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Complexing of Bacterial Lipopolysaccharide with Lung Surfactant

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Lipopolysaccharides (LPS) from *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Serratia marcescens*, or *Pseudomonas aeruginosa* were mixed with pulmonary surfactant to investigate their in vitro interaction. After 6 h of incubation at 37°C, LPS-surfactant mixtures were examined by sucrose density gradient centrifugation. The *E. coli* LPS-surfactant mixture was examined by immunoelectron microscopy with protein A-colloidal gold. The binding that occurred between LPS and the surfactant vesicles resulted in a complex with a density higher than the density of the surfactant alone. The protein A-colloidal gold identified LPS in the LPS-surfactant complexes. The toxicity of *E. coli* LPS was enhanced by complexing with the surfactant when compared with the intraperitoneal injection into CF1 mice, even at a 64:1 ratio of surfactant to LPS. The complexing of LPS and surfactant in the lung may alter the physiologic properties of surfactant that contribute to the physiopathological changes observed with some types of pneumonia.

Bacterial lipopolysaccharide (LPS) has been implicated as a determinant in the pathogenesis of gram-negative bacterial pneumonias (8, 26, 27). In the lung, LPS reacts with both humoral and cellular components (26), causing the release of mediators that induce inflammation. Pulmonary inflammation with edema, hyperemia, hemorrhage, and focal necrosis can result (8). In the alveolus, LPS may absorb to cell membranes (13, 14), including macrophage membranes (15), resulting in the death of cells and the activation of the complement system (26, 27). It has been suggested that LPS may interact with surfactant in the alveolus (26, 27). Phospholipids are the main constitutents of surfactant, and LPS is known to readily complex with similar phospholipids in liposomes (4, 16), cell membranes (13, 14), and high-density lipoproteins in blood plasma (33, 34).

Abnormalities in pulmonary surfactant concentration (19) and composition (3) have been reported with bacterial pneumonia (3, 30), gram-negative sepsis with shock (3), and endotoxin-induced sepsis with shock in animals (5, 19). Baughman et al. (3) reported a change in the ratio of palmitic to oleic fatty acids in the phospholipids of bronchoalveolar lavage fluid in patients with pneumonia. Sutnick and Soloff (30) postulated that a surfactant deficiency was responsible for the atelectasis observed with pneumonia and that bacterial products might be responsible for altering the surface tension of the surfactant. The sheep lung has been proven to be useful for the study of respiratory disease (8, 21), and our preliminary research has shown the *Escherichia coli* O26:B6 LPS binds with sheep lung surfactant to form a complex. The purpose of this study was to survey the in vitro interaction of LPS from five bacterial species with sheep lung surfactant and to describe the physical and morphological characteristics of these complexes with sucrose density gradient centrifugation and immunoelectron microscopy with protein A-colloidal gold.

MATERIALS AND METHODS

LPS. *E. coli* O26:B6 LPS and *Salmonella typhi* O901 LPS were obtained from Difco Laboratories (Detroit, Mich.). *Klebsiella pneumoniae* ATCC 10031 LPS, *Pseudomonas aeruginosa* FD (Fisher Devlin) type 1 LPS, and *Serratia marcescens* ATCC 14756 LPS were obtained from List Biological Laboratories, Inc. (Campbell, Calif.). Each LPS was assayed for protein (7) and 2-keto-3-deoxyoctulonic acid (22) concentrations before use and was found to be acceptable for this study without further purification.

Sheep lung surfactant. Surfactant was recovered by lavaging the excised lungs of healthy adult sheep as described previously (21). Briefly, crude surfactant was isolated from 10 liters of lavage fluid after two differential centrifugation steps: centrifugations at 200 × *g* for 15 min at 4°C to pellet the lavage cells, followed by centrifugation at 9,150 × *g* for 30 min at 4°C to pellet the surfactant. The surfactant pellet was suspended in 0.01 M phosphate-buffered saline (pH 7.2), layered over 0.7 M sucrose in phosphate-buffered saline, and centrifuged at 8,000 × *g* for 30 min at 4°C. The surfactant band was collected, centrifuged at 27,000 × *g* for 30 min at 4°C, washed twice in distilled water, and stored at −70°C. Lyophilized aliquots of surfactant were weighed and found to contain an average of 9.4 mg of solids per ml. Phospholipids were identified by thin-layer chromatography as described by Touchstone et al. (32) by using a serum lipid mixture (Supelco, Inc., Bellefonte, Pa.) as the standard.

LPS-surfactant mixture. LPS and surfactant were mixed to a final concentration of 5 mg of LPS per ml and 4.7 mg of surfactant per ml in 0.025 M Tris buffer (pH 7.2) with 0.001% NaN_3. This mixture was incubated in a 37°C water bath for 6 h with shaking at 15-min intervals. Surfactant alone was prepared similarly.

Density gradient centrifugation. After incubation, LPS-surfactant complexes were layered over 28 ml of discontinuous sucrose gradient containing 4 ml each of 0.15, 0.29, 0.37, 0.44, 0.51, 0.58, and 0.88 M sucrose in 0.025 M Tris buffer (pH 7.2) and centrifuged at 4°C for 16 h at 8,112 × *g*. Fractions (1 ml) were collected, and the sucrose density in each fraction was determined after refractometer readings. Total lipids were extracted from each density gradient fraction as described previously (6), and the phosphorus content was determined with KH_2PO_4 and phosphatidylcholine as standards (11) to detect surfactant phospholipids.

Antiserum. Antiserum to LPS was prepared in rabbits as described by Marks et al. (24) and used to detect LPS in fractions in a gel diffusion precipitin test (9). Rabbit anti-

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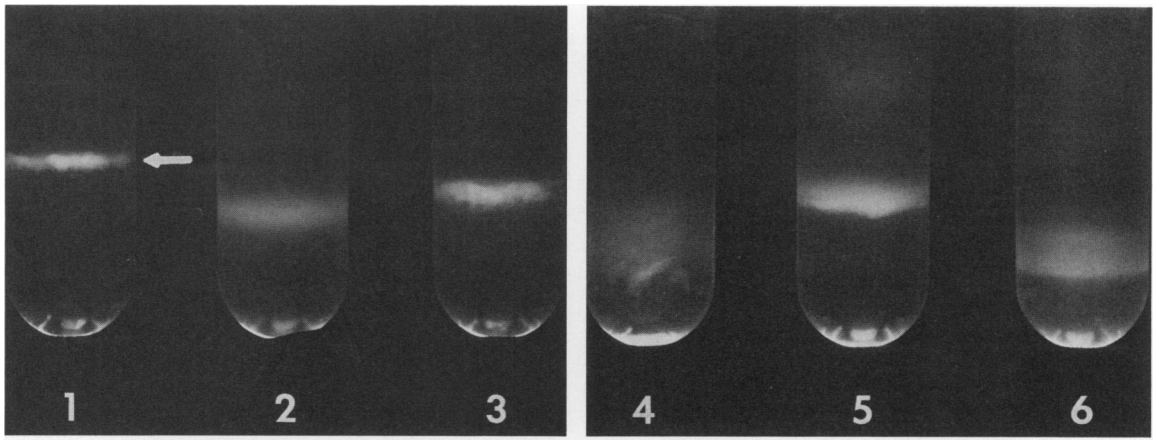

FIG. 1. Sucrose gradient bands of pulmonary surfactant (tube 1) and surfactant plus LPS from *E. coli* (tube 2), *S. typhi* (tube 3), *K. pneumoniae* (tube 4), *S. marcescens* (tube 5), and *P. aeruginosa* (tube 6). The density at the band of surfactant was 1.05 to 1.06 g/ml (arrow).

serum to *E. coli* O26 LPS was obtained from Difco Laboratories for immunoelectron microscopy. Of the *E. coli* LPS antiserum, 3 ml was absorbed with 1 ml of sheep lung surfactant for 1 h at 37°C to remove possible heterophile antibody. After incubation, the surfactant was pelleted by centrifugation at 26,300 × *g* for 20 min at 4°C, and the antiserum was removed and filtered through a 0.22-μm (pore size) filter.

Electron microscopy. *E. coli* LPS, surfactant, and *E. coli* LPS-surfactant complexes were prepared and incubated. After sucrose density gradient centrifugation, the bands and LPS pellet were removed, suspended in 0.025 M Tris buffer (pH 7.2), and centrifuged at 27,000 × *g* for 20 min at 4°C.

Surfactant, LPS-surfactant complexes, and LPS pellets were suspended in 0.025 M Tris buffer and diluted twofold to 1:8; 0.75 ml of each dilution was placed in polypropylene microcentrifuge tubes (VWR Scientific, Inc., San Francisco, Calif.). The *E. coli* LPS antiserum was diluted twofold to 1:4, and 0.50 ml was added to each microcentrifuge tube containing diluted surfactant, LPS-surfactant complexes, or *E. coli* LPS. The mixtures were incubated for 2 h at 4°C and centrifuged (Microfuge 12; Beckman Instruments, Inc., Palo Alto, Calif.); the pellets were then washed three times in 0.025 M Tris buffer (pH 7.2). The final pellets were suspended to their original volume with 0.025 M Tris buffer (pH 7.2), and 150 μl of protein a-colloidal gold (PA-Au; E-Y

TABLE 1. Composition of fractions collected after sucrose density gradient centrifugation of surfactant and LPS-surfactant mixtures

Sample	Density of fractions containing phosphorus (g/ml)	Surfactant phospholipids[a]	LPS[b]
Surfactant alone	1.05–1.06	+	–
Surfactant + LPS			
E. coli	1.01–1.03	–	+
	1.06–1.08	+	+
S. typhi	1.06–1.07	+	+
	1.08–1.09	–	+
K. pneumoniae	1.08–1.10	+	+
S. marcescens	1.04–1.05	+	ND
	1.06–1.07	+	ND
	1.08–1.09	–	ND
	1.09–1.10	–	ND
P. aeruginosa	1.00–1.03	–	+
	1.07–1.10	+	+

[a] Identified by thin-layer chromatography.
[b] Identified with specific antiserum in a gel diffusion precipitin test. ND, Not done.

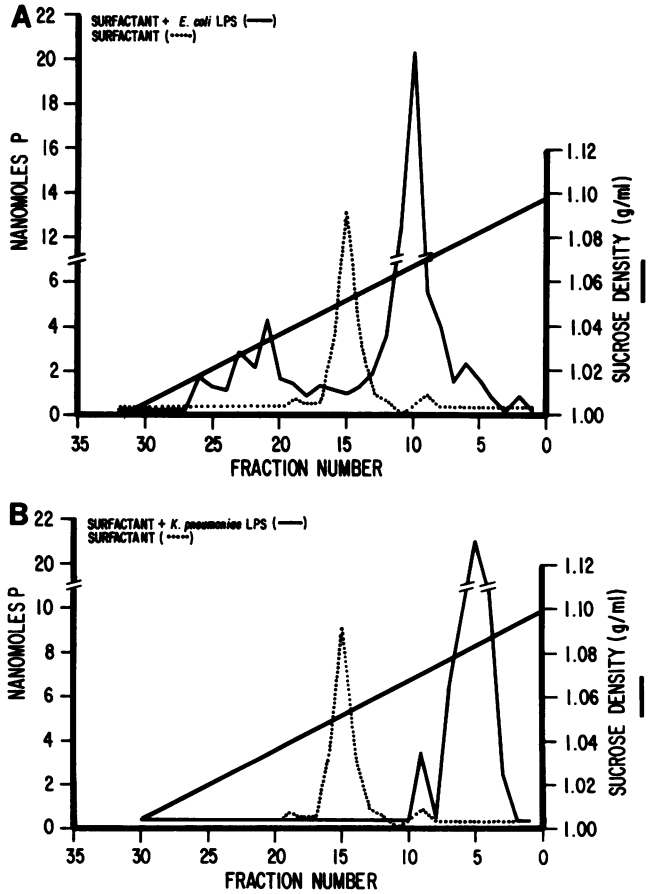

FIG. 2. Nanomole amounts of phosphorus in fractions collected after sucrose density gradient centrifugation of surfactant alone and LPS-surfactant complex from *E. coli* (A) and surfactant alone and LPS-surfactant complex from *K. pneumoniae* (B).

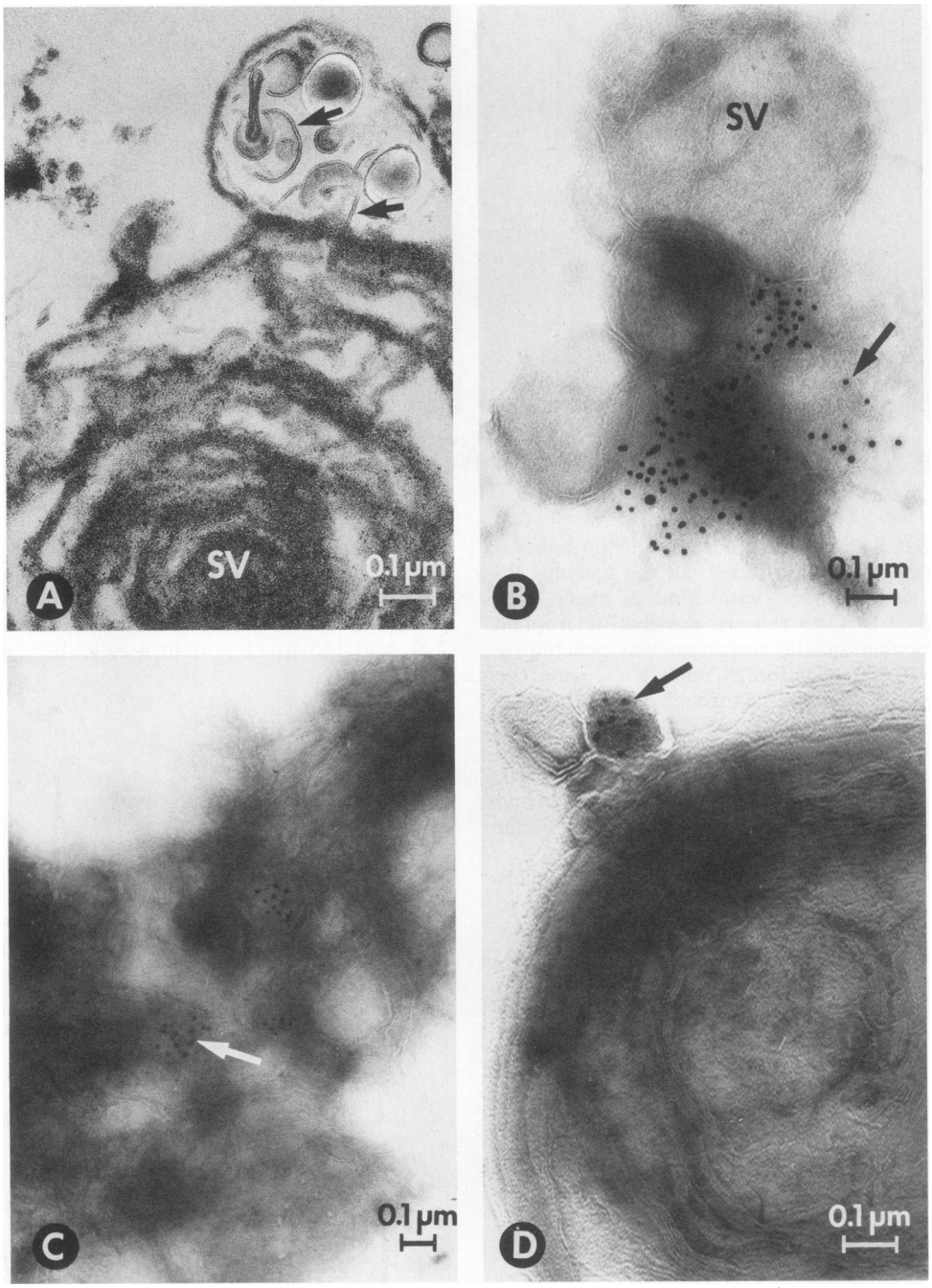

FIG. 3. Electron micrographs of the interaction between *E. coli* LPS and surfactant vesicles. (A) Thin-section preparation showing LPS structures (arrow) within the outer layer of the surfactant vesicle (SV). (B) Negative-stained preparation showing PA-Au label (arrow) attached to *E. coli* LPS antibody-agglutinated surfactant vesicles (SV). (C and D) Negative-stained preparations showing PA-Au label (arrows) attached to LPS-specific antibody in LPS-surfactant complexes.

Laboratories, Inc., San Mateo, Calif.) was added to each preparation. The mixtures were again incubated 2 h at 4°C and centrifuged, and the pellets were washed three times in 0.025 M Tris buffer (pH 7.2). The final pellets were then suspended to their original volume, negatively stained with 1% uranyl acetate, and examined in a Phillips EM-410 electron microscope.

Duplicate LPS-surfactant complexes were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.4) for 1 h at 4°C and pelleted by centrifugation. The pellets were washed twice in sodium cacodylate buffer and prepared for electron microscopy as previously described (10). Thin sections were cut and stained with lead citrate and uranyl acetate.

Toxicity of LPS alone and LPS-surfactant complexes in mice. To determine whether LPS-surfactant complexes were as toxic as LPS alone, *E. coli* LPS was serially diluted from 2 mg/ml twofold to contain 62.5 μg/ml. Each dilution was

TABLE 2. Toxicity of *E. coli* O26:B6 LPS and *E. coli* O26:B6 LPS-sheep lung surfactant in galactosamine-treated CF1 mice[a]

LPS (μg)	Surfactant (mg)	Surfactant/ LPS ratio	Lethality at 24 h (no. dead/total no.)
500	0		5/5
250	0		5/5
125	0		4/5
62.5	0		2/5
31.25	0		2/5
15.63	0		3/5
500	1	2	5/5
250	1	4	5/5
125	1	8	5/5
62.5	1	16	5/5
31.25	1	32	5/5
15.63	1	64	4/5
0.0	0		0/10
0.0	1		0/10

[a] All mice received 8 μg of galactosamine.

split and mixed with an equal part of a suspension containing 16 mg of galactosamine hydrochloride with or without 2 mg of surfactant per ml. The treatment of mice with D-galactosamine increases their sensitivity to the lethal effects of LPS (17). Preparations without LPS were used as controls. All mixtures were incubated for 6 h in a 37°C water bath with stirring at 15-min intervals. Groups of five CF1 mice were injected intraperitoneally with 0.5 ml of each LPS-surfactant suspension. Groups of 10 mice were injected with 0.5 ml of the control suspensions. All mice were observed for 24 h. The 50% toxic dose (TD_{50}) of LPS in each group was calculated as described by Reed and Muench (25).

RESULTS

Density gradient centrifugation. Sheep lung surfactant formed a single white flocculent band at a density of 1.05 to 1.06 g/ml. The density and appearance of this band changed after the incubation of surfactant with each LPS (Fig. 1). The phospholipid content of fractions from each of these tubes indicated that the density of surfactant was 1.06 to 1.08 g/ml when mixed with *E. coli* LPS, 1.06 to 1.07 g/ml when mixed with *S. typhi* LPS, 1.08 to 1.10 g/ml when mixed with *K. pneumoniae* LPS, 1.04 to 1.07 g/ml when mixed with *S. marcescens* LPS, and 1.07 to 1.10 g/ml when mixed with *P. aeruginosa* LPS (Table 1). A minor increase in the density of surfactant was observed when it was incubated with *S. typhi* LPS, and major increases in the density of surfactant were observed when it was incubated with *E. coli* LPS, *K. pneumoniae* LPS, or *P. aeruginosa* LPS. In Fig. 2, graphs of the phosphorus content in fractions show the density of *E. coli* LPS-surfactant complexes compared with the density of surfactant alone (Fig. 2A) and of *K. pneumoniae* LPS-surfactant complexes compared with the density of surfactant alone (Fig. 2B). Note the greater density of phospholipids containing LPS plus surfactant compared with phospholipids of surfactant alone.

At the concentrations used in this study, the binding of LPS to surfactant generally was not equivalent and resulted in an excess of LPS. Unbound LPS was detected in the gradient by specific antibody with the gel diffusion precipitin test and the phosphorus test and found to be at densities different than that of the LPS complexed to surfactant. Unbound LPS was at a density of 1.01 to 1.03 g/ml for *E. coli* LPS, 1.08 to 1.09 g/ml for *S. typhi* LPS, and 1.00 to 1.03 g/ml for *P. aeruginosa* LPS (Table 1). Unbound LPS was not detected in sucrose gradients containing *K. pneumoniae* LPS-surfactant mixtures.

Electron microscopy. LPS from *E. coli* was arranged in both ribbonlike structures approximately 10 nm wide and spherical structures 0.04 to 0.20 μm in diameter (arrows, Fig. 3A). Surfactant contained both multilamellar membranous vesicles and unilamellar membranous vesicles 0.17 to 2.10 μm in diameter (Fig. 3). When LPS was mixed with surfactant and incubated, LPS ribbons and spherical structures could be seen attached to and around multilamellar and unilamellar vesicles and vesicle fragments (Fig. 3A). In undiluted and diluted (1:2) *E. coli* LPS antiserum, LPS-surfactant complexes agglutinated and regions of LPS could not be seen with the PA-Au (Fig. 3B). In antiserum diluted 1:4, the PA-Au label identified regions in which specific antibody had attached to LPS in surfactant-LPS complexes (Fig. 3C and D). This reaction was specific, and the PA-Au label was observed attached to the control LPS in the surfactant and to LPS in the gelatinous pellet only. No PA-Au label was observed on the surfactant-*E. coli* LPS antiserum controls. The label was observed in small clusters on and around spherical structures (0.06 to 0.27 μm) attached to the surfactant vesicles (Fig. 3C and D). The spherical structures with attached antibody and PA-Au label were the same size as the spherical structures observed in the *E. coli* LPS preparation.

Lethal toxicity of LPS and LPS-surfactant in mice. At the 500- and 250-μg concentrations, *E. coli* LPS-surfactant mixtures were as toxic for mice as were the corresponding *E. coli* LPS concentrations alone (Table 2). At the 125- to 15.63-μg concentrations, LPS-surfactant mixtures were more toxic than were the corresponding LPS concentrations alone. Overall, the TD_{50} for *E. coli* LPS alone was 62.1 μg, whereas the TD_{50} for the LPS-surfactant mixture was less than 15.6 μg. This toxicity was not due to any component in either the surfactant or galactosamine.

DISCUSSION

The results of the present study demonstrate a binding between bacterial LPS and surfactant. The binding was observed with surfactant and LPS from five bacterial species and resulted in a complex with a density higher than that of surfactant alone.

The properties of LPS-surfactant complexes were similar to the LPS-phospholipid complexes reported by Weiser and Rothfield (35) in studies on *Salmonella typhimurium* LPS and membrane phospholipids. In the present study, we also observed that LPS partially pellets in the 1.02- to 1.12-g/ml density gradient and that the surfactant phospholipids had a density of 1.05 to 1.06 g/ml. When they were mixed together, heated, and then centrifuged, the two components were found in a new band in a position different from that of either component. The 1.1:1-mg ratio of LPS to surfactant in this study resulted in an LPS excess. The centrifugation speed and time used to examine changes in surfactant were not sufficient to entirely pellet the excess LPS through the gradient, and LPS was detected at other densities with specific antiserum in the LPS-surfactant mixtures. Further studies on these complexes will determine the concentration of LPS, the type and concentration of phospholipid, and the incubation requirements necessary for optimal binding. Weiser and Rothfield (35) found that *S. typhimurium* LPS and phosphatidylcholine formed complexes that were less discrete and more heterogeneous than complexes formed between LPS and phosphatidylethanolamine.

The ultrastructural morphology of *E. coli* LPS was typical of the LPS extracted from *E. coli* and other bacterial species (2, 28, 29). The ultrastructural morphology of purified surfactant was also typical of the surfactant in other investigations (18, 23). When LPS was mixed with surfactant and incubated, LPS ribbons and spherical structures were observed on and possibly in unilamellar and multilamellar surfactant vesicles. In other LPS-phospholipid interactions, LPS inserts into the phospholipid bilayer as a single domain (28, 31, 35), resulting in localized patches of molecules. In the present study, those patches could be identified by clusters of PA-Au labels in the areas of specific antibody attachment to LPS in the complexes. The clusters of PA-Au labels were the same size as the spherical structures that were observed in the *E. coli* LPS preparation. The LPS-surfactant complexes also agglutinated in the presence of antibody and had to be dispersed before the PA-Au label was added. This suggests that the insertion of LPS into the surfactant vesicle results in an orientation of the LPS that leaves the core or the specific side chain polysaccharides or both exposed for antibody reactivity.

The toxicity of *E. coli* LPS in galactosamine-treated mice was dependent on the dose, for which the TD_{50} was 62.1 μg. However, when incubated with surfactant, the same concentrations of LPS were more toxic, the TD_{50} being less than 15.6 μg. The role of this surfactant-enhanced LPS toxicity in the pathogenesis of pneumonia is not known. The nature of other LPS-phospholipid reactions has been well described (4, 13, 14, 28, 33). The phospholipid in sheep lung surfactant is composed of 73 to 79% phosphatidylcholine (18, 21, 23), 11 to 12% phosphatidylglycerol (18, 21), and 8 to 9% phosphatidylinositol, phosphatidylserine, lysophosphatidylcholine, and sphingomyelin (18, 21, 23). The hydrophobic lipid moiety of LPS combines with phospholipids in monolayers and bilayers (4, 14, 16), with phospholipids in mammalian (13, 14) and bacterial (28, 31, 35) cell membranes, and with lipids in plasma high-density lipoproteins (33, 34). These interactions have resulted in altered phospholipid morphology (28), altered surface charge on liposomes (16), and altered surface tension properties (28).

Severe pulmonary exposure to LPS may result in the extensive reaction of LPS, with surfactant altering its surface tension properties and physiologic function. The physiologic function of surfactant has been reported to be altered in animals parenterally exposed to endotoxin (19, 20). Decreased or altered surfactant can result in pulmonary edema, hemorrhage (1), and atelectasis (12, 30). Altered or decreased concentrations of surfactant could contribute to the pathophysiologic changes observed with some bacterial pneumonias.

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