JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1988, p. 778–780
0095-1137/88/040778-03$02.00/0

Vol. 26, No. 4

# *Enterobacter agglomerans* Lipopolysaccharide-Induced Changes in Pulmonary Surfactant as a Factor in the Pathogenesis of Byssinosis

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Received 17 August 1987/Accepted 29 December 1987

**Lipopolysaccharide (LPS) from *Enterobacter agglomerans* and pulmonary surfactant mixtures were centrifuged in discontinuous sucrose gradients to determine whether LPS bound to surfactant and examined in a Langmuir trough with a Wilhelmy balance to determine whether LPS altered the surface activity of surfactant. The LPS was found to bind to the surfactant and altered its surface tension properties. The binding of LPS to surfactant in the lung may change the physiological properties of surfactant and be a possible mechanism for the pathogenesis of byssinosis.**

Byssinosis is an occupational respiratory disorder caused by the inhalation of flax, cotton, or hemp dust during processing of cotton (8). Though reports to date do not indicate any one etiological agent as the sole cause of this disease, several studies indicate that gram-negative bacterial endotoxins are responsible for the decrease in respiratory function in the byssinosis syndrome (10, 20, 21). Haglind et al. (13) found that the main species of gram-negative bacteria in cotton dusts found in mills is *Enterobacter agglomerans*, while others (10) found that during a single growing season *E. agglomerans* predominated on the leaf and bract of pre- and postsenescent cotton plants.

The mammalian lung contains a lipid-proteinacious lining material (surfactant) which acts by reversibly reducing the surface tension at the air interface with the alveolus (9, 14) to impart alveolar stability and homeostasis to the lung. The sheep lung and its secretions have been proven to be useful as a model for the study of respiratory disease, and previous work has shown that bacterial lipopolysaccharide (LPS) binds with sheep lung surfactant to form a complex with properties different from those of either component (5, 6). In this study, we were interested in determining whether LPS from *E. agglomerans* would bind similarly with surfactant in vitro and alter its physical and physiological properties. Even minor alterations in the physiological function of surfactant by LPS may be a mechanism by which LPS in cotton dust can induce the pathophysiological changes and clinical symptoms observed in byssinosis.

*E. agglomerans* ATCC 27996 was grown on nutrient agar (Difco Laboratories, Detroit, Mich.) in Roux flasks and incubated at 30°C. After 24 h, the cultures were harvested in cold, sterile, distilled water and centrifuged (4,080 × *g*) for 30 min at 4°C. The cells were washed once with distilled water, once with acetone, and twice with diethyl ether and then dried. LPS was extracted from 2.3 g (dry weight) of cells by the hot phenol-water procedure of Westphal and Jann (24). The combined water extracts were dialyzed against distilled water at 4°C for 3 to 4 days and centrifuged (5,000 × *g*) to remove any insoluble particles. The LPS solution was diafiltrated with 0.025 M Tris buffer, pH 7.2, and concentrated to 45 ml (YM10; Amicon Corp., Danvers, Mass.). RNase A (type 111A; Sigma Chemical Co., St. Louis, Mo.) and DNase I (Sigma Chemical Co.) were added to the solution at a final concentration of 100 μg/ml and 10 μg/ml, respectively, and incubated at 37°C in a waterbath for 30 min. Trypsin (Worthington Biochemical Group, Bedford, Mass.) was then added to a final concentration of 10 μg/ml and incubated at 37°C in a waterbath for 1 h. The LPS was pelleted from the solution by centrifugation at 105,000 × *g* for 1 h and suspended in distilled water. The LPS was washed twice, resuspended in distilled water, and lyophilized.

This product was assayed for total protein (4), total carbohydrates (11), and 2-keto-3-deoxyoctonic acid (KDO) (16) and contained less than 0.25% protein, 52.9% carbohydrate, and 4.6% KDO, respectively.

Surfactant was recovered by lavage of excised lungs of 12 healthy adult sheep as described previously (15). Phospholipids were extracted from the surfactant by the method of Bligh and Dyer (3), and the chloroform phase was evaporated under nitrogen. The phospholipids were resuspended in chloroform-methanol-hexane (5:4:1) solution and separated by an Ultrasphere S1 column (Beckman Instruments, Inc., San Ramon, Calif.) by high-pressure liquid chromatography (pump 2350 and Z4 absorbance detector; ISCO, Inc., Lincoln, Neb.). The composition of phospholipids was typical of that reported for natural surfactant from sheep (15).

LPS and surfactant were mixed to final concentration of 5 mg of LPS and 5.0 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 and LPS alone were prepared similarly.

The above solutions of *E. agglomerans* LPS, surfactant, and *E. agglomerans* LPS plus surfactant were layered over 28 ml of discontinuous sucrose gradient and centrifuged (8,112 × *g*) as described previously (5). 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 by Bligh and Dyer (3), and the phosphorus content was determined with $KH_2PO_4$ and phosphatidylcholine as standards (8). Thin-layer chromatography (TLC) was used to identify phospholipids (surfactant) as described by Touchstone et al. (23), with a serum lipid mixture (Supelco Inc., Bellefonte, Pa.) as the standard. The KDO concentration was used to identify LPS in fractions as described by

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TABLE 1. Composition of fractions collected after sucrose density gradient centrifugation of sheep lung surfactant, *E. agglomerans* LPS, and LPS-surfactant

| Sample (tube no.) | Sucrose density (g/ml) of fractions containing phosphorus | Fraction no. | Surfactant phospholipids[a] | LPS[b] |
|---|---|---|---|---|
| Surfactant (1) | 1.050–1.060 | 11–15 | + | − |
| LPS-surfactant | 1.101 | 1 | − | + |
| | 1.055–1.072 | 7–12 | + | + |
| | 1.010–1.041 | 17–24 | − | + |
| LPS | 1.101 | 1–2 | − | + |
| | 1.010–1.047 | 15–24 | − | + |

[a] Identified by TLC.
[b] Tested for the presence of KDO.

Karrhanis et al. (16). Fractions containing surfactant, LPS, or LPS-surfactant complexes were dialyzed with 0.025 M Tris buffer, pH 7.2, with 0.001% $NaN_3$ to remove the sucrose, and their surface tension was determined.

Surface tension data were obtained from both the density gradient fractions and the original mixtures of surfactant, LPS, or LPS-surfactant. In addition, the effect of LPS concentration on the surface tension of surfactant was examined. Analysis of surface tension from the density gradient fractions was performed by the Wilhelmy method (9) with a platinum flag in a Langmuir trough. The limiting (minimum) surface tension was measured after compression.

The above solutions of *E. agglomerans* LPS, surfactant, and *E. agglomerans* LPS-surfactant were put in petri dishes (35 by 10 mm; Falcon, Oxnard, Calif.), and the surface tension was measured (12) with a platinum ring attached to a surface tensiometer (model 20; Fisher Scientific Co., Pittsburgh, Pa.). The tensiometer was calibrated with weights on the torsion arms before use. When checked with 0.025 M Tris buffer, pH 7.2, the tensiometer gave a reading of 74.3 ± 0.1 (standard error of the mean) dynes/cm.

The significance in the data was found by analysis of variance (22) and Fisher's least-significant-difference test (*T* test). The confidence intervals for the true mean surface tensions were also determined. It was necessary to transform the data because of the need to equalize the variances.

After centrifugation, surfactant formed a single, white flocculent band (tube 1) that shifted position within the sucrose gradient when it was incubated with *E. agglomerans* LPS (tube 2). A gelatinous pellet and diffuse band were also observed in sucrose gradients containing LPS-surfactant (tube 2) and LPS (tube 3). The phosphorus content and TLC profiles of fractions from tubes 1 and 2 indicated that the density of surfactant phospholipids had increased from the original 1.050 to 1.060 g/ml to a new 1.055 to 1.072 g/ml (Table 1). Surfactant phospholipid was not detected in tube 3.

LPS was detected by the presence of KDO in all fractions collected from sucrose gradients of LPS-surfactant (tube 2) and LPS (tube 3), but not in fraction collected from sucrose gradients of surfactant alone (tube 1). Phosphorus concentrations were used initially to detect the presence of either phospholipids or LPS in fractions collected at each density. Each was then confirmed by TLC or KDO analysis. In tube 1, elevated phosphorus concentrations were seen in fractions 11 to 15, and phospholipids were confirmed by TLC. In tube 2, elevated phosphorus concentrations were seen in fractions 1, 7 to 12, and 17 to 24. Phospholipids were detected by TLC only in fractions 7 to 11. KDO was detected in all fractions but elevated in fractions 1 and 17 to 24, indicating that some LPS had pelleted and some had banded at the density. In tube 3, elevated phosphorus concentrations were seen in fractions 1, 2, and 15 to 24, which correlated with elevated KDO concentrations. This pattern of LPS banding was similar to the excess LPS that was in tube 2.

TABLE 2. Means and results of significant-difference test (*T* test) for surface tension values for sheep lung surfactant, *E. agglomerans* LPS, and LPS-surfactant

| Sample | No. of replicates | Mean surface tension[a] (dynes/cm) | 95% Confidence interval[b] |
|---|---|---|---|
| Control (buffer) | 10 | 74.3 A | 74.14–74.39 |
| LPS (mg) | | | |
| 0.625 | 5 | 74.1 A, B, C | 73.76–74.32 |
| 1.25 | 5 | 74.1 A, B | 74.01–74.22 |
| 2.50 | 10 | 73.9 B, C | 73.58–74.28 |
| 5.00 | 10 | 73.8 C | 73.60–74.02 |
| Surfactant + LPS (mg of LPS) | | | |
| 0.625 | 5 | 34.2 D, E | 31.32–36.95 |
| 1.25 | 7 | 34.6 D | 33.45–35.75 |
| 2.50 | 10 | 34.3 D, E | 32.64–35.91 |
| 5.00 | 13 | 34.4 D | 33.58–35.29 |
| Surfactant alone | 10 | 32.2 E | 30.95–33.36 |

[a] Means with the same letter are not significantly different.
[b] The lower and upper limits are not equidistant from their respective means due to the necessary transformation performed on the data.

The limiting (minimum) surface tension of the density gradient fractions in the Langmuir trough was 42.0 dynes/cm for surfactant but 44.0 dynes/cm for *E. agglomerans* LPS-surfactant and 72.0 dynes/cm for LPS alone. The surface tension of the solutions measured with the tensiometer was 32.2 ± 0.5 dynes/cm for surfactant but 34.4 ± 0.4 for *E. agglomerans* LPS-surfactant and 73.8 ± 0.1 dynes/cm for LPS alone. Decreasing the concentration of LPS in the LPS-surfactant mixtures did not alter the surface tension from the 34 dynes/cm observed with 2.5 mg of LPS per ml.

An analysis of variance (22) was performed on the sets of surface tension data to determine the statistical significance of comparisons between the sets. These sets consisted of the surface tension values for sheep lung surfactant, *E. agglomerans* LPS, the complex of surfactant and *E. agglomerans* LPS, and the control (buffer). The contrasts between the control set of data and those for all other samples, surfactant, and LPS-surfactant each gave *P* values of 0.0001. For the control set versus LPS, *P* was 0.0018, while for the surfactant versus LPS-surfactant *P* was 0.0074. The *P* value for the contrast between the surface tension data for LPS and surfactant complexed with LPS was 0.0001.

Fisher's least-significant-difference test (*T* test) was performed to determine whether there was a significant difference in the surface tension mean values for LPS-surfactant complex, as well as for the isolated LPS and surfactant sample sets (Table 2). The data indicated that there were significant differences among the respective sample set groupings, although there were individual members of the sample sets whose means were not significantly different from those of another set.

The results of the present study demonstrate a binding between *E. agglomerans* LPS and surfactant that was similar to that observed with surfactant incubated with LPS from other bacterial species (3, 4). In addition, LPS induced changes in the surface tension properties of surfactant that were similar to those observed with LPS from *Pseudomonas*

*aeruginosa* incubated with surfactant (K. A. Brogden and B. Hills, Abstr. XIV Int. Cong. Microbiol. 1986, abstr. B9-12, p. 64). The difference in surface tension between surfactant and *E. agglomerans* LPS-surfactant in the present study was not as great but may still have physiological consequences resulting in the symptoms of byssinosis.

Although cotton dust is accepted as the cause of byssinosis among exposed workers, the causative agent(s) in the dust and mechanisms of etiology of the disease have not yet been identified with certainty. Proposed mechanisms have included histamine release (2), allergic hypersensitivity (19), the involvement of complement (17, 19), 5-hydroxytryptamine-induced smooth muscle contraction (18), chemotoxins (1), and tromboxane and prostaglandin release (18). Serum immunoglobulins and complement concentrations have also been examined for an immune complex etiology (17).

The increase in surface tension in the present study suggests that the pulmonary tissues may be "stiffer" after interaction with this LPS, impairing normal function. Although the concentrations of LPS used in the present study were considerably higher than those seen in mill air, they were used to demonstrate the binding of LPS to surfactant. It is possible that considerably lower concentrations of LPS may have the same effect on surfactant. Additional studies of the stoichiometry of the LPS-surfactant interaction and the interaction of LPS with different components of surfactant will have to be conducted before an exact mechanism involving surfactant for the pathogenesis by byssinosis can be proposed.

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