JOURNAL OF BACTERIOLOGY, Sept. 1986, p. 893–904
0021-9193/86/090893-12$02.00/0

Vol. 167, No. 3

Serospecific Antigens of *Legionella pneumophila*

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Received 30 October 1985/Accepted 9 June 1986

Serospecific antigens isolated by EDTA extraction from four serogroups of *Legionella pneumophila* were analyzed for their chemical composition, molecular heterogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunological properties. The antigens were shown to be lipopolysaccharides and to differ from the lipopolysaccharides of other gram-negative bacteria. The serospecific antigens contained rhamnose, mannose, glucosamine, and two unidentified sugars together with 2-keto-3-deoxyoctonate, phosphate, and fatty acids. The fatty acid composition was predominantly branched-chain acids with smaller amounts of 3-hydroxymyristic acid. The antigens contain periodate-sensitive groups; mannosyl residues were completely cleaved by periodate oxidation. Hydrolysis of the total lipopolysaccharide by acetic acid resulted in the separation of a lipid A-like material that cross-reacted with the antiserum to lipid A from *Salmonella minnesota* but did not comigrate with it on sodium dodecyl sulfate gels. None of the four antigens contained heptose. All of the antigen preparations showed endotoxicity when tested by the *Limulus* amebocyte lysate assay. The results of this study indicate that the serogroup-specific antigens of *L. pneumophila* are lipopolysaccharides containing an unusual lipid A and core structure and different from those of other gram-negative bacteria.

Shortly after McDade et al. isolated the first strain of *Legionella pneumophila* in 1977 (23), additional strains of this organism were isolated in Togus, Maine, Bloomington, Ind., and Los Angeles, Calif. Although these strains had the same nutritional requirements and shared DNA homology, antisera to one isolate did not react with others in direct fluorescent antibody assays. Thus, the Togus, Bloomington, and Los Angeles isolates of *L. pneumophila* were assigned to serogroups SG2, SG3, and SG4 of *L. pneumophila* (24), respectively. Since then, four additional serogroups of *L. pneumophila* and 21 additional species of *Legionella* have been identified from several sources (2). The cell surface antigens of each species are unique and serve as the basis for the serological identification of members of the family *Legionellaceae* (18). Both *L. pneumophila* and *Legionella longbeachae* have multiple serogroups.

Despite the importance of the species and serogroup-specific antigens in the serological identification of *Legionellaceae*, little is known concerning the composition and chemical structure of these antigens. Initial studies have focused almost exclusively on the serogroup-specific antigen of serogroup 1 (SG1) *L. pneumophila*, and many of these studies have suggested similarities to endotoxin found in other gram-negative bacteria. Wong et al. identified a serogroup-specific antigen (38) that induced skin reactions in sensitized guinea pigs and a lipopolysaccharide (39) similar to the endotoxin isolated from other gram-negative bacteria. The antigen contained 2-keto-3-deoxyoctonate (KDO) but was devoid of hydroxy fatty acids, which are commonly associated with endotoxin. This antigen was positive in the *Limulus* lysate assay for endotoxin, but gave a very weak response in the rabbit pyrogenicity assay. Johnson et al. (17) reported the isolation of a serogroup-specific antigen that was also similar to lipopolysaccharide. This antigen was reported to contain 35% carbohydrate, 2.6% protein, 1.8% phospholipid, and 1% KDO. The antigen was positive in a pyrogenicity assay and gave a weak localized Schwartzman reaction. Further studies by Johnson et al. (18) resulted in the isolation of the serogroup-specific antigens of *L. pneumophila* SG1, SG2, SG3, and SG4. The serogroup-specific antigen isolated from *L. pneumophila* SG1 by Flescher et al. (8) contained <10% carbohydrate, 15% protein, and 1.1% phosphate, and the remainder was lipid of unknown composition and was also similar to endotoxin. However these investigators were unable to detect either KDO or heptose, both of which are present in classical endotoxin.

Schramek et al. (31) reported the isolation of endotoxin-like material by phenol extraction of SG1 *L. pneumophila*. Mouse toxicity studies confirmed the previous findings of Wong et al. (39) that the endotoxin isolated from *L. pneumophila* has a relatively low toxicity for mice. Serological studies showed that the endotoxin of *L. pneumophila* contains a lipid which cross-reacts with antisera to lipid A isolated from other gram-negative organisms. Although these results suggest antigenic similarities between lipid A and the lipid isolated from *L. pneumophila*, no attempts were made either to ascertain the chemical composition of the lipid or to determine whether the serotypic antigen was present on the endotoxin molecule. Collins et al. (5), using crossed immunoelectrophoresis, have identified a serogroup-specific antigen with the characteristic electrophoretic mobility of endotoxin. This antigen was selectively precipitated with serogroup-specific antibody and was highly reactive in the *Limulus* amebocyte assay for endotoxin.

Because of the discrepencies concerning the chemical composition and structure of the serogroup-specific antigens of *L. pneumophila*, we initiated a comparison of the antigens isolated from *L. pneumophila* SG1, SG2, SG3, and SG4. The results of our studies on the composition of the serogroup-specific antigens indicate that the antigens from all four serotypes are lipopolysaccharides that differ from the classical lipopolysaccharides of other gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and growth. The strains of *L. pneumophila* used for this work were obtained from the Centers for Disease Control, Atlanta, Ga., and include Philadelphia 2 (SG1), Togus (SG2), Bloomington (SG3), and Los Angeles

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(SG4). The bacteria were grown on charcoal-yeast extract agar (7) at 37°C for 2 to 4 days. The cells were harvested from agar plates by washing with phosphate-buffered saline, pH 7.4 (PBS), and the cells were pelleted by centrifugation (26,000 × *g* for 20 min). The cell pellet was washed three times with PBS to remove medium contaminants.

Antigen isolation. The washed cells were suspended in 0.01 M EDTA in PBS, pH 7.4 (10 ml of buffer per g of wet cells). After extraction at 45°C for 1 h with shaking, the cells were removed by centrifugation (12,000 × *g* for 20 min), and the supernatant was dialyzed extensively against distilled water (48 h, 6 times with 20 liters). The dialyzed supernatant was centrifuged as described above, passed through a 22-μm Millex filter, and lyophilized.

The antigen was further purified by Folch extractions (9). The EDTA-extracted material (100 mg) was dissolved in 4 ml of PBS (pH 7.4) and extracted with 75 ml of $CHCl_3$-methanol (2:1) for 1 h at room temperature with stirring. The mixture was filtered through a sintered-glass filter, and the insoluble residue was washed with a few milliliters with $CHCl_3$-methanol (2:1). The filtrate was made up to 80 ml with $CHCl_3$-methanol and extracted with 16 ml of PBS. The mixture was stored at –20°C overnight and then centrifuged (10,000 × *g* for 1 h) to separate the phases. The layers were separated and reextracted with 40 ml of theoretical upper layer ($CHCl_3$-methanol-PBS, 3:48:47) or theoretical lower layer ($CHCL_3$-methanol-PBS, 86:14:1). The phases were separated as described above and concentrated to a small volume with a rotary evaporator. The concentrated upper and lower layers were dialyzed against distilled water for 48 h and lyophilized.

Antigens were also extracted by the phenol-water method of Westphal et al. (37) and by the phenol-chloroform-petroleum ether method of Galanos et al. (11).

Chromatographic analysis. The samples of lipopolysaccharide antigens were analyzed by thin-layer chromatography and high-pressure liquid chromatography with both an anion-exchange column and a molecular sieving column.

(i) Thin-layer chromatography. Precoated silica gel 60 thin-layer plates (support thickness, 0.25 mm) from E. Merck AG were washed chromatographically with methanol and dried. The antigens were analyzed by using a developing system of isobutyric acid-concentrated NH_4OH-water (66:1:33, vol/vol). After chromatography and drying, the plates were sprayed with 3% cupric acetate in 15% phosphoric acid and heated at 170°C for 30 min.

(ii) Molecular exclusion. High-pressure liquid chromatography on a column (7.5 by 300 mm) of Spherogel TSK SW-3000 (Altex) was conducted by using 0.15 M sodium chloride (flow rate, 1 ml/min), with monitoring at 210 nm. Fractions (0.5 ml) were collected and analyzed for antigenic activity by the Western blot procedure.

(iii) Anion-exchange chromatography. High-pressure liquid chromatography with a column (4.1 by 250 mm) of Synchropak AX300 and a gradient of 0 to 1 M sodium chloride in 0.01 M phosphate buffer (pH 7.4 and finally at pH 8.0) failed to elute any material that absorbed at 210 nm.

Preparation of anti-*L. pneumophila* antisera. Antiserum to whole cells of *L. pneumophila* SG1, SG2, SG3, and SG4 were prepared as previously described (18). Each of the antisera was tested for specificity for reaction with homologous and heterologous serogroups. In addition, each of the antiserum preparations was also tested for reactivity to a minimum of four separate isolates to insure that antiserum preparation detected the major serogroup-specific antigen.

Preparation of anti-lipid A serum. Lipid A-coated bacteria were prepared by the method of Galanos et al. (12). *Salmonella minnesota* Re595 was grown overnight in brain heart infusion broth. Phenol was added to the culture at a concentration of 1 g/ml. The culture was washed twice with distilled water, twice with acetone, once with ether and finally dried in vacuo. The dried bacteria were washed twice with 1% acetic acid and suspended in 1% acetic acid at a concentration of 0.1 g of cells per 5 ml of acid. The suspension was heated at 100°C for 2 h and then washed with distilled water and dried in vacuo.

Lipid A isolated from *S. minnesota* was suspended in distilled water (1 mg/ml) and solubilized by the addition of triethylamine (0.5 μl/ml). This solution was mixed with 2 ml of a suspension of hydrolyzed bacteria in distilled water (0.5 mg/ml), and the mixture was dried by rotary evaporation. Rabbits were immunized by intravenous injection of the antigen on days 1 (100 μg), 4 (200 μg), and 11 (400 μg).

Immunodiffusion. Ouchterlony double immunodiffusion was conducted in 1% agarose (type A; Calbiochem) prepared in 0.05 M barbital buffer (pH 8.6) containing 3% polyethylene glycol 2000 and 0.02% NaN_3. The melted agar was cooled to 60°C, and 2.5 ml was poured into 35- by 20-mm petri dishes. Wells were cut in the agar with a gel punch, and 20 μl of antigen or antiserum was added to the wells. The plates were incubated in a humidity chamber at room temperature for 24 to 48 h.

Gas-liquid chromatography of neutral sugars. Antigens (1 mg) were hydrolyzed in sealed ampoules with 1 N trifluoroacetic acid at 100°C for 1, 2, 4, 8, 18, or 24 h. The acid was removed with a rotary evaporator, and the residue was dissolved in 1 ml of H_20. Xylose (0.166 μmol) was added as an internal standard, and the mixture was reduced with 10 mg of $NaBH_4$ for 2 h at room temperature. The excess $NaBH_4$ was destroyed by acidification to pH 3.5 with glacial acetic acid, and the boric acid was removed as methyl borate by concentrating three times with 0.5% acetic acid in methanol with a rotary evaporator. The alditols were acetylated with 1 ml of redistilled acetic anhydride-pyridine (1:1, vol/vol) at 100°C for 1 h. Excess reagents were removed by codistillation with toluene on a rotary evaporator (three times). The alditol acetates were dissolved in chloroform, filtered through glass wool, dried under nitrogen, and redissolved in 0.5 ml of chloroform. The solutions were analyzed with a Hewlett-Packard model 5710A gas chromatograph equipped with a flame ionization detector and with an SE-54 capillary column (Hewlett-Packard) at 180°C. The alditol acetates were identified by comparison with authentic standards and by gas chromatography-mass spectroscopy. Total carbohydrate was determined by the phenol-sulfuric acid method (6).

Determination of amino sugars. Antigens (100 μg) were hydrolyzed in sealed ampoules with 4 N HCl at 100°C for 8 h. The acid was removed with a rotary evaporator, and the amino sugars were determined on a Beckman model 121MB amino acid analyzer. In addition, amino sugars were determined by gas-liquid chromatography of the alditol acetates as described above.

Analysis of heptose and KDO. Heptose was determined by the method of Wright and Rebers (40) with glucoheptose as a standard and by gas-liquid chromatography of the alditol as described above. KDO was determined by the method of Karkhanis (19).

Fatty acid analysis. Mild alkaline hydrolysis (0.2 M NaOH, 1 h, 100°C) of the antigen preparations (5 mg) released the ester-bound fatty acids, and the total fatty acids were released by alkaline (0.2 M NaOH, 1 h, 100°C) and subse-

quent acidic (4 N HCl, 5 h, 100°C) hydrolysis (36). The samples were cooled to room temperature, and pentadecanoic acid (0.415 μmol) was added as an internal standard. The fatty acids were extracted with 1 ml of chloroform (three times), and the chloroform extract was dried over anhydrous sodium sulfate and concentrated to dryness under nitrogen. The residue was methylated with 0.5 ml of 14% boron trifluoride in methanol in a boiling-water bath for 2 min. The reaction mixture was cooled to room temperature, 0.3 ml of water was added, and the fatty acid methyl esters were extracted (three times) with 0.5 ml of petroleum ether. The combined petroleum ether extract was concentrated to dryness under nitrogen and dissolved in 0.1 ml of chloroform. The solutions were analyzed by gas-liquid chromatography on an SE-54 capillary column at 150 to 230°C at a rate of 2°C/min with a 4-min initial hold and a 16-min final hold. Fatty acids were identified by comparison to authentic standards and by gas chromatography-mass spectroscopy.

Determination of phosphate and protein. Total phosphate was determined by the method of Ames (1), and protein was determined by the method of Lowry et al. (22).

PAGE. Polyacrylamide gel electrophoresis (PAGE) was conducted by the method of Laemmli (20) with 14- by 10- by 0.75-mm slabs. The separating gel contained 10 or 12.5% acrylamide, and the stacking gel contained 3% acrylamide. The gels and running buffer contained 0.1% sodium dodecyl sulfate (SDS), and the sample buffer contained 1% SDS (final concentration). The gels were electrophoresed at a constant current of 23 mA and were stained by the silver staining method of Merril et al. (25).

Western blots. Western blots on SDS-polyacrylamide gels were prepared as previously described (3, 34). The gels were electrotransferred with a model TE Transphor Electrophoresis apparatus (Hoefer Scientific Instruments) at a constant voltage of 100 V for 1 h. The electrode buffer contained 192 mM glycine, 25 mM Tris, and 20% methanol. The nitrocellulose blots were blocked for 1 h at 37°C with a solution of Tris-saline (0.9% NaCl in 10mM Tris hydrochloride, pH 7.4) containing 3% bovine serum albumin and 10% heat-inactivated fetal bovine serum. The blocking solution was removed by washing with five portions of Tris-saline for 5 min each. The blot was then incubated for 2 h at room temperature with antiserum diluted 1:500 in blocking solution, washed as described above, and finally incubated for 2 h at room temperature with horseradish peroxidase-linked goat anti-rabbit immunoglobulin G diluted 1:1,000 in blocking solution. After removal of the conjugate by washing, the bands were visualized by incubation for 5 to 15 min with a freshly prepared solution containing 30 mg of 4-chloro-1-naphthol in 10 ml of methanol, 50 ml of Tris-saline, and 20 μl of 30% H_2O_2. The reaction was terminated by washing twice with distilled water.

Preparation of labeled antigens. The growth from a charcoal-yeast extract agar slant was suspended in approximately 1 ml of sterile PBS, and [1,2-^{14}C]sodium acetate (120 to 125 μCi), D-[U-^{14}C]glucose (100 to 125 μCi), or [^{35}S]methionine (260 μCi) was added. The suspension was used to innoculate four or five petri dishes (14 cm) containing charcoal-yeast extract agar. After incubation for 24 to 48 h at 37°C, the cells were harvested by flooding each plate with approximately 5 ml of PBS and loosening the cells with a rubber policeman to form a cell suspension that was removed with a pipette. The mixture was centrifuged (12,000 × *g* 20 min), and the cell pellet was washed twice with 10 ml of PBS. The washed cells were extracted with 0.01 M EDTA in PBS (10 ml of buffer per g of wet cells) at 45°C for 1 h. The mixture was centrifuged as described above, and the supernatant was filtered through a 0.22-μm membrane filter (Millex), desalted, and concentrated to approximately 2.5 ml by ultrafiltration (Amicon PM10 filter).

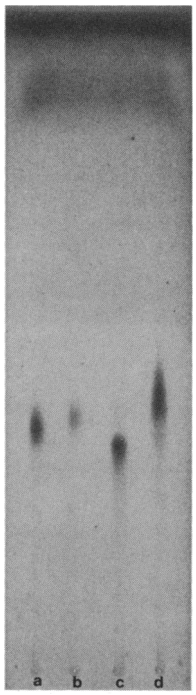

FIG. 1. Thin-layer chromatography of the purified antigens. Lanes: a, SG1; b, SG2; c, SG3; d, SG4. EDTA antigen preparations were subjected to Folch extraction. Lanes contained 10-μg samples.

Pronase treatment of antigens. *L. pneumophila* antigens were digested with pronase as described by Spiro (33). The antigen samples were suspended in a buffer containing 0.2 M Tris hydrochloride and 5 mM $CaCl_2$ (pH 7.8). Pronase solution (10 μl, 25 μg) was added to each sample, and the mixtures were incubated at 37°C with shaking. Additional samples of pronase were added at 8, 24, and 48 h. After 96 h of incubation, the pronase-treated antigens and control antigens were subjected to immunodiffusion and SDS-PAGE.

Periodate oxidation. Antigens (10 mg) were oxidized with 10 ml of 0.05 M $NaIO_4$ at 4°C in the dark for 6 days. The excess $NaIO_4$ was destroyed by reaction with 80 μl of 50% ethylene glycol for 2 h at room temperature. The samples were dialyzed four times against distilled water (24 h, 2 liters) and lyophilized. The oxidized antigens were subjected to SDS-PAGE and immunodiffusion. The oxidized antigens were dissolved in water to give a solution of 1 mg/ml and reduced with 100 mg of $NaBH_4$ for 4 h at room temperature. The excess $NaBH_4$ was destroyed with glacial acetic acid, and the samples were dialyzed against distilled water and lyophilized. The periodate-oxidized antigens were subjected to trifluoroacetic acid hydrolysis and derivation as given above. The alditol acetates were subjected to gas chromatography-mass spectroscopy.

Mild acid hydrolysis. The lipid A was precipitated by boiling each antigen preparation for 2 h with 1% acetic acid (27). The resulting mixture was centrifuged, and the insoluble residue was washed two times with hot distilled water, the washings being combined with the supernatant. The combined supernatants and the residue were each lyophilized.

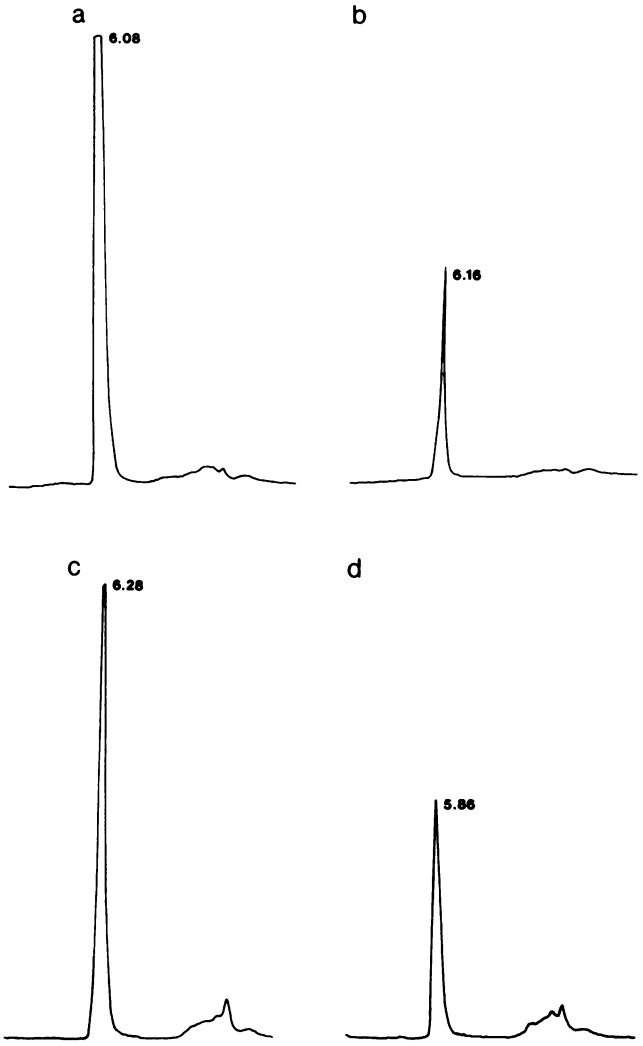

FIG. 2. High-pressure liquid chromatography of EDTA-Folch-extracted antigens on a Spherogel TSK SW-3000 (7.5 by 300 mm) column. Panels: a, SG1; b, SG2; c, SG3; d, SG4. The sample size in each chromatogram was 50 μg; elution was monitored at 210 nm.

For SDS-PAGE analysis of the hydrolyzed fractions, samples were withdrawn at various time intervals (0, 10, 20, 30, 45, 60, 90, and 120 min) during the hydrolysis and stored frozen, after centrifugation to remove the lipid.

Limulus lysate activity. The *Limulus* lysate gelatin assay as a measure of endotoxic potential in vitro was carried out by using the single-vial gelatin kit supplied by Whittaker M.A. Bioproducts, according to their instructions.

RESULTS

Antigen isolation. Serospecific antigens were isolated from SG1, SG2, SG3, and SG4 strains of *L. pneumophila* by extraction with 0.01 M EDTA in a procedure similar to that described by Leive and Morrison (21) for the extraction of *Escherichia coli* lipopolysaccharide. After dialysis, the extracts were centrifuged to remove insoluble material and lyophilized to yield approximately 0.6% of the wet cell weight for SG1, SG2, and SG3 strains and approximately 0.25% for the SG4 strain. The crude antigen preparations were assayed for antigenic activity by Ouchterlony immunodiffusion. When assayed for antigenic activity by Ouchterlony immunodiffusion, the crude antigen preparations revealed a major precipitin line near the antigen well with homologous whole cell antiserum. When the antigen preparations were tested against heterologous antisera, some minor cross-reacting lines were observed, but no cross-reaction of the major line was detected. This result indicates that the major precipitin line represents a serospecific antigen.

The EDTA antigen preparations were subjected to Folch extraction (9) to purify further the serospecific antigen. After dialysis and lyophilization, three fractions were obtained: the residue that was insoluble in $CHCl_3$-methanol (2:1), the Folch upper layer, and the Folch lower layer. With SG1, SG2, and SG3 strains, approximately 45% of the starting material was recovered in the upper layer, 5% was in the lower layer, and 35% was in the residue. The SG4 strain, however, yielded approximately 25% in the upper layer, 5% in the lower layer, and 60% in the residue. These fractions were tested for antigenic activity against the homologous whole cell antiserum by Ouchterlony immunodiffusion. The upper layer from SG1, SG2, and SG3 strains contained most of the activity, and a major precipitin line was observed near the antigen well. Little or no antigenic activity was observed in the lower layer or the residue. When fractions from the SG4 strain were tested, the residue contained most of the antigenic activity. The upper layer and lower layer contained only slight activity. Some fractionation of the major

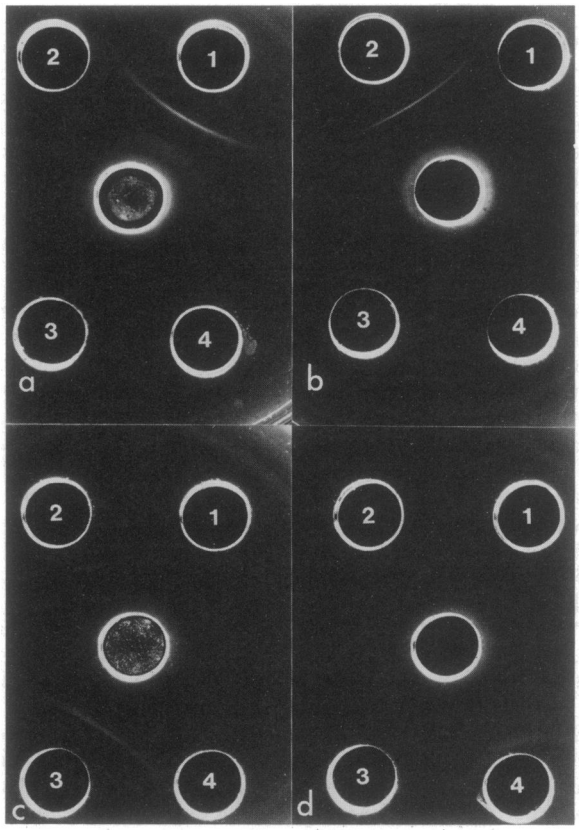

FIG. 3. Double-diffusion gel immunoprecipitation of serospecific antigens from *L. pneumophila*. Numbered wells: 1, SG1; 2, SG2; 3, SG3; 4, SG4. Center wells: a, anti-SG1 whole cell antiserum; b, anti-SG2 whole cell antiserum; c, anti-SG3 whole cell antiserum; d, anti-SG4 whole cell antiserum.

TABLE 1. Chemical composition of antigen preparations

Antigen	Prepn	% wt distribution[a]	% of wt of total antigen		
			Carbohydrate[b]	Protein	Glucosamine[c]
SG1	Total		6.1	7.3	0.68
	Supernatant[d]	70	7.4	2.5	NA[e]
	Residue[f]	22	NA	NA	NA
SG2	Total		6.4	7.0	0.54
	Supernatant	72	6.4	2.5	NA
	Residue	27	NA	NA	NA
SG3	Total		8.2	5.5	0.96
	Supernatant	62	8.6	2.4	NA
	Residue	21	NA	NA	NA
SG4	Total		7.3	5.8	0.55
	Supernatant	60	9.6	4.6	NA
	Residue	21	NA	NA	NA

[a] After weak acid hydrolysis.
[b] Carbohydrates determined by the phenol-sulfuric acid method (6).
[c] Glucosamine was determined with the autoanalyzer.
[d] Supernatant after weak acid hydrolysis.
[e] NA, Not analyzed.
[f] Residue after weak acid hydrolysis.

precipitin line from the second minor line was detected. Folch extraction of EDTA extracts from SG4 that contained the additional precipitin line usually produced upper layers containing both lines. The Folch extraction resulted in approximately a twofold increase in activity by weight for all four strains tested.

Chromatographic analysis of the antigen preparations showed that they were homogenous by thin-layer chromatography (Fig. 1) and showed trace amounts of nonantigenic material separated by high-pressure liquid chromatography (Fig. 2). The antigens were irreversibly bound to an amine or DEAE anion-exchange column when chromatographed with a sodium chloride gradient in phosphate buffer.

When the Folch-purified antigens were tested by immunodiffusion against heterologous whole-cell antisera, no cross-reaction of the major precipitin line was detected (Fig. 3). The purity of the antigen preparations was determined by immunodiffusion against antisera prepared against whole cells (Fig. 3). The SG1 antigen showed a single major line of precipitation and a slight diffuse band located near the antigen well. The antigens isolated from SG2, SG3, and SG4 (Fig. 3b, c, and d, respectively) showed a single line of precipitation with the homologous antiserum. Antiserum prepared against SG2 (Fig. 3) showed a very faint line of precipitation with the SG1 antigen, suggesting some minor cross-reactivity between these two antigens.

SG1 cells were also extracted by the phenol-water method of Westphal and Jann (37) and by the phenol-chloroform-petroleum ether method of Galanos et al. (11). When cells were subjected to extraction by the phenol-water method, no antigenic activity was detected in the water phase, which would be expected to contain the lipopolysaccharide. The phenol phase, however, yielded an antigen extract (0.1% of wet cell weight) with an activity at 125 μg/ml. This result suggests the possible presence of a hydrophobic lipopolysaccharide which is not extractable into the water phase (11). The phenol-chloroform-petroleum ether extraction yielded an antigen extract (0.8% of the wet cell weight) which was active at 32 μg/ml. This result is consistent with results of the phenol-water extraction and also indicates the presence of a hydrophobic lipopolysaccharide molecule. Immunodiffusion against anti-SG1 serum indicated that the antigens obtained by the phenol-water method and the phenol-chloroform-petroleum ether method were antigenically identical to the antigen extracted with EDTA. The EDTA and Folch extraction procedures were used to obtain quantities of antigen for further work. This method was chosen since it gave antigen in as good or better yield and purity as the other two methods and was simple to carry out.

Chemical composition. The chemical composition of Folch-purified antigens from SG1, SG2, SG3, and SG4 strains was determined (Table 1). The carbohydrate composition of the antigens from all four strains was principally rhamnose, mannose, glucosamine, and two unidentified sugars (Table 2). SG4 antigen also contained galactose in addition to the other four sugars. The sugar composition was verified by gas chromatography-mass spectroscopy, which gave mass spectra consistent with the assigned components. The mass spectrum of the unidentified sugar X_1 (Fig. 4) suggested that it may be a 2-aminodideoxyhexose, but a unique structure could not be assigned on the basis of these data.

The amino sugar content of the antigens was also determined by amino acid analysis with glucosamine, mannosamine, and galactosamine as standards; the antigens contained 0.6 to 1.0% glucosamine. The total sugar content as determined by the phenol-sulfuric acid method was about 6 to 8% for the four preparations, and that determined by gas-liquid chromatography and amino acid analysis was approximately 6 to 7% for the SG1, SG2, and SG3 preparations and about 3% for SG4. The sugars were present in similar relative amounts, except that the SG3 antigen had a slightly higher rhamnose and glucosamine content. In addition, the SG4 antigen had a lower rhamnose content and was the only antigen containing galactose.

The antigens were analyzed for heptose by the method of Wright and Rebers (40) with glucoheptose as a standard and by gas-liquid chromatography of the alditol acetate. No heptose was detected in the antigens by either of these methods. Determination of nucleic acid by the ratio of

TABLE 2. Carbohydrate, phosphate, and KDO content of antigen preparations

Antigen	Content [μmol/g (molar ratio[a])]								
	Rhamnose	X_1[b]	Mannose	Glucose	Galactose	Glucosamine	X_2[c]	KDO	Phosphate
SG1	140 (4.7)	60 (2.0)	130 (4.3)	Trace		40 (1.3)	30 (1.0)	140 (4.7)	250 (8.3)
SG2	160 (5.3)	70 (2.3)	130 (4.3)	Trace		30 (1.0)	30 (1.0)	144 (4.8)	240 (8.0)
SG3	190 (6.3)	60 (2.0)	140 (4.7)	10 (0.3)		40 (1.3)	30 (1.0)	154 (5.1)	220 (7.3)
SG4	30 (1.0)	20 (0.7)	30 (1.0)	10 (0.3)	50 (1.7)	15 (0.5)	30 (1.0)	32 (1.1)	200 (6.7)

[a] Molar ratios were calculated by taking X2 as 1.
[b] X_1, Unidentified sugar 1; retention time, 17.74 min.
[c] X_2, Unidentified sugar 2; retention time, 29.39 min.

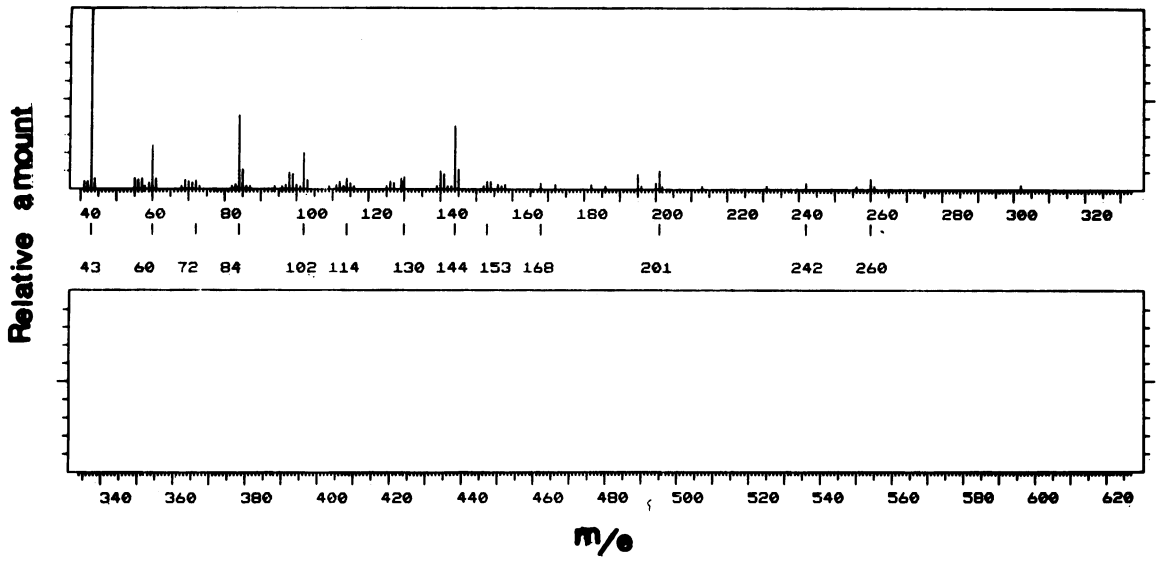

FIG. 4. Mass spectrum of the unknown sugar X1.

absorbances at 280 and 260 nm showed values for each antigen preparation of less than 4%, whereas the method of Setaro and Morley (32) detected no DNA in the antigens. The KDO content was determined by the method of Karkhanis et al. (19); the spectrum of the resulting chromophore showed the required maximum absorbance at 549 nm. KDO was approximately 3.5% (wt/wt) for SG1, SG2, and SG3 but only 0.8% (wt/wt) for SG4. Total phosphate as determined by the ashing procedure gave a value of approximately 2% of the dry weight of the antigens. The antigens were analyzed for fatty acids by gas-liquid chromatography of the fatty acid methyl esters (36) with pentadecanoic acid as an internal standard. The fatty acids were identified by comparison to standards and by gas chromatography-mass spectroscopy (Table 3). The results from gas chromatograms of the fatty acid methyl esters obtained by mild alkaline hydrolysis, releasing ester-bound fatty acids, and alkaline and subsequent acidic hydrolysis, releasing total fatty acids, are shown in Table 4. Of the total acids released, SG2 and SG3 have around 50% ester-linked acids (SG2, 55%; SG3, 47%), whereas SG1 and SG4 have over 70% ester-linked acids (SG1, 78%; SG4, 71%). The fatty acid composition is characterized by the presence of large amounts of branched-chain acids (over 80% of the total), with the iso-16:0 or the 2-methyl-16:0 acids being the major components.

Labeled antigens. SG1 and SG4 strains were grown in the presence of various radioactive substrates to determine what kinds of biosynthetic precursors would lead to incorporation of radioactivity into the serospecific antigen. When SG1 cells were grown in the presence of [^{35}S]methionine (260 μCi), 0.05% of the added radioactivity was recovered in the EDTA extract. When cells were labeled with [1,2-^{14}C]sodium acetate (120 to 250 μCi), 0.4% of the added radioactivity was recovered in the EDTA extract of SG1, and 0.2% was recovered in the EDTA extract of SG4. Cells labeled with D-[U-^{14}C]glucose (100 to 125 μCi) led to incorporation of 3.8% of the activity in the EDTA extract of SG1 and 1.4% of

TABLE 3. Fatty acid composition of antigen preparations

Antigen	Hydrolysis with:	% Composition of the following fatty acids[a]:																
		i:14:0	14:0	x	a-15:0	15:0	3-OH14:0	i-16:0	2-Me16:0	16:1	16:0	a-17:1	a-17:0	17:0	18:2	18:1	18:0	Br19:0	20:0
SG1	0.2 M NaOH	0.5	0.1		0.8		0.1	2.9		0.4	0.5		0.3				0.4	0.1	
	4 N HCl	0.7	Trace[b]		1.2		Trace	4.6		0.2	0.4		0.5				0.2	Trace	
SG2	0.2 M NaOH	0.3	0.1		0.5	Trace	Trace	1.7		0.2	0.6		0.3		Trace	Trace	0.4	Trace	
	4 N HCl	0.8	0.1		0.2	Trace	Trace	4.7		0.2	0.5		0.7		Trace	Trace	0.2	Trace	
SG3	0.2 M NaOH	0.2			0.4			1.1		0.2	0.3		0.2				0.4		
	4 N HCl	0.1		0.1	0.3		0.2	1.0	1.7		1.4	0.8	0.2	0.1			0.1		
SG4	0.2 M NaOH	0.2	0.1		0.7		Trace	1.6		0.2	0.7		0.4				0.5		Trace
	4 N HCl	0.2	Trace	0.1	0.7		0.3	1.7	1.0	Trace	0.4	0.8	0.6	0.2			0.1		0.1

[a] In fatty acid designations, the number to the left of the colon refers to the number of carbon atoms; the number to the right refers to the number of double bonds. i and a indicate methyl branches at the iso and anteiso carbons, respectively. x, Unidentified fatty acid.

[b] Less than 0.1%.

TABLE 4. Carbohydrate composition after periodate treatment

Antigen	Treatment	Content (μmol/g)[a]						
		Rhamnose	X_1	Mannose	Glucose	Galactose	Glucosamine	X_2
SG1	Untreated	140	60	130	Trace		40	30
	$NaIO_4$	120	40	0	Trace		30	30
SG2	Untreated	160	70	130	Trace		30	30
	$NaIO_4$	100	50	0	Trace		30	30
SG3	Untreated	190	60	140	10		40	30
	$NaIO_4$	120	50	0	10		40	30
SG4	Untreated	30	20	30	10	50	10	30
	$NaIO_4$	60	20	0	10	50	10	30

[a] For definitions of X_1 and X_2, see footnotes *b* and *c* of Table 2.

that in the extract from SG4. The labeled EDTA extracts were subjected to immunodiffusion against the homologous whole cell antisera and were found to be antigenically identical to the unlabeled antigen. The precipitin line from radiolabeled extracts was cut from the gel, melted, and subjected to scintillation counting. Cells labeled with D-[U-^{14}C] glucose and [1,2-^{14}C] sodium acetate contained radioactivity in the serospecific antigen. The autoradiogram of the SDS-PAGE gels showed that the radioactivity was incorporated into the various bands with equal intensity, very similar to the pattern obtained with the silver staining (Fig. 5).

SDS-PAGE. Antigens from SG1, SG2, SG3, and SG4 strains (2.5 μg) were subjected to SDS-PAGE by the method of Laemmli (20). Gels visualized with Coomassie blue stain revealed only a few poorly stained bands. However, gels visualized by the silver staining method (25) revealed numerous, distinct bands that were spaced at regular intervals (Fig. 6). The SG1 antigen was subjected to SDS-PAGE with increased detergent concentration to determine whether the multiple bands resulted from incomplete dissociation of aggregates. The final SDS concentration in the sample buffer was increased to 2 or 10%, and the SDS concentration in the gel was increased to 1%. These variations resulted in no alteration in the pattern of bands. These results suggest that the bands represent unique molecular complexes that vary in a regular fashion.

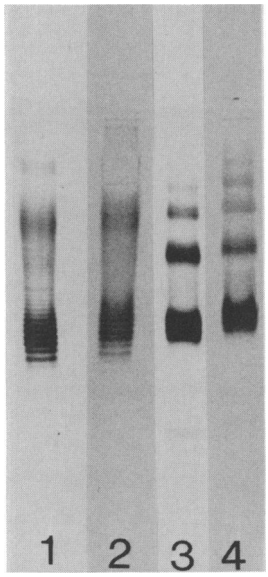

FIG. 5. Autoradiography of SDS-PAGE of EDTA extracted SG1 and SG4 antigens. Lanes: 1, SG1 antigen from SG1 cells grown with [U-^{14}C]glucose; 2, SG1 antigen from SG1 cells grown with [^{14}C]acetate; 3, SG4 antigen from SG4 cells grown with [U-^{14}C]glucose; 4, SG4 antigen from SG4 cells grown with [^{14}C]acetate.

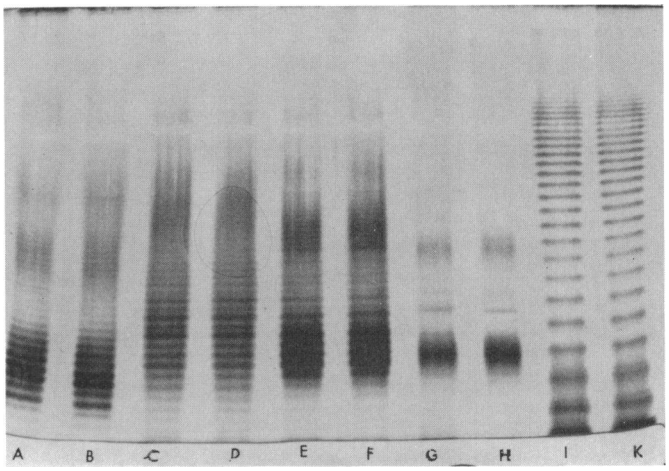

FIG. 6. PAGE (10% polyacrylamide) of the purified antigens. Lanes: A, C, E, and G, SG1, SG2, SG3, SG4 EDTA-Folch-extracted antigens, respectively, at 2.5 μg; B, D, F, and H, the respective antigens treated with pronase; I and K, standard *S. minnesota* (Sigma) lipopolysaccharide at 2.5 and 5.0 μg.

When the gel in the first dimension was run in a second dimension, either as a strip or as individual gel slices, the electrophoretic pattern contained numerous bands with varied migration compared with those in the first-dimension gels (Fig. 7). Such aberrant migration has been observed in the two-dimensional gels with lipopolysaccharide preparations from *Salmonella typhimurium* and *S. minnesota* (14).

Western blots. The antigen preparations were subjected to Western immunoblotting (3, 34) to determine the antigenic activity of the many bands observed on SDS gels. Reaction with homologous antiserum indicated that all of the bands on the gels were antigenically active (Fig. 8). When the blots were incubated with heterologous antiserum, only two cross-reacting bands were observed. These bands probably represent minor contamination with a common antigen. In general, the bands were found to be serospecific, except that SG1 antigen showed significant cross-reaction with SG4 antiserum. The reciprocal reaction between SG4 antigen and SG1 antiserum showed no cross-reaction. This result would not be predicted from the results of Ouchterlony immunodiffusion and may indicate that SG1 and SG4 antigens share a common antigenic determinant, which is detected only on Western blots due to the high sensitivity of this technique.

Pronase and periodate treatment. After 96 h of incubation, the pronase-treated antigens and controls analyzed by SDS-PAGE (Fig. 6) showed no change in the pattern of bands in the antigens from SG1, SG2, and SG3 strains. Pronase treatment of the SG4 antigen resulted in the disappearance of many minor bands, but had no effect on the major bands. The pronase-treated antigens were also tested for antigenic activity by immunodiffusion against the homologous antiserum. No change in activity relative to the control antigens was observed.

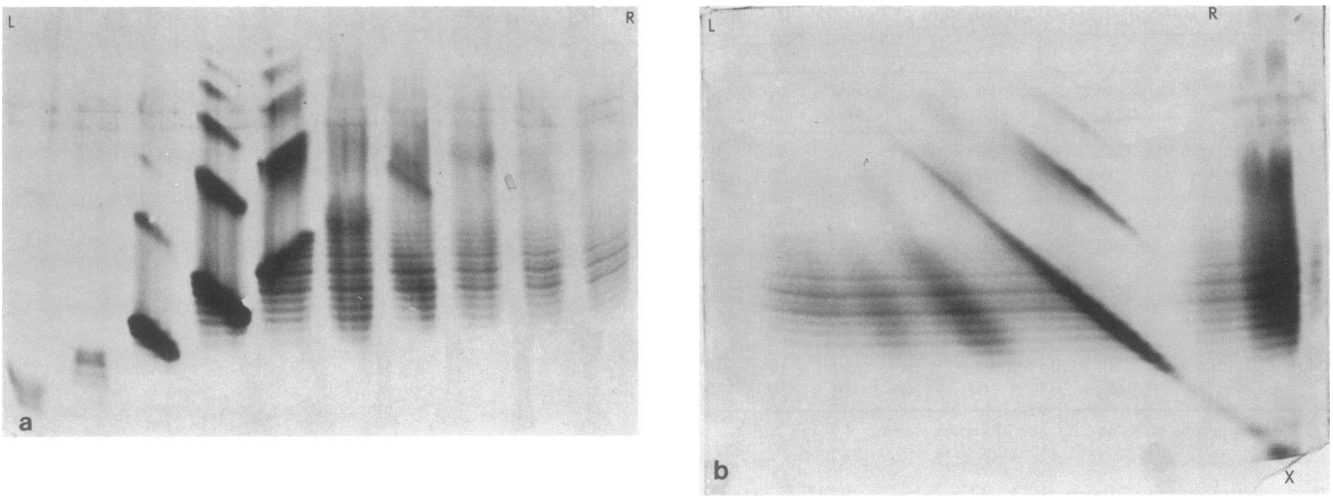

FIG. 7. (a) Reelectrophoresis on 10% gel. A lane from the first-dimension slab gel was cut into 10 equal pieces, and each piece was loaded onto a well of the second gel with the bottom of first gel to the left (L) and the top to the right (R). (b) Second-dimension electrophoresis on 10% gel of a whole lane from the first dimension gel with the top of the first gel to the left (L) and the bottom to the right (R).

The periodate-treated antigens when analyzed by SDS-PAGE revealed substantial changes in the pattern of bands (Fig. 9). In addition, silver staining resulted in many bright green and yellow bands. These bands presumably resulted from the presence of free aldehyde groups in the periodate-oxidized antigens. The oxidized antigens were examined for antigenic activity by immunodiffusion against the homologous antiserum. The oxidized SG1, SG2, and SG3 antigens were antigenically active, but only at high concentrations (250 to 500 μg/ml). The oxidized SG4 antigen was inactive. In addition, two precipitin lines were observed with oxidized SG1 and SG3 antigens. One line was identical to the serospecific antigens found in unoxidized antigen. The other precipitin line was partially identical to the serospecific antigen. Oxidized SG2 antigen gave only one line which was partially identical to the unoxidized serospecific antigen. Preliminary gas chromatographic analysis of the oxidized antigens indicated that the mannosyl residues were completely cleaved by the periodate oxidation (Table 4).

Mild acid hydrolysis. SG1 antigen was hydrolyzed at 100°C with 1% acetic acid (27). Samples were removed at various time intervals and examined by SDS-PAGE (Fig. 10), which showed that the antigen was rapidly degraded by this treatment. After 30 min of reaction, the characteristic multiple bands had disappeared, and a new band was observed near the tracking dye. The hydrolysate was centrifuged to remove the precipitated lipid, which was washed with water and suspended in 0.2 M Tris buffer (pH 7.8). SDS-PAGE of the lipid and aqueous fractions showed that the fast-migrating band that was generated by acid hydrolysis was in the lipid

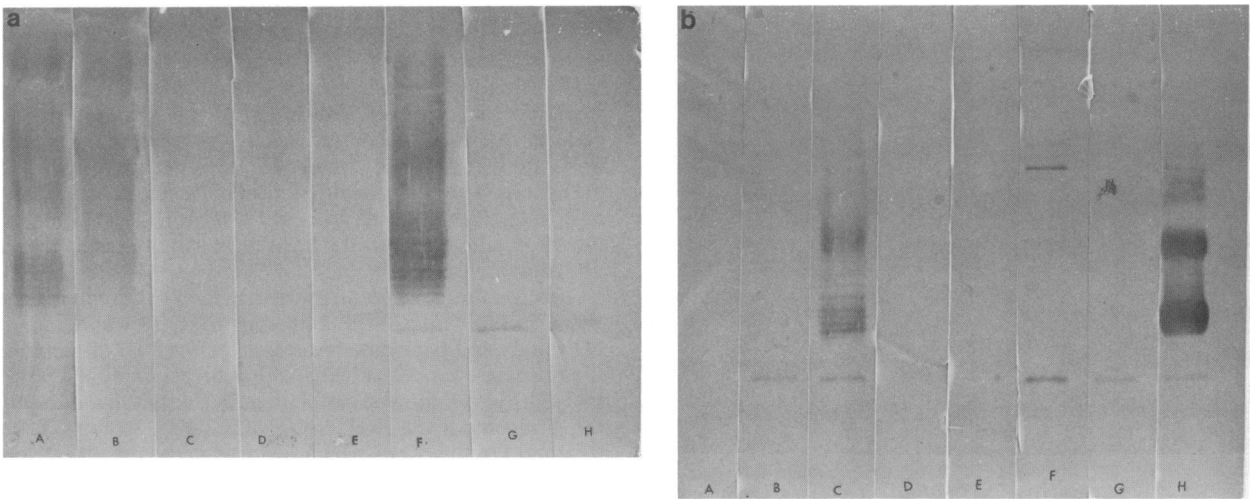

FIG. 8. Western blots of the EDTA-Folch-extracted antigens against homologous and heterologous antisera. (a) Lanes: A, B, C, and D, SG1 reacted against anti-SG1, anti-SG4, and anti-SG3, and anti-SG2, respectively; E, F, G, and H, SG2 reacted against anti-SG1, anti-SG2, ?anti-SG3, and anti-SG4, respectively. (b) Lanes: A, B, C, and D, SG3 reacted against anti-SG1, anti-SG2, anti-SG3, and anti-SG4, respectively; E, F, G, and H, SG4 reacted against anti-SG1, anti-SG2, anti-SG3, and anti-SG4, respectively.

precipitate. SG2, SG3, and SG4 antigens and *S. minnesota* and *E. coli* lipopolysaccharides were hydrolyzed with 1% acetic acid (Fig. 11) and gave analogous results, except that the SG4 antigen contained many minor protein bands that were stable to this treatment. These bands were probably the protein contaminants in the SG4 antigen preparation.

Authentic lipid A from *S. minnesota* migrated in the same region as the fast-migrating band generated by acid hydrolysis of lipopolysaccharides or the serospecific antigens. The antigens and lipopolysaccharides are presumably cleaved at the acid-labile KDO linkage to release lipid, which would be expected to migrate rapidly in SDS-PAGE.

The serospecific antigens and lipopolysaccharides from *S. minnesota* and *E. coli* were analyzed by Western immunoblotting against anti-lipid A (Re 595) serum. The antibody was bound to authentic lipid A and to all of the bands of *S. minnesota* lipopolysaccharide. With *E. coli* LPS, however, the anti-lipid A bound only to the last two low-molecular-weight bands. In addition, the Western blot of *S. minnesota* and *E. coli* revealed a band that migrated at the same distance as lipid A. This band was not detected on the corresponding silver-stained gel. With the serospecific antigens, only a single band was observed on the Western blot. This band migrated slightly above the lipid A band and was not detected on the silver-stained gel. Apparently, anti-lipid A binds well only to *S. minnesota* lipopolysaccharide from which this lipid A was prepared. All preparations, however, appear to contain free lipid A that was not detected by silver staining but was easily detected by Western blotting with anti-lipid A due to the greater sensitivity of this technique. The serospecific antigens were hydrolyzed with 1% acetic acid and then analyzed by Western blotting against anti-lipid A. The lipid released by the hydrolysis readily bound anti-lipid A (Fig. 12) and appeared to migrate slightly above *S. minnesota* lipid A on the SDS gel.

Endotoxicity as determined by the *Limulus* amebocyte lysate gelation showed that all the preparations formed a firm gel at 1 ng/ml compared with a commercial human albumin preparation showing no gelation at 20 μg/ml.

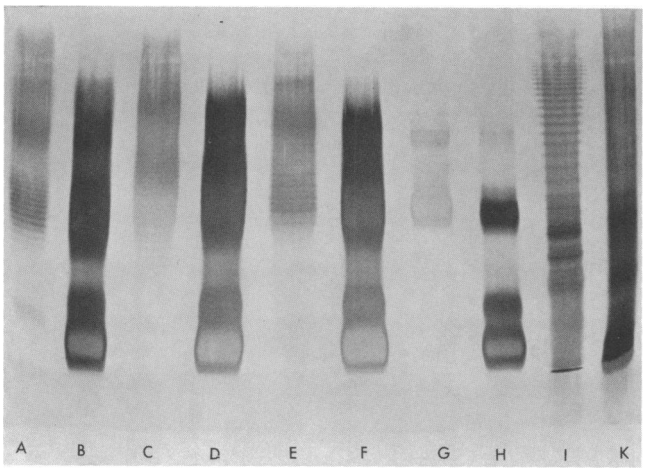

FIG. 9. PAGE (12.5% polyacrylamide) of the periodate-treated antigens. Lanes: A, C, E, and G, SG1, SG2, SG3, and SG4 EDTA-Folch-extracted antigens, respectively, at 2.5 μg; B, D, F, and H, the respective antigens treated with $NaIO_4$; I, standard *S. minnesota* lipopolysaccharide (control); K, standard *S. minnesota* lipopolysaccharide (periodate treated).

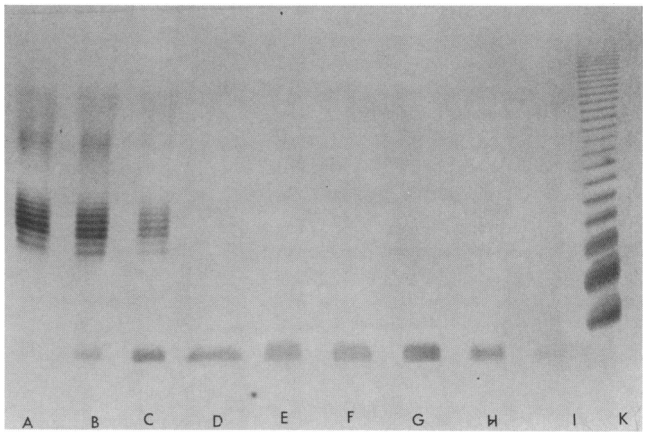

FIG. 10. PAGE (12.5% polyacrylamide) during hydrolysis of SG1 antigen (2.5 μg) with 1% acetic acid. Lanes: A, unhydrolyzed; B, 0 min; C 10 min; D, 20 min; E, 30 min; F, 45 min; G, 60 min; H, 90 min; I, 120 min; K, standard *S. minnesota* lipopolysaccharide.

DISCUSSION

The results of this study confirm that the serospecific antigens isolated from SG1, SG2, SG3, and SG4 strains of *L. pneumophila* are lipopolysaccharides that can be obtained from cells by extraction with EDTA. In addition, the SG1 antigen was isolated in the phenol phase of the phenol-water extraction (37) and was readily extracted by the phenol-chloroform-petroleum ether method (11). Gabay and Horwitz (10) had noted that the lipopolysaccharide of the outer membrane of *L. pneumophila* was not found in the water phase of a phenol-water extraction.

Chemical analysis showed that the antigens contained carbohydrate, KDO, phosphate, and fatty acids. The absolute and relative amounts of KDO, glucosamine, and phosphate detected in the antigens is consistent with a lipopolysaccharide structure. The antigens also contained rhamnose and mannose residues, which have been found frequently in lipopolysaccharides. However, total carbohydrate was less than 10% of the antigens. The major differences in the composition of the antigens isolated from all four serogroups are the molar ratios of the carbohydrate residues and the presence of galactose in the SG4 antigen (Table 2). A number of fatty acids, primarily i-16:O, were detected including small amounts of 3-hydroxymyristic acid, which is a characteristic component of lipopolysaccharides. The fatty acid composition shows the presence of large amounts of branched-chain acids, similar to the findings of Moss et al. (28) on the cellular fatty acid composition of the isolates from Legionnaires disease. Like the sugars, the total amount of fatty acid was also unexpectedly low; the total analyzed material was approximately 15 to 20%, in spite of analyses for all known groups of biochemicals. The results were similar to those of Flescher et al. (8). The analyzed material could reasonably account for the lipid and core portions of a lipopolysaccharide, except that a higher fatty acid content would be expected. This suggests that the antigens contain unusual groups in the side-chain, which were not detected by the methods used in this study.

Despite the similarities to lipopolysaccharides of other gram-negative bacteria, there were several important differences. The SG1 antigen was isolated in the phenol phase of the phenol-water extraction and was readily extracted by the

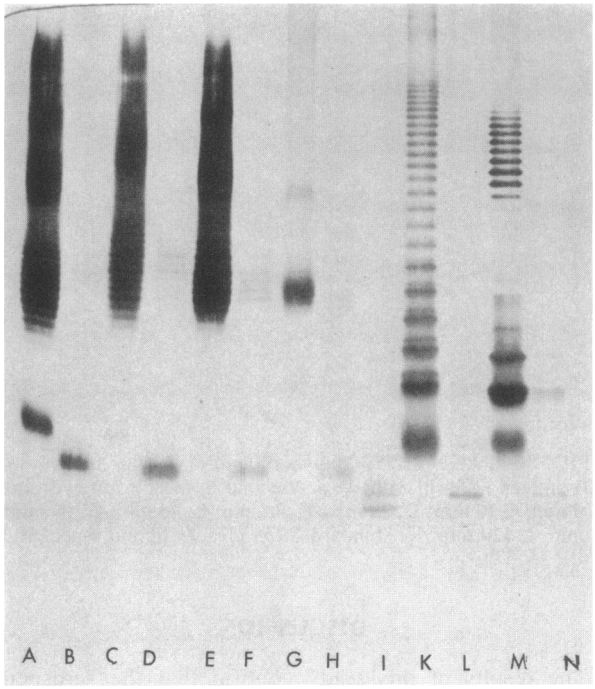

FIG. 11. PAGE (12.5% polyacrylamide) after 30 min of hydrolysis of the antigens with 1% acetic acid. Lanes: A, C, E, and G, SG1, SG2, SG3, and SG4, respectively, unhydrolyzed (5 μg each); B, D, F, and H, SG1, SG2, SG3, and SG4, hydrolyzed (2.5 μg each); K and M, standard *S. minnesota* and *E. coli* lipopolysaccharides, respectively, unhydrolyzed (2.5 μg each); L and N, standard *S. minnesota* and *E. coli* lipopolysaccharides, respectively, hydrolyzed (2.5 μg each); I, lipid A (Calbiochem) (1.25 μg).

phenol-chloroform-petroleum ether method, suggesting that the antigen is more hydrophobic than typical lipopolysaccharides. In addition, our studies confirm the finding of Flescher et al. (8) that the SG1 antigen is devoid of heptose and extend this observation to show that none of the four lipopolysaccharide antigens analyzed contains heptose. In this respect they are also similar to the lipopolysaccharides isolated from *Brucella abortus* (26) and *Microcystis aeruginosa* (30).

The properties of the SG4 antigen are somewhat different from those of the other antigens. This was most evident in the Folch extraction. The SG4 antigen was insoluble in $CHCl_3$-methanol (2:1) and was recovered in the residue, whereas the other antigens were extracted into the Folch upper layer. The SG4 antigen also exhibited a somewhat different pattern of bands on SDS-PAGE. Relative to SG1, SG2, and SG3 antigens, the SG4 antigen had lower antigenic activity and lower KDO, phosphate, and fatty acid content and is the only antigen that contains galactose. The lower carbohydrate content detected by gas chromatography is difficult to explain.

The antigens can be separated by SDS-PAGE to give many regularly spaced bands. This result is consistent with previous studies (13, 15, 16, 29, 35) in which lipopolysaccharides from a variety of sources were shown to contain numerous components that vary in an orderly manner. It has been proposed that this heterogeneity is due to variation in the number of repeating units in the O side chain of the lipopolysaccharide molecule (13, 16). The many bands observed on SDS gels were shown to be serospecific by Western immunoblotting and were equally labeled when the cells had been grown in the presence of radioactive precursors. There was a small percentage of incorporation of radioactive label into the antigens, as would be expected from the growth of cells on agar plates. However, it is particularly significant that the bands from the radiolabeled antigens on SDS-PAGE gels were the same whether detected by autoradiography, immunoblotting, or silver staining, which would not be the case if the antigens were grossly contaminated with other materials that were not part of their molecular structure. Pronase treatment of the antigens resulted in no alteration in the pattern of bands on SDS-PAGE and had no effect on the antigenic activity, indicating that proteinaceous constituents are not the determinants in the serospecific antigens. Oxidation of the antigens with periodate, however, resulted in substantial changes in the SDS gels and antigenicity. The antigenic activity of the oxidized antigens was either abolished or significantly reduced, and a new precipitin line was generated that was partially identical to the serospecific antigen. These results indicate that the antigens contain periodate-sensitive antigenic determinant groups. Gas chromatographic analysis of the periodate-treated antigens revealed that the mannosyl residues were completely cleaved by the periodate oxidation (Table 4).

Mild acid hydrolysis resulted in rapid degradation of the antigens and liberation of a lipid that migrated near the tracking dye on SDS gels. The lipid appeared to be structurally similar to lipid A from *S. minnesota* since it readily bound anti-lipid A antibody on Western blots. The lipid, however, is not identical to this lipid A since the two materials did not comigrate on SDS-PAGE. Previous studies (4, 38) have shown that the serospecific antigens of *Legionella* species possess only weak endotoxin activity. In the present study the various preparations were endotoxic

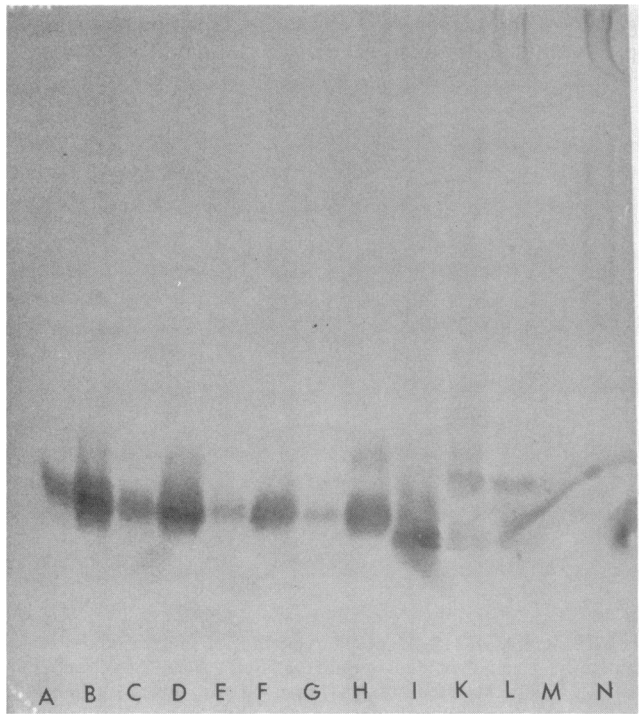

FIG. 12. Western blot against anti lipid A (Calbiochem) antiserum. Lanes are as in Fig. 11.

showing a firm gelation in the *Limulus* amebocyte lysate gelation assay for endotoxicity at 1 ng/ml.

The results of this study indicate that the serospecific antigen from *L. pneumophila* is a lipopolysaccharide molecule with a modified structure. The antigen appears to have an unusual lipid A and core structure, since no heptose was detected. The nature of the side chain is largely unknown, but it appears likely that it contains periodate-sensitive groups. Further work is required to elucidate the structure of the antigen and to determine how these structural features are related to the biological properties of the molecule.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI17159 from the National Institutes of Health.

We thank Richard Saur for his technical assistance and G. W. Wilson, Director of the University of Iowa Protein Structure Facility, for the amino acid analyses.

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