Effects of endotoxin on surfactant protein A and D stimulation of NO production by alveolar macrophages

J O RAE WRIGHT,1,2 DANIEL F. ZLOGAR,1 JULIE C. TAYLOR,1 THOMAS M. ZLOGAR,1 AND CLARA I. RESTREPO2
Departments of 1Cell Biology and 2Medicine, Duke University Medical Center, Durham, North Carolina 27710

Wright, J O Rae, Daniel F. Zlogar, Julie C. Taylor, Thomas M. Zlogar, and Clara I. Restrepo. Effects of endotoxin on surfactant protein A and D stimulation of NO production by alveolar macrophages. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L650–L658, 1999.—Surfactant protein (SP) A and SP-D affect numerous functions of immune cells including enhancing phagocytosis of bacteria and production of reactive species. Previous studies have shown that SP-A and SP-D bind to a variety of bacteria and to the lipopolysaccharide (LPS) components of their cell walls. In addition, purified preparations of SPs often contain endotoxin. The goals of this study were 1) to evaluate the effects of SP-A and SP-D and complexes of SPs and LPS on the production of nitric oxide metabolites by rat alveolar macrophages and 2) to evaluate methods for the removal of endotoxin with optimal recovery of SP. Incubation of SP-A or SP-D with polymyxin, 100 mM N-octyl-β-D-glucopyranoside, and 2 mM EDTA followed by dialysis was the most effective method of those tested for reducing endotoxin levels. Commonly used storage buffers for SP-A, but not for SP-D, inhibited the detection of endotoxin. There was a correlation between the endotoxin content of the SP-A and SP-D preparations and their ability to stimulate production of nitrite by alveolar macrophages. SP-A and SP-D treated as described above to remove endotoxin did not stimulate nitrite production. These studies suggest that the functions of SP-A and SP-D are affected by endotoxin and illustrate the importance of monitoring SP preparations for endotoxin contamination.

nitric oxide; lipopolysaccharide; C-type lectin; collectin

ALTHOUGH THE MOST WELL-ESTABLISHED FUNCTION of surfactant is the reduction in surface tension at the air-liquid interface, surfactant proteins (SPs) and lipids have also been shown to be involved in the innate or non-antibody-mediated host defense system in the lungs (reviewed in Ref. 34). Recent studies (reviewed in Ref. 3a) have focused on SP-A and SP-D, which are members of a family of proteins known as collectins. The term collectins was coined for these proteins, which include SPs and the liver-derived serum mannose-binding protein and conglutinin, because they contain an NH2-terminal collagen-like domain and a carboxy-terminal lectin (carbohydrate binding) (reviewed in Ref. 29). Both the serum and lung collectins have been implicated in host defense.

Many of the collectins have been shown to interact with pathogens including viruses and bacteria. For example, SP-A has been shown to bind to a large number of organisms including gram-negative and gram-positive bacteria (25, 30) as well as influenza A virus (1, 2, 9), herpes simplex virus (32), Mycobacterium tuberculosis (5, 7), Cryptococcus neoformans, and Pneumocystis carinii (33, 37). SP-D also binds to Escherichia coli, Salmonella paratyphi, Klebsiella pneumonia (15), C. neoformans (27), Pseudomonas aeruginosa (26), Aspergillus fumigatus (17), P. carinii (21), and influenza virus A (9). At least part of the binding of SP-A (31) and SP-D (15) to gram-negative bacteria appears to be mediated via interaction with the lipopolysaccharide (LPS) component of the bacterial cell wall. Although the mechanism of the interaction of SP-A and SP-D with LPS is not completely understood, it has been shown that SP-A can bind directly to lipid A (31) as well as to carbohydrate components of LPS (12). SP-D binds rough LPS to a much greater extent than smooth LPS and binding is calcium dependent and inhibited by sugars, suggesting that the lectin domain of SP-D mediates LPS binding (15).

Some functional consequences of the binding of SP-A and SP-D to bacteria and LPS have been previously investigated. For select organisms, binding of SP-A and SP-D facilitates phagocytosis (reviewed in Ref. 34). However, binding is not sufficient to stimulate phagocytosis of all organisms. For example, SP-D binds to P. carinii, although it does not stimulate its uptake (21). In contrast, SP-D both binds to and enhances the uptake of P. aeruginosa (26) and A. fumigatus (17). In addition, Kalina et al. (12) reported that both SP-A and LPS stimulated secretion of colony-stimulating factor from alveolar type II cells. However, when SP-A and LPS (in the µg/ml range) were both added to the cells, the stimulatory effects of both were diminished.

The goal of this study was to investigate further the effects of LPS on SP-A- and SP-D-mediated functions, specifically the production of nitric oxide metabolites by alveolar macrophages, and to establish a procedure for reducing endotoxin contamination while maintaining optimal protein recovery.

METHODS

Materials. Unless otherwise indicated, all chemicals and reagents were obtained from Sigma (St. Louis, MO). A Picosystem water purification system (Hydro Water Systems, Research Triangle Park, NC) was used for the preparation of all reagents and buffers. The typical water quality specifications for this system include a specific resistance > 18 MΩ·cm and pyrogen content < 0.25 endotoxin unit (EU)/ml.

Animals. All animals received humane care in compliance with the National Institutes of Health Guide for the Care and
Use of Laboratory Animals. Male Sprague-Dawley rats were obtained from Charles River Laboratories (Raleigh, NC).

Purification of SP-A with butanol extraction. SP-A was purified from the therapeutic lavage fluid of patients with alveolar proteinosis by sequential extraction with butanol and 30 mM N-octyl-β-D-glucopyranoside (OGP), 150 mM NaCl, and 5 mM Tris, pH 7.4 (35). For routine treatment to reduce endotoxin, the resulting pellet was resuspended in 9 mM Tris, pH 7.4, 100 mM OGP, and polymyxin-agarose at a ratio of 1:4 (volume of polymyxin-agarose to protein solution). The mixture was dialyzed against 5 mM Tris, pH 7.4, for 48 h, with at least four changes. The polymyxin-agarose and insoluble material were removed by centrifugation at 100,000 g for 1 h in a type 40 Beckman ultracentrifuge rotor. Additional methods that tested for reduction of endotoxin content are described in Treatment of SP-A and SP-D to remove endotoxin.

Purification of SP-A by calcium chelation (nonbutanol-extracted SP-A). SP-A was purified from the therapeutic lavage fluid from patients with alveolar proteinosis or lavage fluid from rats treated with silica (4) by a method that does not entail extraction with butanol (28). Briefly, the surfactant pellets were washed sequentially with calcium-containing buffers and with buffers containing magnesium, with subsequently lower concentrations of calcium and magnesium than the magnesium-containing buffers that release SP-A (and SP-D). SP-A was purified by size-exclusion chromatography.

Purification of rat SP-D. SP-D was purified from rats treated with silica to induce surfactant accumulation as described by Dethloff and co-workers (4). Rats were anesthetized by inhalation of halothane and intubated with a modified pediatric fiber-optic laryngoscope manufactured by the Duke University Surgical Shop (Durham, NC). The rats received 25 mg of silica in 0.5 ml of saline prepared according to the method of Miles et al. (20). Approximately 4 wk later, the rats were anesthetized with an overdose of pentobarbital sodium and killed by exsanguination, and the lungs were lavaged six times to apparent total lung capacity with 150 mM NaCl and 5 mM Tris, pH 7.4. The lung lavage fluid was centrifuged at 27,150 average g, in a Beckman type 19 ultracentrifuge rotor for 30 min at 10°C. The supernatant was mixed with 8–10 ml of maltose-Sepharose prepared as described (6) and 1.3 mM magnesium and no EGTA. The cell lysate fractions were collected, and any unattached cells were removed by centrifugation at 27,150 average g, at 10°C for 15 min. The cells were pooled and lysed with 150 mM NaCl, 50 mM sodium phosphate, 0.5% Nonidet P-40, and 2 mM EDTA. The cell lysate fractions were assayed for protein content by the BCA method (BCA kit, Pierce, Rockford, IL) with bovine serum albumin as a standard.

Viability assay. At the time of medium collections, viability was assessed by erythrosin B dye exclusion. None of the treatments decreased viability.

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Isolation of alveolar macrophages. Adult male Sprague-Dawley rats were maintained with free access to water and food. To isolate macrophages, the rats were killed by a pentobarbital sodium overdose, and the tracheae were isolated, cannulated, and sutured in place. The lungs were removed and lavaged to total lung capacity (~10–15 ml) six times with a macrophage isolation buffer containing 140 mM NaCl, 6 mM glucose, 2.5 mM phosphate buffer, pH 7.4, 10 mM HEPES, and 0.2 mM EGTA. This was followed by two lavages with the same isolation buffer containing 2 mM calcium and 1.3 mM magnesium and no EGTA. The cell suspensions were centrifuged at 188 g at 25°C for 10 min in a Beckman model GS-6R centrifuge. The alveolar macrophages were resuspended at 1.25 × 10⁶ cells/ml nitrate-free basal medium Eagle (GIBCO BRL, Gaithersburg, MD) containing 110 mg/l of L-glutamine, 50 U/ml of penicillin, 50 µg/ml of streptomycin, 15 mM HEPES, and 10% heat-inactivated fetal calf serum (Atlanta Biologicals, Norcross, GA). The cells were plated at 250,000 cells/well in 96-well plates (Costar, Cambridge, MA) and allowed to adhere for 2 h. Subsequently, the unattached cells were removed and replaced with medium containing LPS, SP-A, SP-D, or a combination of LPS and either SP. After incubation for 24 h, the medium was collected, and any unattached cells were removed by centrifugation at 27,150 g, at 25°C for 10 min. The cells were pooled and lysed with 150 mM NaCl, 50 mM sodium phosphate, 0.5% Nonidet P-40, and 2 mM EDTA. The cell lysate fractions were assayed for protein content by the BCA method (BCA kit, Pierce, Rockford, IL) with bovine serum albumin as a standard.
Fig. 1. Effects of N-octyl-β-D-glucopyranoside (OGP), EDTA, and NaCl on recovery of surfactant protein (SP) A incubated with polymyxin-agarose. Conditions for treatment of SP-A to remove endotoxin with high recovery of protein were evaluated by incubating SP-A with polymyxin-agarose in presence (+) of various combinations of these compounds. After 6 h of incubation at room temperature, polymyxin-agarose was removed by centrifugation, and SP-A remaining in supernatant was evaluated by Western blot. Recovery was improved when SP-A was incubated with polymyxin in presence of 2 mM EDTA and 100 mM OGP. Nos. at left, molecular mass.

<table>
<thead>
<tr>
<th>OGP (100 mM)</th>
<th>EDTA (2 mM)</th>
<th>NaCl (50 mM)</th>
<th>Tris, pH 7.4 (5 mM)</th>
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ENDOTOXIN AND SURFACTANT PROTEIN STIMULATION OF NO

mM EDTA, 5) polymyxin-agarose, 100 mM OGP, 2.5 mM EDTA, and 50 mM NaCl, and 6) polymyxin-agarose and 2.5 mM EDTA. The treatment groups were incubated for 6 h at room temperature, and the polymyxin-agarose was removed by centrifugation at 13,350 g, in a Tomy MRX-152 refrigerated microcentrifuge. The samples were transferred to Spectrapor 4 dialysis tubing (Spectrum Quality Products, Gardena, CA) that had been sprayed briefly with a dilute solution of an endotoxin-reducing agent (PyroCLEAN eluting buffer, Alerchek, Portland, MA), then soaked and washed extensively in Picopure water. All groups were dialyzed against 1 liter of 5 mM Tris, pH 7.4, at room temperature for 48 h, with four buffer changes.

SP-D (0.5 mg), purified from the lavage fluid of rats treated with silica as described in Purification of rat SP-D (generally 0.5 EU/ml) were diluted in the manufacturer's directions. Buffer controls (e.g., equal volumes of buffer in which the protein was suspended, usually ~1–30 µl) were always analyzed for positive or negative effects on the assay. For these inhibition assays, known concentrations of endotoxin (generally 0.5 EU/ml) were diluted in the manufacturer's diluent (Limulus amebocyte lysate (LAL) water) or protein storage buffer with and without SP-A or SP-D. The signal obtained in the presence of buffer or protein was compared with that obtained from the known concentration of endotoxin diluted in LAL water. Protein stocks containing detectable levels of endotoxin were diluted serially and analyzed to obtain a reading within the linear portion of the standard curve. SP-A and SP-D that had the lowest concentrations of endotoxin were routinely analyzed at 1 or 5 µg/well (total volume in the well was 50 µl) of the QCL-1000 assay and usually contained endotoxin levels at or below the minimal detection levels of the assay (0.1 EU/ml). The assay was linear over a range of 0.1–1.0 EU/ml.

Lipid aggregation assay. Lipids were prepared from surfactant-like lipids (Avanti Polar Lipids, Birmingham, AL) with a French pressure cell as previously described (36). The liposomes were diluted to 100 µg/ml in 5 mM HEPES-150 mM NaCl, pH 7.4, 5 µg/ml of SP-A were added, and a baseline absorbance at 400 nm was measured in a Hitachi U-2000 spectrophotometer. The change in absorbance induced by the addition of 2 mM CaCl₂ was measured over 200 s.

Optimization of endotoxin removal and protein recovery. Purified SP-A and SP-D isolated both from recombinant expression systems (data not shown) and from lung lavages of humans and animals often contained significant and variable levels of endotoxin. Initial attempts to reduce the endotoxin content of SP-A and SP-D by incubation with polymyxin-agarose were unsuccessful because huge losses of protein were incurred (Fig. 1, lane 2 vs. lane 1, which contains an untreated sample of SP-A), presumably due to association of SP-A with polymyxin-agarose. Recovery was also low if either OGP, NaCl, or OGP and NaCl were included in the incubation. Recoveries were higher when EDTA alone was included with the polymyxin treatment; however, as described below, this treatment did not effectively reduce endotoxin. Recovery of SP-A was improved when the protein was incubated with polymyxin, OGP, and EDTA (Fig. 1, lane 6).

To obtain preparations of SPs containing various amounts of LPS and to determine the optimal conditions for the removal of endotoxin, SP-A was incubated with LPS and treated by dialysis with various combina-
tions of EDTA, OGP, NaCl, and polymyxin-agarose as described in Methods. The most effective method of those tested for reducing endotoxin was incubation of SP-A with polymyxin, EDTA, and OGP followed by dialysis (Table 1). After this treatment, endotoxin levels were often below the limit of sensitivity of the assay even when 5 or 10 µg of SP-A were analyzed in the QCL lysate system. Although endotoxin levels were reduced by the other treatments (dialysis, incubation with polymyxin and OGP followed by dialysis, or incubation with polymyxin and EDTA followed by dialysis), the level of endotoxin remaining was ~2,000 times that remaining when SP-A was treated with polymyxin, EDTA, and OGP. When SP-A was incubated with polymyxin alone, a highly variable amount of endotoxin was recovered with SP-A; however, it should be noted that the SP-A recoveries were very low, with losses averaging >90%. Thus treatment with OGP, EDTA, and polymyxin-agarose followed by dialysis yielded both good recovery (~50%) and a reduction in endotoxin.

Most of the loss of protein occurred during dialysis (data not shown). Most of the SP-A utilized in this study was isolated by butanol extraction and detergent solubilization of sedimentable material from the lavage fluid of patients with alveolar proteinosis. However, because a previous study (31a) has shown that butanol-extracted SP-A from rats and dogs is inactivated by the butanol extraction process, a recently described method (28) of isolation of SP-A from rats that does not entail extraction with butanol was utilized to purify SP-A from alveolar proteinosis lavage samples. This procedure, which involves EDTA extraction of the surfactant pellet and size-exclusion chromatography, yielded non-butanol (NB) SP-A that contained varying levels of endotoxin. Treatment of NB SP-A with polymyxin, OGP, and EDTA also reduced endotoxin levels from 444 to 7 pg endotoxin/µg SP-A in one experiment and from 7 to 0.06 pg/µg in a second experiment.

Table 1. Effects of different treatments on reducing endotoxin levels of SP-A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Endotoxin Levels, pg/µg SP-A</th>
<th>Protein Recovery, %starting material</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>3</td>
<td>23,218</td>
<td>100</td>
</tr>
<tr>
<td>Dialysis</td>
<td>3</td>
<td>2.787 ± 1.145</td>
<td>10 ± 3.5</td>
</tr>
<tr>
<td>Polymyxin</td>
<td>3</td>
<td>41,064 ± 22,367</td>
<td>10 ± 8</td>
</tr>
<tr>
<td>Polymyxin + OGP</td>
<td>2</td>
<td>15.5</td>
<td>66</td>
</tr>
<tr>
<td>Polymyxin + EDTA</td>
<td>2</td>
<td>4.028</td>
<td>70</td>
</tr>
<tr>
<td>Polymyxin + OGP + EDTA</td>
<td>3</td>
<td>0.58 ± 0.41</td>
<td>43 ± 11</td>
</tr>
</tbody>
</table>

Values are means ± SE for n = 3 experiments and averages for n = 2 experiments. Surfactant protein (SP) A was purified from lavage fluids of patients with alveolar proteinosis as described in METHODS. SP-A (5 µg/ml) in 5 mM Tris-1 mM CaCl2, pH 7.4, was incubated for 24–48 h with lipopolysaccharide (LPS; from Escherichia coli serotype 026:B6) at a ratio of 20:1 (SP-A to LPS; wt/wt) at 4°C on a rotator. Mixture was then divided into 5 treatment groups, incubated with the appropriate reagents for 6 h at room temperature, and then centrifuged to remove polymyxin-agarose beads. Samples were then dialyzed against 5 mM Tris, pH 7.4, for ~48 h with 4 buffer changes and analyzed for protein and endotoxin. OGP, N-octyl-β-d-glucopyranoside.

Only the selected methods for reducing endotoxin described in Treatment of SP-A and SP-D to remove endotoxin were tested for SP-D because it is much more expensive and difficult to purify. As shown in Fig. 2, incubation of SP-D with polymyxin, EDTA, and OGP also resulted in maximal recovery (~50% as quantitated by protein assay) of SP-D. This treatment also reduced endotoxin levels of SP-D ~10-fold (from 68 to 7 pg endotoxin/µg SP-D in one experiment and from 1,677 to 126 pg/µg in a second experiment).

Effects of SP-A, SP-D, and buffers on endotoxin detection. The endotoxin assay is sensitive to ionic strength, pH, divalent cations, and various proteins (QCL Technical Bulletin, BioWhittaker); therefore, we analyzed the effects of SP-A, SP-D, and the buffers routinely used to isolate and store the proteins on the endotoxin assay. Known amounts of endotoxin were added to the buffers, and the measured levels were compared with those obtained with standards diluted in the manufacturer’s recommended medium ("LAL water"). SP-A storage buffer (5 mM Tris, pH 7.4) did not inhibit the detection of endotoxin by this assay nor did 10 µg of purified SP-A (data not shown). In contrast, buffers that were routinely used to elute SP-D from saccharide-affinity columns and sizing columns, which contain either 2 or 10 mM EDTA, significantly inhibited the assay (Fig. 3). The addition of calcium to the buffers containing 2 mM EDTA but not to those containing 10 mM EDTA partially decreased this inhibition. SP-D did not interfere with endotoxin detection (data not shown).

Stimulation of nitrite production by SP-A and SP-D containing endotoxin. The ability of SP-A containing varying levels of endotoxin to stimulate production of nitrite by rat alveolar macrophages was evaluated (Table 2). For these studies, a preparation of purified SP-A was preincubated with LPS and then treated in various ways to produce preparations of SP-A containing a wide range of endotoxin contamination. SP-A containing 0.61 pg endotoxin/µg SP-A did not stimulate nitrite production at an SP-A concentration of 5 µg/ml. In addition, concentrations of treated SP-A as high as 80 µg/ml also did not stimulate nitrite production (data not shown). However, SP-A containing levels of endotoxin greater than ~20 pg endotoxin/µg SP-A stimulated alveolar macrophage production of nitrite in the medium. When the production of nitrite was plotted as a function of the endotoxin level in SP-A, the correlation coefficient was 0.91 (data not shown).

The ability of SP-D and human NB SP-A containing varying amounts of endotoxin to stimulate nitrite production was also evaluated (Table 3). These preparations of SP-A were not preincubated with LPS; the endotoxin contamination was present at the end of the purification. However, SP-D was incubated with LPS and treated as described methods. When human NB SP-A and SP-D were treated to remove endotoxin as described in Optimization of endotoxin removal and protein recovery, they did not significantly stimulate nitrite production. However, both human NB SP-A and SP-D containing endotoxin did stimulate nitrite produc-
tion. Rat NB SP-A also did not stimulate nitrite production at a final SP-A concentration of 5 µg/ml. When the production of nitrite stimulated by several preparations of butanol-extracted SP-A, NB-extracted SP-A, and SP-D was plotted as a log function of the endotoxin in the protein, the correlation coefficient was equal to 0.88 (Fig. 4).

Polymyxin inhibits stimulation of nitrite production by SP-A containing endotoxin. Polymyxin was added to SP-A preparations that contained endotoxin, and the mixture was incubated with alveolar macrophages. After 24 h, the levels of nitrite in the medium were analyzed. Polymyxin inhibited the production of nitrite stimulated by 200 ng/ml of LPS used as a positive control as well as by SP-A that contained an estimated endotoxin concentration of 1.44 ng/µg SP-A (Table 4).

SP-A and SP-D treated to reduce endotoxin retain aggregation-inducing ability. To determine whether treatment to reduce endotoxin altered protein function, the ability of SP-A to induce lipid aggregation and the ability of SP-D to induce bacterial aggregation were tested. For these studies, a single preparation of SP-A or SP-D was divided into treated and untreated groups.

As shown in Fig. 5, both treated and untreated SP-A enhanced lipid aggregation to comparable extents. Furthermore, both treated and untreated SP-D enhanced aggregation of E. coli to comparable extents (Fig. 6).

DISCUSSION

The results of this study show that there is a correlation between the levels of endotoxin in SP-A and SP-D preparations and their ability to enhance production of nitrite by rat alveolar macrophages. Preparations of SP-A and SP-D that have been treated to remove endotoxin do not stimulate production of nitrite by alveolar macrophages. Furthermore, polymyxin B, a decapeptide that binds to and inhibits the action of LPS, inhibits the induction of nitrite production by the SP-A preparations containing endotoxin. In addition, a method for treating SP-A and SP-D to remove endotoxin while maximizing protein recovery is reported.

The optimal method for removing endotoxin from both SP-A and SP-D was incubation with EDTA, poly-

Table 2. Effects of SP-A containing various amounts of endotoxin on production of nitrite by alveolar macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Endotoxin, pg/µg SP-A</th>
<th>Nitrite Levels, %control</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td>28,213</td>
<td>428 ± 52</td>
</tr>
<tr>
<td>Dialysis</td>
<td>4,113</td>
<td>457 ± 37</td>
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<tr>
<td>Polymyxin</td>
<td>34,261</td>
<td>466 ± 59</td>
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<tr>
<td>Polymyxin + EDTA</td>
<td>1,578</td>
<td>388 ± 32</td>
</tr>
<tr>
<td>Polymyxin + OGP</td>
<td>22</td>
<td>140 ± 21</td>
</tr>
<tr>
<td>Polymyxin + OGP + EDTA</td>
<td>0.61</td>
<td>91 ± 6</td>
</tr>
</tbody>
</table>

Values for nitrite are means ± SE from 3 experiments. Purified SP-A was incubated with E. coli LPS serotype 026:B6 at a ratio of 20:1 (SP-A to LPS wt/wt) at 4°C on a rotator for 24–48 h. Mixture was then divided into 5 treatment groups. Protein-LPS mixture was either dialyzed against 5 mM Tris, pH 7.4 (Dialysis) for 48 h with 4 changes or mixed with polymyxin, polymyxin + EDTA, polymyxin + OGP, or polymyxin + OGP + EDTA for 6 h, after which time polymyxin was removed by centrifugation and supernatants were dialyzed for 48 h with 4 changes against 5 mM Tris, pH 7.4. Effects of SP-A on production of nitrite by alveolar macrophages were tested by incubating SP-A (5 µg/ml) with isolated, adherent alveolar macrophages in culture for 24 h. Supernatants were collected and centrifuged to remove any detached cells, and nitrite was measured with Greiss assay. Data are expressed as optical density of samples treated with SP-A as percentage of signal obtained with cells incubated with medium alone (control).
During treatment to remove endotoxin. Most of the loss, results is that significant amounts of protein were lost in protein, the disaggregation of LPS, or both. Detergent facilitated the dissociation of LPS from the protein-agarose, and the detergent OGP. OGP was tested based on the previous observations of Karplus et al. (13) that the detergent facilitated removal of endotoxin from catalase solutions. Although the mechanism by which detergent facilitates endotoxin removal is not entirely understood, it was speculated that the detergent facilitated the dissociation of LPS from the protein, the disaggregation of LPS, or both.

An important caveat in the interpretation of these results is that significant amounts of protein were lost during treatment to remove endotoxin. Most of the loss, which was ~50%, occurred during the dialysis step (data not shown). In an attempt to reduce this loss, dialysis membranes were soaked in low endotoxin-BSA, but significant losses were still incurred. It is possible that specific functional forms of protein were being selectively lost during the treatment, although we know of no data that support this possibility.

We found that many preparations of purified SP-A and SP-D isolated from lung lavage fluid contained significant amounts of endotoxin. It is not surprising that SP-A and SP-D isolated from lung lavage fluid contain endotoxin because both proteins have been reported to bind to bacteria and bacterial endotoxin (reviewed in Ref. 34). Because recovery of surfactant from normal rodents is very low and is prohibitively expensive, many laboratories (including this one) routinely utilize lavage fluid obtained from the therapeutic lavage of patients with alveolar proteinosis or from rats treated with silica to enhance surfactant production. Pulmonary infections are relatively common in patients with alveolar proteinosis (8), and the lungs of silica-treated rats usually exhibit aberrant gross morphology. Thus, in many preparations of SP, the endotoxin contamination may be due to interaction of the proteins with bacteria or bacterial LPS in vivo.

Although it seems likely that recombinant proteins would have low levels of endotoxin because they are purified from tissue culture medium that should have low levels of endotoxin, we have found that many commercial saccharide columns contain significant amounts of endotoxin as do preparations of agarose used to prepare such columns (data not shown). Furthermore, many sources of distilled or deionized water...
contain significant amounts of endotoxin. Thus even recombinant proteins can easily become contaminated with endotoxin during purification.

To test the effects of endotoxin contamination of SP-A on macrophage function, purified SP-A was incubated with LPS and treated in a number of ways to produce protein preparations with endotoxin concentrations ranging from \( \text{<} 1 \text{ to } \sim 30,000 \text{ pg endotoxin/µg SP} \). The midrange levels are similar to those we measured in some purified SP-A and SP-D preparations that were not treated to remove endotoxin. The source of these endotoxin-containing proteins include rat lung lavage fluid, alveolar proteinosis lung lavage fluid, and medium of Chinese hamster ovary cells transfected with cDNAs for SPs that were then purified by saccharide-affinity chromatography (data not shown). SP-A and SP-D that were treated with polymyxin in the presence of OGP and EDTA and contained very low amounts of endotoxin (less than \( \sim 20 \text{ pg endotoxin/µg SP} \)) did not stimulate nitrite production by alveolar macrophages. However, protein preparations containing higher levels of endotoxin stimulated nitrite production, and there was a significant correlation between the levels of nitrite produced and the endotoxin content.

Another important caveat in interpreting these results is that the measured levels of endotoxin in the SP preparations might be more or less than the actual levels if the proteins or buffers affect the endotoxin analysis. We cannot totally exclude this possibility. We have, however, attempted to optimize the assay so that buffer components do not inhibit the detection of endotoxin. In addition, purified SP-A and SP-D did not inhibit detection of a known quantity of endotoxin in the midrange of the standard curve where we attempted to make our measurements. Finally, we treated the SP preparations with an agent, Polydisperse (BioWhittaker), recommended by the manufacturer as an additive to enhance the detection of endotoxin in various protein preparations. The addition of Polydisperse only slightly increased the signal elicited by a known amount of endotoxin in the presence of SP-A, but it also slightly increased the signal elicited by a known concentration of endotoxin in the absence of SP-A (data not shown). Thus the enhanced detection is likely due to a slight increase in solubility of the endotoxin by Polydisperse.

We also cannot exclude the possibility that some component of the treatment or the purification technique has altered the function of the SP. However, SP-A that had not been exposed to butanol behaved in a manner similar to that of SP-A extracted with butanol; the presence of endotoxin in both types of preparations enhanced nitrite production. To more rigorously address this question, batches of SP-A and SP-D that were not treated or were treated to reduce endotoxin were compared for their ability to aggregate lipid and bacteria, respectively. Both treated and untreated proteins were effective in inducing aggregation. In addition, we have previously found (unpublished observations) that SP-A treated to reduce endotoxin retains all functions examined thus far, including the ability to stimulate...
lipid uptake by macrophages and type II cells, to stimulate macrophage chemotaxis and phagocytosis, and to inhibit tumor necrosis factor (TNF-α) production by LPS-stimulated alveolar macrophages (data not shown). Both treated and untreated SP-D stimulated macrophage phagocytosis of P. aeruginosa (26). Thus all of the available data suggests that treatment of SP-A and SP-D to reduce endotoxin does not alter their function. However, it is possible that the treatment may affect other as yet untested functions.

The studies described here were carried out with an LPS from a smooth serotype of E. coli, 026:B6. SP-A has been reported in one study to bind to both smooth and rough LPS (12) and in another study to bind to only rough LPS (31). The reasons for these apparent conflicts are not known but may involve different methodologies used in the binding assays, which were significant, or differences in the SP or LPS preparations. Kuan et al. (15) carried out an in-depth investigation of the binding of SP-D to various mutant forms of E. coli and reported that the highest binding occurred to rough LPS. Future investigations will be required to determine whether a direct interaction between LPS and SP-A or SP-D is required for the observed effects on nitrite production to determine whether the effects are serotype dependent.

Previous studies (3, 12, 18) have reported that LPS and microorganisms affect SP-mediated immune cell responses. For example, Blau et al. (3) reported that both SP-A and LPS (E. coli 55:135), as well as the combination of the two, stimulated production of nitrite by alveolar macrophages. To address the possibility that the SP-A response was due to endotoxin contamination, the effects of polymyxin B were analyzed. The addition of polymyxin B inhibited release of nitric oxide by ~20% in that study, suggesting that most of the induction of nitrite by SP-A was not due to contaminating endotoxin. These results are in apparent conflict with the data reported here that SP-A that has been treated to remove endotoxin does not stimulate production of nitrite. The reasons for these contradictory data are not known. The SP-A used in the study by Blau et al. was not treated to remove endotoxin, but the measured endotoxin contamination was low. The method for measurement of endotoxin and the sensitivity of the assays may be different. However, the fact that polymyxin inhibited only 20% of the SP-A-induced response suggests that this effect is not entirely mediated by endotoxin. Furthermore, there were other methodological differences in the study, including the fact that Blau et al. measured the production of nitrite after 48 h and our measurements were made after 24 h. In addition, Blau et al. used pathogen-free rats for most, but not all, of their studies, and all of our experiments were carried out with pathogen-free animals.

The effects of SP-A on immune cell function may also depend on the type of organism present or the state of activation of the immune cells. For example, Hickman-Davis et al. (10) reported that SP-A stimulated production of nitrite by alveolar macrophages that were pretreated with interferon-γ and then incubated with Mycoplasma pulmonis. Pasula et al. (22) found that SP-A inhibited nitrite production by macrophages incubated with M. tuberculosis. These studies considered together suggest that the effects of SP-A may vary significantly depending on the organism and the type of LPS to which the cell is exposed.

SP-A has also been shown to affect other LPS-mediated immune cell functions including production of colony-stimulating factor and TNF-α. For example, Kalina et al. (12) reported that both SP-A and LPS enhanced the release of colony-stimulating factor by alveolar macrophages and cultured alveolar type II cells. However, when SP-A and LPS were added together with the cells, the stimulatory effect was reversed. McIntosh et al. (18) reported that SP-A inhibits the production of TNF-α by alveolar macrophages stimulated by LPS. Krenlev and Phelps (14) reported that SP-A, which was prepared by a methodology that is very different from that used in other studies, stimulated production of several cytokines including TNF-α. Thus the effects of SP-A on endotoxin function may depend on several factors including the state of activation of the cells, the time point at which the data are analyzed, the method of preparation of the SPs, and the concentrations of LPS and SPs.

The observations reported here raise the possibility that some previously reported effects of SP-A on the production of free radicals and cytokines may be attributable to low levels of endotoxin in the purified surfactant preparations. As discussed above, it seems possible that SP-A may bind endotoxin in vivo, especially when pulmonary infections are present. Therefore, it seems important to evaluate the effects of SP-A containing endotoxin on immune cell function. However, it is also important to be able to correctly attribute the reported function to either SP-A, endotoxin, or the complex. All of these studies showing that LPS may have multiple effects on SP-A and SP-D function provide a strong rationale for routine quantitation of endotoxin in preparations of SP and analysis of the function of low-endotoxin-containing preparations.

We thank M. Tino, P. Borrari, W. Marencheck, K. Brinker, E. Walsh, and J. Herbein for critical evaluation of the manuscript. This work was supported by National Heart, Lung, and Blood Institute Grants HL-30923 and HL-51134 (both to J. R. Wright) and a supplement to National Heart, Lung, and Blood Institute Grant HL-30923 from the Office of Research on Minority Health (to C. I. Restrepo). Address for reprint requests and other correspondence: J. R. Wright, Box 3709, Dept. of Cell Biology, Duke Univ. Medical Center, Durham, NC 27710 (E-mail: J.Wright@cellbio.duke.edu). Received 10 August 1998; accepted in final form 27 January 1999.

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