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Biological Activities of Fragments Derived from *Bordetella pertussis* Endotoxin: Isolation of a Nontoxic, Shwartzman-Negative Lipid A Possessing High Adjuvant Properties

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Endotoxin from freshly sedimented *Bordetella pertussis* cells, isolated by the phenol/water procedure, when submitted to kinetically controlled, mild acidic hydrolysis released a polysaccharide (polysaccharide 1), a complex lipid (lipid X), and a glycolipid. When treated with somewhat stronger acid, the glycolipid yielded a second polysaccharide (polysaccharide 2) and another complex lipid (lipid A). The intact pertussis endotoxin had all the usual properties of endotoxins extracted from enteric bacteria. Lipid X and the intermediary glycolipid retained all the endotoxic properties of the unfractionated endotoxin. In lipid A, pyrogenicity was reduced to a very low level and toxicity and Shwartzman reactivity were absent; however, this fraction retained most of the endotoxin's antiviral activity, and its adjuvant power was considerably higher than that of the intact endotoxin. Lipid A elicited nonspecific resistance against challenge with certain bacteria, but not against others.

It is generally admitted that the endotoxic activities of lipopolysaccharides extracted from gram-negative bacteria reside in the complex lipid moiety of these macromolecules, and the carbohydrate moiety is responsible for the O-antigenicity. There is increasing evidence available to show that at least some of these endotoxic activities are not dependent on the same chemical structure. Thus, upon acetylation, lethally toxic activity and pyrogenicity were reduced, but capacity to elicit stimulation of nonspecific resistance to bacterial challenge was not affected (8, 34); since the reduction of pyrogenicity and lethal toxicity of acetylated derivatives was of a different order of magnitude, it was concluded that these properties were not dependent on the same chemical groupings or conformations of a given structure of the native endotoxin (33). These reports corroborated an earlier observation (28) that alkaline detoxification reduced lethality but not pyrogenicity of *Escherichia coli* lipopolysaccharide. It has also been shown that in vitro treatment of endotoxin with homologous hyperimmune antiserum (30) or with reagents capable of removing esterified fatty acids (4) abolished the local Shwartzman, pyrogen, and animal lethality properties, but left the nonspecific resistance-enhancing capacity intact.

Early attempts to obtain selective retention of certain biological activities, which made use of 1 M (35) or 0.1 M (25) mineral acid, led to insoluble lipid A preparations which retained most of the biological activities of the intact endotoxin, but at considerably reduced levels. Biological potencies of lipid A preparations with similarly low activities obtained by hydrolysis with 0.1 M acetic acid (12, 17, 23, 25) were found to be enhanced upon solubilization of the material by complexing with triethylamine (25) or with bovine serum albumin (11, 15, 22). Activity of lipid A preparations derived from the endotoxin of *Serratia marcescens* was restored by bovine serum albumin when the hydrolysis was carried out with 0.1 M acetic acid, but not when 0.1 M HCl was used (3). Water-soluble sodium and triethylammonium salts of lipid A preparations, obtained by autohydrolysis of endotoxin previously submitted to electrodialysis (13), had activities comparable to those of intact endotoxin as regards lethality, mitogen B, and local Shwartzman tests (12), and it was observed that, whereas in the complement inactivation test the sodium salt was as effective as the starting endotoxin, the triethylamine salt was almost completely inactive. Isolation of fragments retaining selectively any given property of the intact endotoxin has, to our knowledge, not been reported.

It has been shown recently (21) that by the kinetically controlled fragmentation sequence of

the *B. pertussis* endotoxin, shown in Fig. 1, two polysaccharide fragments and two complex lipid fragments were released and could be isolated. Although their structure has not been completely elucidated yet, it is known that at their reducing end both polysaccharides are terminated by a single molecule of 3-deoxy-oct-2-ulosonic acid; they can be differentiated by their molecular weights, their phosphate content, and the substitution pattern of their terminal 3-deoxy-oct-2-ulosonic acid molecule. The molecular composition of the two lipid fragments is similar to those reported for lipid A preparations obtained from enterobacterial endotoxins; they could be differentiated by the presence of 2-methyl-3-hydroxy-decanoic and 2-methyl-3-hydroxy-tetradecanoic acids (16) in the minor lipid X fragment and by the absence of these in the major lipid A fragment. Selective retention of certain biological activities by some fragments of the *B. pertussis* endotoxin is reported in this paper.

MATERIALS AND METHODS

B. pertussis endotoxin was extracted, purified, and fractionated as described previously (21). Briefly, the endotoxin was treated with trifluoroacetic acid (pH 3) at 50°C until response in the thiobarbiturate test reached a constant value. The precipitate formed was exhaustively extracted with a mixture of toluene and methanol, leaving the insoluble fraction called "glycolipid"; removal of the solvent from the extract gave the complex lipid named lipid X (see Fig. 1). Attention is drawn to a novel observation that, after extraction of the endotoxin with organic solvents, the residual, delipidated endotoxin should only be air-dried. Thorough removal of solvents, for instance by keeping the substance in vacuo at 30 to 50°C, led to material that required prolonged hydrolysis (~200 h) with trifluoroacetic acid to obtain release of polysaccharide 1 and lipid X. In such cases the lipid X fragment was isolated in much lower yields, and its pyrogenicity and potency in the mouse weight gain test was considerably diminished.

Pyrogenicity. Pyrogenicity was measured by the standard procedure (31) using New Zealand albino rabbits (2.5 to 2.7 kg) of the same sex in groups of three for each substance to be tested. Each animal received intravenously, per kg, 1 ml of saline containing 2 μg of the substance to be tested per ml; controls received saline only. Rectal temperatures were monitored continuously for 3 h, and the maximal increase in temperature ($\Delta t°$) was recorded for each animal. Results are expressed as the sum (Σ) of the $\Delta t°$ values for the three rabbits.

General toxicity. General toxicity was estimated by the standard mouse weight gain test (7). Inbred (OF1) female mice (18 to 20 g) were used in groups of 10 for each substance to be tested and for each of the three dose levels used. The dose range to be used was determined by preliminary experiments. Substances were injected intraperitoneally (i.p.) in 0.5 ml of saline. Controls were injected with saline alone. The mean values of the variation of weight (Δp) observed 24 h after injection, plotted against the log of the injected dose, give a straight line; the "limit dose," for which Δp equals zero, is obtained by interpolation.

Local inflammation and local Shwartzman reaction. A number of New Zealand albino rabbits (2.2 to 2.5 kg) of the same sex received randomly injected duplicates of 12.5, 50, and 200 μg of the substance to be tested in 0.2 ml of saline intradermally on the shaved abdomen. The diameter (millimeters) of the strongest lesion observed was measured 17 h later. After 16 h, the animals were given 40 μg of *Shigella dysenteriae* endotoxin (Difco) per kg in 0.5 ml of saline intravenously, and, another 6 h later, the necroses were evaluated visually and marked from 1 to 4 (strongest). Results are expressed as the sum (Σ) of the six [2 × (12.5 + 50 + 200)] values obtained for each substance tested.

Histamine hypersensitivity test (26). Groups of 10 CFW mice (19 to 21 g) received i.p. 100 μg of the substance to be tested in 0.5 ml of saline. Controls received saline. Five days later, 1/30 50% lethal dose of histamine dihydrochloride (i.e., 0.45 mg of histamine base per mouse) was given i.p. in 0.5 ml of saline. Mortality was recorded 2 and 24 h later. Positive controls obtained by injection of 1.3×10^9 *B. pertussis* organisms 5 days before histamine challenge, and neg-

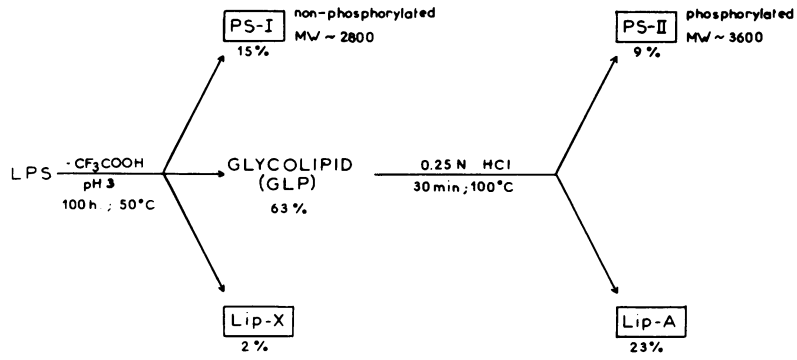

FIG. 1. *Fragmentation sequence of the B. pertussis endotoxin.*

ative controls which received the histamine challenge only, were included.

Increase of nonspecific host resistance to bacterial infection. Groups of 10 female OF1 mice (20 to 22 g) received i.p. increasing amounts (12.5, 50, and 200 μg) of the substance to be tested in 0.5 ml of saline. Three days later, the animals were challenged i.p. with suspensions of the following virulent strains of bacteria: *Salmonella typhi* Ty2, *E. coli* O:111, *Klebsiella pneumoniae* b, *Pseudomonas aeruginosa* Habs serotype 6, and *Staphylococcus aureus*. The level of challenge was between 5 and 15 50% lethal doses and chosen to ensure no survival in the group of controls. Deaths were recorded until no further mortality was observed. The 50% protective dose was calculated from the survival rate plotted against the log of the dose.

Increase of host resistance to viral infection. Groups of 10 female mice (OF1, 27 days old) received a single i.p. injection of 200 μg of the substance to be tested and were challenged 24 h later by a subcutaneous injection of Semliki Forest virus (250 50% lethal doses) or by an i.p. injection of encephalomyocarditis virus (1.7 50% lethal doses). The challenge dose gave a mortality of at least 75% of the controls. Eleven days later, the number of surviving animals was recorded.

Adjuvant effect. The adjuvant effect was measured in mice by the increase of circulating antibodies to *Myxovirus influenzae* vaccine. The dose of human bivalent (A and B) influenza vaccine (Institut Mérieux) that elicited a small but statistically significant amount of circulating antibodies in mice (OF1, female, 20 to 22 g) on day 21 after a single i.p. injection was determined first; it was found to be about 10 International Units. A mixture of this amount of antigen and 200 μg of the substance to be tested, in a total volume of 0.5 ml, was injected into groups of eight mice. Controls received the antigen in saline only. Three weeks later the animals were bled, sera were decomplemented, nonspecific inhibitors were removed by treatment with RDE (Duphar), and the antibody titer was determined as the reciprocal of the last serum dilution able to inhibit hemagglutination. The adjuvant effect is given as the ratio mean value of the titers of antigen + product/mean value of the titers of antigen alone.

Lipids, which had low solubility, were suspended in saline, and the mixture was shaken mechanically for the time required (1 to 2 h) to obtain an apparently homogeneous dispersion.

RESULTS

Clinically undesirable properties of the pertussis endotoxin and its fragments are shown in Table 1. For the purpose of comparison, we have also recorded properties of the polysaccharide released from the endotoxin of *Salmonella typhimurium* LT2 by kinetically controlled hydrolysis with trifluoroacetic acid (pH 3, 50°C) and purified by gel filtration. The whole pertussis endotoxin has biological properties comparable to those obtained from Enterobacteria, as has been observed by previous authors (1, 2, 5, 6, 8, 12, 19, 20, 24, 27, 32). As regards the fragments, although neither polysaccharide is toxic, pyrogenic, or sensitizing (and thus both conform in their physiological properties to polysaccharides isolated from enterobacterial endotoxins), only the minor lipid X of the pertussis endotoxin exhibits the usual toxic, pyrogenic, and sensitizing properties of the complex lipid moiety (lipid A) of enterobacterial endotoxins, whereas the major complex lipid isolated from the pertussis endotoxin (lipid A) lacks all of these properties. It is noteworthy that enterobacterial lipid A is usually obtained after exposure of the endotoxin to acid treatment (acetic acid, pH 3.4; 100°C; 1 to 2 h) milder than that (HCl, 0.25 M; 100°C; 25 min) required to release pertussis lipid A from the toxic, pyrogenic, and Shwartzman-positive glycolipid complex.

The values reported appeared to be readily reproducible for a number of preparations, except for two properties of the fraction lipid X; the pyrogenicity and general toxicity of this fraction decreased when the time of hydrolysis of the endotoxin at pH 3 was increased.

Because of the well-known property of *B.*

TABLE 1. *Clinically undesirable properties of the B. pertussis endotoxin and its fragments*

Endotoxin fragment[a]	MWGT[b] limit dose (μg)	Pyrogenicity[c] (°C)	Local inflammation (mm diam)	Local Shwartzman reaction[d]	Histamine hypersensitivity (deaths)
LPS	1.07	5.9	20	8	0
PS-1	500	0.45	0	0	0
GLP	1.73	6.3	9	9	0
Lipid X	124[e]	4.3[e]	9	2	0
	397[f]	1.3[f]			
PS-2	500	0.65	0	0	0
Lipid A	500	1	7	0	0
PS from *S. typhimurium* LT2	500	0.45	1	0	ND[g]

[a] LPS, Lipopolysaccharide; PS, polysaccharide; GLP, glycolipid.
[b] MWGT, Mouse weight gain test.
[c] 2-μg/kg dose.
[d] Σ of six values as given in the text.
[e] 200-h hydrolysis at pH 3, 50°C.
[f] 60-h hydrolysis at pH 3, 50°C.
[g] ND, Not done.

pertussis to sensitize the host to histamine, all preparations were submitted to the histamine hypersensitivity test; all gave negative results and were thus not contaminated by the histamine sensitizing factor.

The antiviral and adjuvant actions of the pertussis endotoxin and of its fragments are shown in Table 2. (For purposes of comparison, data obtained simultaneously with formolated *Corynebacterium parvum* cells, as used in clinical practice, are also recorded.) Polysaccharide 2 excepted, all of the substances tested had considerable activity against both the challenging viruses. Similarly, in the adjuvancy test both the intact endotoxin and its fragments, including the polysaccharides, appeared to be active. Polysaccharide 2, lipid X, and lipid A exhibited unexpectedly high potencies; these fragments were, in fact, much more active than the intact endotoxin. As far as we know, fragments of endotoxins with biological activities higher than the parent macromolecule have not been described as yet.

Five different pathogens were used to evaluate the antibacterial activity of the pertussis endotoxin and of its fragments. From the data shown in Table 3, it appears that against *S. typhi* and *K. pneumoniae* the nontoxic lipid A fragment gave as good protection as the toxic endotoxin, glycolipid, and lipid X preparations. Against *S. aureus* endotoxin, glycolipid and lipid A showed activity, but lipid X was inactive, whereas against *P. aeruginosa* only the intact endotoxin and the glycolipid conferred protection, while both lipid fractions were inactive. Extensive protection was obtained with the endotoxin, the glycolipid, and lipid X against challenge with *E. coli*, but none with the lipid A fragment. Spurious, statistically not significant protection sometimes appeared with one or another polysaccharide fragment against all five microorganisms. Since both polysaccharides were purified by gel chromatography, it is unlikely that this activity should have its origin in contamination from the endotoxin itself or from the lipid fragments. Results obtained with the intracellular pathogen *Listeria monocytogenes* are complex and will be reported separately.

TABLE 2. *Antiviral and adjuvant activities of the B. pertussis endotoxin and its fragments*

Endotoxin fragment[a]	Antiviral activity[b] (% survival)		Adjuvant activity (factor of increase)	
	EMC virus	SF virus	Human influenza valence A	Human influenza valence B
LPS	100	100	2.4	2.7
GLP	100	100	2.7	2.7
PS-1	ND[c]	ND	1.8	2.3
Lipid X	100	100	5.1	6.7
PS-2	60	10	2.6	4.8
Lipid A	100	70	4.6	3.7
C. parvum	25	ND	3.5	3.2
Control	25	10	1	1

[a] LPS, Lipopolysaccharide; GLP, glycolipid; PS, polysaccharide.
[b] EMC, Encephalomyocarditis; SF, Semliki Forest.
[c] ND, Not done.

DISCUSSION

It has been observed previously that endotoxin preparations obtained from *B. pertussis* possess all of the usual biological properties associated with endotoxins: they are pyrogenic and toxic, they elicit the Shwartzman phenomenon and nonspecific immunity in mice, they have adjuvant properties, and they provoke interferonemia. It was therefore of interest to investigate the biological properties of the major fragments resulting from the controlled hydrolytic cleavage of the pertussis endotoxin, especially since two different polysaccharides, both terminated by a single molecule of 3-deoxy-oct-2-ulosonic acid, and two complex lipid-containing fragments were obtained, rather than, as is the case with enterobacterial endotoxins, a single chain of polysaccharide (O-specific side chain + core) and a single lipid fraction (lipid A).

In enterobacterial endotoxins, lipid A is considered to be the region responsible for the endotoxic properties of the molecule (22). Enterobacterial lipid A preparations, obtained by the acid-catalyzed cleavage of the glycosidic bond of 3-deoxy-oct-2-ulosonic acid present in most endotoxins, are heterogeneous (3, 29), and it was observed that separated lipid A fragments had different potencies in various biological tests (3). Whereas both polysaccharide fragments of the pertussis endotoxin, being purified by gel permeation chromatography, are likely to be homogeneous, nothing is as yet known about the homogeneity of the insoluble lipid A and lipid X fragments and the intermediate glycolipid. Since pertussis lipid A was obtained by treatment with relatively strong acid (0.25 M HCl, 100°C, 30 min), it is unlikely to be homogeneous. It is known, for instance, that in the conditions used the glycosidic bond of a 2-*N*-acylamido-2-deoxyglucopyranoside would be broken (9); we have also observed that crude lipid X preparations contained free fatty acids. This clearly indicates that even in far milder conditions (pH 3, 50°C, 60 to 70 h) cleavage of ester bonds does occur. There are thus at least two types of covalent bonds known to be present in the complex lipid part of the endotoxins, whose cleavage is likely

TABLE 3. *Increase of nonspecific host resistance to bacterial infection elicited by the B. pertussis endotoxin and its fragments*[a]

Endotoxin fragment[b]	Dose (μg)	*S. typhi*		*P. aeruginosa*		*S. aureus* (5.9 LD_{50})	*E. coli* (5 LD_{50})	*K. pneumoniae* (14.7 LD_{50})
		11 LD_{50}[c]	4 LD_{50}	8 LD_{50}	8.6 LD_{50}			
LPS	12.5	0	10	0	0	10	60	60
	50	30	70	0	10	20	60	100
	200	70	100	30	100	80	100	100
	PD_{50}[d]	97	36.7	>200	78.4	84.8	9.9	<12.5
GLP	12.5	0	10	0	10	20	90	30
	50	60	90	20	20	80	90	90
	200	90	100	70	100	—	100	100
	PD_{50}	50	29.9	110	61.4	—	≪12.5	<12.5
PS-1	12.5	20	10	0	10	0	30	20
	50	0	0	10	0	0	40	40
	200	20	10	10	10	0	10	20
	PD_{50}	≫200	≫200	≫200	≫200	≫200	≫200	≫200
PS-2	12.5	0	10	0	0	0	10	0
	50	0	0	0	0	0	20	0
	200	0	0	0	0	0	30	30
	PD_{50}	≫200	≫200	≫200	≫200	≫200	≫200	≫200
Lipid A	12.5	0	20	0	20	0	10	70
	50	30	10	0	10	10	10	90
	200	70	60	10	10	90	0	100
	PD_{50}	97	200	≫200	≫200	100	≫200	<12.5
Lipid X	12.5	0	30	0	10	10	50	ND[e]
	50	40	50	10	0	0	50	ND
	200	100	100	0	0	20	100	100
	PD_{50}	55	33.6	≫200	>200	>200	19.8	<12.5

[a] Groups of 10 mice were treated with 12.5, 50, and 200 μg of the material to be tested and challenged 3 days later with the bacterial suspensions. Survivors are expressed in percentages.

[b] LPS, Lipopolysaccharide; GLP, glycolipid; PS, polysaccharide.

[c] LD_{50}, 50% lethal dose.

[d] PD_{50}, 50% protective dose.

[e] ND, Not done.

to contribute to the heterogeneity of these fragments, including those isolated from *B. pertussis*.

Unexpectedly, biological properties of the fragments lipid X and lipid A were found to be very different: lipid X retained all of the endotoxic properties of the intact endotoxin, whereas in lipid A pyrogenicity was reduced to a very low level and toxicity and the capacity to elicit the Shwartzman phenomenon were absent. Lipid A retained, however, adjuvanticity and the faculty to induce nonspecific immunity against bacterial and viral challenge. It is noteworthy that the glycolipid, lipid X, and lipid A had, in some tests, quantitatively increased biological potencies as compared to the intact endotoxin.

The relatively low activity of crude, heterogeneous lipid A preparations was ascribed (15) to the low solubility of the material and to partial degradation occurring during the hydrolytic procedure (3). Lipid A and lipid X fractions of the pertussis endotoxin are both insoluble in water, at any rate much more insoluble than the intact pertussis endotoxin. It is therefore unlikely that the lack of solubility should be a generally valid explanation for the low biological activities usually observed with other lipid A preparations. In the present work no attempt was made to solubilize the lipid A and lipid X fractions of the pertussis endotoxin.

The fact that lipid X retained more of the intact endotoxin's biological activities than did lipid A probably does not mean that the molecular structures responsible for the biological activities that were not recovered after the fragmentation procedure were originally absent from the lipid A, i.e., while lipid A was still part of the unfragmented endotoxin. Indeed, all of the biological activities of the unfragmented endotoxin can be elicited, in some cases more

efficiently, by the glycolipid that remains after removal of polysaccharide 1 and lipid X and which contains polysaccharide 2 and lipid A. The structure carrying these activities must, therefore, have survived the first mild acidic treatment.

Isolation, after treatment of the glycolipid with mineral acid, of a lipid fragment that possesses no or negligible pyrogenic, toxic, and sensitizing properties, but retains adjuvanticity and the faculty to elicit nonspecific resistance against bacterial or viral challenge, may be due to chemical destruction of the effectors of the former but not of the latter activities. It has been noted previously (3) that the lethal toxicity (chicken embryo) of enterobacterial lipid A preparations obtained with 1 M HCl decreased as a function of the time of hydrolysis; as a corollary, whereas the endotoxicity of lipid A preparations obtained by hydrolysis with acetic acid could be considerably enhanced by complex formation with bovine serum albumin, no such effect was observed with lipid A prepared by treatment with mineral acid. Since, during the chromatographic procedure employed to recover polysaccharide 2, the presence of water-soluble fragments different from polysaccharide 2 was detected, and since the lipid A fragment was purified by extraction with organic solvents, it is also possible that fragments carrying the biological properties that were lost in fact survived the hydrolytic procedure, but have, as yet, not been identified. Alteration of a number of biological activities of endotoxins as a result of chemical transformations other than acidic hydrolysis of the macromolecule has also been observed (34); it was proposed (33) that this alteration reflected dependence of the activities concerned on different chemical structures or conformations. These conclusions are born out by the present study. The considerable difference in resistance to treatment with acid very likely reflects dependence on chemically different effectors.

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