INFECTION AND IMMUNITY, Sept. 1970, p. 229-236

Vol. 2, No. 3
Printed in U.S.A

Isolation and Biological Characterization of *Pasteurella pestis* Endotoxin

JOHNNIE M. ALBIZO AND MICHAEL J. SURGALLA
Medical Sciences Laboratories, Fort Detrick, Frederick, Maryland 21701

Received for publication 22 April 1970

Endotoxin containing 2.1% nitrogen, 1.6% phosphorus, 22.5% neutral hexose, 15% hexosamine, 25% esterified and amide-linked fatty acids, and 1.4% protein was isolated from *Pasteurella pestis* strain Alexander by slight modification of a method adapted by Tauber and Russell. The lipopolysaccharide exhibited classical endotoxic biological properties including: (i) toxicity in mice, guinea pigs, and rabbits; (ii) antigenicity in rabbits; (iii) capacity to evoke a biphasic pyrogenic response in rabbits; (iv) capacity to induce tolerance in mice to the lethal effect of endotoxin; (v) capacity to stimulate rapidly acquired resistance in mice to bacterial infection, and (vi) the capacity to produce the localized and generalized Shwartzman phenomena in rabbits. Findings obtained during the study concerning the occurrence, isolation, toxicity, and other biological properties of *P. pestis* endotoxin provide new evidence that endotoxin could contribute to death in plague.

Early plague investigators proposed that *Pasteurella pestis* produced an endotoxin that contributed to death in plague (14). Early efforts to isolate the endotoxin by classical methods were unsuccessful (8, 9); however, in more recent attempts the phenol extraction method of Westphal et al. (26) has been used, and considerable success has been achieved (7, 24, 25). It is now known collectively from the studies of Davies (7), Walker et al. (25), Walker (23, 24), and Larrabee et al. (11) that *P. pestis* produces a lipopolysaccharide that kills mice, guinea pigs, rabbits, and monkeys with symptoms and pathological changes characteristic of endotoxin shock at death. These recent findings lend direct support to the endotoxic death concept in plague proposed by earlier investigators.

The present investigation, which involved isolating *P. pestis* endotoxin and testing the toxic and biological properties of the endotoxin, was undertaken to provide additional knowledge concerning *P. pestis* endotoxin and to determine whether endotoxin could contribute significantly to the pathogenesis of plague.

MATERIALS AND METHODS

Stock culture. Fully virulent (guinea pig-passed) *P. pestis* strain Alexander ($LD_{E0} < 20$ cells for mice and guinea pigs) possessing fraction I antigen, VW antigens, pigmentation, and pesticinogeny was used as a stock culture.

Cultivation of organisms. Blood Agar Base slants (Difco, *p*H 6.8) were inoculated with stock *P. pestis* culture and were incubated at 26 C for 24 hr. Bacterial growth from each slant was washed into 500-ml flasks, each containing 50 ml of 2.5% Heart Infusion Broth (Difco, *p*H 7.4) supplemented with 0.5% xylose. After primary broth cultures were grown on a shaker for 24 hr at 26 C, 10-ml quantities of these cultures were transferred to 2-liter flasks (secondary broth cultures), each containing 250 ml of 2.5% Heart Infusion Broth (Difco, *p*H 7.4) supplemented with 0.5% xylose. Secondary broth cultures were grown on a shaker at 26 C for 24 hr. After growth, secondary broth cultures were tested for purity and possession of bacterial properties characteristic of *P. pestis*. Bacterial cells were sedimented by centrifugation at 27,000 × *g* for 15 min in a Sorvall RC-2B refrigerated centrifuge at 4 C, and the cells were washed twice with three volumes of sterile 0.85% NaCl. Packed, washed cells were weighed in 100-g quantities and stored at −20 C until used for the extraction of endotoxin.

Extraction and isolation of endotoxin. Endotoxin was extracted and isolated by a slight modification of a general method adapted by Tauber and Russell (21) from the earlier procedure described by Westphal et al. (26). Our particular application of Tauber and Russell's general extraction and isolation procedure is described in some detail, because their procedure has not been previously used for the isolation of *P. pestis* endotoxin and because plague endotoxin obtained by their method was considerably more toxic than plague endotoxin previously reported (7, 24, 25). A 90-ml amount of cold, sterile, double-distilled water was added to each 100-g quantity of frozen, packed, washed *P. pestis* cells, and the thawed cell suspension (12 to 14 C) was stirred in a Waring Blendor for 2 min at low speed (final temperature, 17 to 19 C).

Blending speed was controlled with a rheostat. All blending operations were conducted in a plastic bag housed within a sealed safety hood maintained under negative pressure. Phenol-water (354 ml, cooled to 4 C) containing 110 ml of cold, sterile, double-distilled water and 244 ml of Fisher Certified Liquefied Phenol Reagent was added to the blended cell suspension. The phenol-water bacterial cell suspension (12 to 14 C) was stirred in a Waring Blendor for 8 min at low speed to effect proper mixing without excess foam. The resulting mixture (18 to 19 C) was cooled in an ice bath to 4 C and was centrifuged at 3,000 × *g* for 10 min. The aqueous upper phase was aspirated carefully with a sterile 50-ml volumetric pipette and a pipette filler. The phenol phase was discarded and not re-extracted with water as described by Tauber and Russell (21). The aqueous phases obtained by extracting several 100-g quantities of packed, washed cells with phenol-water were pooled and centrifuged at 3,000 × *g* for 20 min, although very little sediment was obtained with this step. The supernatant liquid was dialyzed against several 10-liter quantities of cold (4 C) double-distilled water with frequent changes of water over a period of 40 hr.

Sterile NaCl (1 mg/ml) from a 10% stock solution (cooled to 4 C) was added with stirring to the dialyzed supernatant, followed by two volumes of cold acetone (−20 C). The resulting mixture was cooled to 4 C and centrifuged at 5,000 × *g* for 5 min. The supernatant was poured off, and the endotoxin sediment was dissolved in 50 ml of sterile double-distilled water per each 100 g of starting packed, washed bacterial cells. The next step in the procedure described by Tauber and Russell (21) called for centrifugation at 3,800 × *g* for 5 min. This was omitted because a considerable quantity of endotoxin sedimented with this step. Two-tenths volume of cold acetone (−20 C) was added to the dissolved sediment, which was cooled to 4 C and centrifuged at 48,000 × *g* for 1 hr in a Sorvall RC-2B refrigerated centrifuge at 4 C. The endotoxin sediment was dissolved in 30 ml of sterile double-distilled water per each 100 g of starting packed, washed cells, and the mixture was centrifuged at 48,000 × *g* for 1 hr at 4 C. The endotoxin sediment was dissolved in 10 ml of sterile double-distilled water per each 100 g of starting washed, packed cells and was lyophilized. To achieve further purification, endotoxin was dissolved in sterile double-distilled water (90 C) to attain a final concentration of 2 mg/ml, cooled to 4 C, and reprocessed. NaCl (1 mg/ml at 4 C) and two volumes of cold acetone (−20 C) were added with stirring to the dissolved endotoxin, which was cooled to 4 C and centrifuged at 48,000 × *g* for 1 hr at 4 C. NaCl (1 mg/ml) and cold acetone (0.2 volumes at −20 C) were added with stirring. The mixture was cooled to 4 C and centrifuged at 48,000 × *g* for 1 hr at 4 C. The endotoxin sediment was again suspended in an original volume of sterile double-distilled water, cooled to 4 C, and centrifuged at 48,000 × *g* for 1 hr at 4 C. The endotoxin sediment was dissolved in one-fourth the original volume of sterile double-distilled water, lyophilized, and stored over Drierite in a vacuum desiccator at 22 C. Lyophilized endotoxin was dissolved in sterile double-distilled water at a concentration of 4 mg/ml to prepare stock solutions for conducting chemical and biological tests.

Chemical analysis. Nitrogen was determined by the micro-Kjeldahl method as modified by Campbell et al. (5). Phosphorus was determined by the method described by Ames (2). Neutral hexose was determined by the method described by Spiro (19) with glucose used as a standard. Hexosamine was determined by the method described by Williams and Chase (27) with glucosamine HCl used as a standard. Esterified fatty acids were determined by the method of Snyder and Stephens (18) as modified for endotoxins by H. Tauber (Fed. Proc. **19:** 245, 1960) with tripalmitin used as a standard. Amide-linked fatty acids were determined by the procedure described by Haskins (10). Protein was determined by the method of Lowry et al. (12) with bovine serum albumin as a standard.

Toxicity in mice, guinea pigs, and rabbits. Lyophilized endotoxin (4 mg/ml) was dissolved in sterile double-distilled water, passed several times through a 25-gauge needle, and diluted serially (twofold dilutions) in sterile double-distilled water. Endotoxin was injected in 0.25-ml quantities into Swiss-Webster mice (18 to 22 g), Hartley guinea pigs (250 to 300 g), and New Zealand white rabbits (2 to 2.5 kg). Thirty animals of each of the three species were injected at each dose level. The animals were equally mixed by sex and were selected at random. Mice and guinea pigs were injected intraperitoneally. Rabbits were injected intravenously through the marginal ear vein. All animals were housed at 22 C, and deaths were recorded over a period of 48 hr.

Biphasic pyrogenic response in rabbits. Endotoxin (1 μg) in 0.1 ml of sterile double-distilled water was injected intravenously into each of eight New Zealand white rabbits (2 to 2.5 kg). Eight control rabbits were injected intravenously with 0.1 ml of sterile double-distilled water. The rabbits were of mixed sex and had been checked for base line temperature stability for 2 days preceding the pyrogenicity test. The rabbits were housed two per cage at 22 C. Temperatures were taken rectally with clinical mercury thermometers before injection with endotoxin and at 15-min intervals up to hr 4. Thereafter, temperatures were taken at 30-min intervals up to hr 6.

Induction of tolerance in mice to the lethal effect of endotoxin. *P. pestis* endotoxin (1 μg in 0.1 ml of sterile double-distilled water) was injected intraperitoneally into each of 80 Swiss-Webster mice of mixed sex (18 to 22 g), on day 1 of the experiment. Each of the 80 mice was injected intraperitoneally with 10 μg of *P. pestis* endotoxin on day 3. Eighty mice used as controls were injected with 0.1 ml of sterile double-distilled water on days 1 and 3. On day 5, 40 experimental mice and 40 control mice were injected intraperitoneally with 1,000 μg of *P. pestis* endotoxin contained in 0.1 ml of sterile double-distilled water. The remaining group of 40 experimental mice and 40 control mice were injected by the same route with 1,000 μg of *Salmonella typhosa* endotoxin 0901 W (Difco). Mice were housed at 22 C. Deaths were recorded over a period of 48 hr.

Rapidly acquired resistance in mice to bacterial

infection. *P. pestis* endotoxin (1 μg in 0.1 ml of sterile double-distilled water) was injected intraperitoneally into each of 80 Swiss-Webster mice of mixed sex (18 to 22 g). An additional group of 80 mice used as controls each received 0.1 ml of sterile double-distilled water by the same route. Twenty-four hours later, 40 experimental and 40 control mice were injected intraperitoneally with 300 *P. pestis* cells (strain Alexander) contained in 0.1 mg of sterile 2.5% Heart Infusion Broth (Difco). The remaining group of 40 experimental mice and 40 control mice were injected intraperitoneally with 90 *S. typhimurium* cells (strain California) contained in 0.1 ml of sterile 2.5% Heart Infusion Broth (Difco). Mice were housed at 22 C, and deaths were recorded over a period of 21 days.

Antigenicity in rabbits. Groups of 10 New Zealand white rabbits (2 to 2.5 kg) of equally mixed sex were injected intravenously with a single inoculation of either 500 or 1,000 μg of *P. pestis* endotoxin contained in 0.25 ml of sterile double-distilled water. Rabbits surviving the single injection of endotoxin were bled from the ear once each week for 8 weeks. Another group of 10 rabbits were injected intravenously with three series of inoculations containing increasing quantities of endotoxin. Rabbits received doses of 15.62, 31.25, and 62.5 μg of endotoxin on alternate days during the first week. The rabbits were rested for 1 week, bled from the marginal ear vein to obtain a sample of blood, and injected with a second series of inoculations containing 62.5, 125, and 250 μg of endotoxin. Rabbits were again rested for 1 week, bled, and injected with a third series of inoculations containing 250, 500, and 1,000 μg of endotoxin. The rabbits were bled from the ear once each week thereafter for 8 weeks. Sera were tested in gel diffusion plates for the presence of antibody against *P. pestis* endotoxin.

Production of the localized Shwartzman reaction in rabbits. Each of eight New Zealand white rabbits (2 to 2.5 kg) of equally mixed sex was injected intradermally (in a shaved abdominal area) with 25 μg of *P. pestis* endotoxin contained in 0.1 ml of sterile double-distilled water. An adjacent area on the opposite side of the ventral abdominal line was injected intradermally with 0.1 ml of sterile double-distilled water as a control. Twenty-four hours later, each rabbit was injected intravenously via the marginal ear vein with 25 μg of *P. pestis* endotoxin in 0.1 ml of sterile double-distilled water. The original intradermal injection sites were examined and photographed 4 hr after the intravenous injection.

Production of the generalized Shwartzman reaction in rabbits. Each of eight New Zealand white female rabbits (1 to 2 kg) was injected intravenously via the marginal ear vein with 6.25 μg of *P. pestis* endotoxin contained in 0.1 ml of sterile double-distilled water. Twenty-four hours later, each of the eight rabbits was injected intravenously with 25 μg of *P. pestis* endotoxin. A group of four rabbits used as controls received two injections of sterile double-distilled water. At 24 to 48 hr after the second intravenous injection (provoking injection), rabbits were killed with chloroform vapor and necropsied immediately. Representative tissues were selected and fixed in 10% neutral Formalin. Tissue sections were embedded in paraffin and cut at 6 μm. Sections were routinely stained with hematoxylin and eosin. Selected tissue sections were stained with Mallory's phosphotungstic acid-hematoxylin (PTAH) for the demonstration of fibrin (13). Tissue sections were photographed at magnifications of 42, 130, and 420 times.

TABLE 1. *Quantitative analysis of Pasteurella pestis endotoxin*

Component	Percentage of dry wt
Nitrogen	2.1
Phosphorus	1.6
Neutral hexose (as glucose)	22.5
Hexosamine (as glucosamine HCl)	15.0
Esterified and amide-linked fatty acids (as tripalmitin)	25.0
Protein	1.4
Undetermined	32.4

RESULTS

Yield of endotoxin. The yield of *P. pestis* endotoxin obtained from 800 g of packed, washed cells representing 2.1×10^{14} organisms (based on viable-cell count) was 1.2 g, or approximately 1% based upon the dry weight (120 g) of the starting material. This represents a ratio of 1 mg of endotoxin extracted from approximately 1.8×10^{11} organisms.

Quantitative analysis. Quantitative chemical analysis conducted on *P. pestis* endotoxin revealed that the endotoxin was typical with respect to percentages of components normally occurring in gram-negative bacterial lipopolysaccharides. The quantitative chemical analysis of *P. pestis* endotoxin is shown in Table 1.

Toxicity in mice, guinea pigs, and rabbits. *P. pestis* endotoxin was lethal for mice, guinea pigs, and rabbits at doses comparable to toxic levels reported for classical endotoxins (4, 15). The intraperitoneal LD_{50} for mice and guinea pigs was 414 and 537 μg, respectively. The intravenous LD_{50} for rabbits was 32 μg. Toxicity titration data of *P. pestis* endotoxin are shown in Table 2. Animals injected with a single toxic dose of endotoxin became visibly ill within 1 to 2 hr after injection. Most of the deaths occurred within 6 to 12 hr after the injection of endotoxin. Symptoms exhibited by endotoxin-poisoned animals included extreme weakness, irregular and difficult breathing, and cyanosis. Some impairment of muscular coordination was observed in mice and guinea pigs. Muscular incoordination in mice appeared to be more pronounced in the posterior quadrants. In guinea pigs, the body temperature dropped below 93 F (33.9 C) before death. Often, 4 to 6 ml of clear to blood-colored fluid could be

aspirated from the peritoneal cavity of guinea pigs immediately after death.

Production of a biphasic pyrogenic response in rabbits. Intravenous injection of 1 μg of *P. pestis* endotoxin in a single dose elicited a classical biphasic pyrogenic response (3) in each of the eight rabbits tested. Fever peaks occurred at 1.25 and 3 hr after a single injection of endotoxin. Temperatures were back to normal after 6 hr. None of eight control rabbits injected intravenously with sterile double-distilled water developed fever. Average fever curves of eight experimental and eight control rabbits are shown in Fig. 1.

TABLE 2. *Toxicity of Pasteurella pestis endotoxin in mice, guinea pigs, and rabbits*

Animal and route of injection	Amt of endotoxin injected	Animals dead of 30 injected	LD_{50}[a]
	μg		μg
Mouse, intraperitoneal	125	3	
	250	6	
	500	21	
	1,000	24	414 (330–520)
Guinea pig, intraperitoneal	125	3	
	250	8	
	500	17	
	1,000	19	537 (386–749)
Rabbit, intravenous	15.62	12	
	31.25	14	
	62.5	16	
	125.0	26	32 (21–49)

[a] Numbers in parentheses show the 95% confidence limits of the LD_{50}.

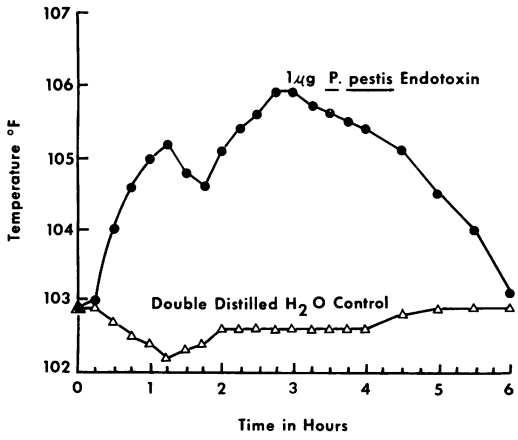

FIG. 1. *Biphasic pyrogenic response produced in rabbits after the intravenous injection of 1 μg of Pasteurella pestis endotoxin. Each point represents the average temperature of eight rabbits.*

TABLE 3. *Induction of tolerance in mice to the lethal effect of endotoxin*

Injection material	Mice surviving of 40 challenged[a] with	
	P. pestis endotoxin	*S. typhosa* 0901W endotoxin
Sterile double-distilled water on days 1 and 3.....	0	0
P. pestis endotoxin, 1 μg on day 1 and 10 μg on day 3..	27	37

[a] Challenged on day 5 with 1,000 μg of endotoxin.

TABLE 4. *Induction of rapidly acquired resistance in mice to bacterial infection*

Injection material	Mice surviving of 40 challenged with virulent bacteria	
	P. pestis Alexander (300 cells)	*S. typhimurium* California (90 cells)
Sterile double-distilled water.....	0	22
P. pestis endotoxin, 1 μg.	29	36

Induction of tolerance to mice to the lethal effect of endotoxin. Mice receiving intraperitoneal injections of 1 μg of *P. pestis* endotoxin on day 1 and 10 μg of *P. pestis* endotoxin on day 3 developed a degree of tolerance (3) against challenge with either homologous *P. pestis* endotoxin or heterologous *S. typhosa* 0901W endotoxin (Difco) injected on day 5 (Table 3).

Induction of rapidly acquired resistance in mice to bacterial infection. Mice injected intraperitoneally with 1 μg of *P. pestis* endotoxin developed a degree of resistance (M. Landy, Fed. Proc. **15**:598, 1956) against small doses of either *P. pestis* (strain Alexander) or *S. typhimurium* (strain California) injected intraperitoneally 24 hr after the endotoxin (Table 4).

Antigenicity in rabbits. Rabbits that managed to survive either large single injections of *P. pestis* endotoxin (500 to 1,000 μg) or three series of injections containing increasing quantities of endotoxin, beginning at 15.6 μg and ending at 1,000 μg, developed antibody that reacted with *P. pestis* endotoxin to produce one precipitin band in gel diffusion tests (Fig. 2).

Production of the localized Shwartzman reaction. All of eight rabbits injected intradermally with 25 μg of *P. pestis* endotoxin, and subsequently

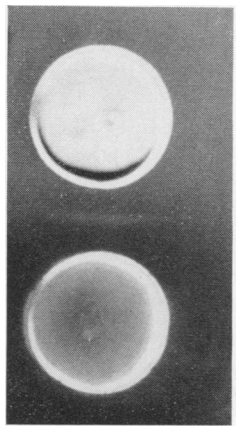

FIG. 2. *Ouchterlony plate showing single precipitin band forming between P. pestis endotoxin (top) and serum obtained from a rabbit after three series of injections with P. pestis endotoxin (bottom).*

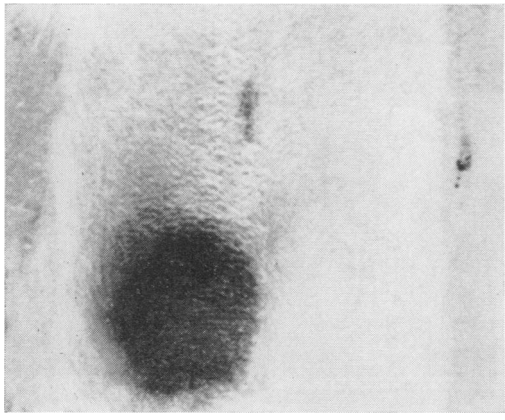

FIG. 3. *Shaved abdominal area of rabbit exhibiting the localized Shwartzman reaction. Photo taken 4 hr after the provoking injection. The left half of the photograph shows the area injected with endotoxin. The right half shows the control area (injected with distilled water).*

injected intravenously with 25 μg of *P. pestis* endotoxin 24 hr later, developed the localized Shwartzman reaction (16, 17). The reaction was maximal within 4 hr after the provoking injection. An adjacent control area on each rabbit was negative in each case (Fig. 3).

Production of the generalized Shwartzman reaction. Rabbits injected intravenously with 6.25 μg of *P. pestis* endotoxin and 24 hr later with 25 μg developed the generalized Shwartzman reaction (17, 22). Of eight rabbits injected with endotoxin, two died within 4 hr after the provoking injection. Four of the remaining six rabbits developed the generalized Shwartzman reaction within 24 to 48 hr after the provoking injection. Four of four control rabbits injected with sterile double-distilled water were negative. The generalized Shwartzman reaction in rabbits was characterized by disseminated intravascular coagulation demonstrable in tissue sections stained with PTAH. Fibrin thrombi and associated necrosis were observed in the kidney, liver, spleen, heart, lung, and skin. The kidney was the most extensively involved organ, and exhibited marked bilateral renal cortical necrosis (Fig. 4). The salient histological feature in the kidney was marked cortical necrosis with only a few islands of viable tubules remaining. Fibrin thrombi were observed in the capillaries of the glomerular tufts and less frequently in the intertubular capillaries. The histological features of the generalized Shwartzman reaction produced in the rabbit kidney by *P. pestis* endotoxin are shown in Fig. 5.

DISCUSSION

Although it was known prior to this study that *P. pestis* produced a lipopolysaccharide (7, 11) that kills mice, guinea pigs, and monkeys with symptoms and pathological changes characteristic of endotoxic shock (24, 25), the lipopolysaccharide had not been studied extensively and it had not been established convincingly that endotoxin could contribute significantly to death in plague. At least three considerations prevented a positive

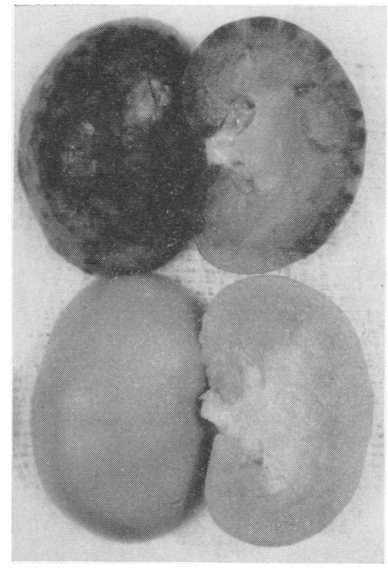

FIG. 4. *Kidneys from rabbit exhibiting the generalized Shwartzman reaction (top) and from control rabbit (bottom). Photograph taken after kidneys had been fixed in neutral Formalin.*

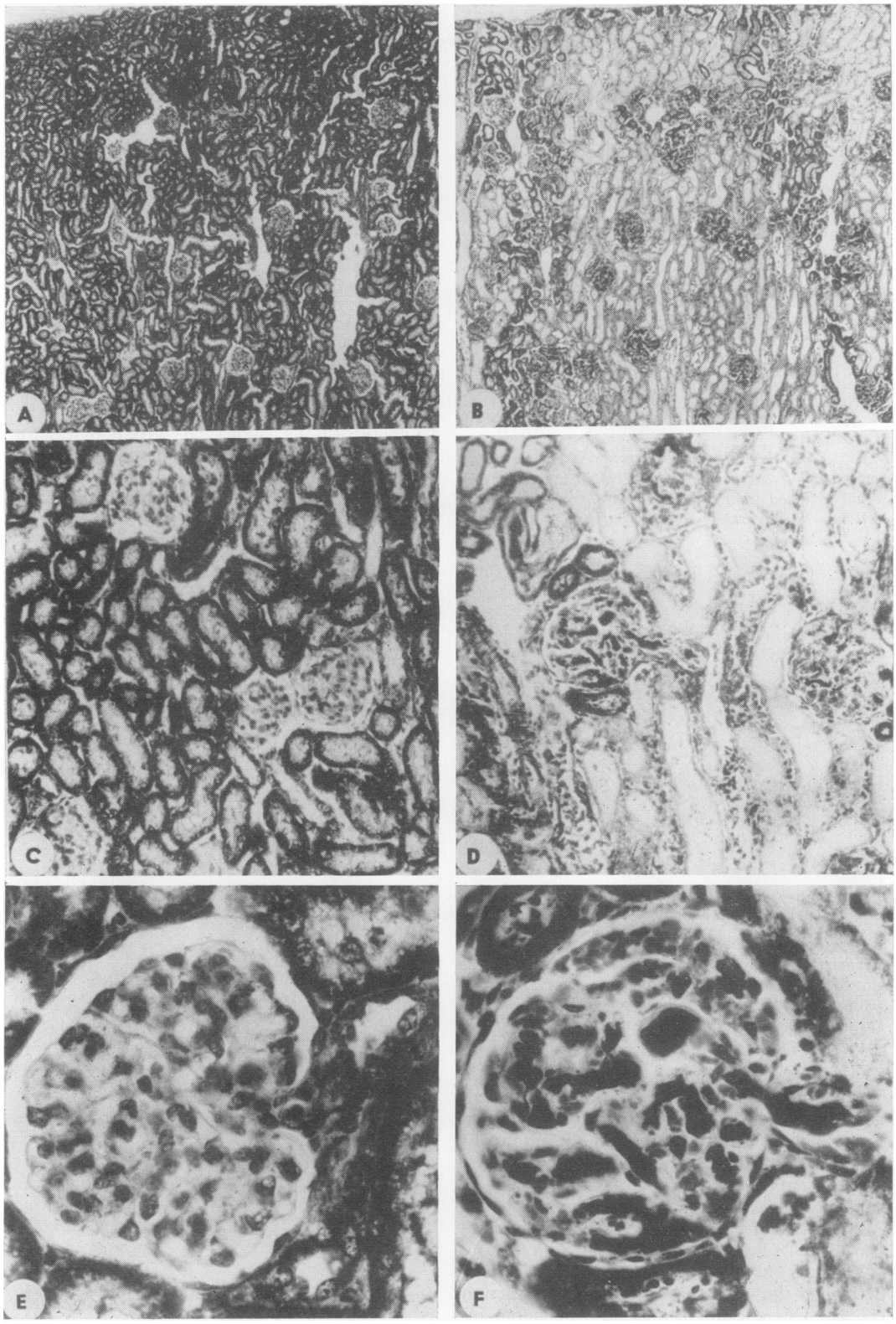

FIG. 5. *PTAH-stained sections of cortical area of rabbit kidneys. (A, C, and E) Normal kidney demonstrating typical glomeruli and intact tubule epithelial cells. (B, D, and F) Kidney from rabbit exhibiting the generalized Shwartzman reaction. Notice the dense fibrin deposits in the glomeruli capillaries and the disruption and fragmentation of tubule epithelial cells. Control and experimental tissue sections received equal light exposure. A and B, × 42; C and D, × 130; E and F, × 420.*

conclusion regarding the importance of endotoxin in plague infections. First, the lipopolysaccharide was considerably less toxic for mice, guinea pigs, and rabbits than were classical endotoxins (7). Second, it had not been shown to possess the classical biological properties characteristic of bacterial endotoxins. A third factor was perhaps the uncertainty created by the report of Cocking et al. (6) describing the isolation of a guinea pig toxin from *P. pestis* that, according to Stanley and Smith (20), was neither lipopolysaccharide (7) nor murine toxin (1), but rather a synergistic mixture of protein components. The present study resolves the first two factors and perhaps provides some insight into the third factor. Concerning the first two factors, we have demonstrated that *P. pestis* lipopolysaccharide possesses classical biological endotoxic properties and toxicity comparable to that of classical endotoxins. Also, we have established that plague endotoxin possesses sufficient toxicity to contribute to or account for death in plague. This is based upon our findings that approximately two LD_{50} (1 mg) of endotoxin can be extracted from 1.8×10^{11} *P. pestis* organisms, a number approximating the number of organisms (up to 1.6×10^{11}) reported by Cocking et al. (6) to be present in guinea pigs dying of plague. Regarding the third consideration, it is conceivable that lipopolysaccharide could have contributed significantly to the toxicity of the guinea pig toxin described by Cocking et al. (6) and partially purified by Stanley and Smith (20). This would appear possible if one considers the intraperitoneal LD_{50} of the present endotoxin preparation in relation to the total quantity of lipopolysaccharide conceivably present within the two components of the partially purified synergistic protein mixture described by Stanley and Smith (20). However, we do not discount the possibility that lipopolysaccharide can act synergistically with proteins or that other toxins, yet to be fully elucidated, are present in the plague bacilli and can act singly or synergistically to exert a lethal effect.

The present study complements the findings of earlier workers (7, 23, 24, 25) and firmly establishes the production by *P. pestis* of a lipopolysaccharide that exhibits classical endotoxic properties and possesses sufficient toxicity to contribute to or account for death in small laboratory animals. The work opens the way for future studies concerning the mechanism of action of *P. pestis* endotoxin in animals and man, which in turn may lead to the development of improved methods for managing the endotoxemia accompanying plague and other gram-negative bacterial infections.

ACKNOWLEDGMENTS

We thank Ross A. Swenerton, Charles L. Warnick, Jerry D. Gordon, and Leonard J. Stack for technical assistance; Robert M. Kovatch, Garrett S. Dill, Jr., William C. Hall, and Steven E. Wikse for histological examination of tissues; John D. Stewart for nitrogen determination; and Brucy C. Gray for statistical analysis. We also thank L. Joe Berry, Karl F. Meyer, Martin S. Wilder, and Earl D. Beesley for their interest and helpful suggestions.

LITERATURE CITED

1. Ajl, S. J., J. S. Reedal, E. L. Durrum, and J. Warren. 1955. Studies on plague. I. Purification and properties of the toxin of *Pasteurella pestis*. J. Bacteriol. 70:158–169.
2. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases, p. 115–118. *In* E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
3. Bennett, I. L., Jr., and L. E. Cluff. 1957. Bacterial pyrogens. Pharmacol. Rev. 9:427–475.
4. Burrows, W. 1951. Endotoxins. Annu. Rev. Microbiol. 5: 181–196.
5. Campbell, D. H., J. S. Garvey, N. E. Gremer, and D. H. Sussdorf (ed.). 1963. Methods in immunology, p. 53–57. W. A. Beniamin, Inc., New York.
6. Cocking, E. C., J. Keppie, K. Witt, and H. Smith. 1960. The chemical basis of the virulence of *Pasteurella pestis*. II. The toxicity for guinea pigs and mice of products of *Pasteurella pestis*. Brit. J. Exp. Pathol. 41:460–471.
7. Davies, D. A. L. 1956. A specific polysaccharide of *Pasteurella pestis*. Biochem. J. 63:105–116.
8. Girard, G. 1941. Absence d'antigène glucido-lipidiaue chez le bacille de la peste et le bacille de la pseudo-tuberculose des rongeurs. Compt. Rend. Soc. Biol. Paris 135:1577–1579.
9. Girard, G. and G. Sandor. 1947. La nature de la toxine pesteuse. Compt. Rend. Acad. Sci. Paris 224:1078–1080.
10. Haskins, W. T. 1961. Spectrophotometric determination of fatty acid amides in lipides. Anal. Chem. 33:1445.
11. Larrabee, A. R., J. D. Marshall, and D. Crozier. 1965. Isolation of antigens of *Pasteurella pestis*. I. Lipopolysaccharide protein complex and R and S antigens. J. Bacteriol. 90:116–119.
12. 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.
13. Luna, L. G. (ed.). 1968. Mallory's posphotungstic acid hematoxylin method (PTAH), p. 85–86. *In* Manual of histological staining methods of the Armed Forces Institute of Pathology, 3rd ed. McGraw Hill Book Co., Inc., New York.
14. Pollitzer, R. 1954. Plague. World Health Organ. Monogr. Ser. 22, p. 121.
15. Ribi, E., R. L. Anacker, K. Fukushi, W. T. Haskins, M. Landy, and K. C. Milner. 1964. Relationship of chemical composition to biological activity, p. 16–28. *In* M. Landy and W. Braun (ed.), Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.
16. Shwartzman, G. 1928. A new phenomenon of local skin. Proc. Soc. Exp. Biol. Med. 25:560.
17. Shwartzman, G. 1937. Phenomenon of local tissue reactivity and its immunological pathological and clinical significance. Paul B. Hoeber, Inc., New York.
18. Snyder, F., and N. Stephens. 1959. A simplified spectrophotometric determination of ester groups in lipids. Biochim. Biophys. Acta 34:244–245.
19. Spiro, R. G. 1966. Analysis of sugars found in glycoproteins, p. 3–26. *In* E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
20. Stanley, J. L., and H. Smith. 1967. The chemical basis of the virulence of *Pasteurella pestis*. IV. The components of the guinea pig toxin. Brit. J. Exp. Pathol. 48:124–129.
21. Tauber, H. and H. Russell. 1961. General method for the

isolation of endotoxins. Study of lipid and polysaccharide moieties. Exp. Med. Surg. 19:161–170.
22. Thomas, L., and R. A. Good. 1952. Studies on the generalized Shwartzman reaction. I. General observations concerning the phenomenon. J. Exp. Med. 96:605–625.
23. Walker, R. V. 1967. Plaque toxins: a critical review. Cur. Top. Microbiol. Immunol. 41:23–42.
24. Walker, R. V. 1968. Comparative physiopathology of plague endotoxin in mice, guinea pigs, and monkeys. J. Infec. Dis. 118:188–196.
25. Walker, R. V., M. G. Barnes, and E. D. Higgins. 1966. Composition of and physiopathology produced by plague endotoxins. Nature (London) 209:1246.
26. Westphal, O., O. Lüderitz, and F. Bister. 1952. Über die Extraction von Bacterien mit phenol/Wasser. Z. Naturforsch. 7b:148–155.
27. Williams, C. A., and M. W. Chase. 1968. Elson-Morgan method (modified), 296–298. *In* C. A. Williams and M. W. Chase (ed.), Methods in immunology and immunochemistry, vol. 2. Academic Press Inc., New York