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Chemical Composition and Biological Activities of a Phenol-Water Extract from *Haemophilus influenzae* Type a

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Ribonucleic acid was removed from a phenol-water extract of *Haemophilus influenzae* type a by streptomycin sulfate. This preparation was called purified preparation or PP. It contained neutral sugars (glucose, galactose, mannose, pentose), glucosamine, amino acids, and fatty acids. Heptose and 2-keto-3-deoxyoctonic acid were not present. The biological properties and immunogenicity were compared with the activities of lipopolysaccharide of *Escherichia coli* or *Salmonella typhimurium*. Higher doses were necessary to obtain lethality in mice and Sanarelli and Shwartzman reactions with our preparations than were necessary with lipopolysaccharide. The *Limulus* test and pyrogen assay in rabbits gave the same results with purified preparation and lipopolysaccharide, but pyrogenicity of purified preparation was not destroyed by NaOH treatment. Purified preparation was not as immunogenic at low doses for rabbits as lipopolysaccharide. The results were different from those obtained with lipopolysaccharide but similar to those known from peptidoglycan studies. The contamination of purified preparation with peptidoglycan was negligible and cannot explain the biological activities of purified preparation. We suggest that the phenol-water extract from *H. influenzae* is not a classical endotoxin, but rather an endotoxin-like substance.

In patients with chronic bronchitis, infections of the bronchial tree occur frequently. These infections are probably the result of a preexisting bronchial obstruction, or a preceding virus infection, or both. More studies have been made with bronchial sputum washed to prevent contamination by pharyngeal commensals. In these cases and for subjects not treated by antibiotic therapy, the bacteria most commonly found in the bronchial sputum are, in order, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Branhamella catarrhalis* (24). Tracheal punctures made to obtain uncontaminated bronchial secretions confirm these results. *H. influenzae* type b is an etiological agent of several human diseases, including meningitis, epoglottitis, arthritis, osteomyelitis, and pneumonitis (30). The pathogenesis of these diseases is generally admitted to have a relation with capsular polysaccharide. The capsular antigen of serotype b is a polyribophosphate which is used to immunize humans (2). However, in patients with chronic bronchitis, *H. influenzae* is most commonly found without the capsule (18). Experimental studies suggest that the lipopolysaccharide (LPS) from *H. influenzae* is an etiological agent for chronic nonspecific lung disease. This endotoxin causes early and late bronchial obstruction in these patients (31). Branefors-Helanders (4) demonstrated the presence of toxic products and Denny (5) suggested a paralyzing action of endotoxin produced on the ciliated respiratory epithelium by *H. influenzae*.

The present study was performed to elucidate the chemical constitution and some biological activities of LPS extracted from *H. influenzae* type a. LPS is a major component of the cell wall of gram-negative bacteria and therefore can be extracted from any serological type. Its biological properties and immunogenicity were compared with the activities of a "classical" endotoxin (LPS of *Salmonella typhimurium* or *Escherichia coli*).

MATERIALS AND METHODS

Bacteria. The strain of *H. influenzae* (no. 52151, Institut Pasteur, Paris) type a was kindly provided by the Cassenne Laboratories (Osny, France). Twenty liters of fresh medium with X and Y factors was poured into a fermentor vessel. After inoculation with a cell suspension in broth, *H. influenzae* was cultivated for 16 h at 37°C. The pH of the culture was maintained at 7.5 during cultivation. Organisms were harvested in a late logarithmic phase by centrifugation at 10,000 rpm for about 2 h. The bacterial sediment was washed three times with physiological saline and four times with cold acetone and dried in vacuo.

LPS extraction. LPS was extracted by the method of Westphal (34). Acetone-dried cells of *H. influenzae* suspended in water were treated with an equal volume of a 90% (wt/vol) phenol solution at 65°C for 30 min and then centrifuged at 3,000 rpm for 30 min. The water and phenol phases were then clearly separated. An equal volume of distilled water was added to the phenol phase, and the mixture was treated again by the same method. The combined aqueous phases were dialyzed against distilled water and lyophilized. Nonpurified preparation was obtained and called NPP. LPS of *E. coli* (O55:B5) and *S. typhimurium* were purchased from Difco (Detroit, Mich.).

Purification. Ribonucleic acid was present in NPP and removed by a streptomycin sulfate treatment (33). Streptomycin (2.5%) sulfate in water was added slowly to NPP in aqueous solution (2 mg/ml) (1 vol/5 vol). After homogenization by stirring for 45 min and centrifugation, the supernatant was dialyzed against distilled water and lyophilized. This preparation was called "purified preparation" or PP. The elimination of streptomycin sulfate was checked by the method of the *Pharmacopée* (16).

Chemical determinations. Nucleic acid evaluation was performed by ultraviolet absorption at 260 nm.

For quantitative analysis, the water content was determined after infrared drying by the method of double weighing. The carbon, hydrogen, and nitrogen contents were determined by the Carlo Erba apparatus. The phosphorus content was estimated by the method of Kabat (10). Neutral sugars and uronic acids were determined by the method of Montreuil (19). Glucose and glucuronic acid were used as standards. The hexosamine content was determined by the method of Elson-Morgan (20) with D-glucosamine as the standard. The heptose content was determined by the cysteine-sulfuric reaction of Dische (6). The content of 2-keto-3-deoxyoctonic acid (KDO) was determined by the thiobarbituric acid method of Warren (32). The protein content was determined by the Folin-Ciocalteau method of Lowry (14). The muramic acid content was determined by the method of Strominger (28).

For qualitative analysis, gas-liquid chromatography was done in a Varian apparatus (model 3700) with the column (200 cm by 0.2 cm) packed with Varaport 30-5% O.V. 210 and nitrogen flow at 7.5 ml/min. The samples were heated for 20 h at 80°C in 0.5 N HCl-methanol and then vacuum dried over nitrogen flow at 150°C for 5 min by Zanetta's process (37). Amino sugars (8) and ethanolamine (9) were detected after high-voltage paper electrophoresis by ninhydrin (0.1% in acetone). Amino acids were separated by thin-layer chromatography in two dimensions and detected by ninhydrin (35). The preparations were hydrolyzed by Adams' procedure (1) for KDO determination. They were then heated in 0.02 N H_2SO_4 at 100°C for 20 min, cooled, and diluted. After centrifugation, the presence of KDO was determined in the upper methanol-water layer by paper chromatography by Novotny's procedure (22). Evaporation of the chloroform layer yielded a lipid A which was dried in vacuo and weighed.

For lipids, PP was separated into chloroform-soluble ($CHCl_3S$) and chloroform-insoluble ($CHCl_3IS$) fractions by the lipid extraction procedure of Adams (1). PP was also saponified by heating a portion (10 mg) in 5% methanolic KOH (50 ml) at 50°C for 5 h (7). The ether fraction obtained by saponification, $CHCl_3S$ and $CHCl_3IS$, and the lipid A fraction were chromatographed on thin-layer chromatography plates. Fatty acids, glycolipids containing osamine, amino compounds, and phosphorus compounds were detected with iodine vapor, Elson-Morgan reaction, ninhydrin, and Dittmer reagents, respectively. These operations were performed by the method described by Konno (12). Diaminopimelic acid was chromatographed on thin-layer chromatography plates by the semi-quantitative Zalisch procedure (personnal communication). The preparation was hydrolyzed in a sealed glass tube in 6 N HCl (2 ml) for 16 h at 100°C. After evaporation, the hydrolyzed preparation was applied to the plate. The migration solvent was methanol-pyridine-acetic acid-water (18:50:4:28). Diaminopimelic acid (R_f 0.23) and amino acids (R_f from 0.25 to 0.83) were detected by ninhydrin.

Biological activities. Lethality to mice was determined in the usual manner, and 50% endpoint (50% lethal dose) was done by the method of Reed and Muench (25).

The pyrogenicity test in rabbits was done by the usual method (15) with NPP and PP treated or not with 0.25 N NaOH for 60 min at 56°C (17).

The *Limulus* assay was performed as described by Levin (13). Equal volumes of *Limulus* lysate and test solution in 0.9% sodium chloride were incubated at 37°C for 3 h and allowed to stand at room temperature for 18 h. A gel or floculation in the tube indicated a positive result.

The Shwartzman and Sanarelli reactions were done by the routine assay method (23).

For immunogenicity in rabbits, rabbits were immunized intravenously with NPP or PP daily for 5 days with a total dose of 3.7 μg/rabbit (21). Each rabbit received on successive days 0.33, 0.33, 0.67, 0.67, and 1.7 μg in 2 ml. The rabbits were bled on days 0, 6, 8, and 11. A booster injection was made on day 11 (0.7 μg in 2 ml intravenously), and specimens of serum were obtained on days 14, 15, and 18. Antibodies were evaluated by immunoprecipitation and quantified by passive hemagglutination. This measurement was possible because NPP and PP could be fixed on sheep erythrocytes by the glutaraldehyde method (3).

RESULTS

Chemical analysis. The yields from acetone-dried cells were 17.5% for PNP and 1.2% for PP. After purification, ultraviolet absorption was lower, but proteins were also removed by this treatment (Fig. 1). Most of the LPS preparations from gram-negative bacteria are found to have similar compositions, the major components being neutral sugars, hexosamines, fatty acids, ethanolamine, KDO, and phosphate. The analytical data on NPP and PP are shown in Table 1. After treatment with streptomycin, the decrease in the protein content was associated with a decrease both in the nitrogen level and in the

protein-sugar ratio. Amino acid presence was also reduced after purification. The composition of NPP and PP was further explored by gas-liquid chromatography of trifluoroacetate derivatives of acid hydrolysates. Table 2 shows the molar ratios of various sugars. Uronic acid was not found in PP by gas-liquid chromatography. The presence of neutral sugars in the preparation could explain the results obtained by the carbazol method in the quantitative determination of uronic acids. KDO, heptose, and ethanolamine are characteristic components of LPS from various enterobacteria. Heptose and KDO were not found, but ethanolamine was present in NPP and PP. *N*-acylmuramic and diaminopimelic acid are specific components of peptidoglycan, which is also present in the bacteria cell wall. We did not find muramic acid. The level of diaminopimelic acid was less than 0.25% by semi-quantitative chromatography. Therefore, the contamination by peptidoglycan could be neglected. Fatty acids were detected by thin-layer chromatography only in $CHCl_3S$ and ether fractions of NPP and PP. The lipid A part was weighed after mild hydrolysis. NPP and PP contained 25 and 8% of lipid fractions, respectively. Spots obtained by the thin-layer chromatography of lipid A were stained with various reagents. Fatty acids, glucosamine, and phosphorus compounds were detected.

Biological activities. The lethality (50% lethal dose) of each preparation (NPP, PP, LPS) for mice was determined. Three groups of 10 mice each were injected intraperitoneally with increasing dilutions of each of these preparations. The 50% lethal doses were as follows: LPS *E. coli*, 8.80 ± 0.60 mg/kg; NPP, 47 ± 4 mg/kg; PP, 26.5 ± 1.5 mg/kg. Nucleic acids were not

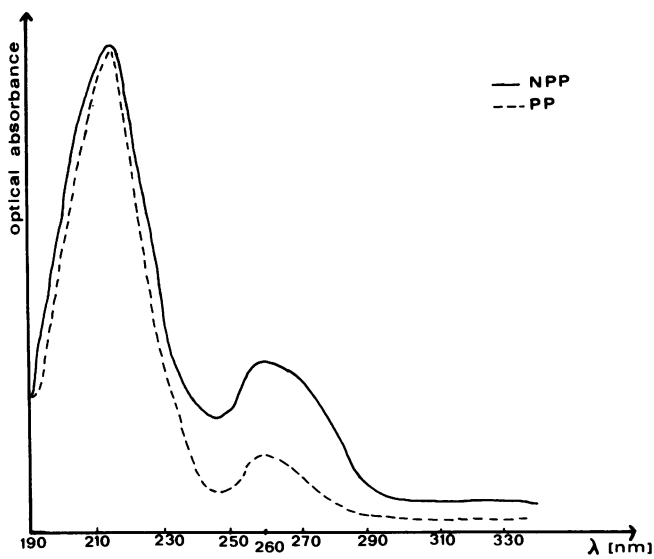

FIG. 1. *Optical absorbance of NPP and PP at 260 nm.*

TABLE 1. *Chemical analysis of NPP and PP extracted from H. influenzae type a*[a]

Prepn	Water	C	H	N	P	Neutral sugars	Uronic acids	Osamine	Protein	Lipid fraction[b]	Sugar/protein
NPP	8	35	5.2	7.4	3.3	38	2.5	1	19.5	25	2
PP	7	37	6	2.9	2.2	37	6	5.6	9.2	8	4

[a] All values are expressed as milligrams per 100 milligrams of preparation.
[b] Lipid fraction was obtained by Adams procedure and called lipid A.

TABLE 2. *Molar ratios of sugars found by gas-liquid chromatography in NPP and PP extracted from H. influenzae type a*

Prepn	Rhamnose	Fucose	Ribose	Glucose	Galactose	Mannose	Glucosamine	Uronic acid
NPP	1	1	1.5	6	2	6	1	—
PP	1	0	0	6	2	2	2	—

removed from LPS of *E. coli*. Therefore, the 50% lethal doses of LPS and NPP could be compared: NPP was about 5.4 times less toxic than LPS. Another difference in the toxicity was found between LPS and NPP: mice died 18 h after injection of LPS, but 4 days were necessary to obtain the same result with NPP.

For the Shwartzman and Sanarelli reactions, the results of the local Shwartzman reaction induced by LPS of *S. typhimurium* and PP are presented in Table 3.

The allergizing intravenous injection was 10 μg/kg for LPS. The Shwartzman reactivity was similar for PP with 200 μg/kg. The general Sanarelli reaction was obtained with 2 μg/kg for LPS and 5,000 μg/kg for PP (Table 4). The same results were obtained with identical doses of PNP.

In the *Limulus* test the minimum to gel was 10^{-9} g/ml for LPS of *E. coli* and 10^{-8} g/ml for NPP and PP.

For pyrogenicity, the minimal pyrogenic dose is the dose giving an increase in rectal temperature of 0.6°C for rabbits weighing 2 to 2.5 kg. NPP, PP, and LPS of *E. coli* and *S. typhimurium* had the same minimal pyrogenic dose (1 μg/kg). Endotoxin is destroyed by a treatment with 0.25 N NaOH at 56°C for 60 min, and it does not give a pyretic response. In this way, Rotta showed the difference between endotoxin and an endotoxin-like substance (26, 27). In this study experiments were made with the dose used by Rotta (5 μg/kg).

Figure 2 shows the thermal curves in rabbits injected intravenously with 5 μg of LPS of *E. coli* per kg and 5 μg of NPP and PP per kg. Each plot of temperature increment shows the average for three rabbits. LPS and NPP or PP provoked comparable fever intensity for equal dosage. However, the NPP fever curve was not similar to those obtained with LPS in their biphasic course. Moreover, the development of the fever response after NPP or PP was retarded as shown on this figure. PP was treated with NaOH, and PPT was obtained. After this treatment, the pyretic response was lower but still present (Fig. 2).

TABLE 3. *Production of local Shwartzman reaction in rabbits*[a]

Concn of prepn i.d. (mg/ml)	Reaction[b]	
	LPS *S. typhimurium*	PP
2.5	−	−
10	+	−
20	+	−
40	NP	−
100	NP	−
200	NP	+
300	NP	+
600	NP	+

[a] The interval between the intradermal (i.d.) and intravenous injections was 24 h. Challenge doses were 20 μg/kg intravenously.

[b] Symbols: +, necrosis; −, negative; NP, not performed.

TABLE 4. *Production of general Sanarelli reaction in rabbits*[a]

Concn of preparation i.v. (μg/kg)	Reaction[b]	
	LPS *S. typhimurium*	PP
2	+	−
5	+	−
10	+	−
40	+	−
100	+	−
400	+	−
1,000	NP	−
2,000	NP	−
4,000	NP	−
5,000	NP	+

[a] The interval between the two intravenous (i.v.) injections was 24 h.

[b] Symbols: +, died; −, negative; NP, not performed.

Immunogenicity in this connection is defined as the capacity of LPS, when injected intravenously in rabbits, to induce the formation of circulating antibodies. LPS of *E. coli* is a potent immunogen, and antibodies were present after primary immunization with a total dose of 3.7 μg/rabbit. The antibody titers by passive hemagglutination were 1:640 on day 11 with this LPS. They were lower than 1:10 with NPP or PP for the same injected dose. After a booster dose, the titers of antibodies in the sera of rabbits immunized by NPP or PP were also negative.

DISCUSSION

LPS is essentially a component of the characteristic outer membrane of the gram-negative cell envelope. It can be extracted from any serotype of *H. influenzae*. Moreover, this bacteria is found without capsule in patients with chronic bronchitis not treated by antibiotic therapy. In this study, we used *H. influenzae* type a provided in large quantities. Chemical analysis of NPP and PP were performed by methods commonly used for studies on the composition of bacterial LPS. Therefore, it was not necessary to use LPS of *Salmonella-Escherichia* group as standard preparation. On the other hand, the activities of the *H. influenzae* phenol-water extract were always studied in relation to LPS of *E. coli* or *S. typhimurium* for a better comparison. The basic structure proposed for LPS of the

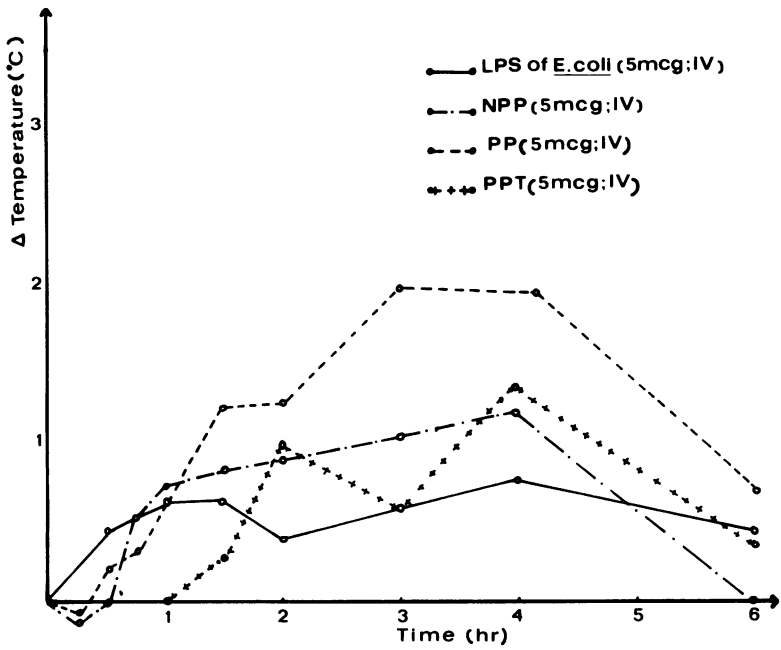

FIG. 2. *Pyrogenic responses of rabbits to intravenous injections of various preparations (LPS, NPP, PP, PPT) in 1 ml of normal saline. Each point is the average of three rabbits.*

Salmonella-Escherichia group consists of a backbone of heptose, phosphate, and KDO to which are attached side chains containing glucose, galactose, and *N*-acetylglucosamine. Attached to the backbone core, through KDO, is the lipid A moiety, which is a phosphorylated polyglucosamine mainly acylated with hydroxy fatty acids. The chemical composition of phenol-water extract from *H. influenzae* was different. It contained neutral sugars (glucose, galactose, mannose) and glucosamine. These common hexoses and hexosamine are widely distributed in bacterial LPS. Uronic acids were not found, but some authors maintain that they are sometimes indicative of contaminants (36). Ethanolamine was also present in NPP and PP. It is a well-recognized component of LPS. Furthermore, these preparations did not contain the essential components common to LPS of the gram-negative groups, KDO and heptose. However, on one hand the detection of small amounts of KDO and heptose can be difficult, and on the other hand, some authors have found LPS in which one or both of these sugars are lacking (36). A contamination of PP by loosely bound lipid can explain the presence of fatty acids in $CHCl_3S$. But fatty acids were also detected in the ether fraction obtained by saponification and in another $CHCl_3$ fraction called lipid A. Glucosamine and phosphorus were also present in this lipid A fraction. These components are commonly found by similar methods in lipid A obtained from various bacteria. Further investigation is necessary to define lipid composition of NPP and PP.

Toxic properties were found by Dubos in a rather undefined extract from *H. influenzae* (R. J. Dubos, J. Bacteriol. **43**:77-78, 1942). This toxic activity was not reproduced by Tunevall (29). Recently, Branefors has isolated a toxic precipitogen from *H. influenzae* (4). The serotype b was used by both of these authors, but neither used strain type a.

Under the same experimental conditions, 50% lethal dose determination and lethality time were different for NPP, PP, and LPS of *E. coli* and *S. typhimurium*. Among biological tests, the ability of endotoxins to cause gelation of the lysate from amaebocytes of *Limulus* is an ultrasensitive method. No significant variation was observed by this method. Pyrogenicity is also one of the most striking properties of endotoxin, but it is not specific: many bacterial extracts have a pyrogenic activity. The minimal pyrogenic dose of these various preparations was the same, but their fever curves were different. Furthermore, PP was not destroyed by NaOH. PPT produced only a slightly reduced fever compared with that of the untreated material. The pyrogenicity of LPS of *E. coli* was destroyed by the same treatment. Therefore, the fever effect of PP does not seem to be caused by endotoxin.

Higher doses were necessary to obtain Shwartzman reaction and Sanarelli phenomena with NPP and PP than with LPS of *S. typhimurium*. LPS from gram-negative bacteria have immunological characteristics that distinguish them from most other antigens. Thus, the immune response is induced by low doses of endotoxin. In this study, as usual, rabbits immunized with a total dose of 3.7 μg of LPS of *E. coli* produced antibodies rapidly and to a significantly greater extent than nonimmunized controls. No modification was found in sera of rabbits immunized with an identical total dose of PP. Thus, the immunogenic response is different for the phenol-water extract from *H. influenzae* than for "classical" LPS. These properties of NPP and PP are similar to those known from studies about peptidoglycan. Peptidoglycan can be responsible for endotoxin-like properties. But our analytical study showed that the contamination of NPP and PP by peptidoglycan was negligible and so could not explain their biological activities. Indeed, muramic acid was not found, and the level of diaminopimelic acid was about 0.25%.

This investigation has revealed that the phenol-water extract from *H. influenzae* type a is not a classical endotoxin, but rather a preparation responsible for endotoxin-like properties. Similarly, other studies have shown that chemical and biological characteristics of endotoxins vary considerably, depending on such factors as bacterial strains and extraction procedures (11).

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