Analysis of Unmodified Endotoxin Preparations by $^{252}$Cf Plasma Desorption Mass Spectrometry

DETERMINATION OF MOLECULAR MASSES OF THE CONSTITUENT NATIVE LIPOPOLYSACCHARIDES*

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Nine unmodified endotoxin preparations constituted of Re-, Rd-, and Re-type lipopolysaccharides (2 to 5 glycoses), representing four species of enterobacteria were analyzed by $^{252}$Cf plasma desorption mass spectrometry. The constituent lipopolysaccharides were characterized by the ion pair: (M – H$^-$) and its corresponding lipid fragment ion. The lipid fragment ion is produced by cleavage of the glycosidic bond of the 3-deoxy-$\alpha$-manno-oct-2ulosonic acid unit that substitutes 0-6' of the glucosaminyl6'-6glucosamine ("lipid A backbone") disaccharide of the lipid A moiety. These lipid fragment ions were identical to the (M – H$^-$) ions seen in the spectra of homologous isolated lipid A preparations that were obtained by hydrolysis (pH 4.5, 100 °C) promoted by sodium dodecylsulfate. Since the molecular components present in the endotoxin preparations analyzed are known, the ion pair (M – H$^-$)-lipid fragment ion defines the molecular compositions of each individual lipopolysaccharide. Heterogeneity of the R-type endotoxin preparations analyzed was due almost exclusively to differing lipid A moieties. In three Salmonella minnesota 595 Re endotoxin preparations 10 different lipopolysaccharides were identified, only two of which were common to all three preparations. Of the nine lipopolysaccharides identified in two S. minnesota R7 endotoxin preparations, only two were present in both.

Endotoxins are amphipathic macromolecules that are obligate (1, 2), major (3) antigens (4, 5) of the outer membrane of Gram-negative bacteria. The fundamental elements of their hydrophobic ("lipid A") domains is a bis-phosphorylated, $\alpha$1,6-linked disaccharide of $\alpha$-glucosamine substituted by fatty acids (6), 3R-3-hydroxytetradecanoic, dodecanoic, tetradecanoic, and hexadecanoic acids being frequently encountered. In the hydrophilic domain which is constituted of various acidic, basic, and neutral glycoses two regions can be distinguished: the "core," proximal to lipid A and the "side-chain." The biosynthetic pathways and the overall structure of these regions are different (7, 8). Core and lipid A are invariably joined by the glycosidic bond of a 3-deoxy-$\alpha$-manno-2-octulosonic acid (KDO)$^1$ which, in certain strains, may be the sole component of the hydrophilic domain.

Being among the most efficient activators of the human immune system, endotoxin preparations are extensively used to induce immunological reactions in whole animals and isolated cells, and numerous attempts have been made to establish structure/activity relationships. However, because of the heterogeneity of the inducer, the data obtained cannot be considered as unambiguous. It has been demonstrated by chromatography (9) and by electrophoresis (10, 11); that endotoxin preparations ("endotoxin," "LPS"), as obtained by extraction (12) of bacterial cells, are heterogeneous and represent ensembles of related, but constitutionally distinct, molecular species. Chemical analysis of fragments produced by hydrolysis revealed that heterogeneity may be due to the variability of both domains (13, 14). Depending on the genus, the species, the strain, and the culture conditions of the cells, the molecular masses of the individual lipopolysaccharides present in any given endotoxin preparation may vary considerably (15). As estimated by indirect methods, they appear to range from 2 to 20 kDa (16); however, none have been determined accurately. So far no endotoxin preparation has been described in terms of unmodified individual chemical structures of its constituent lipopolysaccharides. Different forms of mass spectrometry (fast atom bombardment, laser desorption, $^{252}$Cf PDMS) have been used to determine molecular masses and exact chemical structures of major components of hydrophobic domains ("diphosphoryl lipid A") or fragments ("monophosphoryl lipid A") of endotoxic lipopolysaccharides (17–23). The material thus analyzed was obtained from endotoxin preparations by treatment with acid, followed by chromatographic purifications. With one exception (21) for all such studies the material was derivatized with diazomethane. $^{252}$Cf PDMS was used to establish (19) the molecular mass and to confirm previously proposed structures, based on degradative (24) and NMR (25) studies, of a single Re-type (i.e. constituted of lipid A and 2 KDO residues) lipopolysaccharide (26). To this end a major component of the endotoxin preparation was isolated by column chromatography and derivatized.

1 The abbreviations and trivial name used are: KDO, 3-deoxy-$\alpha$-manno-2-octulosonic acid; C12, dodecanoic acid; C14, tetradecanoic acid; C14OH, hydroxytetradecanoic acid; C16, hexadecanoic acid; Ettn, etanolation (2-amino-ethanol); lipid A, the hydrophobic domain of endotoxic lipopolysaccharides; isolated lipid A, the heterogeneous precipitate isolated after treatment of endotoxin preparations with acid; lipid base, a group of constituents found to be common to most of the lipopolysaccharides present in the endotoxin preparations analyzed in this study; LPS, endotoxic lipopolysaccharide of bacterial origin; n.i., not identified; n.o., not observed; obs., observed; PDMS, plasma desorption mass spectrometry; PenN, pentosamine; Phosph., phosphoric acid, HPLC, high performance liquid chromatography.
The hexamethyl ester of the lipopolysaccharide thus obtained was then analyzed by $^{252}$Cf PDMS in the positive ion mode. Isolated lipopolysaccharides with glycosyl chains consisting of more than two KDO units have not been analyzed by this technique so far, nor have endotoxin preparations been defined in terms of their constituent lipopolysaccharides.

It has now been found that unmodified Re- to Re-type (27) endotoxin preparations, the constituent lipopolysaccharides of which have short glycosyl chains (up to 5 sugars), can be analyzed by $^{252}$Cf PDMS, and the preparations described as ensembles of lipopolysaccharides of defined composition. The results thus obtained accounted for all constituents previously shown to be present in the endotoxin preparations and agree with the proposed general architecture of endotoxic lipopolysaccharides. The interpretation of lipopolysaccharide spectra was confirmed by spectra obtained for homologous isolated lipid A preparations.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Negative-ion PDM spectra (Fig. 1) were obtained for nine different, commercially available, unmodified endotoxin preparations derived from Re-, Rd-, and Re-type mutant cells, and for their respective "isolated lipid A" fragments. Since the molecular components that are released upon acid-, or base-catalyzed hydrolysis of such enterobacterial endotoxins are well known, possible molecular masses of individual lipopolysaccharides could be computed. They were found to account, almost without exception, for signals observed at high (m/z > 2200) values. Consequently, these signals were taken to represent (M − H)$^-$ ions. Those appearing in the lower range of the spectrum were taken to be due to fragment ions: indeed, almost all of them coincided with (M − H)$^-$ ions observed in spectra of homologous isolated lipid A preparations. The spectra thus interpreted revealed that all endotoxins examined were mixtures of a variable number of lipopolysaccharides (Table 1). The expression "lipid base" used here refers to a group of nine constituents (2 GlCN, 2 phosphate, 4 C14OH, 1 C12) that was found to be present in all (save F6) lipopolysaccharides identified. This group is, however, not necessarily the common set of constituents for other endotoxins. Lipid base is not equivalent to lipid A. The latter is the name extensively used to designate the hydrophobic domain of endotoxic lipopolysaccharides.

Since no data are available concerning the fragmentation of unmodified lipopolysaccharides in the conditions of $^{252}$Cf PDMS, a synthetic model, allyl 2-deoxy-2-[3R]-3-hydroxytetradecanamido]-6-O-(3-deoxy-α-D-manno-2-octulopyranosio)-3-D-glucopyranoside(4-phosphate (32), was used to ascertain that in oligosaccharides the glycosidic bond of KDO units was in fact preferentially cleaved (Fig. 2).

Spectra of the endotoxins of *Escherichia coli* D31m4 and *Shigella flexneri* (both Re mutants) (Fig. 1 and Table 1: A and B) were very similar. The two most intense signals of each spectrum (m/z 2318 and 2238), taken to be molecular ions, indicated that the corresponding lipopolysaccharides had, within experimental error, the same molecular masses. Their compositions are given in Table 1. The lipopolysaccharides with (M − H)$^-$ m/z 2318 differed from those with (M − H)$^-$ 2238 only by the presence of an additional phosphate (+80 atomic mass units), that was probably part of a pyrophosphate group (24, 25). Other lipopolysaccharides, apparently minor components, were also present in both endotoxins: e.g. in that of *E. coli* an (M − H)$^-$ ion appeared at m/z 2264 due to a lipopolysaccharide species containing C16 instead of C14. No such minor components were detected by their (M − H)$^-$ in the spectra of the *Sh. flexneri* endotoxins. Their existence was attested however by unresolved signals (at m/z 1810-1825) visible among the "lipid fragment ions" of the spectra of both the *Sh. flexneri* and the *E. coli* endotoxins, and also in the spectra of their isolated lipid A preparations. Fragment ions constituted of lipid base + C16 (m/z 1825.4) or + C140H (m/z 1813.4) appear in the region m/z 1810-1825. Homologs of these ions, containing one additional phosphate group, were found among the lipid fragment ions of the *E. coli* endotoxin 80 atomic mass units higher (m/z 1805 and 1893, incompletely resolved). In the spectra of the isolated lipid A of both endotoxins the signals, due to lipid fragments carrying the presumed pyrophosphate group (m/z 1879-1880), are weak. Since the isolated lipid A preparations were obtained by acid hydrolysis, (31) loss of pyrophosphate in this and other isolated lipid A preparations is not unexpected. Under the same conditions of hydrolysis ADP released 0.5 mol of phosphate per mol of ADP. Relatively weak signals corresponding to ions containing one instead of two KDO units (m/z 2018/2020) were also detected in both endotoxin preparations. This type of ion has been observed repeatedly (see below).

The other endotoxin preparations were far more heterogeneous. Thus, endotoxins of *S. minnesota* 595 Re (Sigma, lots 55 and 28) (C and D, respectively), were mixtures of at least 5 lipopolysaccharides, while that of *S. minnesota* 595 Re List (E) contained at least 7 (Fig. 1 and Table 1). Although some lipopolysaccharides were present in all three endotoxins (m/z 2607.38 and 2237.79), their relative amounts varied considerably. Others appeared in one preparation only: m/z 2731.0 (1 in D); 2856, 2361.5, and 2317.6 (2, 4, and 5 in E).

As expected, all six lipopolysaccharides identified in the endotoxin of *Salmonella typhimurium* SL1181, an Rd2 mutant (27) (erroneously labeled in the 1990 Sigma catalog as an Re mutant), contained one heptose unit (Table 1, F). The hydrophobic domain of lipopolysaccharide 6 of this endotoxin was unusual inasmuch as it contained only 3 instead of 4 C14OH (incomplete lipid base).

Spectra of the two Rd1 endotoxin preparations of *S. minnesota* 3-7 (G, List, H, Sigma) revealed considerable differences between them. Lipopolysaccharides and lipid fragments containing aminopentose or ethanolamine and additional phosphate (1, 3, 4, 4', a) were present in H. In G, molecular species and lipid fragments containing supplementary phosphate (2,3 and 2',3') or an additional C140H (a, 2,2', 4,4') were observed.

Rc-, Rd1-, and Rd2-type lipopolysaccharides were identified among the six detected in the endotoxin of *S. minnesota* R-5, which is described (33) as an Rc mutant, (Fig. 1, Table 1, I). Indeed, while four of these (1, 2, 3, 4) contained 2 heptoses plus 1 hexose, lipopolysaccharide 5 contained only 2 heptoses, and lipopolysaccharide 6 only 1 heptose in addition to the 2 KDO units. Both basic (aminopentose, ethanolamine) and acidic (phosphate) components were present, but no aminopentose-containing lipopolysaccharide was detected by its (M − H)$^-$ ion.

**DISCUSSION**

Characteristics of the Method—While pure, synthetic compounds, constituted essentially of a single molecular species,
gave good spectra rapidly, production and quality of the spectra of endotoxins were critically dependent on the procedures used for the preparation of both sample and target; even for experiments with the same material, the time required to accumulate statistically sufficient data varied considerably (12–44 h). Reasons for these phenomena are not known with certainty, but the presence of cations such as Ca$^{2+}$ and Mg$^{2+}$, invariably present in endotoxin preparations and very difficult to remove quantitatively (34), may be at least partly responsible, as even small amounts of such ions may interfere with the production of $^{252}$Cf-PDM spectra and alter their quality (35). Endotoxins gave useful spectra as negative ions only; positive ion spectra were either not produced or reflected extensive fragmentation. For isolated lipid A preparations, both positive and negative ion spectra were obtained; the latter were better for the purpose of identification of molecular peaks. As a rule, isolated lipid A preparations were ionized/desorbed much faster than endotoxins. Since production and breakdown of charged particles from aggregates was a characteristic feature of PDM spectra, the intensities of signals are merely suggestive of the relative production of molecular peaks. As a rule, isolated lipid A preparations concurred. Hence it was concluded that, in the conditions used to produce the spectra, the glycosidic bonds of KDO units were ruptured easily and selectively and that this fragmentation was a characteristic feature of PDM spectra of endotoxic lipopolysaccharides. The conclusion was corroborated by the PDM spectrum (Fig. 2) of a disaccharide, a close, synthetic analog of the region that embodies the junction core-lipid A in lipopolysaccharides, in which the main signals observed were (M – H)$^-$ and ((M – KDO) – H)$^-$. Taken together these data also prove that the method of hydrolysis (31) used afforded lipid A preparations in which the hydrophobic domains of the lipopolysaccharides were particularly well preserved.

The coherence of all these data justified the assignment of high field signals (m/z > 2200) to molecular peaks of individual lipopolysaccharides of a given endotoxin preparation, and of low field signals to fragment ions corresponding to the hydrophobic domains and fragment ions thereof; it established, moreover, that $^{252}$Cf-PDM was a valid method of analysis of R-type endotoxin preparations.

**Patterns of Fragmentation**—The signals at m/z 2020.2 and at 2018.0 in the endotoxin spectra of *E. coli* D31m4 and of *Sh. flexneri*, respectively, could either be (M – H)$^-$ ions containing one KDO unit instead of the usually encountered two, or may be fragment ions formed from the lipopolysaccharide (2237.6) through loss of a KDO unit. This type of ion was also observed in the endotoxin spectra C (6*, 7**), D (6*), and G (6*). If 6* of spectrum G is in fact a fragment ion, it must have been produced by loss of an extracatenary KDO, as RdI-type lipopolysaccharides have 2 KDO and 2 heptose units. Lipopolysaccharides containing 3 KDO units have not been discovered, but they may have escaped detection either because of the fragility of the glycosidic bond of this constituent (Fig. 2) in the conditions of $^{252}$Cf-PDM or because of their low abundance.

Fragmentation of the hydrophobic domain has also been observed. Indeed, in all spectra in which the ion m/z 1797 (lipid base + C14) appeared, the ion m/z 1587 was also present. The numerical difference (210 atomic mass units) between the two signals corresponds to formal loss of tetradecanoic acid. However, without independent proof, an ion appearing at m/z 1587 should not be interpreted as being the daughter ion of m/z 1797 and taken to establish the presence of an ester-bound tetradecanoic acid in the endotoxin preparation. Indeed, at least in enterobacterial endotoxins, the same signal may also be due to the lipid base produced as a primary fragment. For instance, this was the case for the signal H 6* which could be attributed to that primary fragment without ambiguity.

In all of the individual lipopolysaccharides (*F* 6 of *S. typhimurium* excepted) identified in the endotoxin preparations analyzed, the nine molecular constituents of the lipid base were invariably present. The constant presence of C12 and the frequent absence of C14 were unexpected inasmuch as the latter is considered to be a characteristic component of endotoxic lipopolysaccharides. In one lipopolysaccharide, namely 6 of *S. minnesota R-7* (*H*), the hydrophobic domain
consisted of lipid base alone, and in another 6 of the endotoxin of *S. typhimurium* SL 1181 (E), the lipid base lacked the fourth C14OH and contained no additional constituent. Thus the former had a penta-, the latter a tetra-acyl lipid A region.

**Lipopolysaccharide Composition of the Endotoxins Analyzed**—Heterogeneity appeared in both the hydrophilic and the hydrophobic domains of the *S. minnesota* R-5 (Re type) endotoxin. In all other endotoxin preparations heterogeneity was restricted to the hydrophobic domain and was due solely to those structural elements that are usually referred to as “constituents not present in stoichiometric amounts.” It is nevertheless noteworthy that, although not very numerous, these constituents generate considerable heterogeneity even in endotoxin preparations constituted of lipopolysaccharides that are strictly homogeneous as regards their hydrophilic domain (Table 1: C–H). Moreover, in different batches of nominally identical endotoxin preparations, different lipopolysaccharide species may predominate (Fig. 1: C, D, and E; G and H). This second type of heterogeneity may be due to such parameters as culture conditions, method of extraction, etc.; if so, its appearance is potentially avoidable.

Re-type endotoxins derived from *S. minnesota* 595 Re (Table 1, C–E) were among the most heterogeneous preparations. Of 10 different lipopolysaccharides identified, only two (m/z 2607.28, and 2237.73) were present in all of them, and considerable variation was observed even for different batches of the same strain from the same source (cf. C and D). Hepta-acyl (C, 1; D, 1, 2; E, 1–3) and hexa-acyl (C, 3–5; D, 3–5; E, 4–7) lipopolysaccharide molecular ions, the corresponding lipid fragment ions and isolated lipid A molecular ions have been recognized. Hepta-, hexa-, and penta-acyl monophosphoryl lipid A fragments have been obtained previously from *S. minnesota* 595 Re endotoxin by hydrolysis with 0.1 M HCl; they were isolated by silicic acid chromatography, purified by HPLC, and their structures established by fast atom bombardment–mass spectrometry (17). The dimethyl monophosphoryl heptapeptide A ([M + H]+ = m/z 2237.73) observed by these investigators corresponds to the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions m/z C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′ and the lipid A (M – H)– ions C 5′, 7′, D 5′ and E 7′.

This lipopolysaccharide, one of the simplest, was present in all 3 of *S. minnesota* 595 endotoxin preparations. No penta-acyl lipopolysaccharide or isolated lipid A fragment was detected in the spectra of the three endotoxin preparations of *S. minnesota* 595 analyzed, although one such lipopolysaccharide was present in *S. minnesota* R-7 endotoxin (H 6). Aminopeptidase (36), ethanolamine (37), and additional phosphate (24) are frequently encountered components of *Salmonella* endotoxin preparations. Lipopolysaccharide molecular ions having hepta-acyl (C, 1; D, 1, 2; E, 1, 2) or hexa-acyl (C, 3; D, 3, 4; E, 4, 5) lipid A regions and one or several of these constituents were also found in the spectra.

Because of their very small, simple, and well characterized hydrophilic domain, Re-type endotoxins are extensively used as immunological tools. Studies with synthetic compounds identical to those known (38) to be part of isolated lipid A preparations have demonstrated that seemingly small differences may alter considerably their biological potencies (38). In view of the heterogeneity and variability of their hydrophobic domains, Re-type endotoxin preparations should be used with caution for immunological studies.

While lipopolysaccharides of S-type endotoxins are, for the time being, not amenable to analysis by 252Cf PDMS, molecular compositions of their hydrophobic domains can easily be established. Indeed, as the lipid fragment ions that appeared in the spectra of untreated endotoxin preparations almost invariably coincided with those detected in the isolated lipid A preparations, it follows that hydrolysis of the glycosidic bond of the KDO unit at pH 4.5 in the presence of sodium dodecyl sulfate (31) is not accompanied by noticeable destruction and/or loss of constituents of the hydrophobic domain. This method of hydrolysis can even be applied to lipopolysaccharides in which the glycosidic bond of KDO is relatively stable, e.g. LPS-2 of the *Bordetella pertussis* endotoxin (39).

In all other endotoxin preparations heterogeneity and/or loss of constituents of the hydrophobic domain. This method of hydrolysis can even be applied to lipopolysaccharides in which the glycosidic bond of KDO is relatively stable, e.g. LPS-2 of the *Bordetella pertussis* endotoxin (39).

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**References**


SPECIAL MATERIALS

1. Analysis of endotoxin preparations by 2H/2H or 2H/1H exchange mass spectrometry: Determination of molecular masses of the component

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EXPERIMENTAL PROCEDURES

Samples were observed using the DFPD time-of-flight mass spectrometer (19). Flight distances were 60 or 90 cm; accelerating voltage was 15 kV, and resolution 8.3 or 1 m/channel. Collection time of data was from 20 min (untreated) to 4 h (treated). The synthesized glycerol (1000 µg) was suspended in 5000 µL of D2O or D2O/D2O mixtures (5:5) and treated with D2O 50 (14N) water and 14N 14N water, 14N ions (9 µg) of the 

Figure 1. Polygram 2H/2H FMB Analysis of commercial and commercial endotoxin preparations.

Endotoxin preparations: (A) calf intestine, (B) rat liver, (C) chicken, (D) human, (E) pig kidney, (F) sperm, (G) sperm, (H) fish, (I) fish, (J) fish, (K) fish, (L) fish, (M) fish, (N) fish, (O) fish, (P) fish, (Q) fish, (R) fish, (S) fish, (T) fish, (U) fish, (V) fish, (W) fish, (X) fish, (Y) fish, (Z) fish.
## PDMS Analysis of Endotoxin Preparations

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*Note: Values represent nanograms of endotoxin per milligram of PDMS.*
$^{252}$Cf PDMS Analysis of Endotoxin Preparations