INFECTION AND IMMUNITY, Feb. 1975, p. 371-379

Vol. 11, No. 2
Printed in U.S.A.

Characterization of Endotoxin from *Fusobacterium necrophorum*

M. M. GARCIA,* K. M. CHARLTON, AND K. A. McKAY

Animal Pathology Division, Health of Animals Branch, Animal Diseases Research Institute, (E), Box 11300, Station H, Ottawa, Ontario K2H 8P9 Canada

Received for publication 2 December 1974

Endotoxic lipopolysaccharide (LPS) was obtained from phenol-water extraction of cell walls prepared from mass-cultivated *Fusobacterium necrophorum*. The LPS was relatively free of nucleic acids and low in protein, and constituted about 4% of the cell walls. Upon acid hydrolysis, some of the components detected were hexosamines (7.0%), neutral and reducing sugars (50.5%), heptose (6.4%), 2-keto-3-deoxyoctonate (0.8%), lipid A (21.0%), and phosphorus (1.7%). Under electron microscopy the LPS appeared mainly as ribbon-like trilaminar structures, and upon chemical treatment it displayed a behavior resembling that reported in certain enterobacterial LPS. The LPS was lethal to mice, 11-day-old chicken embryos, and rabbits. Endotoxicity in mice was enhanced at least 1,380-fold by the addition of 12.5 μg of actinomycin D. Induced tolerance to lethal effect of the endotoxin and rapidly acquired resistance to infection by *F. necrophorum* viable cells were also demonstrated in mice. The endotoxin produced both localized and generalized Shwartzman reactions as well as biphasic pyrogenic responses in rabbits. These results firmly establish the presence of a classical endotoxin in *F. necrophorum*, thus providing strong support to our recent suggestion that cell wall-associated components may contribute significantly to the pathogenicity of *F. necrophorum*.

Until recently, evidence for the presence of a toxic lipopolysaccharide (LPS) in *Fusobacterium necrophorum* cultures has been based primarily on the findings of Kirchheiner (9), who showed that trichloroacetic acid extracts from *F. necrophorum* cells were toxic to mice. About 3 years ago, Hofstad and Kristoffersen (7) described biochemically LPS preparations extracted with phenol-water from three strains of *F. necrophorum*. However, these preparations did not provoke a localized Shwartzman reaction consistently, leaving some doubt as to the relative endotoxicity of the preparations. More recently, Sonnenwirth et al. (20) detected endotoxins from members of the family *Bacteroidaceae*, including clinical isolates of *F. necrophorum* by the rapid and sensitive *Limulus* endotoxin assay. Information on the biological characteristics and the relative endotoxicity of *F. necrophorum* LPS for experimental animals, however, remains unknown.

The present report describes a biologically potent LPS in *F. necrophorum* cell walls that may play a prime role in *F. necrophorum* infections. Data from this investigation are useful in our continuing study of the pathogenesis of liver abscesses in cattle.

MATERIALS AND METHODS

Culture. *F. necrophorum* (*Sphaerophorus necrophorus*) LA19 was isolated from a bovine liver abscess and was positively identified by procedures described in a previous study (5). The organism was cultivated in bulk with modified Casitone medium (4).

Preparation of cell walls. Cell walls were prepared as described elsewhere (submitted for publication). Saline-washed whole cells were adjusted to 15 mg (dry weight)/ml and disrupted in an MSE sonic vibrator (Measuring & Scientific Equipment Ltd., West Lake, Ohio) for 18 min at 4 C. The sonically treated material was centrifuged at 18,000 × *g* for 15 min, and the resultant sediment, the crude cell envelopes, was twice washed with 1 M NaCl and at least five times with distilled water. This series of washings was terminated only after samples of the cell walls, examined with an ultraviolet spectrophotometer, no longer showed a discrete absorbance peak at 260 nm. Uniformity of the cell walls was ascertained by electron microscopic examination of samples negatively stained with 1% phosphotungstic acid (PTA). Infrared spectra of cell wall preparations from different batches exhibited a principal band with an extinction at 1,660 cm^{-1} corresponding to an amide-1-band (2).

Extraction of endotoxin. An initial comparison of various extracting agents, using both whole cells and cell walls, revealed that the phenol-water extraction

of cell walls yielded the most homogeneous and potent preparation. The endotoxin was associated with the aqueous layer. This method essentially follows that used by Clarke et al. (2) for the extraction of *Pseudomonas aeruginosa* endotoxin. Briefly, this technique involved the treatment of approximately 200 mg of cell walls with 30 ml of aqueous 45% (wt/vol) phenol at 74 C for 30 min. The aqueous layer was separated from the phenol layer by centrifugation at 2,000 × *g* for 1 h at 4 C. Residual phenol was removed by dialysis for 3 days at 4 C, and the phenol-free LPS preparation was stored by freeze-drying.

Electron microscopy. Freeze-dried LPS was suspended in double-distilled water at a concentration of 1 mg/ml. Drops of the suspension were placed on Formvar carbon-coated grids and stained with either 2% uranyl acetate, pH 4.5, or 1% phosphotungstic acid. The stained specimens then were examined with an electron microscope (Philips EM201) operated at 60 kW. LPS preparations treated with water-saturated ether (18) and sodium deoxycholate (17), or sonically treated for 1 min, were likewise examined to assess the behavior of the LPS in comparison to other enterobacterial LPS.

For thin sectioning, a solution of LPS was spun at 100,000 × *g* for 3 h at 4 C, and the resulting pellet was fixed overnight in 3% glutaraldehyde, pH 7.2. After the LPS pellets were washed with Veronal buffer, pH 6.0, they were postfixed with 2% OsO_4 for 6 h. The fixed sample then was dissolved in distilled water, stained with 2% uranyl acetate for 2 h, dehydrated in ethyl alcohol, and embedded in Spurr low-viscosity embedding media (21). Ultrathin sections were post-stained with uranyl acetate for 5 min and lead citrate for 10 min.

Chemical analyses of LPS preparation. Protein content was determined by the Folin-Ciocalteau method of Lowry et al. (10), with bovine albumin fraction V as a standard. Hexosamine was estimated by the method of Rondle and Morgan (16) after acid hydrolysis for 4 h. The amount of reducing sugars was determined by the Nelson test (12), whereas the neutral sugars were analyzed by the Winzler orcinol method (23). Anthrone determination (6) was carried out for hexose from unhydrolyzed samples. Heptose and 2-keto-3-deoxyoctonate (KDO) were assayed by Osborn's modified procedures (13). Phosphorus was estimated by the Raabe colometric method (14) after ashing. To obtain crude lipid A, the LPS preparation was dissolved in 1% (vol/vol) acetic acid and hydrolyzed in a sealed ampoule, under nitrogen, at 100 C for 4 h (22). The resultant white precipitate was washed several times with distilled water and lyophilized.

Mouse L_{50}. Groups of six Swiss albino female mice, each weighing 20 ± 1 g, were injected with twofold dilutions of the LPS preparation. Each animal received an intraperitoneal (i.p.) injection of 1.0 ml of the preparation. Mice injected with pyrogen-free saline served as controls. The mice were observed up to 5 days post-inoculation, and the mean lethal dose (LD_{50}) was calculated by the method of Reed and Muench (15).

Chicken embryo LD_{50}. Eleven-day-old White Leghorn chicken embryos were inoculated intravenously (i.v.) with 0.1 ml of various LPS dilutions. The embryos were checked after 24 and 48 h. Chicken embryo LD_{50} ($CELD_{50}$) was then estimated (11).

Potentiating effect of actinomycin D. Mixtures of LPS and actinomycin D (Merck, Sharpe and Dohme) were injected i.p. into groups of 10 mice, each receiving 1.0 ml of the mixture. Actinomycin D was added in amounts of 10, 12.5, 15 or 20 μg/ml of mixture. Control mice were injected with actinomycin D alone. LD_{50} calculations were made after a 5-day observation period.

Induced tolerance to endotoxin effect and acquired resistance to infection. Both tests were carried out by the procedure of Albizo et al. (1). For the acquired resistance test, the mice were inoculated i.p. with *F. necrophorum* LPS and challenged 24 h later with 0.5 ml of an actively growing *F. necrophorum* culture containing a direct microscopic count of 1.15 × 10^9 cells.

Pyrogenicity in rabbits. Two groups of four female rabbits were monitored for body temperature with a rectal thermistor. One group was injected i.v. with 0.2 ml of pyrogen-free saline and the other was injected with 0.2 ml of saline containing 1 μg of LPS. Readings were recorded at 20-min intervals from 2 h before to 6 h after LPS administration.

Localized Shwartzman reaction. Before the demonstration of the localized Shwartzman reaction, a skin test was carried out on 12-week-old New Zealand female rabbits. Dilutions of LPS were injected intradermally on the sides of the animals. Within 24 h post-inoculation, erythematous to necrotic lesions appeared at the injection sites.

The localized Shwartzman test (19) was done on 4-, 8-, 12-, 16-, and more than 16-week-old rabbits. An initial dose consisting of 5 to 50 μg of LPS in 0.1 ml was administered intradermally at various places on the animals' sides. The challenge dose, consisting of 10 to 20 μg of LPS in 0.1 ml, was injected into the marginal ear veins 24 h later. Rabbits that developed local lesions typical of the Shwartzman phenomenon within 4 h post-inoculation were considered positive reactors.

Generalized Shwartzman reaction. Eight New Zealand rabbits (2.5 kg) were injected i.v. with 10 to 40 μg of LPS in 0.1 ml and challenged 24 h later with an i.v. injection of 20 to 40 μg of the same preparation. Control animals were injected twice with pyrogen-free saline. At 24 and 48 h after challenge, animals from each group were killed and necropsied immediately. The viscera were removed and selected tissues were fixed in 10% formalin in phosphate-buffered saline, pH 7.1. Pieces of tissue were dehydrated, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

RESULTS

Electron microscopy. Uranyl acetate-stained *F. necrophorum* LPS preparation appeared mainly as a matrix of freely branched

filaments with an average diameter of 9.5 nm (Fig. 1). At various places, shorter LPS segments (arrows) revealed a distinct trilaminar dense-light-dense appearance. These segments were narrow (5.0 nm) and appeared to be the thin edges of the twisted LPS ribbon which, for the most part, tended to lie on its widest surface (18). A few disks ranging in diameter from 70 to 200 nm showed varying intensity of staining. Similar forms were observed from *F. necrophorum* N167 LPS with sizes ranging from 40 to 100 nm and were thought to be aggregated LPS ribbons (8). Thin sections of the LPS revealed the predominance of trilaminar rods (Fig. 2). The addition of 2% sodium deoxycholate dissociated the LPS rods into subunits of short rods or vesicular structures (Fig. 3), but this effect was reversed upon removal of sodium deoxycholate by dialysis. Drastic agitation or brief sonic treatment of the LPS in water saturated with diethyl ether produced occasional splitting of the trilaminar LPS into monolaminar subunits similar to those obtained by Shands et al. (18).

Chemical composition. Data presented in Table 1 indicate that *F. necrophorum* LPS contains the major components found in classical endotoxins. These include: reducing and

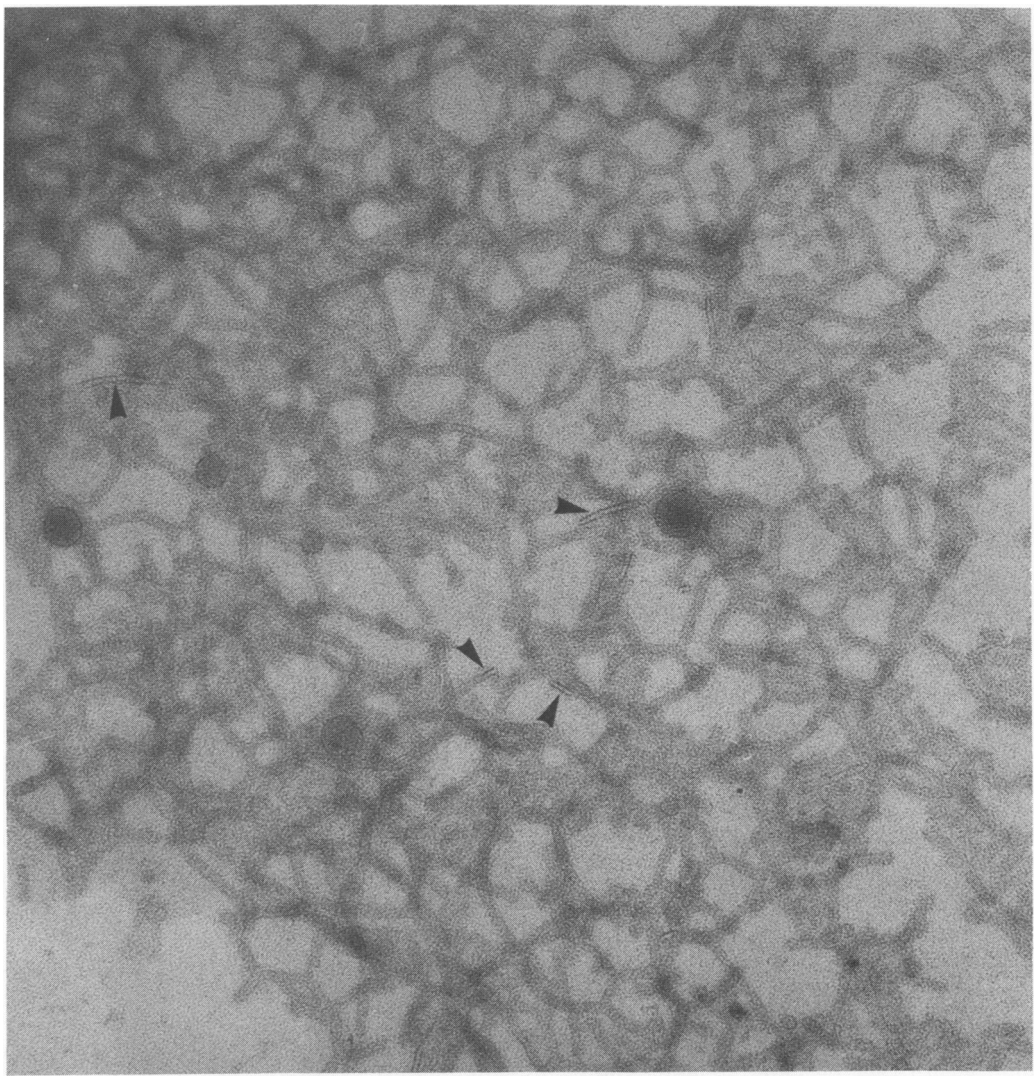

FIG. 1. *LPS from F. necrophorum LA19 stained with uranyl acetate.* ×*124,420*

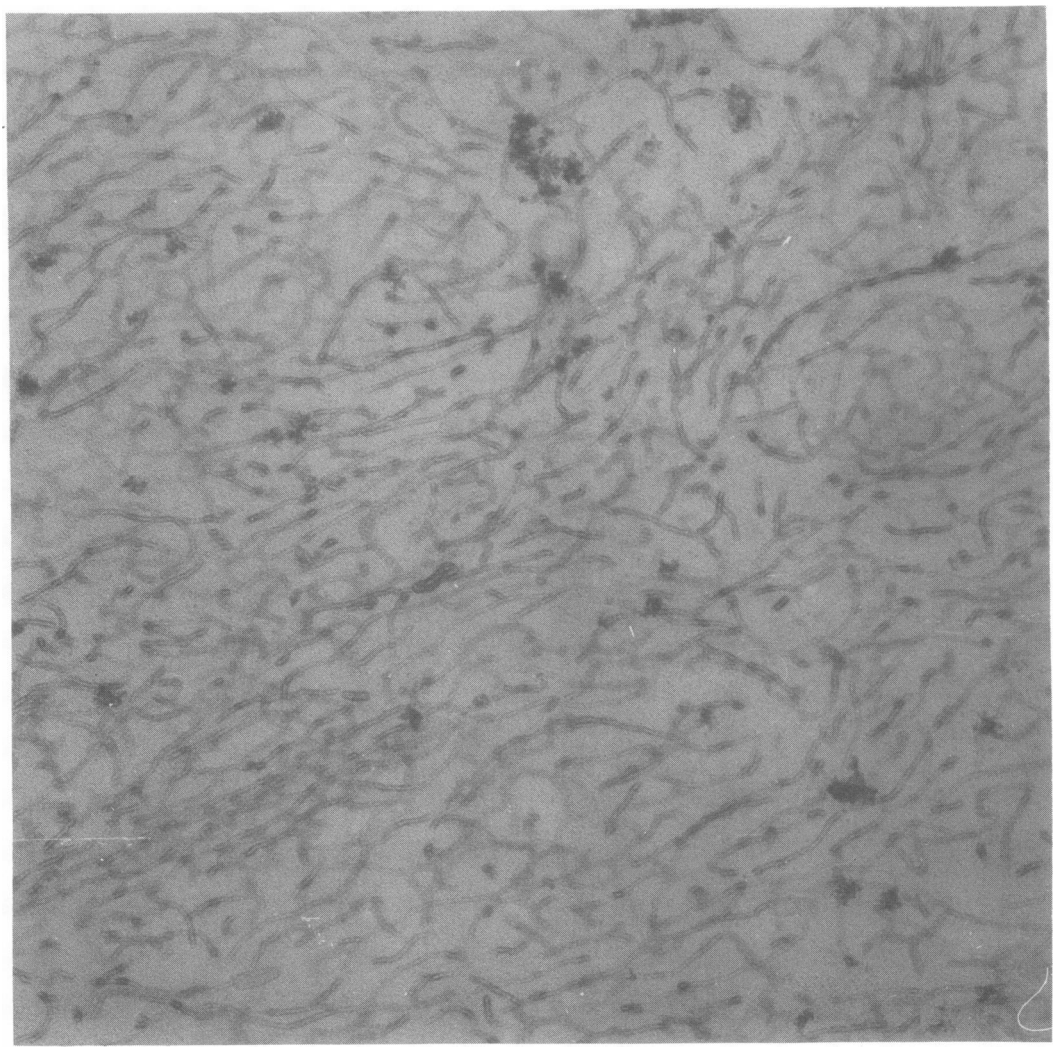

FIG. 2. *Thin section of LPS from F. necrophorum LA19 stained with uranyl acetate and lead citrate.* ×*122,880*

neutral sugars (50.5%), hexosamines (8.5%), KDO (0.8%), and lipid A (21.0%). The protein content was low (2.4%) and there were no detectable nucleic acids, as indicated by the absence of an absorption peak at 260 nm in the ultraviolet spectrum. Two of the major sugars determined were hexose (23.5%) and heptose (6.4%).

Toxicity of F. necrophorum LPS. Mice injected with lethal doses of LPS showed initial signs of illness within 2 h after injection. The animals generally displayed labored breathing, uncoordinated movement, and loss of appetite. Before death they became immobile and were cyanotic. Most of the mice died within 48 h after injection. A mouse LD_{50} value of 584 μg was obtained for this endotoxin. A parallel test using *Escherichia coli* O111:B4 LPS (Difco) produced an LD_{50} of 555 μg.

Eleven-day-old chicken embryos proved to be highly susceptible to the effect of endotoxin and thus were used extensively to test endotoxin activity. The $CELD_{50}$ for *F. necrophorum* LPS was 0.002 μg (Table 2). Heated LPS (100 C, 1 h) showed somewhat reduced toxicity ($CELD_{50}$ = 0.016 μg). Free lipid A was relatively aggregated and nontoxic; however, this effect was reversed when lipid A solubilized with bovine serum albumin (3) was used. The toxicity of the latter ($CELD_{50}$ = 0.071 μg), however, was still less

than the parent LPS ($CELD_{50}$ = 0.002 μg).

Potentiating effect of actinomycin D on endotoxicity. Actinomycin D enhanced the lethal effect of *F. necrophorum* endotoxin in dose-effect relationship. Mice injected with 10 to 20 μg of actinomycin D became more susceptible to the endotoxin by 11.6 to 29,200 times (Table 3). There were no obvious signs of illness in mice injected with 12.5 μg of antibiotic alone, but those receiving higher doses suffered up to 20% mortality. Notwithstanding the difference in the antibiotic lot and mouse strain used, the present results agree well with those of Wong et al. (24), who observed 23% mortality among mice receiving 20 μg of actinomycin D alone.

Induced tolerance in mice to endotoxins and acquired resistance to infection. Mice inoculated with small doses of *F. necrophorum* endotoxin tolerated subsequent challenge (after 3 days) of a lethal dose of the endotoxin (Table 4). Similarly treated mice also became resistant to a challenge of a 24-h viable culture of *F. necrophorum* (Table 5). In contrast, saline-injected mice succumbed to both harsh challenges. Rapidly acquired resistance in mice to infection was induced by injecting as little as

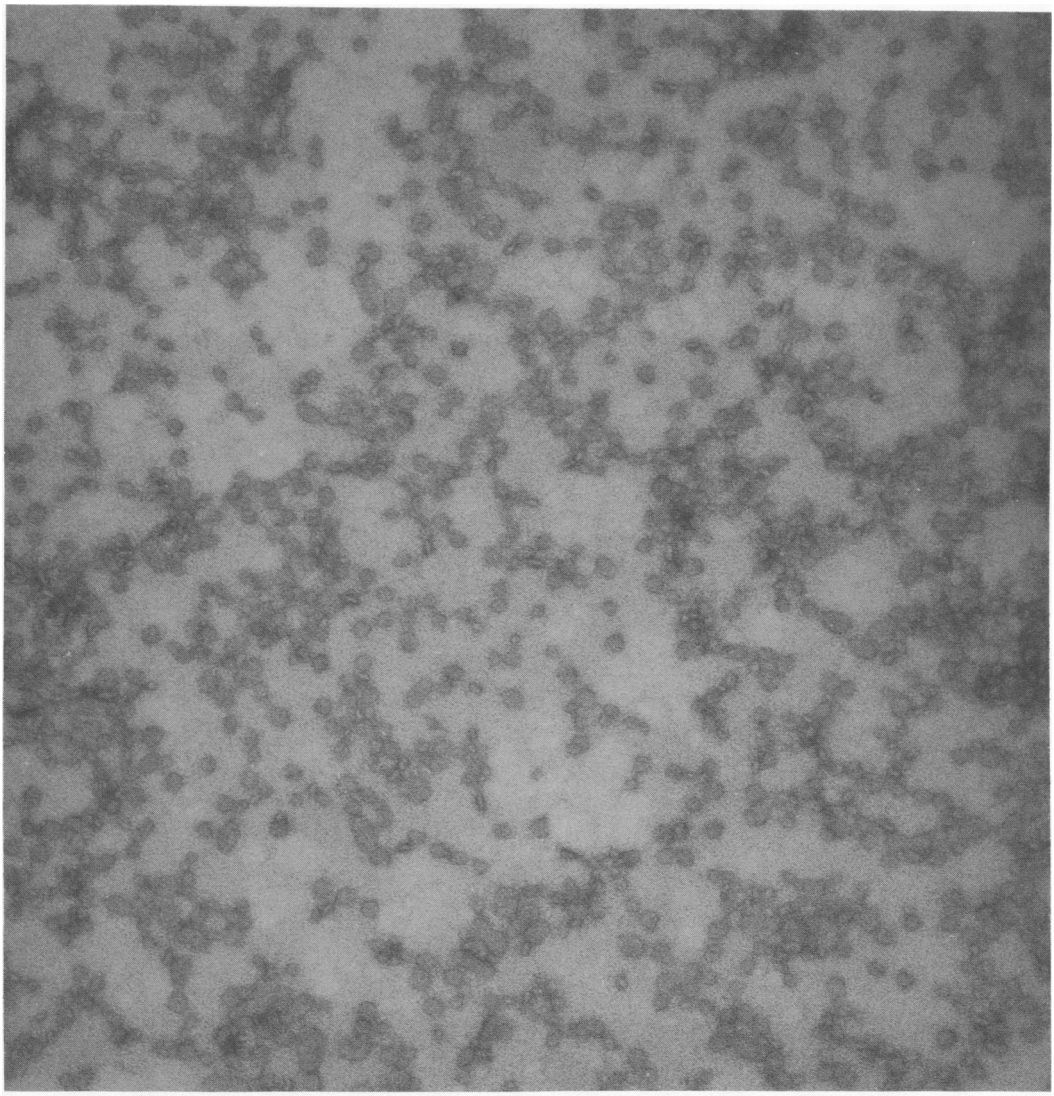

FIG. 3. *LPS from F. necrophorum LA19 treated with 2% sodium deoxycholate followed by alcohol extraction; LPS stained with uranyl acetate.* ×127,720.

TABLE 1. *Percentage major chemical composition of lipopolysaccharide preparation extracted from Fusobacterium necrophorum cell walls*

Nitrogen	2.1
Protein	2.4
Neutral sugars	23.4
Reducing sugars	27.1
Hexosamines	8.5
KDO	0.8
Lipid A	21.0
Phosphorus	1.7

TABLE 2. *Lethal effect of F. necrophorum lipopolysaccharide and lipid A for chicken embryos*

Material	$CELD_{50}$ (μg)
Lipopolysaccharide	0.002
Heated lipopolysaccharide, 100 C, 1 h	0.016
Free lipid A	>10.000
Lipid A, complexed with BSA (1:0.5)	0.071
BSA alone	—

TABLE 3. *Effect of actinomycin D on the lethality of F. necrophorum endotoxin*

Actinomycin D added (μg)	Mouse LD_{50} (μg)	Enhancement factor	Dead from antibiotic alone (%)
None	584		
10	50	11.6	0
12.5	0.422	1,383.9	0
15	0.195	2,994.8	10
20	0.002	29,200.0	20

TABLE 4. *Induction of tolerance in mice to the lethal effect of F. necrophorum and Escherichia coli endotoxins*

Material	No. of mice surviving/ total no. challenged[a]	
	F. necrophorum	*E. coli*
Saline on days 1 and 3	0/20	0/10
F. necrophorum endotoxin, 1 μg on day 1 and 10 μg on day 3	16/20	10/10

[a] Challenge dose: 1,000 μg of endotoxin on day 5.

0.05 μg of endotoxin, or approximately 10,000 times less than its LD_{50} value. Sera from mice surviving the challenge of *F. necrophorum* culture produced a precipitating antibody against the pathogen, suggesting an adjuvant action of the endotoxin on the immune response at the latter stage of resistance.

Pyrogenicity in rabbits. A biphasic response was obtained in rabbits injected with 1 μg of endotoxin (Fig. 4). The fever curve indicates the difference in the average temperature of the endotoxin-injected rabbits over the saline-injected rabbits. Typical temperature peaks were observed at 1.25 and 3 h after inoculation. A higher body temperature was maintained for more than 6 h with endotoxin-injected animals compared to the saline-injected animals.

Production of localized and generalized Shwartzman reactions. Results of the localized Shwartzman reaction induced by *F. necrophorum* endotoxin are presented in Table 6. All experimental animals reacted positively to the test. However, one rabbit older than 16 weeks did not show any response on one side to the initial injection of 5 and 10 μg and challenged with 15 μg of endotoxin. The overall result appears to be an improvement over that obtained by Hofstad and Kristoffersen (7), who used 6-month-old rabbits and did not obtain consistent dermal Shwartzman reaction with their *F. necrophorum* LPS. In the present experiment, we observed that 4- to 12-week-old

TABLE 5. *Rapidly acquired resistance in mice to F. necrophorum LA19 infection*

LPS (μg)	No. of survivors from 20 mice injected with bacterial culture[a]		
	1 week PI[b]	2 weeks PI	3 weeks PI
1.0	20	18	17
0.5	20	17	17
0.25	20	18	18
0.10	20	19	19
0.05	20	14	13
Saline	10	2	0

[a] Twenty-four-hour culture, 1.15×10^9 cells.
[b] PI, Postinoculation.

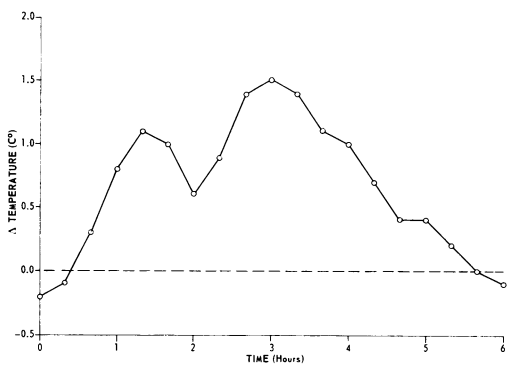

FIG. 4. *Biphasic pyrogenic response in New Zealand rabbits to the injection of 1 μg of LPS.*

rabbits showed better response than older ones. Figure 5 illustrates lesions resulting from a typical localized Shwartzman reaction assay. All lesions appeared within 4 h after challenge.

Rabbits subjected to successive i.v. administration of endotoxin developed a severe generalized Shwartzman reaction. In fact, one of the four rabbits injected initially with 20 μg of LPS and provoked with the same dose 24 h later later suffered endotoxic shock and died 3 h after provocation. No microscopic changes occurred in the organs of saline-injected control animals. By contrast, livers of rabbits which received LPS injections were swollen and dark red-brown with several gray foci of necrosis surrounded by hemorrhage. The kidneys were swollen and dark red; the lungs were dark red and contained many petechial hemorrhages, especially in the diaphragmatic lobes. The spleens were slightly enlarged.

Microscopic examination revealed extensive intravascular coagulation with fibrin thrombi in capillaries of the renal glomeruli, in the central veins of the liver, and in the sinuses of the spleen. In the kidney, the fibrin occurred as fibrillar dark pink material in the capillaries of the glomeruli (Fig. 6). Extensive areas of necrosis of tubules were observed in the cortex. Thrombi in the liver were attached to the endothelium of hepatic veins and were partially covered by endothelial cells (Fig. 7). Many of the thrombi in these organs contained a few necrotic cells and heterophils.

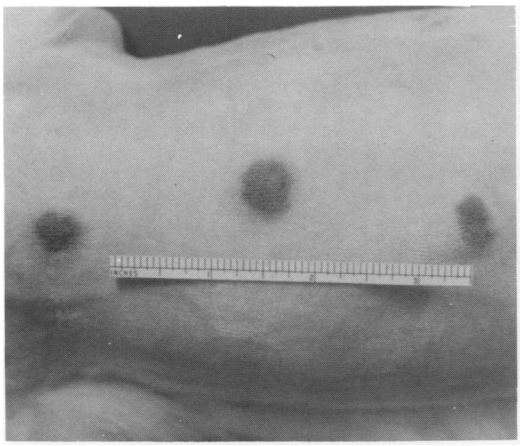

FIG. 5. *Typical localized Schwartzman reaction 4 h after the provoking dose. Saline-injected sites did not show any reaction.*

DISCUSSION

Data from this study prove that *F. necrophorum* produces an active LPS that resembles the physicochemical and biological be-

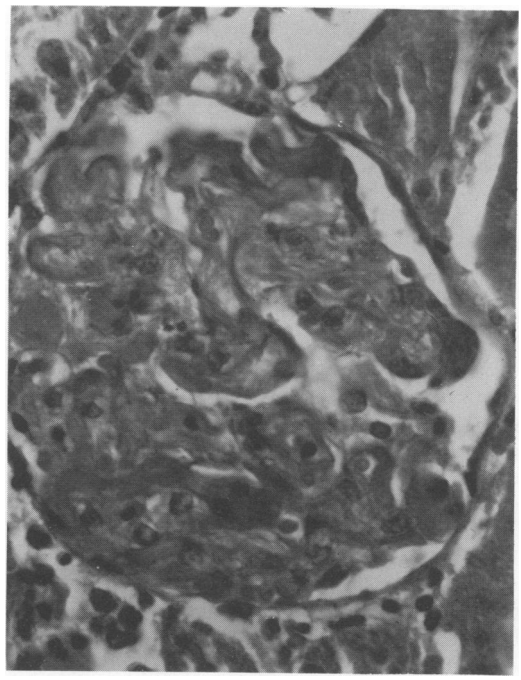

FIG. 6. *Generalized Shwartzman reaction. Rabbit injected i.v. with 10 μg of LPS and challenged 24 h later with 20 μg of LPS. Fibrin thrombi in capillaries of renal glomerulus.* ×510.

TABLE 6. *Production of local Shwartzman reaction in rabbits*

Age of rabbits (weeks)	No. of animals with positive reactions/no. injected[a]						
	50 μg	40 μg	30 μg	20 μg	10 μg	5 μg	2.5 μg
4	5/5	5/5		5/5	5/5		
8	5/5	5/5	5/5	5/5	5/5	5/5	
12	5/5	5/5	5/5	5/5	5/5	5/5	
16 weeks and over	4/4	6/6	4/4	6/6	5/6[b]	5/6[b]	2/2

[a] Challenge doses: 4-week-old rabbits, 10 μg i.v.; 8- and 12-week-old rabbits, 20 μg i.v.; 16 weeks and over, 15–30 μg i.v.

[b] In one animal, only one of two sides injected showed positive response.

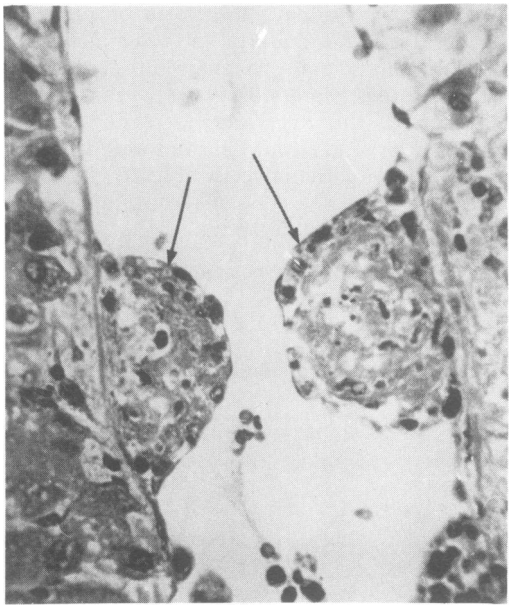

FIG. 7. *Generalized Shwartzman reaction. Rabbit injected i.v. with 10 μg of LPS and challenged 24 h later with 20 μg of LPS. Fibrin thrombi containing debris of necrotic cells (arrows) in a hepatic central vein.* ×420.

havior of classical endotoxin. Its potency is comparable to those of certain enterobacterial endotoxins and it is capable of inducing both primary and secondary toxicity in experimental small animals. Lipid A appears to be a major component responsible for this toxicity.

Presumably, the characteristic necrosis manifested in a number of infections associated with *F. necrophorum* is due partly to the activity of this endotoxin. In the development of bovine hepatic abscesses, the establishment of septic foci could be a prime requisite before any significant endotoxin action. Once this requirement is fulfilled, the extent of endotoxic damage may depend on several factors such as (i) the rate and extent of *F. necrophorum* multiplication, (ii) the ability of fibrous connective tissue to encapsulate the focus of infection, and (iii) the effective clearance of the endotoxin by the liver reticuloendothelial system.

Although previous workers (7-9, 20) have reported the presence of endotoxin in *F. necrophorum* strains, this study provides a more comprehensive description of the activity of the endotoxin and thus a better insight into those properties that are linked directly to the pathogenicity of *F. necrophorum*. Based on these data, further work can be devoted to elaborate the mechanism of *F. necrophorum* endotoxic action and to determine whether the endotoxin acts independently or collaborates with protein antigens (e.g., hemolysins) to enhance host damage. Results from such approaches will help clarify the etiology and pathogenesis of bovine liver abscesses.

ACKNOWLEDGMENTS

We wish to thank Ruth Robertson and Blake Stewart for their excellent technical help, Susie Becker for valuable assistance and advice in the electron microscopy, and the staff of the Animal Care Section for support in the animal experiments.

LITERATURE CITED

1. Albizo, J. M., and M. J. Surgalla. 1970. Isolation and biological characterization of *Pasteurella pestis* endotoxin. Infect. Immun. **2**:229-236.
2. Clarke, K., G. W. Gray, and D. A. Reaveley. 1967. The cell wall of *Pseudomonas aeroginosa*. Biochem. J. **105**:749-754.
3. Galanos, C., E. T. Rietschel, O. Luderitz, and O. Westphal. 1971. Interaction of lipopolysaccharides and lipid A with complement. Eur. J. Biochem. **19**:143-152.
4. Garcia, M. M., and K. A. McKay. 1972. A satisfactory medium and technique for the mass cultivation of *Sphaerophorus necrophorus*. Can. J. Microbiol. **19**:296-298.
5. Garcia, M. M., D. H. Neil, and K. A. McKay. 1971. Application of immunofluorescence to studies on the ecology of *Sphaerophorus necrophorus*. Appl. Microbiol. **21**:809-814.
6. Herbert, D., P. J. Phipps, and R. A. Strange. Chemical analyses of microbial cells, p. 266-272. *In* J. R. Norris and D. W. Ribbons (ed.), Methods of microbiology, vol. 5B. Academic Press Inc., New York.
7. Hofstad, T., and T. Kristoffersen. 1971. Preparation and chemical characteristics of endotoxic lipopolysaccharide from three strains of *Sphaerophorus necrophorus*. Acta Pathol. Microbiol. Scand. Sect. B **79**:385-390.
8. Hofstad, T., T. Kristoffersen, and K. A. Selvig. 1972. Electron microscopy of endotoxic lipopolysaccharide from *Bacteroides*, *Fusobacterium* and *Sphaerophorus*. Acta Pathol. Microbiol. Scand. Sect. B **80**:413-419.
9. Kirchheiner, E. 1940. Rescherches biochimiques et immunologiques comparées sur *Sphaerophorus necrophorus* et *Sphaerophorus fundiliformia*. Ann. Inst. Pasteur Paris **64**:238-254.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265-275.
11. Milner, K. C., and R. A. Finkelstein. 1966. Bioassay of endotoxin: correlation between pyrogenicity for rabbits and lethality for chick embryos. J. Infect. Dis. **116**:529-536.
12. Nelson, N. 1944. A photometric adaptation of the Somagyi method for the determination of glucose. J. Biol. Chem. **153**:375-380.
13. Osborn, M. J. 1963. Studies on the gram-negative cell walls. I. Evidence for the role of 2-keto-3-deoxyoctonate in lipopolysaccharide of *Salmonella typhimurium*. Proc. Nat. Acad. Sci. U.S.A. **50**:499-506.
14. Raabe, S. 1955. Die Bedeutung routinemässiger Phosphatase—Bestimmungen in lelinischen Labors. Recl. Trav. Chim. Pays-Bas **74**:652-660.
15. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**:493-497.
16. Rondle, C. J. M., and W. T. S. Morgan. 1955. The

determination of glucosamine and galactosamine. Biochem. J. **61**:586-589.
17. Rudbach, J. A., R. L. Anacker, W. T. Haskins, A. G. Johnson, K. C. Milner, and E. Ribi. 1966. Physical aspects of reversible inactivation of endotoxin. Ann. N.Y. Acad. Sci. **133**:629-643.
18. Shands, J., J. A. Graham, and K. Nath. 1967. The morphological structure of isolated bacterial lipopolysaccharide. J. Mol. Biol. **25**:15-29.
19. Shwartzman, G. 1928. A new phenomenon of local skin reactivity to *B. typhosus* culture filtrate. Proc. Soc. Exp. Biol. Med. **25**:560-561.
20. Sonnenwirth, A. C., E. T. Yin, E. M. Sarmiento, and S. Wessler. 1972. Bacteroidaceae endotoxin detection by *Limulus* assay. Am. J. Clin. Nutr. **25**:1452-1454.
21. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:21-43.
22. Sutherland, I. W., and J. F. Wilkinson. 1971. Chemical extraction methods of microbial cells, p. 353-354. *In* J. R. Norris and O. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press Inc., New York.
23. Winzler, R. J. 1955. Determination of serum glycoproteins, p. 279-311. *In* D. Glick (ed.), Methods of biochemical analysis, vol. 2. Interscience Publishers Inc., New York.
24. Wong, K. H., C. E. Miller, J. C. Feeley, and Y. K. Chan. 1973. Determination of endoxicity in bacterial vaccines. Appl. Microbiol. **25**:403-407.