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Isolation, Characterization, and Biological Properties of an Endotoxin-Like Material from the Gram-Positive Organism *Listeria monocytogenes*

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The bacterial component responsible for the induction of transient cold agglutinin syndrome in rabbits after intravenous injection of heat-killed *Listeria monocytogenes* type 4B has been purified and biologically and chemically characterized. A purified immunoglobulin M cold agglutinin was prepared from high-titer sera resulting from the immunization of rabbits with heat-killed *L. monocytogenes* type 4B and was subsequently used to monitor the purification of the bacterial component responsible for its induction. The bacterial component was isolated from a hot phenol-water extract of lyophilized *L. monocytogenes* type 4B by multiple molecular sieve chromatography. Upon chemical analysis the purified material was found to be strikingly similar in chemical composition to gram-negative lipopolysaccharide endotoxins. The material contained 15% total fatty acid (of which 50% was β-hydroxymyristic acid), 40 to 45% neutral sugar (glucose, galactose, and rhamnose), 11.5% amino sugar, 12% uronic acid, 2.5% 2-keto-3-deoxyoctonic acid, 2% heptose, 0.87% phosphorus, and 1.6% amino acid, thereby accounting for 85 to 90% of the weight of the component. Electron micrographs of the purified material were similar to those of lipopolysaccharide preparations from gram-negative organisms. The purified material exists in aqueous solutions as large aggregates, but can be dissociated into a single smaller subunit (3.1S) by dialysis against sodium dodecyl sulfate buffer. The listerial component was toxic and pyrogenic to rabbits, producing symptoms typical of gram-negative endotoxins. Activity in the limulus lysate gelation assay and in the carbocyanine dye assay provides a further link of this material with classical gram-negative endotoxins.

The clinical significance of *Listeria monocytogenes*, a gram-positive diphtheroid-like rod of the family *Corynebacteriaceae*, is becoming increasingly recognized (13). Infection in humans may result in encephalitis, meningitis, septicemia, and abortion. A peculiar result of some septicemic listeric infections in humans is the induction of a transient cold agglutinin syndrome in which antibodies are induced that can react with the host's own erythrocytes under appropriate conditions, causing a complement-mediated in vivo lysis (17). The antibodies responsible for this phenomenon are of the immunoglobulin M (IgM) class and appear to be directed against the I blood group system (5).

Costea and co-workers (5) developed an animal model of this response by the intravenous injection of heat-killed *L. monocytogenes* serotype 4B (HKLM) into rabbits. The cold agglutinin syndrome so produced was found to mimic that which occurs in humans in symptomology and pathology and was caused by IgM antibodies. Their results further indicated that this IgM antibody was not only induced by and directed against the HKLM, but was also able to cross-react with the erythrocytes of the host as well as other xenogenic erythrocytes which possess I blood group antigen(s). Costea et al. (7) were subsequently able to show that a crude fraction obtained from HKLM by a hot phenol-water extraction procedure typically used for extracting endotoxin from gram-negative bacteria was active in inhibiting the cold agglutinating activity of the induced antibodies. They described this fraction as a crude lipopolysaccharide (LPS), presumably because of the nature of the extraction procedure, but did not further characterize the component.

A toxic LPS-like component isolated from a phenol-water extract of *L. monocytogenes* was described and characterized by Conklin and co-workers (4). The extraction procedure used was similar to the method used by Costea et al. (7), except for one major distinction: the bacteria

were not heat killed before extraction. Heating in aqueous solution at 80°C may remove surface components from bacteria (such as endotoxin from gram-negative bacteria [30]); thus, the heat treatment used by Costea et al. (7) may have removed a large portion of the LPS material before their phenol extraction procedure. Conklin and co-workers characterized this material and found that it was toxic to chicken embryos and contained carbohydrate, including 2-keto-3-deoxyoctonic acid (KDO), lipid, protein, nucleic acid, and phosphorus.

The present study was undertaken to isolate and characterize the bacterial component responsible for cold agglutinin syndrome in humans and animals. Rabbits were immunized with HKLM, and cold agglutinin purified from the sera so obtained was used to monitor the purification of the component responsible for its induction. The chemical composition, physical properties, and biological activity of this isolated component were found to bear a striking similarity to classical gram-negative LPS endotoxins.

MATERIALS AND METHODS

Bacterial cell cultures. *L. monocytogenes* serotype 4B (American Type Culture Collection no. 19115), a virulent strain (intraperitoneal inoculation of 10^8 organisms caused death in 10 of 10 Swiss HA/ICR mice within 72 h), was grown in a brain heart infusion broth (Difco). Overnite cultures (50 ml) were inoculated into fresh media and grown in a Microferm Laboratory fermentor (New Brunswick Scientific Co.) at 26°C. Log-phase bacteria were harvested by centrifugation at 8,000 rpm in a JA10 rotor in a Beckman J-21C refrigerated centrifuge for 15 min, washed twice in saline and twice in distilled water by centrifugation, and finally lyophilized.

Immunization and serology. Antibody to HKLM was raised in female New Zealand white rabbits (2 to 3 kg) by intravenous injection with 10^{10} HKLM divided into three equal doses given at 0, 4, and 24 h. Blood samples were collected from the ear vein and allowed to clot at 37°C. The serum was subsequently separated by centrifugation at 37°C, filtered through a 0.45-μm membrane filter (Millipore Corp.), heated at 56°C for 30 min, and stored at −20°C without preservative.

Cold hemagglutination assays were performed by serial dilutions of 0.2 ml of serum (or purified IgM antibody) in phosphate-buffered saline (PBS), pH 6.8, to which was added 0.05 ml of a 2% (vol/vol) suspension of either isologous or autologous rabbit erythrocytes washed three times. After mixing, the tubes were incubated at 4°C for 2 h. The agglutination end point was determined macroscopically after centrifugation for 15 s in an Adams serofuge. Titers were recorded as the reciprocal of the highest dilution of serum exhibiting definite agglutination. Hemagglutination inhibition assays were performed similarly by using either a 1% antigen solution or fractions directly off chromatography columns and an antiserum or purified immunoglobulin dilution with a final hemagglutination titer of 4 to 8. All steps of these assays were performed and read at 4°C. The erythrocytes used were either autologous or isologous as indicated. As Lind suggested (18), to evaluate the rise in cold antibody titer during immunization, consecutive sera from one animal, including nonimmune sera, were tested simultaneously in the same titration experiment. Anti-*Listeria* antibody levels were determined by slide agglutination assay with microflocculation slides. Titrations were performed by serial dilutions of 0.1 ml of serum (or purified antibody fraction) in PBS to which an equal volume of HKLM suspension containing 10^{10} organisms per ml in PBS was added. Slides were incubated for 5 min at 22°C on a rotary shaker. The end point was determined macroscopically as the last dilution in which visible agglutination could be observed.

To differentiate IgM from IgG antibody populations, rabbit sera or purified immunoglobulin fractions were incubated at 37°C for 1 h with an equal volume of 0.1 M 2-mercaptoethanol in PBS, pH 7.4, before use in the appropriate assay system. This procedure causes complete inactivation of IgM antibodies but does not affect IgG immunoglobulin (24).

Purification of cold agglutinin. The rabbit IgM cold agglutinin antibody population was purified from high-titer whole sera by a modification of the glutaraldehyde-treated erythrocyte membrane affinity chromatography procedure described by Tsai et al. (40) for the isolation of human IgM cold agglutinin. Glutaraldehyde-treated isologous rabbit papain-treated erythrocyte membrane was prepared as follows. Erythrocytes were washed three times in PBS, pH 7.0, and the buffy coat was removed; 100 ml of packed erythrocytes was then incubated with an equal volume of 0.4% papain in PBS, pH 7.0, at 37°C for 30 min in a shaking water bath, after which the erythrocytes were washed three additional times with PBS. The erythrocytes were lysed in 30 volumes of distilled water, pH 5.8. The resultant membranes were harvested by centrifugation at 8,000 rpm for 10 min in JA10 rotor and washed twice with the lysis solution and once with PBS; the membranes were then suspended in 100 ml of 2.5% glutaraldehyde in PBS and incubated in a 37°C shaking water bath for 2 h. A one-fifth volume of 0.5 M glycine in PBS was then added, and the mixture was allowed to incubate for an additional 5 min. The stromata were washed three times with PBS containing 0.1 M glycine and twice with PBS, pelleting the stroma each time by centrifugation for 5 min at 20,000 rpm. The affinity column was prepared by mixing 25 ml of a loosely packed suspension of the fixed membranes with 50 ml of a 50% suspension of celite in PBS, then pouring the slurry over a 2-cm base of acid-washed sand in a chromatographic column (5 by 20 cm) under mild suction, and finally overlaying with an additional 2 cm of sand. The column was washed extensively with PBS at both 4 and 37°C before use.

Absorption procedures. Purified listerial LPS samples (100 μg) were incubated with 2.5 ml of 2% suspensions of five-times-washed sheep erythrocytes for 1 h at 4, 25 and 37°C in a shaking water bath. After incubation the erythrocytes were washed three times in PBS and resuspended to 2%. Agglutination reac-

tions were then performed by using the high-titer anti-*Listeria* sera produced in rabbits and tanned sheep erythrocytes at 4, 25, and 37°C, as previously described, with autologous rabbit erythrocytes as a control.

Extraction of crude LPS. A crude LPS fraction was obtained from lyophilized cells by using a modification of the phenol extraction procedure described by Conklin (4). Lyophilized cells (7 g) were suspended in 170 ml of distilled water (65°C), mixed with 170 ml of 90% phenol at 65°C, and shaken for 8 min. Phases were separated by centrifugation for 30 min at 10,000 × *g* in a JA10 rotor at 4°C, and the aqueous upper layer was removed. The phenol layer was decanted, filtered, washed with an additional 170 ml of distilled water, and centrifuged as described above. The aqueous phases were combined and dialyzed extensively, first versus cold running tap water and then against distilled water. The dialysate was reduced to one-tenth of its original volume by coating the dialysis tubing with Aquacide I (Calbiochem). The concentrated solution was centrifuged at 10,000 rpm in a JA20 rotor; the supernatant was decanted and saved, and the precipitate was discarded. Crude LPS was precipitated by slowly adding the supernatant to 5 to 10 times its volume of cold methanol (−20°C) containing 1% sodium acetate. The precipitate was collected by centrifugation at 10,000 rpm for 10 min at −10°C, dissolved in water, and lyophilized.

Purification of LPS. Initial purification of LPS was achieved by chromatography on Sephadex G-25. All fractions were monitored for absorbance at 260 nm as well as ability to inhibit the hemagglutinating activity of the purified cold agglutinin. The relevant fractions were pooled and lyophilized. The lyophilized material was resuspended in distilled water (20 mg/ml) and treated with 2 volumes of packed, washed Enzite agarose ribonuclease (Miles Laboratories) on a rotator at room temperature for 3 h. The mixture was passed through a scintered glass filter to remove the beads, which were then rinsed with several volumes of water. The combined filtrates were lyophilized, and the resultant material was suspended in 0.15 M of ammonium carbonate and chromatographed on a Sephadex G-25 fine column. The relevant fractions as determined by inhibitory activity were pooled and lyophilized. Final purification was achieved by chromatography on Sepharose 4B. The eluant in all columns was 0.15 M ammonium carbonate.

Colorimetric assays. Hexose was measured by the anthrone method of Scott and Melvin (32). Hexosamine was determined by the method outlined by Blumenkrantz and Absoe-Hansen (3). Uronic acids were measured by the method of Dische (8). KDO was measured by the method of Anacker et al. (1). Heptose was measured by the procedures described by Dische (8). Methyl pentose was determined by the method of Dische and Shettles (9). Phosphorus was determined by Shwartzkopf Microanalytical Laboratories, Woodside, N.Y.

Total fatty acids were determined by the method of Duncombe (10) by using a chloroform extract of a hydrolysate (2 N HCl, 3 h, 105°C) of the purified material.

Gas-liquid chromatography. Individual sugars were identified and quantitated by gas-liquid chromatography on a model 400 F + M biomedical gas chromatograph. Neutral sugars were identified and quantitated by chromatography as their trimethyl silyl ethers on a 15% Carbowax 20 M column (Applied Science Laboratories) (27, 28). Neutral sugars, as well as glucuronic acid and KDO, were also identified as trimethyl silyl ethers of their methyl glycosides on a 3% SE-30 column (Applied Science) by the method outlined by Sweeley and Walker (39).

Amino sugars were identified on a model 120C amino acid analyzer (Beckman Instruments, Inc.). The material was hydrolyzed (8 h, 4 N HCl, 100°C), and the HCl was evaporated on a rotary evaporator. The residue was dissolved in water, extracted with hexane to remove fatty acids, dried, and redissolved in 0.02 M HCl before introduction into the analyzer. Amino sugar analysis was done in the laboratory of Richard Margolis, Department of Medicine, New York University School of Medicine.

Individual fatty acids were identified and quantitated by gas-liquid chromatography of their methyl esters on polar (10% Silar 10C) and nonpolar (3% SE-30) columns, both obtained from Applied Science. Fatty acid methyl esters were prepared from a chloroform extract of an LPS hydrolysate (2 N HCl, 3 h, 110°C) according to the procedure described by Kates (16). Fatty acid methyl ester standards were purchased from Applied Science.

Amino acid analysis. Amino acids were determined and quantitated on a Durrum D-500 amino acid analyzer by using standard hydrolysis methodology in the laboratory of Edward Franklin, Irvington House Institute, New York University School of Medicine.

Thin-layer chromatography. Thin-layer chromatography was performed on precoated thin-layer chromatography plates (0.25 mm of Silica Gel 60 on glass supports [20 by 20 cm]; E. M. Laboratories, Merck). The chromatography was performed on a lipid extract of listerial LPS obtained by the extraction procedure of Bligh and Dyer (2). The solvent system used was chloroform–methanol–water–acetic acid (65:25:4:1), and development of the plate was carried out by using iodine vapor. The chromatography was performed in the laboratory of Peter Elsbach, Department of Medicine, New York University School of Medicine.

Analytical ultracentrifugation. Analytical ultracentrifugation was carried out with a Beckman model E analytical ultracentrifuge at 22°C by Marylyn Horwitz, New York Blood Center. Lyophilized, purified listerial LPS was either resuspended in PBS, pH 7.0, and directly run or resuspended and dialyzed against tris(hydroxymethyl)aminomethane (Tris)–ethylenediaminetetraacetate buffer (0.05 M Tris-hydrochloride, 0.005 M ethylenediaminetetraacetate, pH 8.0) or Tris-sodium dodecyl sulfate (SDS) buffer (0.05 M Tris-hydrochloride, 0.001 M SDS, pH 8.0) before centrifugation.

Slide and gel electrophoresis. Slide zonal electrophoresis was performed in 0.5% (wt/vol) agarose gels containing barbital–HCl buffer (ionic strength = 0.02, pH 8.4) cast on glass slides (8.3 by 10.2 cm) with a ratio of volume to surface area of 0.132 ml/cm^2. After application of 4-μl samples, gels were subjected to

electrophoresis at a 20-mA constant current per slide for 45 min in a Behring Diagnostic water-cooled immunoelectrophoresis chamber using the barbital-HCl buffer and Whatman no. 1 filter paper wicks. After electrophoresis, slides were dried directly by a stream of hot air and then stained with toluidine blue (0.15% toluidine blue in 2% ethanol containing 1% acetic acid) and destained with 10% acetic acid in 40% ethanol. Polyacrylamide-SDS gel electrophoresis was performed as described by Fairbanks et al. (11). Gels were stained with either periodic acid-Schiff stain (38) or Coomassie brilliant blue (11).

UV spectra. Ultraviolet (UV) spectra were determined on a Carey model 14 recording spectrophotometer (Varian Associates). Samples were dissolved in either 0.1 N HCl, 0.1 N NaOH, or PBS, pH 7.0.

Infrared spectra. The infrared spectra were obtained on a Perkin-Elmer model 421 grating infrared spectrophotometer by using KBr pellets prepared by mixing 0.75 mg of material with 100 mg of KBr and pressed.

Biological assays. The limulus lysate assay was performed with a Pyrostat test kit (Worthington Biochemicals Corp.). Carbocyanine dye assay was performed by the method of Janda and Work (15).

Pyrogenicity was determined in 2-kg New Zealand white female rabbits according to instructions outlined in the United States Pharmacopeia (*United States Pharmacopeia XIX*, p. 613, 1975). Rectal temperatures were determined with a Yellow Springs Instruments model 425-G tele-thermometer. Listerial LPS solutions and a saline control were injected intravenously in the ear vein.

Electron microscopy. Purified LPS was resuspended in distilled water and placed directly on Formvar carbon-coated grids. The dried samples were negatively stained with 2% ammonium molybdate and examined in a Siemens Elmiskop I electron microscope.

RESULTS

Production of cold agglutinin with HKLM. To be able to identify and immunologically monitor the purification of the bacterial component responsible for the induction of the cold agglutinin syndrome, cold antibody was elicited in rabbits and subsequently purified. Rabbits injected with HKLM responded with a parallel increase in both cold agglutinin and anti-listerial activity (Fig. 1), as determined by cold hemagglutination and bacterial slide agglutination assays. In all 10 rabbits tested nonimmune sera exhibited low levels of naturally occurring IgM cold antibody, with titers ranging from 16 to 64. In the nonimmune sera of 2 of 10 rabbits very low levels (titer of 2) of an apparent IgM anti-*Listeria* antibody were also observed. In all of the rabbits increases both in cold agglutinin and in anti-listerial activity followed a 30- to 40-day cycle. Both activities increased within 72 h after intravenous injection, peaked between 9 and 20 days, and subsequently began to decline.

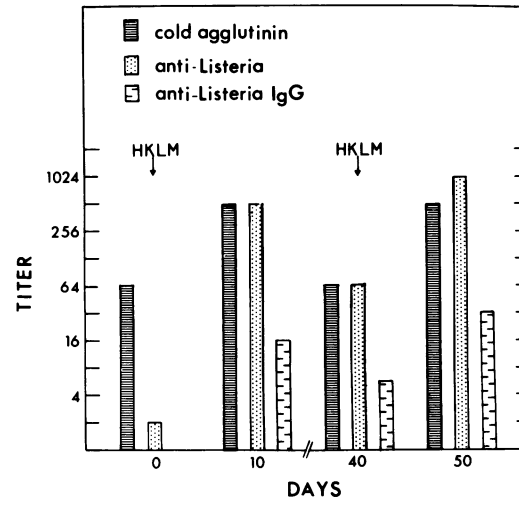

FIG. 1. *Levels of anti-listerial and cold agglutinin antibodies in a typical rabbit injected with HKLM. Arrows indicate injections at days 0 and 40 with 10^{10} HKLM.*

All initial antibody produced was 2-mercaptoethanol sensitive. Anti-listerial IgG antibody (2-mercaptoethanol resistant) was not detected within the first 7 days after inoculation but then began to rise. All cold agglutinin activity was always found to be 2-mercaptoethanol sensitive. After 40 days cold agglutinin activity was approximately back to basal levels, whereas anti-listerial activity was still significantly increased. If, at this time, the rabbits are reinjected with HKLM, a new 30- to 40-day cycle in cold agglutinin and anti-listerial activities was initiated.

Purification of cold agglutinin. The purification of the cold agglutinin from high-titer sera was achieved by using a thermal affinity chromatographic procedure (Fig. 2), followed by molecular sieve chromatography. The affinity column was prepared by using glutaraldehyde-fixed rabbit erythrocyte membrane mixed with sand and celite; high-titer sera were passed through the column at 4°C, and the column was then rinsed extensively with cold buffer. The column was then warmed to 37°C and eluted with buffer at the same temperature. Column fractions were monitored for both absorbance at 280 nm and cold agglutinating activity in the hemagglutination assay. No cold agglutinin activity was eluted from the column at 4°C, but it was eluted at the higher temperature. The fractions containing cold agglutinin activity were pooled and concentrated in an Amicon Diaflo cell. Upon immunodiffusion analysis of this material against anti-rabbit whole sera, it was determined that further purification was needed.

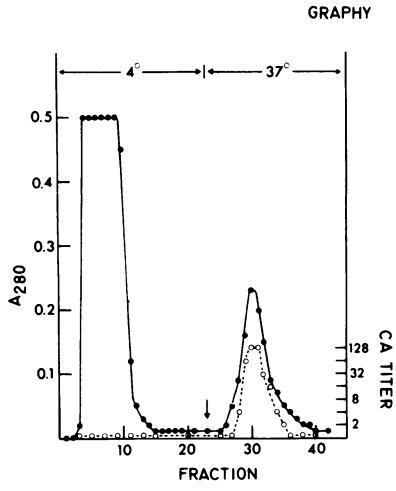

FIG. 2. *Purification of cold agglutinin by thermal affinity chromatography. High-titer immune rabbit sera were titrated to pH 6.8, membrane filtered (Millipore Corp.), and subsequently passed through a rabbit membrane celite column (5 by 20 cm) that was prepared as described in the text. The column was washed extensively with PBS at 4°C and then eluted with PBS at 37°C. Fractions (10 ml) were collected and monitored for absorption at 280 nm (A_{280}; ●) and for cold hemagglutinating activity (CA titer;* ○).

The warm eluted material was therefore chromatographed on a Sepharose 6B column (2.5 by 100 cm) at 30°C with PBS as the eluant, and fractions were monitored for both absorbance at 280 nm and cold agglutinin activity. A purified antibody fraction was obtained, which was shown to be IgM by immunoelectrophoresis assay when developed with anti-whole rabbit serum.

Purification of LPS. The purification scheme of the LPS-like component from *L. monocytogenes* is shown in Fig. 3. A crude LPS fraction was obtained from lyophilized cells by using hot phenol-water extraction as described above. Purification was achieved through a series of molecular sieve chromatographic procedures (Fig. 4A through C) and monitored by cold hemagglutination inhibition assays and by slide gel (Fig. 5) and polyacrylamide gel electrophoresis. Initial purification of the crude extract was obtained on a Sephadex G-25 column (Fig. 4A). The void volume peak contained all of the cold agglutinin inhibitory activity. Material from this peak was found to consist of two major populations of macromolecular species (Fig. 5); one apparently was high-molecular-weight ribonucleic acid. This material was therefore subjected to ribonuclease treatment and then chromatographed on a Sephadex G-25 fine column (Fig. 4B). All of the inhibitory activity was found in material from the void volume peak. Electrophoresis of this material (Fig. 5) again showed the presence of two molecular species. Final purification was achieved by chromatography on a Sepharose 4B column (Fig. 4C). Although the inhibitory material was again found in the void volume peak, electrophoresis (Fig. 5) indicated a single component which was designated purified LPS. The final product was able to inhibit 4 to 8 hemagglutinating doses of the purified cold agglutinin at a concentration of 7.5 μg/ml. As opposed to the void volume peaks from the Sephadex G-25 and G-25 fine columns, which showed UV absorption maxima at 260 nm, material in the Sepharose 4B void volume peak showed increasing UV absorbance as the wavelength was lowered, but it did not exhibit any UV maxima. The second peak from the Sepharose 4B column apparently contained the nucleic acid, judging by its UV absorption spectrum (maximum at 260 nm).

Slide and gel electrophoresis. Figure 5 shows the results of slide electrophoresis of listerial LPS at various stages of purification. The slides were subjected to electrophoresis, dried, and stained with toluidine blue. The purified material migrated as a single band under the conditions employed. The purified material was dissolved and dialyzed against 2% SDS in Tris buffer as described above and subjected to electrophoresis on SDS gels. No bands were noted when the gels were stained with Coomassie brilliant blue, indicating that no contaminating protein was present. Gels stained with periodic acid-

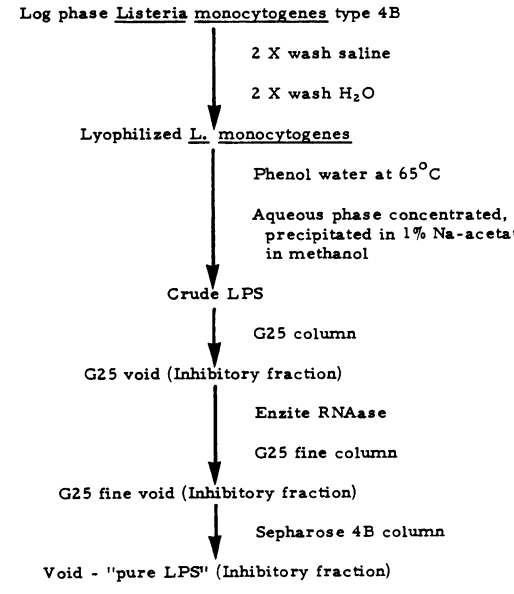

FIG. 3. *Purification scheme for listerial LPS.*

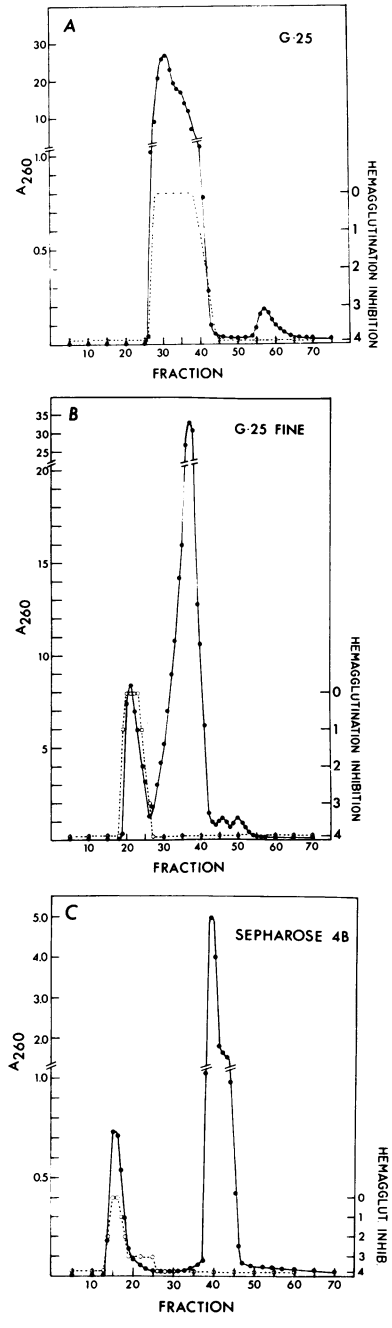

FIG. 4. *Chromatographic purification of listerial LPS. (A) Crude LPS (250 mg) was dissolved in 10 ml of 0.15 M ammonium carbonate and chromatographed on a Sephadex G-25 column (5 by 50 cm). (B) Ribonuclease-treated crude LPS (200 mg) from a Sephadex G-25 column was dissolved in 5 to 10 ml of 0.15 M ammonium carbonate and chromatographed on a Sephadex G-25 fine column (2.5 by 100 cm). (C) The Sephadex G-25 fine void volume fraction material (> 100 mg) was dissolved in 5 ml of 0.15 M ammonium carbonate and chromatographed on a Sepharose 4B column (2.5 by 100 cm). All columns were eluted with 0.15 M ammonium carbonate, and 10-ml fractions were collected. All fractions were monitored for absorbance at 260 nm (A_{260}; ●) and cold agglutinin inhibitory activity (○).*

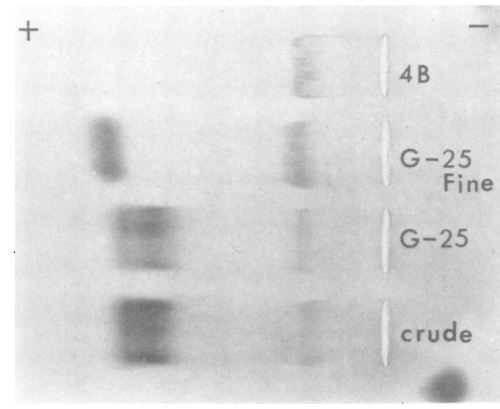

FIG. 5. *Slide electrophoresis of 4-μl samples of fractions from the various stages of purification of LPS. The slide was stained directly after drying with toluidine blue as described in the text.*

Schiff stain, however, showed differing distribution patterns and number of bands (one or three) depending on temperature and length of storage, conditions of solubilization, and mode of dialysis before electrophoresis.

Analytical ultracentrifugation. Figure 6 shows the Schlieren patterns from sedimentation velocity ultracentrifugation analysis of the purifed LPS treated as described above. LPS suspended in PBS (Fig. 6A) showed a fast-moving, diffuse peak of 37S and a slower, sharper peak of 17S. As the material sedimented, a boundary at the bottom of the cell was noted, and, when it was removed from the ultracentrifuge, it was seen that a hard gel had accumulated at the bottom of the ultracentrifuge cell. LPS dialyzed against Tris-ethylenediaminetetraacetate (Fig. 6B, bottom pattern) exhibits two peaks—a small diffuse shoulder which quickly flattened out and a sharp peak of approximately 3.9S. LPS dialyzed against Tris-SDS (Fig. 6B, top pattern) showed a single sharp peak of 3.1S.

Electron microscopy. An electron microscopic examination of the purified listerial LPS, in which negative staining techniques were used, revealed a heterogeneous mixture of forms, primarily ribbons and spheres of various sizes and shapes (Fig. 7) Such structures are very similar to those reported by a number of other investi-

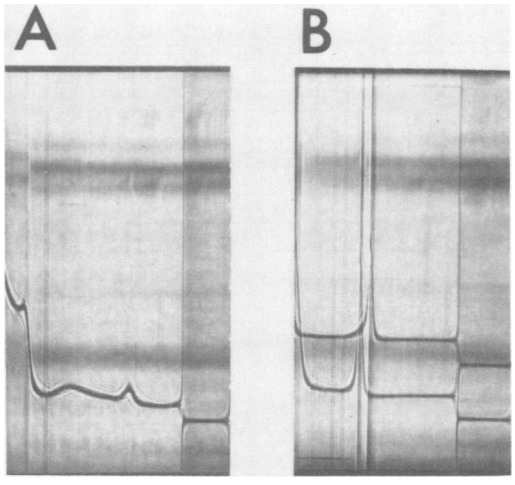

FIG. 6. *Sedimentation velocity patterns of purified LPS under various conditions. (A) PBS buffer, 20 min at 40,000 rpm. (B) Top pattern, Tris-SDS buffer, 104 min at 52,000 rpm; bottom pattern, Tris-ethylenediaminetetraacetate buffer, 104 min at 52,000 rpm. Direction of sedimentation was from right to left.*

gators for LPS from gram-negative bacteria (14, 19, 33, 41).

UV absorption spectra. UV spectra of the purified LPS revealed no UV maxima, but did reveal a steadily increasing optical density as the wavelength was lowered. The absence of an absorption maximum at 258 nm indicated that no contaminating nucleic acid was present. Aqueous solutions of LPS were translucent, and the observed absorbance may have been due to light scattering by the macromolecular aggregate.

Infrared absorption spectra. The infrared spectra of purified LPS from both an *Escherichia coli* K-12 rough strain and a *L. monocytogenes* strain are shown in Fig. 8A and B, respectively. Although there are limitations in interpreting infrared spectra of complex natural products such as LPSs, the striking similarities between the two spectra are apparent.

Chemical composition of listerial component. The purified material was analyzed, and its chemical constituents (Table 1) were carbohydrate, lipid, and phosphorus, with a small amount of amino acid. Sugars were quan-

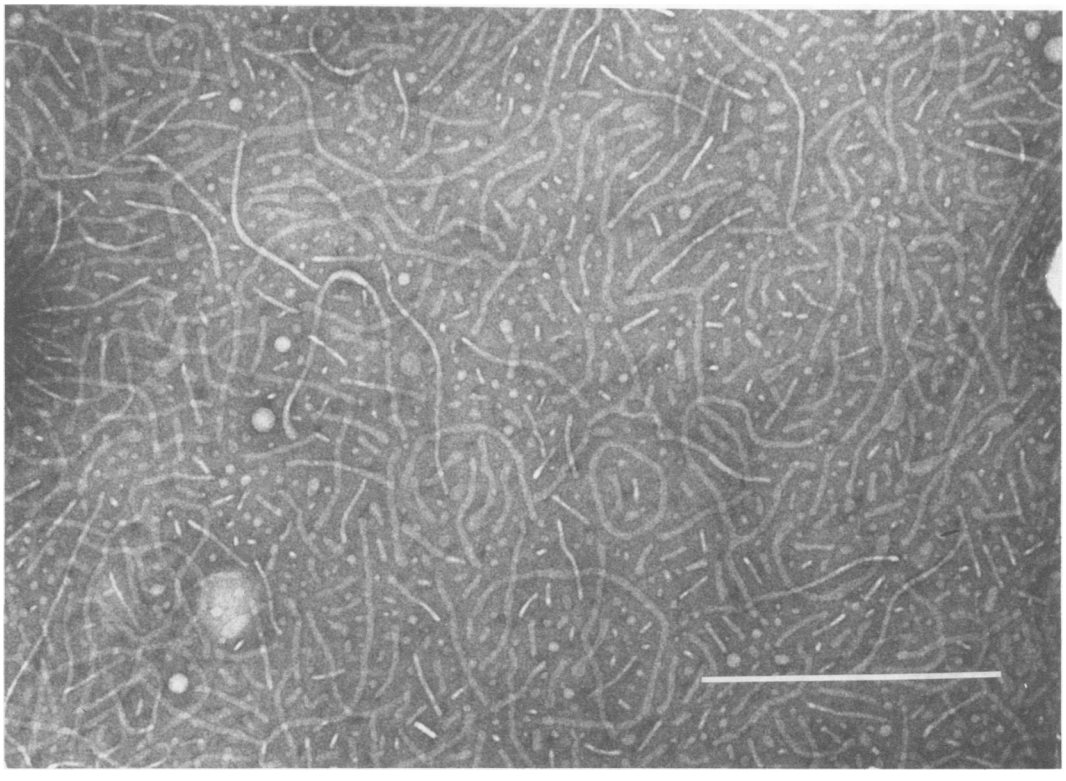

FIG. 7. *Electron micrograph of purified listerial LPS negatively stained with ammonium molybdate. Bar = 0.5 μm.*

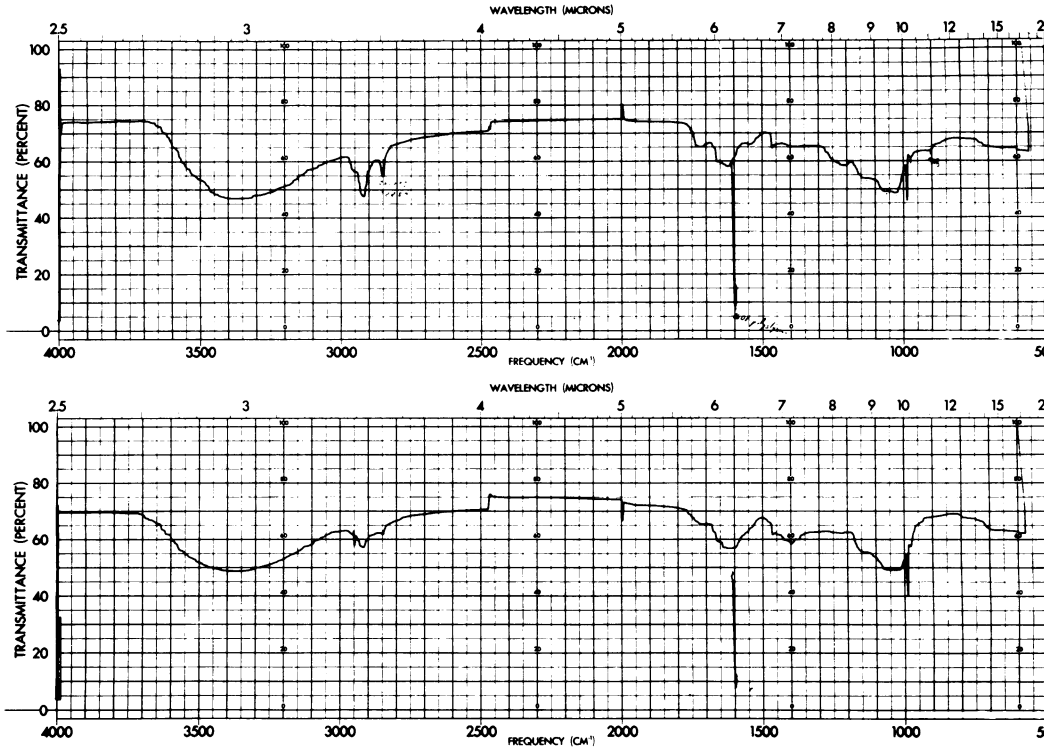

FIG. 8. *Infrared spectra of purified LPS preparations. (A) LPS from a rough strain of E. coli K12. (B) LPS from L. monocytogenes.*

TABLE 1. *Chemical composition of listerial LPS*

Compound	% Of total	% Of fraction
Carbohydrate		
Hexose		
Glucose	20	
Galactose	20	
Methyl-pentose (rhamnose)	12	
Uronic acid (glucuronic acid)	12	
Amino sugars		
N-acetylgalactosamine	9	
N-acetylglucosamine	2.5	
KDO	2.5	
Heptose	2	
Fatty acids	15	
C_{12} saturated		5
C_{14} saturated		17
Unidentified, elutes after C_{14}		15
β-Hydroxymyristic acid		45
C_{16} saturated		17
Phosphorus	0.87	
Amino acids	1.6	

titated by both colorimetric techniques and gas-liquid chromatography. The carbohydrates consisted of neutral sugars (glucose, galactose, and rhamnose), glucuronic acid, KDO, heptose, *N*-acetylgalactosamine, and *N*-acetylglucosamine. The elution patterns in gas-liquid chromatography of the neutral sugars, uronic acid, and KDO as trimethyl silyl ethers of their methyl glycosides are shown in Fig. 9.

The fatty acids are apparently covalently attached to the molecule since they were released only upon hydrolysis and could not be extracted by a total lipid extraction procedure. To ensure that there was no contaminating phospholipid present, a chloroform–methanol–water extraction was performed on the purified material, and the chloroform phase was subjected to thin-layer chromatography. Development of the chromatogram revealed no phospholipid or free fatty acids. The fatty acids were identified by gas-liquid chromatography of their methyl esters on both polar and nonpolar columns. Tracings of their elution patterns are shown in Fig. 10A and B. Methyl laurate (C_{12}), methyl myristate (C_{14}), methyl palmitate (C_{16}), and methyl-β-hydroxymyristate were identified by comparison of their R_f values relative to methyl stearate (C_{18}) with those of standard fatty acid methyl esters.

When listerial LPS was hydrolyzed and the fatty acids were extracted with chloroform and then methylated, the fatty acids noted in Fig.

10A and B were seen. However, when the material was subjected directly to methanolysis (which, unlike hydrolysis, does not break amide linkages), the peak emerging after methyl myristate was absent, indicating that it may be connected by an amide linkage, as is the case with some fatty acids in classical gram-negative LPS (12).

Limulus lysate and carbocyanine dye assays. The purified listerial material was found to be active in two assays normally used for assaying gram-negative endotoxin, the limulus lysate and carbocyanine dye assays. The limulus lysate assay involves the gelation of an extract from *Limulus polyphemus* amoebocytes by nanogram amounts of endotoxin (36). There have been conflicting reports in the literature as to the specificity of this assay; but, although limulus lysate can apparently be gelled by substances other than LPS, the exquisite sensitivity seems to be unique to endotoxins. The listerial LPS-like component was able to induce gel formation in the same concentration range as the *E. coli* endotoxin standard. A 1-ng amount of listerial LPS was as active as 0.4 ng of the *E. coli* reference endotoxin in inducing gel formation. A lipoteichoic acid (LTA) preparation from *Lactobacillus fermentum* was also able to induce gel formation, but only at 1,000 to 2,000 times the concentration at which listerial LPS and the reference endotoxin were active.

Another LPS assay involves the use of a cationic carbocyanine dye. This assay is based on a spectral shift in an acidic solution of a cationic carbocyanine dye. Polyanions, such as proteins, nucleic acids, and acidic polysaccharides cause the dye, which normally absorbs at 510 nm, to undergo shifts to longer wavelengths. Spectral shifts to shorter wavelengths result from reactions of the dye with LPSs from gram-negative bacteria, as well as with their isolated lipid A component (15). The concentration range detected is from 0.5 to 10 μg of LPS. As Fig. 11

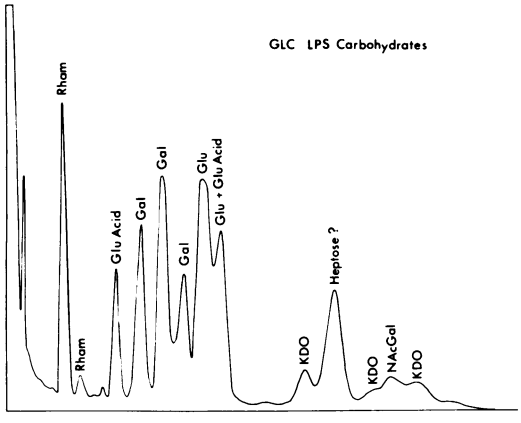

FIG. 9. *Gas-liquid chromatographic elution pattern of purified LPS sugars as trimethyl silyl ethers of their methyl glycosides prepared as described in the text.*

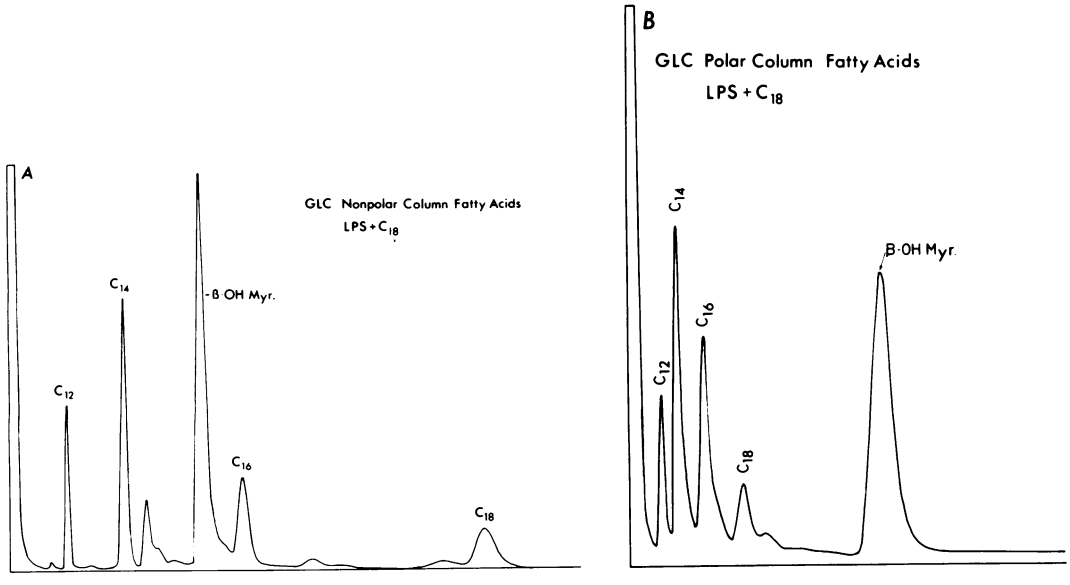

FIG. 10. *Gas-liquid chromatographic elution patterns of purified LPS fatty acids as their methyl esters, with methyl stearate added as an internal standard. (A) Chromatography on a nonpolar (3% SE-30) column. (B) Chromatography on a polar (10% Silar 10 C column). β-OH Myr., β-Hydroxymyristic acid.*

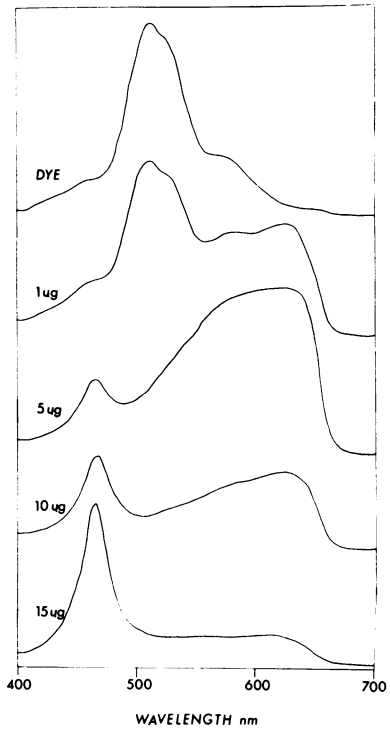

FIG. 11. *Spectra of increasing quantities (0, 1, 5, 10, 15 μg) of purified listerial LPS in the carbocyanine dye assay performed by the method of Janda and Work (15).*

shows, at the lower end of the concentration range, the listerial component shifted the peak to 630 nm, a shift which could possibly be explained by the presence of uronic acid in the listerial material. At the higher end of the concentration range, 10 to 15 μg of a purified listerial LPS caused a spectral shift to 470 nm.

Biological activity. When 2-kg rabbits were injected intravenously with the purified LPS at dosages ranging from 10 to 400 μg, a variety of physiological responses were observed, ranging from a simple pyrogenic response accompanied by malaise to severe diarrhea and death within several hours. All rabbits (four) injected with 10-μg doses exhibited a classical endotoxin type of pyrogenic response (23) (Fig. 12) but nothing more severe. These rabbits showed a general malaise and a 2.5°C rise in rectal temperature, which peaked within 3 h and gradually returned to normal within 6 to 8 h. In one case a biphasic temperature pattern was observed, which peaked at both 1 and 3 h (Fig. 12). A control rabbit similarly injected with pyrogen-free sterile saline showed no response. When given larger doses, rabbits became much sicker, and all exhibited severe diarrhea from which they either recovered or died within 24 h. The rate of death in these animals increased as dosage was increased: when given 50-μg doses, one of four succumbed within 2 h; at a 200-μg dosage two of four died, one after 1 h and the other after 24 h; and with 400 μg three of four expired within a few hours. In those rabbits which died within 2 h autopsy revealed only gastrointestinal and stomach distension, most likely a result of the diarrhea. In the rabbit which survived 24 h before expiring, autopsy also revealed fluid in the lungs.

The purified LPS could also cause localized edema and erythema in rabbits when injected subcutaneously in doses as low as 2.5 μg. Subsequent intravenous injection of the purified material caused enhanced inflammation at the original subcutaneous injection site but did not produce the hemorrhagic or necrotic appearance that would be expected for a localized Shwartzman reaction.

Amphipathic nature. Similar to gram-negative LPS and gram-positive LTA macromolecules, listerial LPS is amphipathic. Incubation of relatively nonreactive sheep erythrocytes with the purified listerial LPS at 4, 25, or 37°C resulted in sensitized erythrocytes which subsequently could be readily agglutinated by immune anti-listerial rabbit sera or purified IgM fractions. The degree of agglutination was dependent on the time of the incubation of the LPS with the sheep erythrocytes, requiring a minimum of 1 h for adsorption, but did not seem to be temperature dependent, since similar results were obtained whether the incubation was performed at 4, 25 or 37°C.

DISCUSSION

The listerial component responsible for the

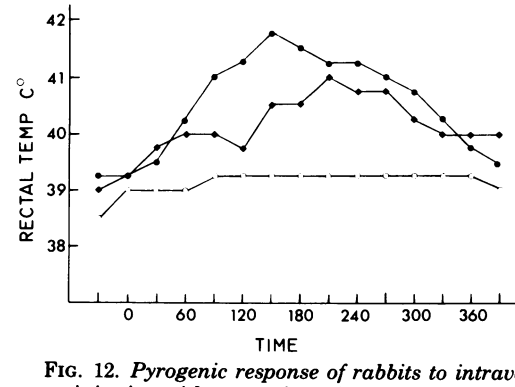

FIG. 12. *Pyrogenic response of rabbits to intravenous injection with 10 μg of purified listerial LPS (● and ◆) and with saline (○). Time is expressed in minutes.*

induction of the cold agglutinin syndrome in rabbit appears to bear a striking similarity, both chemically and biologically, to classical LPS or endotoxin from gram-negative organisms. Chemical analysis of the purified material revealed the presence of 15% total fatty acid (of which 50% was β-hydroxymyristic acid), 40 to 45% neutral sugar (glucose, galactose, and rhamnose), 11.5% amino sugar, 12% glucuronic acid, 2.5% KDO, 2% heptose, 0.87% phosphorus, and 1.6% amino acids (Table 1). KDO, heptose, and β-hydroxymyristic acid are often considered markers for LPS. Whereas KDO and heptose have been identified in bacterial sources other than LPS, β-hydroxymyristic acid has only in a few cases been encountered in bacterial lipids other than lipid A (12), and the presence of all three LPS markers together in this listerial macromolecule is surprising. Physical characteristics of the purified listerial component, such as structural appearance in electron micrographs (14, 19, 33, 41), sedimentation velocity in analytical ultracentrifugation analysis (26), and infrared spectra, are similar to those reported for gram-negative LPSs. Toxic effects in rabbits, as well as activity in two assays specific for LPS (the limulus lysate and carbocyanine dye assays) provide a further link of this material to other gram-negative endotoxins.

To test the specificity of the two LPS assays employed in this study, a comparison was made between the reactivities of listerial LPS and an LTA preparation from *L. fermentum*. A comparison of the properties of gram-negative LPS, listerial LPS, and LTA may be seen in Table 2. Both LPS and listerial LPS are pyrogenic and lethal to rabbits; LTA is not. All three substances are amphipathic and can sensitize erythrocytes to agglutination with specific antibody. LPS and LTA both give positive localized Shwartzman reactions. With listerial LPS, intravenous injection of the material produced enhanced inflammation of the originally subcutaneously injected site, but the area did not become hemorrhagic or necrotic.

LPS and listerial LPS are both active in the limulus lysate assay. The LTA preparation was able to activate the assay, but only at 1,000 to 2,000 times the concentration range required by LPS and the listerial component. In the carbocyanine dye assay, LTA caused a spectral shift of the dye to a longer wavelength, characteristic of the shift caused by polyanions, but did not cause a spectral shift to 470 nm, even at 10 times the concentration at which listerial LPS was active. It is interesting to note the types of shifts caused by the various concentrations of LPS (Fig. 11). At the lower concentrations it appears that the acidic moieties of the macromolecule comprise the available sites for reaction with the carbocyanine dye. At the higher end of the concentration range, it appears that the lipid A-like component (assuming that lipid A, as suggested by Janda and Work [15], is the responsible agent) is available for reaction with the dye. These results suggest that the aggregation of the macromolecule may be concentration dependent, a characteristic which has been noted by others working with various LPS preparations (26). The formation of aggregates also appears to be time and temperature dependent, since the same preparation exhibited various SDS gel patterns, depending on temperature and time of storage, conditions of solubilization, and mode of dialysis before electrophoresis.

TABLE 2. *Comparison of characteristics of bacterial cell surface components*

Characteristic	LPS[a]	Listerial LPS	LTA[b]
Pyrogenicity in rabbits	+	+	−[c]
Lethality in rabbits	+	+	−[c]
Amphipathic (sensitizes erythrocytes)	+	+	+[c]
Localized Shwartzman reaction	+	±	+[c]
Limulus lysate gelation	+	+	−[d]
Carbocyanine dye assay (spectral shift)	+	+	−[d]
Presence of acylated hydroxy fatty acid	+	+	−[c]
Presence of KDO, heptose	+	+	ND[e]

[a] From gram-negative organism.
[b] From gram-positive organism.
[c] From Wicken (42).
[d] LTA from *L. fermentum*.
[e] ND, Not determined.

Since our purified LPS-like material accounted for approximately 6% of the dry weight of the cell and from the immunological data appeared to be located on the outer surface, an electron microscope study was initiated with Kwang Shin Kim, Department of Microbiology, New York University School of Medicine, to determine whether there is (are) any unique cell surface structure(s) associated with the *Listeria* strain used and also whether any structural changes are induced in cells subjected to hot phenol–water extraction. Electron micrographs of cells taken from an actively growing culture revealed typical gram-positive morphology, with no indication of a gram-negative outer envelope. Micrographs of the same cells subjected to the extraction procedure showed drastically altered internal morphology, but cell wall structures appeared intact with approximately the same thickness as in untreated cells.

Although the isolated LPS-like component

was not able on its own to induce a cold agglutinin response in rabbits, when rabbits were first injected with HKLM and subsequently injected with 50 μg of LPS after a specified time interval had elapsed, a rise in both cold agglutinin titer and anti-listerial antibody was observed. In this case, only IgM antibodies were induced; with a booster of HKLM, on the other hand, both IgM and IgG anti-listerial antibodies, as well as IgM cold agglutinins, were induced (Fig. 1). Absorption studies carried out on the test bleed and immune sera indicated that two different IgM populations are induced by the HKLM: the cold agglutinating antibody, which can be absorbed by both erythrocytes and HKLM, and an anti-listerial antibody, which can be absorbed only by HKLM. Nonimmune rabbit sera have low levels of naturally occurring cold agglutinins, with titers ranging from 16 to 64; HKLM was able to absorb out cold agglutinating activity at both 4 and 37°C in all the immune sera tested, but only down to the naturally occurring levels, suggesting that HKLM may be able to absorb out only the induced antibody. Rabbit erythrocytes (autologous and isologous) were able to absorb out all of the cold agglutinating activity, both naturally occurring and induced, at 4°C but not at 37°C.

Other workers, while studying listerial cell walls as well as crude preparations from phenol extracts of *L. monocytogenes* serotype 4B (4, 21, 34), found that these preparations did have some endotoxic activities, such as dermal edema and erythema in rabbits and death in chicken embryos. Indications of KDO and heptose were also noted by these workers in listerial cell wall preparations and phenol extracts. The present study indicates that a component isolated in apparently "pure" form (i.e., with no detectable contaminating nucleic acid, protein, or phospholipid) which migrates as a single band on slide electrophoresis and as a single, small homogeneous subunit in an analytical ultracentrifuge is extremely similar to gram-negative endotoxins in terms of its chemical composition, behavior in LPS assays, and biological activity.

Many workers have attempted to isolate the toxic component of *L. monocytogenes* (4, 20–22, 25, 29, 31, 35, 37), and various cell fractions (lipoidal, carbohydrate, and proteinaceous) have been reported to be biologically active. Our purified LPS-like substance, apparently located on the surface of this gram-positive organism, is extremely toxic to rabbits, may account for some of the toxic effects due to infection with *L. monocytogenes*, and has potent biological activity. The biological properties of listerial LPS include: (i) pyrogenicity; (ii) activity in limulus amoebocyte lysate assay; (iii) death within few hours after injection, if given in sufficiently large doses (death is accompanied by severe diarrhea); (iv) edema and erythema in rabbit skin upon subcutaneous injection; (v) rise in anti-listerial antibody titer; and (vi) rise in cold agglutinin antibody titer.

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