentle agitation. After incubation, the cells were washed three times by centrifugation in PBS with 1 mM EDTA. After the third centrifugation, the cells were resuspended in 200 μl of lysis buffer (150 mM NaCl, 50 mM NaPO_4, 2 mM EDTA, and 0.1% Nonidet P-40) and vortexed vigorously. A 150-μl aliquot was taken for scintillation counting using a United Technologies Packard Minaxi Tri-carb 4000 scintillation counter; protein concentration was analyzed using BCA protein assay reagent.

The contribution of a 10% macrophage contamination to the uptake by the neutrophil preparation was calculated by measuring the lipid uptake per microgram cell protein in the neutrophil preparation and estimating the contribution of macrophages based upon the amount of protein contributed by macrophages to the neutrophil preparation and by the amount of lipid uptake measured with macrophage preparations. This estimated value for macrophage uptake was subtracted from the uptake with the neutrophil preparation. It is important to note that because the content of protein per macrophage is much higher than the protein content per neutrophil, a 10% macrophage contamination translates to a 33% contamination of protein. The data were corrected for macrophage contamination in Table 1, since the data presented in that table are estimates of cell-specific lipid clearance. All other data are presented as measured with no correction as indicated in the table and figure legends.

Table 1. Estimated PC clearance from healthy and 12-h LPS-injured rat lungs

<table>
<thead>
<tr>
<th>SP-A, μg/ml</th>
<th>Injury</th>
<th>Cell Type</th>
<th>10^6 Cells/ Lung</th>
<th>PC Cleared, nmol/10^6 cells</th>
<th>PC Cleared, nmol/lung</th>
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<tbody>
<tr>
<td>0</td>
<td>Control</td>
<td>Macrophage</td>
<td>4.8</td>
<td>0.09</td>
<td>0.43</td>
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<td></td>
<td></td>
<td>Neutrophil</td>
<td>0.1</td>
<td>0.01</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>0</td>
<td>12-h LPS</td>
<td>Macrophage</td>
<td>11</td>
<td>0.19</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neutrophil</td>
<td>62</td>
<td>0.01</td>
<td>0.62</td>
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<td></td>
<td></td>
<td>Total</td>
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<td>0.71</td>
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<td></td>
<td></td>
<td>Neutrophil</td>
<td>0.1</td>
<td>0.36</td>
<td>0.34</td>
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<td></td>
<td></td>
<td>Total</td>
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<td></td>
<td>3.4</td>
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<tr>
<td>2.5</td>
<td>12-h LPS</td>
<td>Macrophage</td>
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<td>2.1</td>
<td>23</td>
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<tr>
<td></td>
<td></td>
<td>Neutrophil</td>
<td>62</td>
<td>0.36</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
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<td></td>
<td>45</td>
</tr>
</tbody>
</table>

Lipid clearance was calculated by multiplying the molar clearance of phosphatidylcholine (PC) over 90 min (as determined by in vitro experiments) by the number of each cell type isolated from the lung by lavage. Neutrophil clearance was corrected for macrophage contamination as described in MATERIALS AND METHODS. Cell types were determined by Hemacolor staining of cytospin samples. LPS, lipopolysaccharide; SP-A, surfactant protein A.

For lipid degradation assays, after the third centrifugation, the cells were resuspended in 800 μl of ethanol and dried under nitrogen. The lipids were extracted by the method of Bligh and Dyer (4a) and separated by two-step, one-dimensional thin-layer chromatography (TLC) (12). The first phase consisted of hexanes-diethyl ether-acetic acid (6:4:1 vol/vol) and was used for the first half of the plate. The second phase consisted of hexanes-diethyl ether-acetic acid (90:10:1) and was used for the remainder of the plate. Iodine vapor was used to visualize the lipids. Individual lipid species were scraped into scintillation vials with a razor blade; radioactivity was determined by liquid scintillation counting. Lipids were identified by comparison to known lipid standards separated under the same conditions.

Confocal microscopy. Alveolar macrophages and neutrophils isolated by lung lavage of control rats or LPS-treated rats were resuspended at a concentration of 2 × 10^6 cells/ml in PBS with 1 mM Ca^{2+} and 0.1% BSA (pH 7.4). The cells were incubated with 40 μg/ml of unilamellar liposomes labeled with BODIPY-PC with and without 2.5 μg of SP-A/ml. After 60 min of incubation, the cells were washed twice by centrifugation with PBS containing 1 mM EDTA and made to make cytospins. The distribution of BODIPY-PC was examined with a Zeiss 410 confocal microscope with a ×63 objective.

Data analyses. Uptake of lipid in picomoles per microgram of cell protein was calculated using the specific activity of DPPC in the liposomes and data from the BCA protein assays. Data were compared by Student’s t-test for unpaired samples, or analysis of variance (ANOVA) and a Tukey test when appropriate. n refers to independent experiments. Error bars not visible in figures are occluded by the data point marker.

RESULTS

In vivo LPS treatment initiates changes in the population of cells resident in the alveolar space. Intratracheal instillation of 026:B6 LPS in rats induced an increase in the number of cells that can be recovered from the alveolar space by lavage (Fig. 1A). By 12 h postinstillation, the number of cells increased from 6.2 ± 0.4 × 10^6 cells/lung in control animals to 73.7 ± 3.1 × 10^6 cells/lung. Cell numbers were also higher than control values at 24 h (52.7 ± 23.7 × 10^6 cells/lung) and at 72 h (50.7 ± 2.97 × 10^6 cells/lung). Vehicle control animals showed no difference from uninstilled animals (data not shown).

LPS treatment also affected the cell types isolated by lavage (Fig. 1B). Under control conditions, 93.2 ± 1.5% of cells isolated by lavage were macrophages. Twelve hours after instillation of LPS, 84.6 ± 1.8% of cells isolated by lavage were neutrophils. By 72 h postinstillation, the percentage of cells that were alveolar macrophages returned to levels similar to control conditions (90.7 ± 1.7%). Vehicle-instilled control animals showed no differences from uninstilled animals (data not shown).

Exposure to LPS in vivo stimulates lipid uptake by macrophages in vitro. The amount of lipid taken up by alveolar macrophages isolated from lungs 12 h after LPS was approximately twofold greater compared with that taken up by alveolar macrophages isolated from control lungs (Fig. 2A). In the presence of 2.5 μg of
SP-A/ml, macrophages isolated from lungs exposed to LPS for 12 h internalized three times more phospholipid than macrophages isolated from control lungs (2,110 ± 430 pmol DPPC/million cells vs. 710 ± 130 pmol DPPC/million cells, respectively) (Fig. 2B). Lipid uptake by neutrophils isolated from the alveolar space or from peripheral blood was fourfold lower than control macrophages with no SP-A present (Fig. 2A), but in the presence of 2.5 μg of SP-A/ml, lipid uptake was approximately the same for control macrophages, lung neutrophils, or PMN (Fig. 2B).

Neutrophils internalize phospholipid in a time-, temperature-, and SP-A-dependent manner. Twelve hours after LPS exposure, the lavage neutrophils outnumbered macrophages by a ratio of six to one. Because the metabolism of surfactant lipids by lavage neutrophils has not been described, studies were carried out to characterize this process. Neutrophils isolated by lung lavage internalized lipids in a time-dependent manner (Fig. 3). SP-A stimulated uptake of DPPC-containing liposomes by alveolar neutrophils in a time-dependent manner (Fig. 3). At 90 min, 19.7 ± 3.2 pmol DPPC/million cells associated with the neutrophils, representing an increase of 300% compared with 0.5 min. In the presence of 2.5 μg of SP-A/ml, the uptake at 90 min (461 ± 60 pmol DPPC/million cells) also represents an increase of 300% compared with 0.5 min.

Alveolar neutrophils bound and internalized greater amounts of lipid at 37°C compared with 4°C, and this uptake was enhanced by SP-A (Fig. 4). The presence of 5 μg of SP-A/ml increased the association of lipid with neutrophils ~40-fold. The stimulation of association

![Fig. 1. Differential cell counts recovered by lavage from lipopolysaccharide (LPS)-treated rat lungs. After intratracheal LPS instillation, rat lungs were lavaged and cells were isolated by centrifugation. The cells were counted (A), and cell type was determined by Hemacolor staining of cytopsins (B). Data are means ± SE. *Significantly different from no LPS exposure, P < 0.05 (n = 3 for t = 0 h and t = 24 h; and n = 4 for t = 12 h and t = 72 h).](http://ajplung.physiology.org/)

![Fig. 2. Lipid uptake by alveolar resident cells in vitro. Macrophages and neutrophils were isolated by lavage and density gradient centrifugation from control lungs (2,110 ± 430 pmol DPPC/million cells vs. 710 ± 130 pmol DPPC/million cells, respectively) (Fig. 2A). Lipid uptake by neutrophils isolated from the alveolar space or from peripheral blood was fourfold lower than control macrophages with no SP-A present (Fig. 2A), but in the presence of 2.5 μg of SP-A/ml, lipid uptake was approximately the same for control macrophages, lung neutrophils, or PMN (Fig. 2B).](http://ajplung.physiology.org/)
observed at 37°C was 700% greater than that observed at 4°C.

Localization of BODIPY-PC in alveolar macrophages and neutrophils by confocal microscopy. Confocal microscopy was used to confirm the internalization of phospholipids by neutrophils isolated from the lungs of LPS-treated rats (Fig. 5). Lipids were internalized in the presence or absence of SP-A by macrophages isolated from the lungs of healthy animals and by both macrophages and neutrophils isolated from LPS-treated lungs. The fluorescence intensity of the cells varied, as some cells were more brightly labeled than others. In all three cell types, the label localized to the interior of the cell. Lack of variation in apparent fluorescence intensity in the presence or absence of SP-A was due to differences in image capture settings between images; the settings were selected to optimize resolution of the internalized lipids, not differences in the levels of lipid uptake.

Estimation of the effect of LPS injury on surfactant lipid clearance by cells isolated by lavage. To estimate the change in lipid clearance due to the contributions of activated macrophages and infiltrating neutrophils in the inflamed lung, we calculated the relative contributions of each cell type based on recovered cell numbers and measured lipid uptake in vitro, and we estimated (as described in MATERIALS AND METHODS) the contribution of a 10% contamination of macrophages to the measured lipid uptake by neutrophils (Table 1). Before correcting for macrophage contamination, the uptake by neutrophils in the absence of SP-A is 0.45 pmol DPPC/µg protein; while in the presence of SP-A, the uptake is 9.8 pmol DPPC/µg protein. After correcting for macrophage contamination, the uptake by neutrophils in the absence of SP-A is 0.30 pmol DPPC/µg cell protein (or 12 pmol/10^6 cells), whereas the uptake in the presence of SP-A is 8.6 pmol DPPC/µg cell protein (or 350 pmol/10^6 cells).

We estimate that macrophages in the normal healthy lung account for >90% of the total clearance carried out by cells from the lavage in the absence or presence of SP-A. In contrast, in the LPS-treated lung lavage, neutrophils contribute substantially to lipid clearance. In LPS-treated lungs, the estimated contribution of neutrophils to clearance was 22% of total clearance in the absence of SP-A and 48% in the presence of SP-A. Importantly, the calculated total lipid clearance in the LPS-treated lung was 6- to 13-fold greater than in the normal lung. Thus our data suggest that, in the injured lung, both the total lipid clearance by macrophages and the contribution of neutrophils to...
the process are augmented. Several assumptions have been made in these calculations, including the assumption that cell recoveries are representative of the populations in the alveolar space and that the uptake we measure in vitro is comparable to that occurring in vivo. Although we cannot validate these assumptions, it does seem reasonable to conclude that lipid clearance is likely to be augmented in the injured lung, although the absolute magnitude may not be accurately reflected in our estimates.

**Phospholipid degradation by neutrophils and activated macrophages is stimulated in the presence of SP-A.** Two-step, one-dimensional TLC was used to analyze the degradation of DPPC by alveolar macrophages and neutrophils and to determine if SP-A affected the amount of lipid degraded (Table 2). In the

<table>
<thead>
<tr>
<th>SP-A, μg/ml</th>
<th>Cell Type</th>
<th>Uptake, pmol DPPC/10^6 Cells</th>
<th>Degradation, % of associated</th>
<th>Degradation, pmol DPPC/10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Control macrophage</td>
<td>90 ± 8</td>
<td>64 ± 7%</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>12-h LPS macrophage</td>
<td>190 ± 22*</td>
<td>45 ± 7%</td>
<td>86 ± 17</td>
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</tr>
<tr>
<td>12-h LPS neutrophil</td>
<td>20 ± 5†</td>
<td>39 ± 9%</td>
<td>7.7 ± 2.1</td>
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<tr>
<td>2.5</td>
<td>Control macrophage</td>
<td>710 ± 130†</td>
<td>16 ± 3%†</td>
<td>110 ± 28</td>
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<tr>
<td>12-h LPS macrophage</td>
<td>2,100 ± 430‡</td>
<td>11 ± 3%†</td>
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</tr>
<tr>
<td>12-h LPS neutrophil</td>
<td>460 ± 98†</td>
<td>17 ± 3%</td>
<td>78 ± 18</td>
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</table>

Data are means ± SE. Alveolar macrophages or neutrophils were suspended in PBS with 2 mM Ca^{2+} and 0.1% BSA. The cells were then incubated with 40 μg of phospholipid/ml labeled with trace amounts of [3H]dipalmitoylphosphatidylcholine (DPPC) for 90 min in the presence or absence of 2.5 μg SP-A/ml. The cells were washed, and lipid uptake was analyzed by scintillation counting and standardized according to total protein. For degradation studies, lipids were extracted and separated by thin-layer chromatography. Individual lipid species were isolated and analyzed for radioactivity. *Significantly different from control macrophage, P < 0.05. †Significantly different from the corresponding 0-μg SP-A/ml control, P < 0.05 (n = 3, except for control macrophage %degradation, n = 4). Molar degradation was calculated by multiplying %degradation by molar uptake (e.g., 710 pmol/million cells × 16% degraded = 110 pmol degraded). Neutrophil clearance was not corrected for macrophage contamination.
presence of 2.5 μg SP-A/ml, control macrophages degraded 16 ± 3% of the internalized lipid; similar results were found for macrophages and neutrophils isolated from lungs 12 h after LPS treatment (11 ± 3% and 17 ± 3%, respectively). In the absence of SP-A, control macrophages degraded 64 ± 7% of the internalized lipid. For macrophages and neutrophils isolated from lungs treated with LPS 12 h prior, 45 ± 7% and 39 ± 9% of the internalized lipid were degraded, respectively. These results show that a significantly lower percentage of cell-associated DPPC is degraded by alveolar macrophages and neutrophils when SP-A is present, although molar amounts of lipid degraded are increased in the presence of SP-A due to increased uptake. In the same manner that we estimated the relative contributions of contaminating macrophages to neutrophil lipid uptake (as described in MATERIALS AND METHODS), we estimated the contribution of a 10% contamination of macrophages to the measured neutrophil lipid degradation. As a percentage of the lipid degraded by neutrophils, contaminating macrophages account for 11% of the lipid degraded in the absence of SP-A and 2% of the lipid degraded in the presence of SP-A.

DISCUSSION

During an acute inflammatory response in the lung, the population of alveolar cells changes dramatically. The number of cells increases by ~12-fold, and the percentage of cells that are neutrophils increases from <5% to ~85%. Because metabolism of alveolar surfactant by lavage cells represents a major route of surfactant clearance, we hypothesized that the change in cell number and cell types could lead to alterations in surfactant clearance and in the surfactant pool size. Therefore, the purpose of this study was to determine if neutrophils entering the lung in response to LPS installation could contribute to alterations in surfactant phospholipid pool sizes and to determine if macrophages were activated for surfactant phospholipid clearance by LPS injury. Our results indicate that neutrophils and macrophages isolated from injured lungs degrade surfactant lipid in vitro. Importantly, our estimates of the contribution of these cells to surfactant clearance suggest that total lipid clearance in this model of acute lung injury could be increased by 6- to 13-fold due to the contributions of these cells to the clearance process. We speculate that changes in alveolar cells and their state of activation may contribute to the altered surfactant pool sizes observed in patients and animals with acute lung injury (31, 35, 52).

LPS exposure altered the lipid uptake characteristics of alveolar macrophages in vitro. For example, macrophages isolated from inflamed lungs exhibited increased lipid uptake and degradation; macrophages from the injured lung degraded ~1.5-fold more DPPC per million cells than macrophages from the normal lung. Based on the increased numbers of macrophages in the inflamed lung and our estimates of their lipid degradation, LPS exposure would result in a population of macrophages capable of clearing seven times more phospholipid in the injured lung compared with the normal lung (Table 2), if indeed the metabolism we measured in vitro mirrors the metabolism that occurs in vivo. Although the mechanism by which this increased metabolism is regulated is not known, other studies have reported that macrophages that are activated by adherence to tissue culture plastic have enhanced ability to degrade PG (40) and SP-A (4). We attempted to determine if LPS was directly activating the cells to increase lipid degradation by incubating macrophages isolated from normal lungs with LPS in vitro and measuring their lipid uptake. Our findings (data not shown) indicate that an in vitro treatment with LPS did not affect the ability of macrophages to take up lipid. Thus we speculate that either other factors are involved in the induction of enhanced lipid uptake in vivo, or the time or dose of LPS treatment in vitro may be insufficient to be effective.

In the presence of SP-A, macrophages from both LPS-treated lungs and healthy lungs degrade a smaller percentage of the lipid that is internalized, but a greater molar amount, compared with cells incubated without SP-A (Table 2). Two possible explanations for this finding are that SP-A may be inhibiting PLA2 activity, as has been previously reported (11), or that the increased lipid uptake may be saturating the degradative pathway. This would result in increased molar amounts of degraded lipid but a decrease in the percentage of internalized lipid that was degraded.

The neutrophils found in the alveolar space were also capable of internalizing and degrading liposomes. To the best of our knowledge, this report is the first to characterize the uptake and metabolism of surfactant-like lipids by neutrophils, although it has been previously reported that peripheral neutrophils degrade SP-A (45) and that neutrophils isolated from the rabbit peritoneal cavity are capable of internalizing liposomes (8, 46). Although lipid uptake by alveolar macrophages from LPS-exposed lungs is greater than lipid uptake by alveolar macrophages from normal lungs, lipid uptake by alveolar neutrophils from LPS-exposed lungs is similar to lipid uptake by neutrophils isolated from peripheral blood. It is possible that the process of isolation of neutrophils from peripheral blood activates them and therefore the activation by LPS exposure would be masked. Though this is conceivable, another group has made comparisons between PMN and LPS-exposed lung neutrophils and observed differences in production of cytokine mRNA (57), suggesting that the process of cell isolation does not result in nonspecific activation of the neutrophils.

It is also possible that macrophage contamination of the neutrophil preparation could account for a significant amount of lipid uptake. If one subtracts the contribution of contaminating macrophages to clearance based on protein recoveries and lipid uptake using purified macrophages, a 10% macrophage contamination in the neutrophils preparation would account for 33% of the lipid uptake by the neutrophils in the absence of SP-A. In the presence of SP-A, the macro-
neutrophils. Experiments using confocal microscopy to examine BODIPY-PC lipid uptake by macrophages and neutrophils confirmed that both cell types internalized lipid in the presence and absence of SP-A (Fig. 5). Thus our data are most consistent with the fact that neutrophils from peripheral blood and the alveolar compartment have similar capacity to internalize and degrade surfactant.

Although the mechanism by which SP-A stimulates lipid internalization by neutrophils is not known, it is likely to happen through one of two mechanisms. SP-A has been shown to bind and aggregate phospholipid vesicles (15), presenting a larger target for internalization. It is also possible that SP-A may bind a receptor (or receptors) on the neutrophil cell surface, leading to direct stimulation of internalization. Receptors on the surface of macrophages and type II cells have been identified (7, 25, 36, 54). Either of these characteristics of SP-A could lead to increased lipid uptake by neutrophils, although it has been demonstrated that SP-A enhanced lipid uptake by type II cells independently of lipid aggregation. Point mutations engineered into SP-A have resulted in a protein that can still aggregate liposomes, although lipid uptake by type II cells is not stimulated (37, 50). Similar studies using isolated alveolar macrophages and neutrophils could discern the role of lipid aggregation in stimulation of lipid uptake.

Although LPS injury does not stimulate lipid uptake in neutrophils, the large number of cells recruited to the lung contributes significantly to lipid clearance. Although both neutrophils and macrophages internalize and degrade surfactant lipid, neutrophils isolated from lavage fluid from inflamed lungs internalized ~20-fold less lipid per cell than macrophages isolated from LPS-treated lungs. However, the population of neutrophils found in the inflamed lung is approximately six times larger than the population of macrophages. Therefore, the total contribution of lavage neutrophils to clearance is estimated to be ~30% that of macrophages in the acutely injured lung in the absence of SP-A. In the presence of SP-A, the total contribution of the neutrophil population to lipid uptake is equal to that of the population of macrophages (Table 1). This is due to the increased SP-A-mediated stimulation of lipid uptake in neutrophils compared with macrophages. Thus the combination of activated macrophages and large numbers of neutrophils in the LPS-treated lung could lead to a 6- to 13-fold increase in lipid clearance. It is possible that these experiments overestimate the amount of phospholipid cleared by macrophages and neutrophils in the alveolar space, as the concentrations of both SP-A and liposomes may be different in vivo. Isolation of the cells may also alter cellular functions relating to phospholipid clearance and degradation. Though these studies may underestimate the amount of lipid internalized and degraded by macrophages or neutrophils, they do demonstrate that these cells are capable of clearing surfactant phospholipids and altering pool sizes in vivo. It is also important to note that the actual numbers of neutrophils that emigrate into the lung vary with the model of lung injury (2, 29, 52, 57), and therefore, the contribution of neutrophils to surfactant metabolism may depend on the animal model or lung disease. However, an influx of neutrophils is a hallmark of acute lung injury in both animals and humans, and it seems likely that their increased numbers will contribute substantially to surfactant degradation.

Although in vitro studies support a role for SP-A in surfactant homeostasis, depletion of SP-A in vivo does not greatly alter surfactant homeostasis in the SP-A (−/−) mice. For example, previous in vitro studies have suggested that SP-A may play a role in maintaining surfactant phospholipid levels through interactions between both alveolar macrophages (56) and type II cells (3, 26, 41, 55). However, SP-A (−/−) mice have similar levels of lavage phospholipid compared with SP-A (+/+) mice (18). The reasons for these discrepant findings are not known, but it is possible that other key regulatory components of the system are not being included in the in vitro systems. Alternatively, SP-A may have a role in regulating metabolism in the lung during non-steady state conditions, which may occur, for example, during acute inflammation. For this reason, we characterized the effects of SP-A on lipid uptake by neutrophils and macrophages from injured lungs and found that SP-A enhanced lipid uptake and degradation by both neutrophils and activated macrophages, although to different extents. For example, SP-A enhanced the molar degradation of DPPC by macrophages from both the normal and inflamed lung by approximately two- to threefold. In contrast, SP-A enhanced degradation of DPPC by neutrophils by ~10-fold (Table 2). Thus our in vitro studies suggest that the effects of SP-A on metabolism may be more evident in lung injury when large numbers of neutrophils emigrate into the lung. However, hyperoxia-induced lung injury, which can result in migration of neutrophils into the alveolar space (9), resulted in increased levels of lavage protein with no change in saturated PC levels in the lavage of both SP-A (+/+) and SP-A (−/−) mice (17). Resolving the role of SP-A in surfactant homeostasis in LPS-induced lung injury will require experiments that examine surfactant synthesis, secretion, and clearance in SP-A (−/−) mice after induction of LPS-induced acute lung injury.

In summary, our results show that neutrophils and activated macrophages degrade surfactant lipids and suggest that both cell types may contribute to the alterations in surfactant lipid pool size that have been observed in acute lung injury. Clearance of large amounts of phospholipid could, over time, lead to decreases in surfactant phospholipid levels, such as those observed in animal models of acute lung injury. For example, McIntosh et al. (31) showed that phospholipid levels recovered by lavage were decreased compared with control rats 72 h after intratracheal instillation of E. coli LPS. In addition, Viviano et al. (52) also reported a decrease in lavage phospholipid levels after LPS exposure. We speculate that lower surfactant phospholipid levels may result in smaller pools of func-
tional surfactant that have diminished ability to reduce surface tension, interfering with normal respiration. If surfactant secretion cannot compensate for increases in phospholipid clearance and/or inactivation of surfactant by chemical modification, then lung inflammation would result in decreases in surfactant phospholipid levels available for reducing surface tension and for participating in pulmonary host defense.

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LIPID CLEARANCE BY CELLS FROM INFLAMED LUNGS

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