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Chemical Structure and Inhalation Toxicity of Lipopolysaccharides from Bacteria on Cotton

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Lipopolysaccharides from different bacteria isolated from cotton were purified and chemically analyzed. Their pulmonary toxicity to animals was tested in inhalation tests. Lipopolysaccharides from *Agrobacterium* and *Xanthomonas* were shown to differ from the others in that they contained no heptose and no non-hydroxylated fatty acids with a chain length of 12, 14, or 16 carbon atoms. Lipopolysaccharides from *Pseudomonas putida*, *Enterobacter agglomerans*, and *Klebsiella oxytoca* were found to cause an influx of polymorphonuclear leukocytes into the airways. Lipopolysaccharides from *Agrobacterium* sp. and *Xanthomonas sinensis* caused no significant invasion. The data point to substances in both the lipid A part and the core part of the lipopolysaccharides being responsible for the capacity to induce leukocyte invasion into the airways.

Gram-negative bacteria are natural contaminants of fibrous plant material. In processes where such material is agitated, e.g., during loading or machining, bacteria on the fibers often become airborne and people involved in these processes can be exposed to aerosols of the bacteria of concentrations of up to and above 10^7 bacteria m^{-3} (9).

Previous experiments have shown that strains of bacteria isolated from cotton induce leukocyte migration into the airways of guinea pigs to varying degrees (15; M. S. Salkinoja-Salonen and R. Boeck, Abstr. 8th Meet. N. W. Eur. Microbiol. Group, 1976, p. 150). Strains of *Enterobacter* and *Klebsiella* were found to be potent leukocyte inducers, whereas those of *Agrobacterium* showed no effect (16). No reaction was found after exposure to gram-positive bacteria or to suspensions of various molds (15).

Lipopolysaccharides (LPS) isolated from strains of bacteria are known to differ in their ability to cause biological reactions. This may be related to the chemical structure of the LPS, but little specific information on this is available at present (10).

In these studies the relation between the chemical structure of LPS isolated from some species of gram-negative bacteria found on cotton and their capacity to induce leukocyte mobilization into the airways was examined. The LPS was prepared from strains of bacteria isolated from cotton. LPS from *Xanthomonas sinensis* and *Escherichia coli* were also tested. Chemical compositions of the LPS were determined. Guinea pigs were exposed to aerosols of the various LPS, and the number of free lung cells was determined 24 h later.

MATERIALS AND METHODS

Bacteria. A water extract was prepared from a sample of bale cotton. The extract was inoculated on agar selective for gram-negative rods (Drigalski) and incubated overnight at 35°C. Strains of *Agrobacterium* sp., *Enterobacter agglomerans*, *Klebsiella oxytoca*, and *Pseudomonas putida* were isolated from colonies on the plate.

The *Agrobacterium* strain was grown in a culture broth containing, per liter of medium (pH 7.0),: 10 g of glucose, 10 g of yeast extract, 1 g of $(NH_4)_2SO_4$, and 250 mg of KH_2PO_4. The other strains were grown in the nutrient broth (Oxoid no. 2). The liquid cultures were grown in a 14-liter New Brunswick fermentor with aeration (0.5 liter of air per liter of medium per min) and agitation (200 rpm). Cultures were harvested by centrifugation at 3,000 × *g* at 4°C and washed once with distilled water. The cells were either freeze-dried or washed with a 95% solution of ethanol, acetone, and diethyl ether, and then dried in a vacuum desiccator (7).

Extraction of LPS. LPS was extracted from the bacterial strains by the method of Westphal and Jann (19). Dried cells were extracted twice with 45% (wt/wt) aqueous phenol at 65 to 68°C for 15 to 30 min. To remove the phenol, we collected the aqueous phases and dialyzed them against distilled water for 3 days (four to six changes) at 4°C. For the depositing of the LPS, the dialyzed solution was treated with ribonuclease (Worthington Biochemical Co., 50 $\mu g\ ml^{-1}$), concentrated in a rotary evaporator, and centrifuged for 3 h at 80,000 × *g* at 4°C. The resulting pellet was resuspended in distilled water twice and centrifuged for 90 min at 105,000 × *g* and 4°C. The pellet was then

suspended in a small amount of distilled water and freeze-dried.

The LPS from *X. sinensis* was a gift from E. Th. Rietschel (Max-Planck Institut für Immunbiologie, Freiburg, West Germany). A commercially available LPS from *E. coli* (O26 from Difco) was also tested.

Chemical analysis. The LPS samples were analyzed for their sugar contents by the phenol-sulfuric acid method (5), for heptose by the cysteine-hydrochloride reagent (4), for 2-keto-3-deoxyoctonate (KDO) by the thiobarbituric acid reagent after periodate oxidation (13), and for phosphorus by the method of Bartlett et al. (1). The qualitative analysis of sugar was performed by thin-layer chromatography as described previously (17).

Fatty acids were released from the LPS samples by hydrolysis in 4 M KOH, converted to their methyl esters with diazomethane, and analyzed at 180°C in a Perkin-Elmer F11 gas-liquid chromatograph with columns of 3% SE-30 and 10% EGSS-X (17).

Protein and amino compounds were analyzed with a Beckman amino acid analyzer.

Animal exposure. Water solutions (5 μg/ml) of the LPS were placed in a Collison atomizer and 300- to 400-g guinea pigs were exposed to aerosols for 40 min. The aerosol was generated with a Collison spray. Of the particles generated, 96% are less than 1.5 μm, which allows the preparations to deposit in the alveolar region. The estimated dose of LPS was about 0.1 μg/kg of body weight. The animals were killed in 24 h with an overdose of sodium pentothal, and a lung lavage was then performed. From a syringe connected to the trachea, 10 ml of saline was slowly injected into the lungs. The fluid was withdrawn into the syringe and injected again. This was repeated 10 times. A sample of the washing fluid was stained with Türk solution, and the total number of cells present was counted with a Bürker chamber. From another sample of the fluid a smear was prepared with a Cytocentrifuge Cytospin, Shandon). The smear was stained with Giemsa, and a differential count was made. The absolute number of different cell types was calculated and expressed as the number of cells $\times 10^6$ per animal lung.

RESULTS

The LPS preparations were found to be pure: protein contamination was less than 1% of the weight in all cases. Nucleic acid contamination was also low: in the LPS from *E. agglomerans*, half of the total phosphorus was accounted for by nucleic acid, in other cases contamination was much less.

Table 1 shows the carbohydrate composition of the LPS that were used in the inhalation experiments. No heptose was contained in the LPS of *Agrobacterium* and *Xanthomonas*. KDO was present in the LPS preparations in quantities ranging from 0 to 6.3%. All LPS contained amino sugar phosphate and glucosamine, and *P. putida* LPS also contained galactosamine and a significant amount of alanine.

Table 2 shows the fatty acid compositions of the LPS. The total fatty acid content varied between 13 and 32% of the dry weight. The major components in every LPS studied were the 3-hydroxy fatty acids.

Table 3 shows the number of free lung cells 24 h after exposure to various LPS. The total number of cells was significantly high in animals exposed to *E. agglomerans*, *P. putida*, *K. oxytoca*, and *E. coli*, mainly due to a high number of macrophages and neutrophils. Lymphocytes were significantly high in animals exposed to *E. agglomerans* and *K. oxytoca*; eosinophils were high in animals exposed to *K. oxytoca*.

Neutrophil reaction was strongest in animals exposed to *K. oxytoca*, *E. agglomerans*, and *P. putida* and weakest in those exposed to *E. coli*. In animals exposed to LPS from *X. sinensis* and *Agrobacterium* sp., no significant cell reactions were observed.

DISCUSSION

Our present results from the inhalation experiments confirm the findings obtained earlier with whole bacteria that the severity of the systemic symptoms caused by inhalation of bacterial aerosols depends not only on the dose but also on the species of bacteria inhaled (16). We also show that the reaction occurring in the lung after inhalation is caused by the LPS part of the gram-negative bacteria. To our knowledge, this has not been demonstrated before, although as early as 1961 Westphal et al. predicted that endotoxins will turn out to be the ultimate cause for the systemic disease that occurred in Swedish Bible-printing houses where India paper was handled in humid air (20).

The chemical composition of the LPS that we isolated from cotton bacteria was similar to that reported earlier for the same genera (8, 17, 21). LPS from *X. sinensis* is known to contain glucose, mannose, glucosamine, and KDO, but not heptose (22).

Exposure to LPS causes a variety of reactions in the body (10). An invasion of neutrophils into the airway epithelium occurs both in animals and in humans acutely exposed to endotoxin-containing dusts (12; R. Rylander, Chest, in press). This reaction, which is likely to be caused by secretion of leukotactic factors from macrophages (2), can be considered as an index of the acute inflammatory reaction in the airways. After a prolonged exposure, the reaction gradually decreases (M.-C. Snella and R. Rylander, submitted for publication), probably due to a development of a local immune defense (R. Rylander, I. Mattsby, and M.-C. Snella, Bull. Eur. Physiopathol. Respir., in press).

Agrobacterium sp. and *X. sinensis* LPS were found to be less potent inducers of the neutrophil migration. These LPS contained no heptose and

TABLE 1. *Chemical composition of the LPS*

Organism	Carbohydrates[a] (mg g [dry wt] of LPS^{-1})							Neutral sugars[a] identified by thin-layer chromatography	Phosphorus (mg g^{-1})	Protein[b] (mg g [dry wt] of LPS^{-1})
	Total[c]	Hep	KDO	GlcN[b]	GalN[b]	QuinN[b]	Aminouronic acid[b]			
Agrobacterium sp.	730	0	14	22	0	4.4	0	Gal, Man, Rha, Xyl	13	11.1
E. agglomerans	365	140	63	72	0	0	0	Gal, Glc, Rha	42	2.3
K. oxytoca	620	34	40	45	0	0	0	Gal, Glc, Man, Rha	14	4.1
P. putida	262	13	50	74	20	0	2.8	Gal, Glc	49	9.0
X. sinensis		0	9[d]					Glc, Man, Rha[d]	34[d]	
Soil bacterium K17		64	0	73	0	0	78	Glc, Rha	80	2.0

[a] Abbreviations for sugars: Gal, galactose; GalN, galactosamine; Glc, glucose; GlcN, glucosamine; Hep, D-manno-glycero-heptose; Man, mannose; QuinN, quinovosamine; Rha, rhamnose; Xyl, xylose.
[b] Analyzed by using an amino acid analyzer.
[c] Estimated by the phenol-sulphuric acid of Dubois et al. (5).
[d] Based on the results of Volk (22).

TABLE 2. *Fatty acid composition of LPS isolated from cotton bacteria*

Organism	Fatty acid content (mg g [dry wt] of LPS^{-1})									
	12:0	14:0	16:0	18:1	3-OH-10:0	2-OH-12:0	3-OH-12:0	3-OH-14:0	3-OH-16:0	Unid 2[a]
Agrobacterium sp.	0	0	0	5	0	0	0	105	23	0
E. agglomerans	28	12	21	0	0	0	0	94	0	27
K. oxytoca	31	25	14	Tr	0	0	0	98	0	25
P. putida	6	0	0	0	86	138	89	0	0	0

[a] An unidentified compound which coincides with 15:1 in the SE-30 column.

TABLE 3. *Mean number of free lung cells 24 h after a 40-min exposure to an aerosol suspension of 5 μg of LPS per ml in sterile water*[a]

LPS	No. of cells (×10^6)				
	Total cells	Macrophages	Lymphocytes	Neutrophils	Eosinophils
E. agglomerans	53.4 ± 16.0[b]	25.7 ± 8.1[b]	3.6 ± 1.6[b]	20.1 ± 9.0[b]	4.1 ± 2.3
P. putida	43.9 ± 19.5[b]	25.8 ± 11.4[b]	2.3 ± 1.0	12.1 ± 5.4[b]	3.6 ± 2.9
K. oxytoca	83.4 ± 22.3[b]	35.8 ± 12.6[b]	5.1 ± 1.0[b]	35.6 ± 20.2[b]	6.9 ± 2.8[b]
Agrobacterium sp.	15.5 ± 7.4	9.2 ± 4.3	1.2 ± 0.5	2.9 ± 2.5	2.2 ± 1.2
E. coli	23.1 ± 7.0[b]	15.2 ± 4.5	1.1 ± 0.7	4.5 ± 2.1[b]	2.2 ± 1.3
X. sinensis	17.8 ± 6.1	13.0 ± 4.1	1.4 ± 0.7	1.5 ± 0.9	1.9 ± 1.0
Control	12.6 ± 4.4	9.7 ± 4.0	1.0 ± 0.5	0.4 ± 0.1	1.6 ± 0.7

[a] Mean ± standard deviation for 10 animals.
[b] $P < 0.001$ (t test, Welch modification).

less KDO than the other LPS that were toxic in the lung. To find out whether the lack of heptose alone would explain the lower toxicity, we tested an LPS known to contain heptose but to lack KDO (Table 1) (Salkinoja-Salonen and Boeck, Abstr. 8th Meet. N. W. Eur. Microbiol. Group, 1976, p. 150). Preliminary tests with this LPS of a nontypical *Agrobacterium* isolated from soil (6) showed that this was nontoxic in the inhalation assay.

The core part of LPS is linked to lipid A, which is thought to be the center of the biological activity in the LPS (10, 11). Lipid A usually contains glucosamine with fatty acids linked by ester and amide bonds (8). All of the three LPS preparations that caused a neutrophil invasion contained lauric acid (C12) and two of them contained also myristic acid (C14) and palmitic acid (C16). The two nonreactive LPS did not contain either of these fatty acids. LPS from *X. sinensis* contained isobranched hydroxy fatty acids of less than 13 carbons and nonhydroxy fatty acids with a chain length of 9 carbon atoms (14). The agrobacterial and the K17 LPS contained little nonhydroxy fatty acid (0.5 to 1.7%, wt/wt) (Table 2) (Salkinoja-Salonen and Boeck, Abstr. 8th Meet. N. W. Eur. Microbiol. Group, 1976, p. 150).

The biological properties of lipids depend on their fatty acid composition. Chain length and hydrophobic properties of the fatty acids determine the fluidity of the membrane lipid (18). The low content of *n*-chain fatty acid of 12 to 16 carbon atoms in the three LPS that failed to induce neutrophil invasion in the lung might therefore be of significance. Consequently, it can be hypothesized that some common characteristics in the lipid A of the LPS molecule are of importance for the mobilizing of neutrophils. To verify this hypothesis, further studies must be undertaken.

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