INFECTION AND IMMUNITY, Mar. 1989, p. 717–723
0019-9567/89/030717-07$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 57, No. 3

Comparison of the Biological Responses Induced by Lipopolysaccharide and Endotoxin of *Treponema hyodysenteriae* and *Treponema innocens*

JAMES M. GREER† AND MICHAEL J. WANNEMUEHLER*

Department of Veterinary Microbiology and Preventive Medicine, Veterinary Medical Research Institute, Immunobiology Program, Iowa State University, Ames, Iowa 50011

Received 25 August 1988/Accepted 18 November 1988

The chemical composition and classical biologic activities of lipopolysaccharide (LPS; phenol-water) and endotoxin (butanol-water) preparations from virulent *Treponema hyodysenteriae* and avirulent *Treponema innocens* were examined. The LPS and endotoxin preparations from *T. hyodysenteriae* B204 contained approximately 80.9 and 35.2% hexose, 0.12 and 0.45% thiobarbituric acid-reactive compound, and <1 and 11.3% protein, respectively. The LPS and endotoxin preparations of *T. innocens* B1555a contained approximately 56.3 and 37.8% hexose, 0.45 and 0.4% thiobarbituric acid-reactive compound, and <1 and 26% protein, respectively. A silver-stained 7.5 to 15% sodium dodecyl sulfate-polyacrylamide gel showed four bands for the *T. hyodysenteriae* preparations, while the *T. innocens* preparations failed to resolve into discrete bands on electrophoresis. We determined by the *Limulus* amebocyte lysate assay that the treponemal preparations had comparable amounts of endotoxin activity when *Escherichia coli* LPS was used as a standard. The 50% lethal doses of LPS and endotoxin from *T. hyodysenteriae* for BALB/cByJ mice were 380 and 80 μg, respectively. The treponemal preparations were poor adjuvants, failed to induce a dermal Shwartzman reaction, and were not pyrogenic. The treponemal LPS preparations, unlike the endotoxin preparations, were not mitogenic for murine spleen cells. Differences in virulence between the two treponemal species could not be associated with the biologic activities of the respective LPS or endotoxin moieties, but the endotoxin preparations were consistently more active than the purified LPS preparations.

Lipopolysaccharide (LPS) is a molecule that is found in the outer membrane of gram-negative bacteria and is associated with numerous biologic effects on the mammalian immune system (25). These responses include B-cell mitogenicity, polyclonal antibody induction, adjuvanticity, macrophage activation, immunogenicity, pyrogenicity, lethality, induction of tolerance, and inflammatory reactions (25, 33, 34, 39).

Different extraction methods for LPS have been reported (5, 8, 24, 46). LPS, when free of contaminating protein, is extracted by the hot phenol-water method of Westphal and Jann (46). Endotoxin preparations, which consist of LPS as well as lipid A-associated protein(s), is extracted by either the trichloroacetic acid methods of Boivin and Mesrobeanu (5) and Staub (36) or the butanol-water method of Morrison and Leive (24). Endotoxin possesses all of the aforementioned biologic activities and the ability to stimulate lymphoreticular cells from LPS-hyporesponsive C3H/HeJ mice (10, 45). This stimulation has been shown to reside with the protein moieties that are associated with endotoxin (10, 23, 25).

In comparison with the LPS from *Escherichia coli*, there are numerous gram-negative organisms which have LPS with various chemical and biologic characteristics (12, 31, 35). These differences include the inability to detect the presence of 2-keto-3-deoxyoctonate (KDO) (9), differences in fatty acid composition (47), and the ability to stimulate the lymphoreticular cells from the LPS-hyporesponsive C3H/HeJ mouse strain (12, 45).

* Corresponding author.
† Present address: Department of Microbiology, University of Iowa, Iowa City, IA 52242.

The gram-negative spirochetes *Leptospira interrogans* and *Borrelia burgdorferi* possess LPS-like components which have various biologic effects on the host when compared with those of classical LPS (3, 7, 42). The presence of an LPS-like material has also been described in outer membrane preparations of *Treponema pallidum*; however, the preparations were not pyrogenic (30).

Treponema hyodysenteriae and *Treponema innocens* have been described as anaerobic, β-hemolytic spirochetes that are found in the porcine large intestine (14, 15). *T. hyodysenteriae* has been shown to be the causative agent of swine dysentery (15), while *T. innocens* has been shown to be avirulent (14). Previous results have implicated LPS in the development of dysenteric lesions (29), have demonstrated that *T. innocens* is more susceptible to serum killing than *T. hyodysenteriae* (M. E. Nuessen, Ph.D. dissertation, Iowa State University, Ames, 1982), and have shown that the lipid composition is different for the two species (21).

To date, there have been no published studies in which the biologic activities of the LPS or endotoxin preparations from *T. hyodysenteriae* and *T. innocens* have been compared. The results of the present study indicate that the treponemal LPS preparations are much less stimulatory than *E. coli* LPS preparations and that the differences in virulence between the two treponemal species is not associated with the biologic activity of LPS or endotoxin.

MATERIALS AND METHODS

Animals. Original C3H/HeJ and BALB/cByJ breeder mice were obtained from Jackson Laboratory (Bar Harbor, Maine), and C3H/HeN breeder pairs were obtained from Harlan Sprague-Dawley (Madison, Wis.). Mice were housed at the Laboratory Animal Resources Facility at the College

of Veterinary Medicine, Iowa State University, Ames, Iowa. The mice were given autoclave-sterilized water and feed (Lab Chow 5010; Purina Mills, Inc., St. Louis, Mo.) ad libitum. New Zealand White rabbits were obtained from Small Stock Industries (Pearidge, Ark.) and housed at the Laboratory Animal Resources Facility.

Bacterial strains. *T. hyodysenteriae* B204 and *T. innocens* B1555a were used throughout these studies. *E. coli* K235 was obtained from Suzanne Michalek (Department of Microbiology, University of Alabama in Birmingham, Birmingham, Ala.). The *Treponema* species were grown in Trypticase soy broth (pH 7.3; BBL Microbiology Systems, Cockeysville, Md.) with glucose and containing 5% horse serum (Hyclone Laboratories, Inc., Logan, Utah), 0.5% yeast extract (BBL), 2.0% each of VPI A and B salt solutions (salt solution A, 0.04% $CaCl_2$, 0.04% $MgSO_4$; salt solution B, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 2.0% $NaHCO_3$), and 0.05% L-cysteine. Cultures were grown under anaerobic conditions in an atmosphere of 10% H_2–10% CO_2–80% N_2 for 18 to 24 h. The cells were harvested by centrifugation at $10{,}000 \times g$ for 20 min and washed twice in phosphate-buffered saline (0.8% NaCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4 [pH 7.2]) and once in distilled water. Whole cells were frozen at −20°C until the LPS or endotoxin extraction was performed.

LPS and endotoxin extraction. LPSs from *E. coli* and both species of *Treponema* were extracted by a modified hot phenol-water extraction procedure (45, 46). Endotoxin was prepared by the butanol-water extraction procedure (24).

The LPS and endotoxin preparations were dissolved in pyrogen-free saline and sterilized by heating the preparations at 100°C for 10 min. These solutions were stored at 4°C until use. The preparations were heated for 1 to 2 min at 100°C before use in the assays.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (17) by using a 1-mm, 7.5 to 15% polyacrylamide gradient separating gel with a 4% stacking gel. Gels were electrophoresed at 35 mA per gel (model SE600; Hoeffer Scientific Instruments, San Francisco, Calif.) for 3 to 4 h. The LPS (40 to 80 μg) and endotoxin (40 μg) samples were visualized by the silver stain procedure of Tsai and Frasch (41) or with Coomassie brilliant blue R250 stain.

Chemical determination. The protein content of the LPS and endotoxin preparations was measured as described by Lowry et al. (19), with bovine serum albumin used as a standard.

Carbohydrate content was determined by the phenol-H_2SO_4 method (1). The KDO content was determined by the thiobarbituric acid procedure of Karkhanis et al. (13). Phosphorus was determined by the method of Bartlett (2). Heptose was determined as described by Nowotny (26), using D-glycero-L-mannoheptose as a standard (kindly provided by R. Rimler, National Animal Disease Center, Ames, Iowa).

Gas-liquid and thin-layer chromatography. Derivation of the LPS was performed by the method of Bryn and Jantzen (6) with slight modifications. Briefly, 2 to 5 mg of bacterial LPS was suspended in 1 ml of 2 M HCl in methanol in a Teflon-lined (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) screw-cap vial and held at 85°C for 18 h. Methanolysates were concentrated to dryness at room temperature with nitrogen gas and were trifluoroacetylated by adding 0.2 ml of 50% trifluoroacetic anhydride (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in acetonitrile (high-pressure liquid chromatography grade; Fisher Scientific Co., Fair Lawn, N.J.) and heating the solution in a boiling water bath for 2 min. After cooling to room temperature, the reaction mixture was diluted with acetonitrile to a final trifluoroacetic anhydride concentration of 10% and injected into the column.

Gas chromatography was carried out in a gas chromatography system (402; Hewlett-Packard Co., Palo Alto, Calif.) equipped with a flame-ionization detector. Fused-silica capillary columns (25 m by 0.25 mm [inner diameter]; GB-1; Foxboro/Analabs, North Haven, Conn.; or 30 m by 0.25 mm [inner diameter]; SPB-1; Supelco, Bellefonte, Pa.) were operated in the split mode, with helium used as the carrier gas. The column temperature was held at 95°C for 5 min and then programmed to increase at 4°C/min up to 230°C. Peaks of individual standards and bacterial LPS were recorded with a recorder (2210; LKB, Bromma, Sweden), and retention times were calculated by determining the distance the individual peak moved from the injection line on the chromatogram. Sugar standards (rhamnose, fucose, ribose, galactose, mannose, glucose, glucosamine, and KDO) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Unhydroxylated fatty acids (C_{12} to C_{17}) were obtained from Alltech Associates, Inc., Applied Science Div. (State College, Pa.), and a bacterial fatty acid mixture was purchased from Supelco (catalog no. 4-7080). Hydroxylated fatty acids (3-OH-$C_{12:0}$, 2-OH-$C_{14:0}$, and 3-OH-$C_{14:0}$) were purchased from Foxboro/Analabs.

An acid-hydrolyzed (1 N HCl, overnight at room temperature) preparation of *T. hyodysenteriae* phenol-water-extracted LPS was applied to K6 silica gel plates (no. 4860-820; Whatman, Inc., Clifton, N.J.). Samples were separated with a solvent system consisting of 1-butanol–acetic acid–water (40:50:7.5). Following separation, plates were sprayed with an anisaldehyde reagent (1 ml of anisaldehyde was added to a mixture of 85 ml of methanol, 10 ml of acetic acid, and 5 ml of sulfuric acid) and heated for 60 min at 100°C to develop the spots. The R_f values for glucose and KDO were 0.47 and 0.38, respectively.

Mitogenesis. Mitogenesis was performed as described previously (45). Briefly, 5×10^5 murine splenocytes per well of a 96-well microtiter plate (no. 3799; Costar, Cambridge, Mass.) were cultured for 48 h in RPMI 1640 medium supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 25 U of penicillin per ml, 25 μg of streptomycin per ml, and 20 mM L-glutamine. Cells were incubated with the indicated doses of LPS or endotoxin at 37°C in 5% CO_2 in air. Cultures were pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine (Amersham Corp., Arlington Heights, Ill.) during the final 8 h of incubation. Cells were harvested onto filter paper disks by using a microharvester (Bellco Glass, Inc., Vineland, N.J.), dried, and counted by liquid scintillation. Cultures were performed in triplicate, and results are expressed as the stimulation index (experimental cpm/control cpm).

Lethality. The 50% lethal dose (LD_{50}) of the treponemal preparations was determined in galactosamine-sensitized (18) BALB/cByJ mice (age, 8 to 16 weeks) by the method of Reed and Muench (32). Briefly, mice (five per group) were provided drinking water containing 2 mg of galactosamine per ml ad libitum for 48 h. Mice were then given an intraperitoneal injection of 16 μg galactosamine followed by an intravenous (i.v.) injection of the indicated dose of LPS or endotoxin. Mice were observed for 48 h and deaths were recorded.

***Limulus* amebocyte lysate assay.** A chromogenic *Limulus* amebocyte lysate (LAL) assay (QCL-1000; Whittaker M.A.

TABLE 1. Chemical analysis of treponemal LPS and endotoxin

Component	% Weight of the indicated components in:			
	T. hyodysenteriae		*T. innocens*	
	LPS	Endotoxin	LPS	Endotoxin
Protein	<1.0	11.3	<1.0	26
Hexose	80.9	35.2	56.3	37.8
KDO[a]	0.12	0.45	0.45	0.4
Heptose	6.4	7.5	13.4	17.8
Phosphorus	1.5	ND[b]	ND	ND

[a] KDO determined as a thiobarbituric acid-reactive compound.
[b] ND, Not determined.

Bioproducts, Walkersville, Md.) was performed as described by the manufacturer.

Adjuvanticity. Both in vivo (45) and in vitro (20) assays were used to determine the adjuvant activity of the stimulants. In vivo, BALB/cByJ mice were given an intraperitoneal injection of saline containing the indicated dose of LPS or endotoxin and a suboptimal dose of sheep erythrocytes (0.5%). After 4 days, mice were sacrificed by cervical dislocation, and spleens were removed and minced to provide a single cell suspension and washed once in Hanks balanced salt solution; the spleen cells were then suspended in Hanks balanced salt solution. The anti-sheep erythrocyte plaque-forming cell (PFC) response was determined by the Cunningham slide method (20).

The in vitro immune response to sheep erythrocytes was examined by using spleen cells from BALB/cByJ and C3H/HeN mice that were previously primed with 0.05% sheep erythrocytes intraperitoneally 3 days earlier, and spleen cells were cultured as described previously (45). Briefly, cells were cultured at 5.0×10^6 cells per well of a 24-well cluster dish (No. 3424; Costar) for 5 days at 37°C in modular incubator chambers (Billups-Rothenberg, Del Mar, Calif.) flushed with a 7% O_2–10% CO_2–83% N_2 gas mixture. The anti-sheep erythrocyte PFC response was determined as described above.

Pyrogenicity. New Zealand White rabbits (weight, 3 to 4 kg) were injected i.v. in the marginal ear vein with LPS or endotoxin (50 to 250 μg) in 0.5 ml of pyrogen-free saline. Rectal temperatures were taken every 15 to 20 min.

Dermal Shwartzman reaction. New Zealand White rabbits (weight, 3 to 4 kg) were shaved 24 h before they received preparative intradermal (i.d.) injections of LPS or endotoxin in 0.1 ml of pyrogen-free saline. Twenty-four hours after the i.d. injection, a provocative dose of 50 μg of *E. coli* LPS in 0.2 ml of pyrogen-free saline was given i.v. into the marginal ear vein. The i.d. injection sites were observed for induration, erythema, and necrosis during the ensuing 24 h. Rabbits were euthanized 24 h after the provocative injections, and tissue samples were taken for routine histologic examination (16).

Reagents. Unless stated otherwise, all reagents and chemicals were obtained from Sigma.

Statistics. The results are expressed as the mean ± standard error of the mean when applicable, and significance was determined by Student's *t* test.

RESULTS

Chemical analysis of LPS and endotoxin. Results of the chemical analyses presented in Table 1 indicate that the treponemal LPS preparations contained negligible amounts of protein and a high percentage of carbohydrate (80.9% for *T. hyodysenteriae* and 56.3% for *T. innocens*). The endotoxin preparations, as expected, had protein concentrations ranging from 11.3 to 26%. The thiobarbituric acid-reactive component was determined to be between 0.12 and 0.45% in the preparations that were tested. In addition, the phenol-water preparation from *T. hyodysenteriae* was shown to contain 1.5% phosphorus.

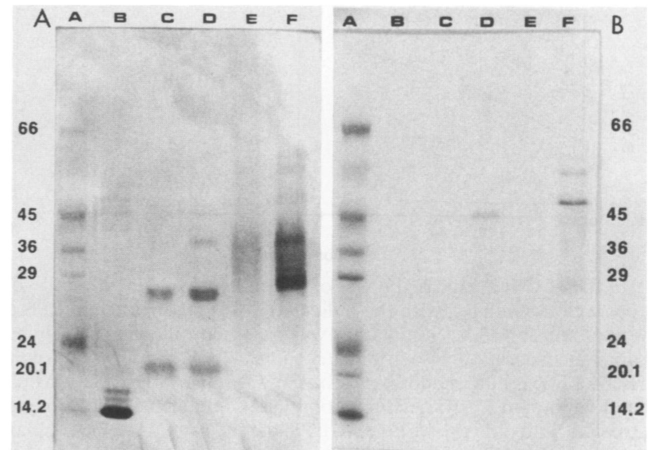

FIG. 1. Electrophoretic separations of LPS and endotoxins from *T. hyodysenteriae* B204 and *T. innocens* B1555a were performed on a 7.5 to 15% SDS-polyacrylamide gel. The LPS and endotoxin were visualized by silver (A) and Coomassie brilliant blue (B) staining. Lanes A, Molecular mass standards; lanes B, *E. coli* LPS (20 μg); lanes C, B204 LPS (80 μg); lanes D, B204 endotoxin (40 μg); lanes E, B1555a LPS (40 μg); lanes F, B1555a endotoxin (40 μg). Apparent molecular masses (in kilodaltons) are indicated on left side of panel A and on the right side of panel B.

The LPS preparation of *T. hyodysenteriae* was analyzed by gas-liquid chromatography, and the components were identified by comparing the retention times with those of known substances (data not shown). The sugars identified included fucose, galactose, glucose, glucosamine, heptose, mannose, rhamnose, and KDO. The retention times for the peaks identified as KDO were 20.27 and 21.36 min, and these peaks were identical to those obtained for KDO from *E. coli* LPS. In addition, the fatty acid constituents identified by retention times included myristic acid, 13-methyl-myristic acid, and 3-hydroxy-hexadecanoic acid (26.1, 27.9, and 33.0 min, respectively).

SDS-PAGE. Following SDS-PAGE, silver-stained polyacrylamide gels (Fig. 1A) indicated that four bands could be resolved from the *T. hyodysenteriae* LPS but not from the *T. innocens* LPS. The *T. innocens* LPS failed to resolve into distinct bands (Fig. 1A, lane E). However, *T. innocens* endotoxin (Fig. 1A, lane F) resolved into distinct bands, but its relative mobility in the gel was less than that of the *T. hyodysenteriae* (Fig. 1A, lanes C and D) or *E. coli* (Fig. 1A, lane B) preparations. Coomassie brilliant blue-stained gels (Fig. 1B) demonstrated the presence of a major protein band with an apparent molecular mass of 43.3 kilodaltons in the *T. hyodysenteriae* endotoxin preparation and proteins of 31, 46, and 57.5 kilodaltons in the *T. innocens* endotoxin preparation.

Mitogenic response. Treponemal endotoxins were observed as very potent mitogens for spleens cells from BALB/cByJ (Fig. 2) and C3H/HeN and LPS-hyporesponsive C3H/HeJ mice (data not shown). The treponemal LPS preparations failed to stimulate a measurable mitogenic response even at doses of 50 μg/ml.

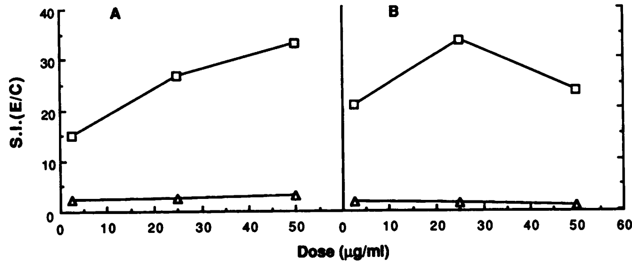

FIG. 2. Mitogenic responses of BALB/cByJ splenocytes (5 × 10^5 per well) stimulated with the indicated doses of treponemal LPS or endotoxin for 48 h. Stimulation was assessed following [^{3}H]thymidine incorporation into triplicate cultures. (A) *T. hyodysenteriae* B204 LPS (△) and endotoxin (□). (B) *T. innocens* B1555a LPS (△) and endotoxin (□). The stimulation index obtained for splenocytes treated with 2.5 μg of *E. coli* LPS per ml was 18.6. Values are expressed as a stimulation index (S.I.), which was defined as the experimental cpm/control cpm (E/C).

Endotoxin activity. The relative toxicities of LPS and endotoxin preparations were examined by mouse lethality and LAL assays. The LD_{50}s of *T. hyodysenteriae* LPS and endotoxin were determined to be 350 and 80 μg per mouse, respectively (data not shown). This is in comparison with results for *E. coli* K235 LPS, which had an LD_{50} of 0.6 μg (45).

Results of the LAL assay indicated that there were 1.2 and 1.4 units of endotoxin activity per ng of *T. hyodysenteriae* LPS and endotoxin, respectively. *T. innocens* LPS and endotoxin had 0.2 and 2.4 units of endotoxin activity per ng, respectively. This was comparable to the 1.2 units of endotoxin activity per ng obtained with LPS from *E. coli* K235.

Adjuvanticity. The adjuvant activity of LPS and endotoxin from both *Treponema* species was determined in vitro (Fig. 3) and in vivo (Fig. 4) by using sheep erythrocytes as the test antigen. The results depicted in Fig. 3 demonstrate that the treponemal LPS did not induce significant adjuvant activity in comparison with *E. coli* LPS or the treponema endotoxins. In vivo (Fig. 4), the *T. hyodysenteriae* LPS and endotoxin preparations enhanced the anti-sheep erythrocyte PFC responses at 100-μg doses ($P < 0.05$). Although the PFC responses were lower than those obtained with 10 μg of *E. coli* LPS, the differences were not significantly different.

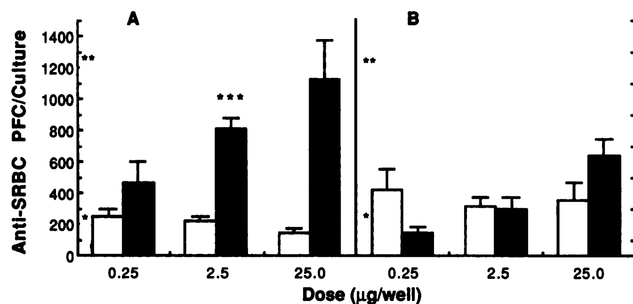

FIG. 3. C3H/HeN spleen cells (5 × 10^6 per well) were cultured in complete minimum essential medium with 10% fetal bovine serum. Spleen cells were cultured for 5 days in the presence of sheep erythrocytes (SRBC) and the indicated dose of LPS or endotoxin. On day 5 cells were harvested and the anti-sheep erythrocyte PFC response was determined. Values are expressed as the mean ± standard error of the mean. (A) Cultures stimulated with LPS (open bars) or endotoxin (shaded bars) from *T. hyodysenteriae* B204. (B) Cultures stimulated with LPS (open bars) or endotoxin (shaded bars) from *T. innocens* B1555a. Symbols: *, anti-sheep erythrocyte PFC response of unstimulated cultures; **, anti-sheep erythrocyte PFC response of cultures stimulated with 10 μg of *E. coli* LPS; ***, $P < 0.05$.

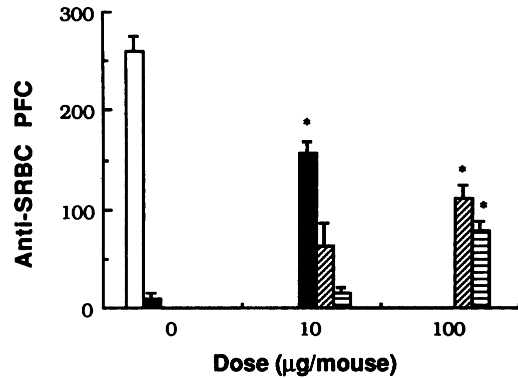

FIG. 4. The in vivo adjuvant activity of LPS or endotoxin from *T. hyodysenteriae* B204 was determined by injecting BALB/cByJ mice intraperitoneally with 0.2 ml of a 0.5% sheep erythrocyte (SRBC) suspension containing the indicated dose of LPS or endotoxin. On day 4, the animals were sacrificed and the anti-sheep erythrocyte PFC response per 10^6 splenocytes was determined. Activities of the following were determined: 10% sheep erythrocytes (open bar), 0.5% sheep erythrocytes (black bar), 0.5% sheep erythrocytes plus *E. coli* LPS (shaded bar), 0.5% sheep erythrocytes plus *T. hyodysenteriae* LPS (diagonally hatched bar), 0.5% sheep erythrocytes plus *T. hyodysenteriae* endotoxin (cross-hatched bar). Values are expressed as the mean ± standard error of the mean. *, $P < 0.05$.

Pyrogenicity. Rabbits received an i.v. injection of 50, 100, or 250 μg of the treponemal LPS or endotoxin preparations. The results presented in Fig. 5 demonstrate that 250 μg of the treponemal preparations was less pyrogenic than 10 μg of *E. coli* LPS. A mild febrile response was noted in rabbits treated with 250 μg of *T. hyodysenteriae* B204 endotoxin (Fig. 5A). The LPS or endotoxin preparation from *T. innocens* was not pyrogenic (Fig. 5B). There was no pyrogenic response observed in rabbits that received 50 or 100 μg of the treponemal preparations (data not shown).

Local Shwartzman reaction. Following i.d. injection of either treponemal endotoxin (50 to 500 μg), there was no

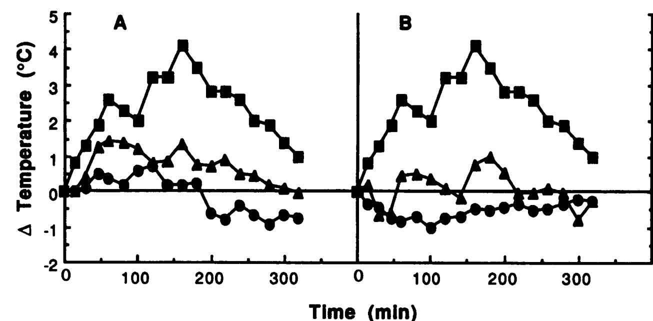

FIG. 5. The febrile responses of rabbits that received an injection of LPS or endotoxin were measured. Rectal temperatures were taken every 15 to 20 min. The results are expressed as the average change in temperature of two rabbits following treatment with either 250 μg of a treponemal preparation or 10 μg of *E. coli* LPS (■). (A) *T. hyosysenteriae* B204 LPS (●) and endotoxin (▲). (B) *T. innocens* B1555a LPS (●) and endotoxin (▲).

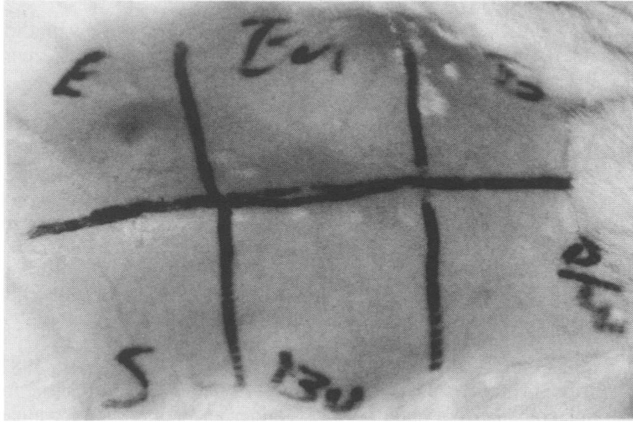

FIG. 6. Dermal Shwartzman reaction was examined in rabbits following an i.d. injection of LPS or endotoxin from each treponemal species or *E. coli* LPS. At 24 h after the i.d. injections, rabbits were given an i.v. injection in the marginal ear vein of either *T. hyodysenteriae* endotoxin or *E. coli* LPS. The rabbit depicted received an i.v. injection of *E. coli* LPS (50 μg). The i.d. injection pattern from left to right on the top row was *E. coli* LPS (100 μg), *T. hyodysenteriae* LPS (100 μg), and *Bacteroides fragilis* LPS (100 μg); on the bottom row the i.d. injection pattern from left to right was saline, *T. hyodysenteriae* endotoxin (250 μg), and *T. hyodysenteriae* LPS (200 μg).

consistent evidence of gross lesions (Fig. 6); however, histological examination of the tissue revealed multifocal areas of inflammation and edema only at sites that were treated with 500 μg of endotoxin (data not shown). No gross or histological lesions were observed at sites that were inoculated with either treponemal LPS preparation. Necrotic lesions were observed both microscopically (data not shown) and macroscopically following i.d. inoculation of 100 μg of *E. coli* LPS (Fig. 6).

DISCUSSION

Several lines of evidence suggest that the LPS of *T. hyodysenteriae* has biologic activity and may contribute to the pathogenesis of swine dysentery (27–29; Nuessen, Ph.D. dissertation). Besides *T. hyodysenteriae*, other spirochetes have also been shown to contain a LPS-like molecule (3, 7, 11, 22, 42). Mergenhagen et al. (22) have demonstrated the presence of endotoxic products in phenol-water extracts of *Borrelia buccalis* and *Borrelia vincentii*, and LPS-like components have been isolated from *Leptospira interrogans* (11, 42). The presence of LPS has also been reported in *B. burgdorferi* (3). However, Takayama et al. (40) have questioned the nature of LPS in *B. burgdorferi* since they were unable to induce prostaglandin E2 production or gelation of the LAL assay; nor could they demonstrate the presence of KDO, glucosamine, and hydroxy fatty acids in either a phenol-water extract or a phenol-chloroform-petroleum ether extract of the organism. Therefore, it appears that LPSs isolated from members of the class *Spirochaetales* may be quite different biologically and chemically from the LPSs isolated from other gram-negative microorganisms (i.e., *E. coli*).

The results of the present study indicate that the phenol-water and butanol-water preparations from *T. hyodysenteriae* and *T. innocens* contain LPS-like molecules. LPSs isolated from members of the family *Enterobacteriaceae* contain hydroxylated fatty acids, glucosamine, KDO, and phosphorus (25, 40). Based on retention times, myristic acid, 13-methyl-myristic acid, and 3-hydroxy-hexadecanoic acid fatty acids, as well as glucosamine, KDO, heptose, rhamnose, mannose, galactose, and glucose, were detected in phenol-water preparations from *T. hyodysenteriae* by gas-liquid chromatography (data not shown). The phenol-water- and butanol-water-extracted preparations from *T. innocens* and *T. hyodysenteriae* were shown to contain hexose, heptose, phosphorus, and a thiobarbituric acid-reactive component (Table 1). Although a thiobarbituric acid-reactive component (i.e., KDO) was detected, it should be stated that compounds other than KDO can be detected by this assay (13, 26). Consistent with results of the gas-chromatographic analysis, an acid-hydrolyzed component with the same R_f value as KDO (0.38) was detected by thin-layer chromatography (data not shown). However, this component developed as brown spots, in comparison with the purple spots obtained with the KDO standard. Therefore, without mass spectroscopic analysis, it may be premature to indicate that the LPSs of *T. hyodysenteriae* and *T. innocens* contain KDO.

Treponemal preparations were analyzed by SDS-PAGE, and two different profiles were obtained for the *T. hyodysenteriae* and *T. innocens* preparations (Fig. 1). In comparison with the *T. hyodysenteriae* LPS (Fig. 1A, lane C), the *T. innocens* LPS (Fig. 1A, lane E) had less relative mobility in a polyacrylamide gel and did not resolve into distinct bands. The endotoxin preparation of *T. innocens* was also less mobile in the gel but resolved into numerous bands (Fig. 1A, lane F). The differences observed in the SDS-polyacrylamide gels may be related to variations in the lipid contents between the two species, as reported previously by Matthews et al. (21). However, these apparent physicochemical differences did not result in various biologic activities between the *T. hyodysenteriae* and *T. innocens* LPS or endotoxin preparations as discussed below.

Neither of the treponemal LPS preparations was mitogenic (Fig. 2), enhanced in vitro antibody responses (Fig. 3), or was pyrogenic (Fig. 5). These results are in contrast to those of a previous report, in which *T. hyodysenteriae* LPS was shown to be mitogenic for murine spleen cells (27), but are consistent with the inability of Takayama et al. (40) to detect biologic activity in the LPS from *B. burgdorferi*. On the other hand, the treponemal endotoxin preparations induced mitogenic and adjuvant responses in murine splenocytes (Fig. 2 and 3) and a mild pyrogenic response in rabbits (Fig. 5). At a dose of 100 μg per mouse, *T. hyodysenteriae* LPS and endotoxin induced significant adjuvant responses ($P < 0.05$), but the magnitude of the response was not as great as that obtained in vivo with *E. coli* LPS (Fig. 4). The protein component(s) of endotoxin preparations has been shown to be biologically active (37, 38, 43, 44), and the data presented here indicate that the protein-containing complex is more biologically active than the protein-free LPS preparations.

In comparison with *E. coli* LPS (LD_{50}, 0.6 μg) (45), the *T. hyodysenteriae* B204 LPS (LD_{50}, 350 μg) and endotoxin (LD_{50}, 80 μg) preparations were at least 100 times less toxic for galactosamine-treated mice. The protein component of endotoxin appeared to augment the toxicity of the *T. hyodysenteriae* LPS molecule, but there is no reported evidence to suggest that lipid A-associated or porin proteins are toxic (37, 38, 43, 44). The endotoxic activity of the treponemal preparations was also indicated by the ability to induce gelation of the LAL assay. In contrast to mitogenesis, the *T. hyodysenteriae* endotoxin did not enhance the gelation of the LAL assay in comparison with LPS (1.4 and 1.2 endotoxin

units per ng, respectively). However, there was a 10-fold difference in the LAL assay activity between LPS and endotoxin from *T. innocens* (0.2 and 2.4 endotoxin units per ng, respectively).

In addition to murine toxicity and gelation of the LAL assay, a local Shwartzman reaction was used to indicate the relative toxicity of an endotoxic preparation. The treponemal preparations were unable to prime rabbits for a dermal Shwartzman reaction regardless of the source of the LPS used for the provocative dose (Fig. 6). On histological examination of the tissue, a mild inflammatory response was induced when 500 μg of treponemal endotoxin was injected intradermally (data not shown). Dermal necrosis was obtained when an *E. coli* LPS priming dose (50 μg) was followed by a provocative dose of *T. hyodysenteriae* endotoxin (>100 μg; data not shown). The ability of the treponemal endotoxin to serve as the provocative but not the priming dose appears somewhat contradictory, but it is likely that the actions of the two injections are not the same (4). It appears that the priming injection renders the host hyperresponsive to the provocative dose (4, 16, 34), and therefore, the host would be secondarily responsive to a less active endotoxin preparation. Even though the treponemal preparations were not as biologically active as *E. coli* LPS, they did possess some endotoxic properties and may have subtly contributed to the development of inflammatory lesions following infection with *T. hyodysenteriae*.

Taken collectively, these results indicate that *T. hyodysenteriae* and *T. innocens* contain a LPS-like molecule which can be extracted by conventional methods. The presence of protein in the endotoxin complex enhanced the biological activity of the LPS-like molecule. Even though the role of LPS in the development of dysenteric lesions is not fully understood, the virulence or avirulence of *T. hyodysenteriae* and *T. innocens* cannot be attributed to differences in the biologic activity of LPS or endotoxin preparations.

ACKNOWLEDGMENTS

We thank Linda Vandemark and S. K. Nibbelink for technical assistance, H. Ryu for performance of the gas-liquid chromatography, H. Mike Stahr for assistance with the thin-layer chromatography, D. Morfitt for histological examination of tissue sections, and F. Chris Minion and R. F. Rosenbusch for critical evaluation of the manuscript.

This study was supported in part by grant CRSR-2-25-83 from the U.S. Department of Agriculture, the National Pork Producers Council, and the Iowa Pork Producers Council.

LITERATURE CITED

1. **Ashwell, G.** 1966. New colorimetric methods of sugar analysis. Methods Enzymol. **8**:85–95.
2. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234**:466–468.
3. **Beck, G., G. S. Habicht, J. L. Benach, and J. L. Coleman.** 1985. Chemical and biologic characterization of a lipopolysaccharide extracted from the lyme disease spirochete (*Borrelia burgdorferi*). J. Infect. Dis. **152**:108–117.
4. **Billiau, A.** 1988. Gamma-interferon: the match that lights the fire? Immunol. Today **9**:37–42.
5. **Boivin, A., and L. Mesrobeanu.** 1935. Recherches sur les antigenes somatiques et sur les endotoxines des bacteries. 1. Considerations generales, et expose des techniques utilisees. Rev. Immunol. (Paris) **1**:553–569.
6. **Bryn, K., and E. Jantzen.** 1982. Analysis of LPS's of methanolysis, trifluoroacetylation, and gas chromatography on a fused-silica capillary column. J. Chromatogr. **240**:405–413.
7. **Cinco, M., E. Banfi, and E. Panfili.** 1986. Heterogeneity of lipopolysaccharide banding patterns in *Leptospira* spp. J. Gen. Microbiol. **132**:1135–1138.
8. **Galanos, C., O. Luderitz, and O. Westphal.** 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. **9**:245–249.
9. **Hofstad, T.** 1974. The distribution of heptose and 2-keto-3-deoxyoctanate in *Bacteroidaciae*. J. Gen. Microbiol. **85**:314–320.
10. **Hogan, M. M., and S. N. Vogel.** 1987. Lipid A-associated proteins provide an alternate "second signal" in the activation of recombinant interferon-gamma-primed, C3H/HeJ macrophages to a fully tumoricidal state. J. Immunol. **139**:3697–3702.
11. **Isogai, E., H. Isogai, Y. Kurebayashi, and N. Ito.** 1987. Biological activities of leptospiral lipopolysaccharide. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **261**:53–64.
12. **Joiner, K. A., K. P. W. J. McAdam, and D. L. Kasper.** 1982. Lipopolysaccharides from *Bacteroides fragilis* are mitogenic for spleen cells from endotoxin responder and nonresponder mice. Infect. Immun. **36**:1139–1145.
13. **Karkhanis, Y. D., J. Y. Zeltner, J. J. Jackson, and D. J. Carlo.** 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram negative bacteria. Anal. Biochem. **83**:595–601.
14. **Kinyon, J. M., and D. L. Harris.** 1979. *Treponema innocens*, a new species of intestinal bacteria and an amended description of the type strain of *Treponema hyodysenteriae*. Int. J. Syst. Bacteriol. **29**:102–109.
15. **Kinyon, J. M., D. L. Harris, and R. D. Glock.** 1977. Enteropathogenicity of various isolates of *Treponema hyodysenteriae*. Infect. Immun. **15**:638–646.
16. **Koga, T., C. Osaka, I. Moro, T. Fujiwara, T. Nishihara, N. Okahashi, and S. Hamada.** 1987. Local Shwartzman activity of lipopolysaccharides from several selected strains of suspected periodontopathic bacteria. J. Periodontal Res. **22**:103–107.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lehmann, V., M. A. Freudenberg, and C. Galanos.** 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. J. Exp. Med. **165**:657–663.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Marbrook, J.** 1980. Liquid matrix (slide method), p. 86–89. *In* B. B. Mishell and S. M. Shiigi (ed.), Selected methods in cellular immunology. W. H. Freeman and Co., San Francisco.
21. **Matthews, H. M., T.-K. Yang, and H. M. Jenkin.** 1980. *Treponema innocens* lipids and further description of an unusual galactolipid of *Treponema hyodysenteriae*. J. Bacteriol. **143**:1151–1155.
22. **Mergenhagen, S. E., E. G. Hampp, and H. W. Scherp.** 1961. Preparation and biological activities of endotoxins from oral bacteria. J. Infect. Dis. **108**:304–310.
23. **Morrison, D. C., S. J. Betz, and D. M. Jacobs.** 1976. Isolation of a lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C3H/HeJ spleen cells. J. Exp. Med. **144**:840–846.
24. **Morrison, D. C., and L. Leive.** 1975. Fractions of lipopolysaccharide from *Escherichia coli* O111:B4 prepared by two extraction procedures. J. Biol. Chem. **250**:2911–2919.
25. **Morrison, D. C., and R. J. Ulevitch.** 1978. The effects of bacterial endotoxins on host mediation systems. Am. J. Pathol. **93**:526–617.
26. **Nowotny, A.** 1979. Basic exercises in immunochemistry. A laboratory manual, 2nd ed., p. 140–185. Springer-Verlag, Berlin.
27. **Nuessen, M. E., J. R. Birmingham, and L. A. Joens.** 1982. Biological activity of a lipopolysaccharide extracted from *Treponema hyodysenteriae*. Infect. Immun. **37**:138–142.
28. **Nuessen, M. E., and L. A. Joens.** 1982. Serotype-specific opsonization of *Treponema hyodysenteriae*. Infect. Immun. **38**:1029–1032.
29. **Nuessen, M. E., L. A. Joens, and R. D. Glock.** 1983. Involve-

ment of lipopolysaccharide in the pathogenicity of *Treponema hyodysenteriae*. J. Immunol. **131**:997–999.
30. **Penn, C. W., A. Cockayne, and M. J. Bailey.** 1985. The outer membrane of *Treponema pallidum*: biological significance and biochemical properties. J. Gen. Microbiol. **131**:2349–2357.
31. **Pier, G. B., R. B. Markham, and D. Eardley.** 1981. Correlation of the biological responses to C3H/HeJ mice to endotoxin with the chemical and structural properties of the lipopolysaccharides from *Pseudomonas aeruginosa* and *Escherichia coli*. J. Immunol. **127**:184–191.
32. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating 50% endpoints. Am. J. Hyg. **27**:493–497.
33. **Roenstreich, D. L., L. M. Glode, L. M. Wahl, A. L. Sandberg, and S. E. Mergenhagen.** 1977. Analysis of the cellular defects of endotoxin unresponsive C3H/HeJ mice, p. 314–320. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
34. **Shwartzman, G.** 1928. Studies on *Bacillus typhosus toxis* substances. I. Phenomenon of local skin reactivity to *B. typhosus* culture filtrate. J. Exp. Med. **38**:247–268.
35. **Spellman, J. M., and N. D. Reed.** 1979. Immune and mitogenic responses by BALB/c, C3H/HeJ, and nude mice to *Brucella abortus* bacteria and lipopolysaccharide. Infect. Immun. **24**: 371–378.
36. **Staub, A. M.** 1965. Bacterial lipido-proteino-polysaccharides ('O' somatic antigens) extraction with trichloroacetic acid. Methods Carbohydr. Chem. **5**:92–96.
37. **Strittmatter, W., and C. Galanos.** 1987. Characterisation of protein co-extracted together with LPS in *Escherichia coli*, *Salmonella minnesota*, and *Yersinia enterocolitica*. Microb. Pathog. **2**:29–36.
38. **Sultzer, B. M., G. W. Goodman, and T. K. Eisenstein.** 1980. Endotoxin protein as an immunostimulant, p. 61–65. *In* D. Schlessinger (ed.), Microbiology—1980. American Society for Microbiology, Washington, D.C.
39. **Sveen, K., T. Hofstad, and K. C. Milner.** 1977. Lethality for mice and chick embryos, pyrogenicity in rabbits and ability to gelate lysate from amoebocytes of Limulus polyphemus by lipopolysaccharides from *Bacteroides*, *Fusobacterium* and *Veillonella*. Acta Pathol. Microbiol. Scand. Sect. B **85**:388–396.
40. **Takayama, K., R. J. Rothenberg, and A. B. Barbour.** 1987. Absence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*. Infect. Immun. **55**:2311–2313.
41. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biol. **119**:115–119.
42. **Vinh, T., B. Adler, and S. Faine.** 1986. Ultrastructure and chemical composition of lipopolysaccharide extracted from *Leptospira interrogans* serovar *copenhageni*. J. Gen. Microbiol. **132**:103–109.
43. **Vordermeier, H. M., and W. G. Bessler.** 1987. Polyclonal activation of murine B lymphocytes in vitro by *Salmonella typhimurium* porins. Immunobiology **175**:245–251.
44. **Vordermeier, H. M., K. Stab, and W. G. Bessler.** 1986. A defined fragment of bacterial protein I (omp F) is a polyclonal B-cell activator. Infect. Immun. **51**:233–239.
45. **Wannemuehler, M. J., S. M. Michalek, E. J. Jirillo, S. I. Williamson, M. Hirasawa, and J. R. McGhee.** 1984. LPS regulation of the immune response: *Bacteroides* endotoxin induces mitogenic, polyclonal, and antibody responses in classical LPS responsive but not C3H/HeJ mice. J. Immunol. **133**:299–305.
46. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol/water and further application of the procedure. Methods Carbohydr. Chem. **5**:83–91.
47. **Wollenweber, H. W., E. T. Rietschel, T. Hofstad, A. Weintraub, and A. A. Lindberg.** 1980. Nature, type of linkage, quantity, and absolute configuration of (3-hydroxy) fatty acids in lipopolysaccharides from *Bacteroides fragilis* NCTC 9343 and related strains. J. Bacteriol. **144**:898–903.