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Variations in the Carbohydrate Regions of *Bordetella pertussis* Lipopolysaccharides: Electrophoretic, Serological, and Structural Features

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Structural and immunological differences between the two components that are usually present in unequal quantities in *Bordetella pertussis* endotoxin preparations and are visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis have been studied by using strains 1414, A100, and 134, all in phase I. According to analyses by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and thin-layer chromatography, the minor (8%) component of the endotoxin of strain 1414 (endotoxin 1414) appeared to be the predominating component of endotoxins A100 and 134. The masses of the carbohydrate chains isolated from endotoxin A100 and from the major component of endotoxin 1414 were 1,649 and 2,311 atomic mass units, respectively, as determined by ^{252}Cf plasma desorption mass spectrometry. Comparison of the ^{1}H nuclear magnetic resonance spectra of these chains established that four *N*-acetyl groups, an *N*-methyl group, and a 6-deoxy function, which characterize the nonreducing, distal trisaccharide of the glycose chain of strain 1414, were absent from that of strain A100. The antigenicity of endotoxin 1414, as measured by enzyme-linked immunosorbent assay, was higher than that of endotoxin A100, but fell below it when the glycose chain of endotoxin 1414 was deprived of seven sugars by treatment with nitrous acid. This observation suggests that at least three (distal, proximal, and intermediate) regions of the glycose chain of endotoxin 1414 carry antigenic determinants. One of these, located in the distal trisaccharide, is absent from both endotoxins A100 and 134.

Bordetella pertussis, the causative organism of whooping cough, produces a number of substances which are capable of interacting not only with the epithelial cells of the respiratory tract, but also with other cells of the host, and thus contribute to the disease syndrome. Adenylate cyclase (24), agglutinogens (48), filamentous hemagglutinin (3), pertussis toxin (53), heat-labile toxin (5), tracheal cytotoxin (22), and endotoxin (36) are some of these biologically active substances.

When grown on Bordet-Gengou agar, *B. pertussis* exhibits changes in colony morphology (28, 45). This phenomenon, termed phase variation by Leslie and Gardner (34), has been shown to coincide with diminished production or even disappearance of some of the above-mentioned substances (16, 28, 34, 42). One of them, the endotoxin, an integral component of the outer membrane, is responsible for the multiple, heat-stable biological activities common to *B. pertussis* and other gram-negative bacteria (4). Two different lipopolysaccharides (LPS-I and LPS-II) can be isolated from the endotoxin of both phase I and phase IV *B. pertussis* cells upon chromatography on hydroxylapatite (33); they differ, at least, by the presence in LPS-II of a phosphate group on OH-4 of that 2-keto-3-deoxyoctulosonic acid unit which is also substituted at C-5 by the polysaccharide chain (8). A different type of heterogeneity of the endotoxin has been observed on analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (43, 46). The ladder-like pattern, characteristic of endotoxins prepared from smooth-type enterobacterial cells (44), is not seen on SDS-PAGE of *B. pertussis* endotoxin. Instead, only two components are revealed: a major (band A) component and a minor (band B) component, the latter moving somewhat faster (46). Band A and band B do not correlate with LPS-I and LPS-II (A. LeDur and R. Chaby, unpublished observations). The aim of the present study was to determine the structural features responsible for this second type of heterogeneity that is revealed by the appearance of bands A and B on SDS-PAGE of the *B. pertussis* endotoxin.

MATERIALS AND METHODS

Organisms and media. *B. pertussis* 1414 in phase I is a vaccine strain of the Institut Mérieux, Lyon, France. Cells of another strain of *B. pertussis* in phase I, designated by us as A100, were also a gift of the Institut Mérieux. Strain 134 was obtained from J. M. Dolby, Lister Institute of Preventive Medicine, Elstree, U.K. The strains were grown at the Institut Mérieux, in Cohen and Wheeler liquid medium (13).

Endotoxins. Endotoxin preparations from *Salmonella minnesota* (smooth), *Escherichia coli* O111:B4, and *S. minnesota* Re-595 were from Sigma Chemical Co., St. Louis, Mo. The endotoxins of *B. pertussis* 1414 and A100 were extracted by the hot aqueous phenol method of Westphal and Jann (57) and that of Galanos et al. (21) (chloroform-petroleum ether-phenol), both without the addition of salt. Endotoxin from strain 134 (endotoxin 134) was prepared by the hot phenol-water procedure only. After removal of phenol by dialysis, the endotoxins were purified by repeated high-speed centrifugation (150,000 × *g* for 2 h) until the UV absorbance due to nucleic acids disappeared. Lipidic contaminants were eliminated by three extractions of the preparations (10 mg/ml) with chloroform-methanol-water (60:30:5, vol/vol/vol).

Endotoxin fragments obtained by nitrous acid deamination.

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Endotoxins 1414 and A100 were deaminated as described previously (32). Briefly, the endotoxins (250 mg) were suspended (5 mg/ml) in water–5% sodium nitrite–30% acetic acid (1:1:1, vol/vol/vol) and maintained at room temperature for 4 h with magnetic stirring. The suspension was centrifuged (200,000 × *g* for 2 h), and the pellet and the supernatant were lyophilized separately. The lyophilized material obtained from the pellet of strain 1414 will be referred to as nad-lipopolysaccharide (nad-LPS). If thin-layer chromatography (TLC) (solvent A) indicated incomplete deaminative cleavage, the pellet was treated with nitrous acid a second time. The lyophilizates of the supernatants containing oligosaccharides were dissolved in water and treated with sodium borohydride, and excess hydride was destroyed with acetic acid. The solution obtained from endotoxin 1414 was desalted by ultrafiltration (Diaflo UM05 membrane; Amicon Corp., Lexington, Mass.), and the concentrated retentate was subjected to chromatography on a Bio-Gel P2 column (Bio-Rad Laboratories, Richmond, Calif.) eluted with water. The fragment released from LPS-A100 was desalted with a mixed-bed resin, concentrated, and purified by column chromatography as described above. In both cases the elution was monitored by analysis for neutral sugars (17). The purity of the isolated oligosaccharides was monitored by TLC in solvent C and by proton nuclear magnetic resonance (NMR) analysis (250 MHz).

Isolation of the glycose chains (PS-1414, PS-A100, and PS-134) of *B. pertussis* endotoxins 1414, A100, and 134. The endotoxins (250 mg) were dephosphorylated with aqueous hydrofluoric acid (48%) as described previously (8) and then hydrolyzed at pH 4.5 (4 mg/ml for 2 h at 100°C) with sodium acetate (10 mM) in the presence of SDS (1%, wt/vol) (9). After lyophilization, SDS was extracted with ethanol. The residual material was suspended in water, and the insoluble fraction (lipid A) was removed by centrifugation (150,000 × *g*). Material containing neutral sugars (17) was recovered from the concentrated supernatant by column chromatography (Sephadex G-50, 80 by 1.5 cm, with 0.05 M pyridinium acetate [pH 5] as eluant).

Quantitative analyses. Total neutral sugars were estimated by the method of Dubois et al. (17) with D-glucose as the standard; amino sugars, after hydrolysis with 4 M HCl at 100°C for 6 h, were estimated by the method of Rondle and Morgan (52) but with acetylacetone dissolved in 0.5 M sodium carbonate; hexoses were estimated by the method of Dische and Danilchenko (15); heptoses were estimated by the cysteine-sulfuric acid method as modified by Osborn (41) with D-*glycero*-L-*manno*-heptose as the standard; hexuronic acids were estimated by the method of Bitter and Ewins (6); 3-deoxy-2-octulosonic acid was estimated by the modified (11) periodate-thiobarbiturate method; and phosphate was estimated by the method of Chen et al. (12).

Gel electrophoresis. Purified LPS samples (0.1 μg) were applied to slabs of 15% acrylamide gels (1 mm) in 0.1 M Tris hydrochloride buffer (pH 6.8) containing 0.4% (wt/vol) SDS by using the system described by Laemmli (31) but without the stacking gel. The samples were subjected to electrophoresis at 13 mA until the tracking dye (bromophenol blue) moved 10 cm. Gels were fixed and stained with silver by the method of Tsai and Frasch (56).

TLC. Silica gel 60 (0.2 mm) on aluminum foil (20 by 20 cm; E. Merck AG, Darmstadt, Federal Republic of Germany) and isobutyric acid–1 M ammonium hydroxide (5:3, vol/vol) (solvent A) or isobutyric acid-ammonium hydroxide (5:4, vol/vol) (solvent B) were used for TLC of endotoxins. Oligosaccharides were analyzed with *n*-butanol–pyridine–water (6:4:3, vol/vol/vol) (solvent C). Developed plates were sprayed with 10% sulfuric acid in ethanol and heated (120°C).

Methylated alditol acetates. Oligosaccharides (3 mg) were treated with $NaBH_4$ (1 mg in 1 ml of water) for 4 h at room temperature. After evaporation to dryness, boric acid was removed by coevaporation with methanol (1 ml) acidified with a few drops of acetic acid. The residual material, which contained sodium acetate, was sequentially acetylated (0.5 ml of acetic anhydride for 2 h at 100°C) and methylated (with methyl iodide in the presence of sodium methyl sulfinate), as described by Lindberg and Lönngren (35). Methoxycarbonyl groups thus formed were then reduced by treatment of the methylated oligosaccharide with sodium borodeuteride (2 mg) in 1 ml of methanol-water (1:2, vol/vol) for 16 h at room temperature; the sequence of acetylation and methylation was repeated. Solvents were evaporated, and the permethylated oligosaccharides were hydrolyzed for 2 h at 100°C with 1 ml of 90% (wt/vol) formic acid. After removal of the formic acid under vacuum, the material was incubated for 16 h at 100°C with 0.5 ml of 0.2 M sulfuric acid. The solution was diluted with water, neutralized with barium carbonate, and filtered. The methylated sugars were then reduced with sodium borodeuteride and peracetylated as described above. Solvents were removed, and the residue, dissolved in ethyl acetate, was used for analysis.

Gas-liquid chromatography–mass spectrometry. Methylated alditol acetates were analyzed by gas-liquid chromatography in a GC 4180 instrument (Erba Science, Massy, France) equipped with a vitreous silica (WCOT type) capillary column (0.32 mm by 25 m) coated with a BP 10 bonded phase (0.5 μm thick). A temperature gradient of 4°C/min from 160 to 240°C was used. The peaks were identified by their retention time and mass spectrum (Delsi/Nermag Spectral 30 instrument).

Rabbit anti-pertussis serum. An outbred Bouscat white rabbit (2 to 3 kg) was given two successive intravenous injections of 3×10^9 and 6×10^9 heat-killed *B. pertussis* bacteria in phase I (Institut Pasteur Production, Paris, France) at 2-day intervals. After a rest period of 2 weeks, the rabbit received three additional intravenous injections of 9×10^9, 15×10^9, and 21×10^9 bacteria at 2-day intervals and was bled 7 days after the last injection. The serum was a gift of Robert Girard.

Human anti-lipid A serum. A human serum of nosocomial origin was selected (optical density, >1 above background, measured by enzyme-linked immunosorbent assay [ELISA] with a 1/100 dilution of the serum) by screening for its reactivity with the LPS of the deep rough mutant of *S. minnesota* Re-595 and with a number of other LPSs from different bacteria (*B. pertussis*, *Pseudomonas aeruginosa*, *E. coli*).

ELISA. The binding of antibodies to LPS was measured by ELISA (18) in 96-well microdilution plates (Dynatech, Marnes la Coquette, France). A suspension of the endotoxins (0.2 to 20 μg/ml) in a 50 mM Tris hydrochloride buffer (pH 9.6) containing 20 mM $MgCl_2$ (26) was added to each well, and the plates were left overnight at room temperature on a rocking tray. The remaining binding sites were blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS). Wells were washed (three times) with 150 μl of PBST (PBS containing 0.02% NaN_3 and 0.05% Tween 20 [Sigma]) and incubated for 2 h at 37°C with 100 μl of human serum diluted 1/500 or 100 μl of rabbit serum diluted 1/5,000 in PBST containing 0.1% bovine serum albumin. Binding of rabbit or human antibodies to LPS was detected with anti-rabbit immunoglobulins (diluted 1/1,000) or anti-human

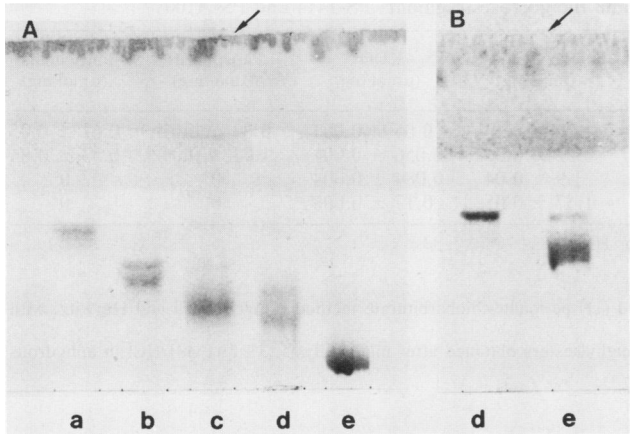

FIG. 1. TLC analysis of endotoxin preparations in solvents A (A) and B (B). Lanes: a, *S. minnesota* Re-595; b, nad-LPS from *B. pertussis* 1414; c, *B. pertussis* 134; d, *B. pertussis* A100; e, *B. pertussis* 1414. Arrows indicate the solvent front.

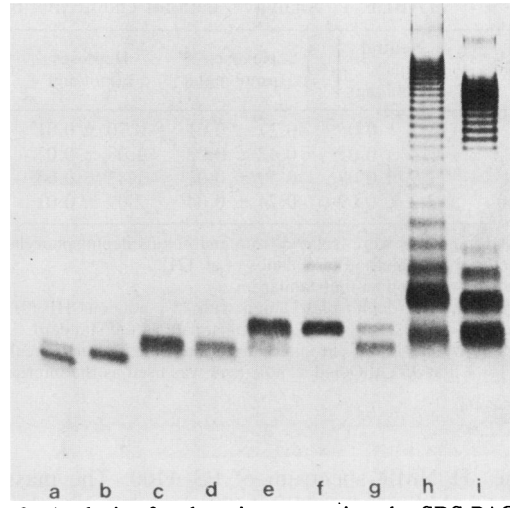

FIG. 2. Analysis of endotoxin preparations by SDS-PAGE. For conditions, see Materials and Methods. Lanes: a, *S. minnesota* Re-595; b, nad-LPS from *B. pertussis* 1414; c, *B. pertussis* 134; d, *B. pertussis* A100; e, *B. pertussis* 1414; f, slow-moving component of *B. pertussis* endotoxin 1414 separated by TLC; g, fast-moving component of endotoxin 1414; h, *S. minnesota*; i, *E. coli* O111:B4.

immunoglobulins (diluted 1/500), both conjugated to alkaline phosphatase (Sigma). After incubation with the conjugate (100 μl in PBST containing 0.1% bovine serum albumin) at 37°C for 1 h, plates were washed again (three times) with PBST (150 μl) and incubated for 1 h at 37°C with 100 μl of 2 mM *p*-nitrophenylphosphate (Sigma) in 1 M Tris hydrochloride buffer (pH 8) containing 1 mM $MgCl_2$. The A_{405} was measured with an MR 700 microplate spectrophotometer (Dynatech).

RESULTS

Preparation, solubility, and chromatographic and electrophoretic characteristics of *B. pertussis* endotoxins 1414, A100, and 134. When extracted by the phenol-water procedure, less endotoxin was recovered from A100 than from 1414 cells (0.85 and 1.00% of the weight of lyophilized cells, respectively), and the solubility in water of the former was considerably lower than that of the latter. When cells of these strains were extracted with petroleum ether-chloroform-phenol (21), only slightly more endotoxin 1414 was recovered (1.25%, by weight), but the endotoxin A100 level increased fourfold (3.5%, by weight). The low solubility of endotoxin A100 in water and its high solubility in petroleum ether-chloroform-phenol suggested some structural analogy with rough-type LPSs of enterobacteria. The absolute quantity of LPS extracted from A100 cells by the petroleum ether-chloroform-phenol technique was three times that obtained from 1414 cells; this could indicate the presence of a higher density of LPS on A100 cells.

Endotoxin preparations of *B. pertussis* 134, A100, and 1414 and nad-LPS were analyzed by TLC and compared with the rough-type endotoxin of *S. minnesota* Re-595. In solvent A, endotoxins A100 and 134 migrated at similar rates and faster than endotoxin 1414 but more slowly than nad-LPS and the Re endotoxin (Fig. 1A). When a more polar eluant (solvent B) was used (Fig. 1B), endotoxin 1414 proved to be a mixture of a major, slow-moving component and a minor, fast-moving component. These constituents were isolated by preparative TLC. Their ratio, as measured by quantitative estimation of their fatty acids by gas-liquid chromatography, was 11:1.

When the isolated (TLC) slow and fast components of *B. pertussis* endotoxin 1414 were analyzed by SDS-PAGE (Fig. 2), they appeared as slow- and fast-migrating bands, respectively, just like those observed by other investigators and called *B. pertussis* LPS-A and LPS-B (43, 46). On both TLC and SDS-PAGE, the rate of migration of the minor (LPS-B) constituent of *B. pertussis* endotoxin 1414 was similar to that of *B. pertussis* endotoxin A100, suggesting that endotoxin A100 and LPS-B were smaller than LPS-A. The material produced by treatment of endotoxin 1414 with nitrous acid (nad-LPS) and the intact endotoxin from *S. minnesota* Re-595 appeared to be even smaller by both methods.

Chemical analyses of endotoxins 1414 and A100. To establish whether their observed physical characteristics could be related to their chemical compositions, endotoxins 1414 and A100 were analyzed comparatively (by colorimetric methods) for constituents previously shown to be present in endotoxin 1414. The results (Table 1) indicated that as regards their glycose composition, the major difference between the two endotoxins is in the ratio of their hexosamine-to-hexose content, which was 1:4 for endotoxin 1414 but only 1:1.2 for endotoxin A100. As regards the hydrophobic region, the fatty acid compositions of the two endotoxins were very similar (Fig. 3): both contained 3-hydroxydecanoic, myristic, and 3-hydroxymyristic acids in similar amounts, but an additional fatty acid (hexadecanoic acid) was present in endotoxin A100.

Analysis of PS-1414, PS-A100, and PS-134 isolated from the respective endotoxins. When analyzed by chromatography on Sephadex G-50 columns, with 0.05 M pyridinium acetate as the eluant, the apparently homogeneous glycose chain (PS-A100) released from endotoxin A100 eluted after that released from endotoxin 1414 (PS-1414) and thus appeared to be of lower molecular weight.

The four singlets (between δ 1.66 and 1.84 ppm) due to *N*-acetyl groups, the one due to an *N*-methyl group (δ 2.6 ppm), and the doublet characteristic of a methyl group of a 6-deoxyhexopyranose (δ 1.2 ppm) seen in the ^1H NMR spectrum (250 MHz) of PS-1414 (Fig. 4A) were all missing

TABLE 1. Analytical data for endotoxins 1414 and A100 and the glycose fragments PS-1414 and PS-A100[a]

Endotoxin[b]	Neutral sugars[c] (μmol/mg)	Hexoses (μmol/mg)	Heptoses (μmol/mg)	Uronic acids (μmol/mg)	Hexosamines (μmol/mg)	KDO[d] (μmol/mg)	Phosphorus (μmol/mg)	Fatty acids[e] (μmol/mg)
1414	1.32 ± 0.05	0.27 ± 0.01	0.70 ± 0.01	0.27 ± 0.01	1.10 ± 0.13	0.05 ± 0.001	0.84 ± 0.01	0.61 ± 0.05
A100	1.14 ± 0.05	0.42 ± 0.02	1.08 ± 0.02	0.34 ± 0.01	0.52 ± 0.06	0.056 ± 0.001	1.03 ± 0.01	0.53 ± 0.04
PS-1414	1.9 ± 0.08	0.59 ± 0.01	1.47 ± 0.05	0.59 ± 0.01	1.9 ± 0.04	0.084 ± 0.004	0	0
PS-A100	2.2 ± 0.09	0.74 ± 0.04	2.09 ± 0.01	0.71 ± 0.03	1.57 ± 0.05	0.17 ± 0.008	0	0

[a] Glycose fragments were isolated from endotoxins dephosphorylated with aqueous HF (8).
[b] Extracted by the method of Galanos et al. (21).
[c] D-Glucose was used as the standard.
[d] Determined, after dephosphorylation with 48% aqueous HF (8), by the modified (11) periodate-thiobarbiturate method of Weissbach and Hurwitz, with crystalline ammonium 2-keto-3-deoxyoctulosonate as the standard.
[e] Estimated by gas-liquid chromatography and integration of peak areas of the methyl esters obtained after methanolysis (3% [wt/vol] HCl in anhydrous methanol for 4 h at 95°C). Octadecanoic acid was used as the internal standard.

from the ^{1}H NMR spectrum of PS-A100. The masses of underivatized PS-1414 and PS-A100, determined with a ^{252}Cf plasma desorption mass spectrometer (14), were 2,311 and 1,649 atomic mass units, respectively. Comparative analytical data—obtained by colorimetric methods—of the glycose compositions of PS-1414 and PS-A100 are presented in Table 1. Analysis by methylation afforded the data shown in Table 2. According to these data, PS-1414 and PS-A100 differ (i) by the presence of a 4,6-disubstituted hexosamine (peak H) in PS-1414 which is absent from PS-A100 and (ii) by the presence of a 4-monosubstituted hexosamine residue (peak F) in PS-A100 which is not detectable in PS-1414. Upon treatment with nitrous acid, each endotoxin gave rise to two main fragments. In both of them, the proximal part of the sugar chain was still attached to the (possibly altered) hydrophobic region by the glycosidic bond of a 2-keto-3-deoxyoctulosonic acid unit. In both cases, the other fragment was an oligo-

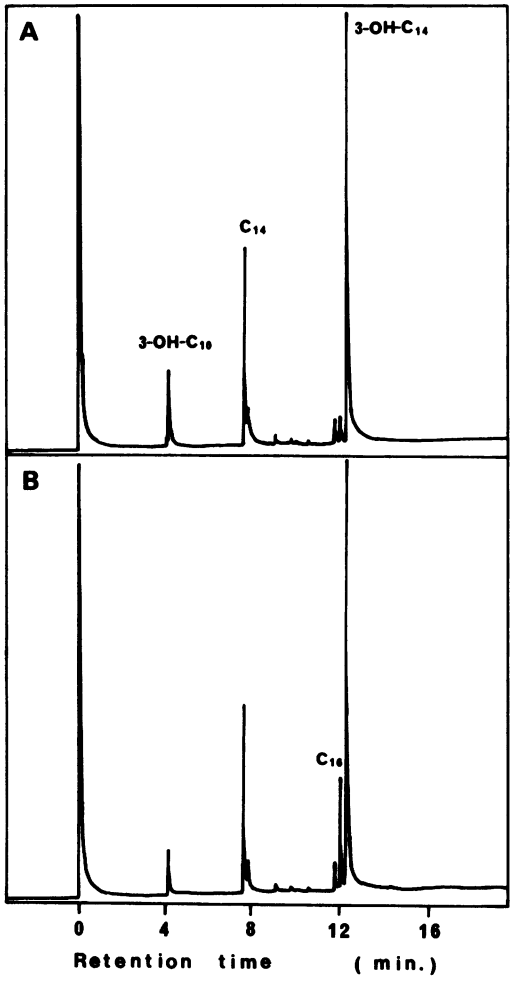

FIG. 3. Gas-liquid chromatogram of the fatty acid methyl esters from *B. pertussis* endotoxins 1414 (A) and A100 (B).

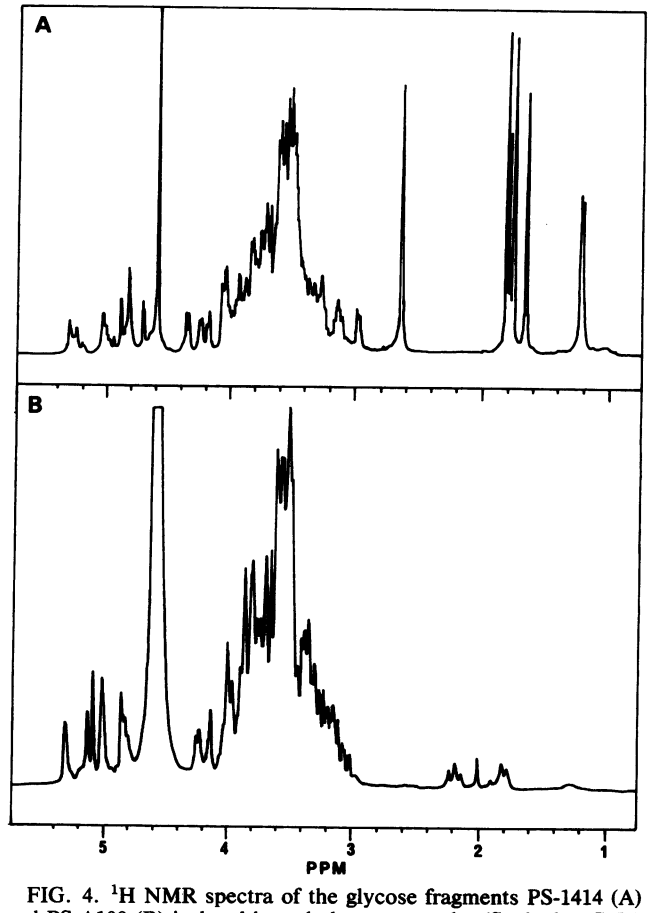

FIG. 4. ^{1}H NMR spectra of the glycose fragments PS-1414 (A) and PS-A100 (B) isolated by gel chromatography (Sephadex G-50) after dephosphorylation and mild acid hydrolysis of the corresponding endotoxins.

TABLE 2. Gas-liquid chromatography–mass spectrometry analysis of methylated alditol acetates obtained from the glycose fragments PS-1414 and PS-A100 isolated from *B. pertussis* endotoxins 1414 and A100

Methylated alditol acetate[a]	Peak	Relative retention time[b]	Relative peak intensity[c]	
			PS-1414	PS-A100
2,3,4,6-*O*-Methylglucitol	A	1.00	1.7	1.2
1,2,4,6,7,8-*O*-Methyl-3-deoxyoctitol	B	1.32	0.4	0.5
2,3,4,6,7-*O*-Methylheptitol	C	1.41	1.0	1.0
3,4,6-*O*-Methyl-*N*-methyl-hexosaminitol	D	1.63	1.5	1.4
2,6,7-*O*-Methylheptitol	E	1.80	1.2	1.0
3,6-*O*-Methyl-*N*-methylhexosaminitol	F	1.87	0	0.3
3,4,6-*O*-Methylheptitol	G	2.07	1.3	1.2
3-*O*-Methyl-*N*-methylhexosaminitol	H	2.85	0.3	0

[a] Acetylated derivatives of the methylated alditols. Assignation of the peaks is based on the corresponding mass spectrum. Peak D comes from unsubstituted glucosamine or galactosaminuronic acid (38).

[b] The retention times are relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, which is set at 1.

[c] The intensities are relative to that of 2,3,4,6,7-*O*-methyl-1,5-di-*O*-acetyl-heptitol, which is set at 1.

saccharide. After treatment with borohydride and purification on Bio-Gel P2, the oligosaccharide released from endotoxin 1414 was characterized by ^{1}H NMR spectroscopy as a pentasaccharide composed of *N*-acetylglucosamine, 2,3-di-*N*-acetamidomannuronic acid, *N*-acetyl-*N*-methylfucosamine, heptose, and 2,5-anhydromannitol (M. Caroff, S. Lebbar, and L. Szabó, XIVth International Carbohydrate Symposium, Stockholm, Sweden, p. 42, 1988). The oligosaccharide released from LPS-A100 was a heptopyranosyl-2,5-anhydromannitol. These data establish the absence of three amino sugars, namely, *N*-acetylglucosamine, 2,3-diacetamidomannuronic acid, and *N*-acetyl-*N*-methylfucosamine, known to be present at the nonreducing end of PS-1414 (Caroff et al., XIVth International Carbohydrate Symposium, 1988).

The ^{252}Cf plasma desorption mass spectrum (Fig. 5) and the ^{1}H NMR spectrum of PS-134 appeared to be identical to those of PS-A100.

Serological reactions of the LPSs. Quantitative analyses of the cross-reactivities between endotoxins of *S. minnesota* Re-595, *B. pertussis* 1414, A100 and 134, and a fragment with a shorter polysaccharide chain obtained by nitrous acid deamination of LPS-1414 (nad-LPS) were performed by estimation (by ELISA) of the binding of these LPSs to the immunoglobulins present in the serum of a rabbit immunized with vaccinal (phase I) *B. pertussis* organisms. Such experiments can be carried out only if the efficiencies of the coating of the various endotoxins to the plastic surface of the wells are identical. However, in a preliminary experiment, we found that after incubation of the wells with identical concentrations of the endotoxins to be tested, the amounts of these endotoxins actually adsorbed on the plastic were significantly different. The amounts of plastic-bound endotoxins were measured with an anti-lipid A serum (a human serum which reacted with a large panel of different endotoxins). To obtain comparable coatings and thus identical reactivities with the human anti-lipid A serum, concentrations of 2.8, 1.4, 1.8, 18.0, and 0.4 μg of 1414, A100, 134, and Re-595 endotoxins and nad-LPS per ml, respectively, had to be used during incubation.

Accurate comparisons of the binding of the different

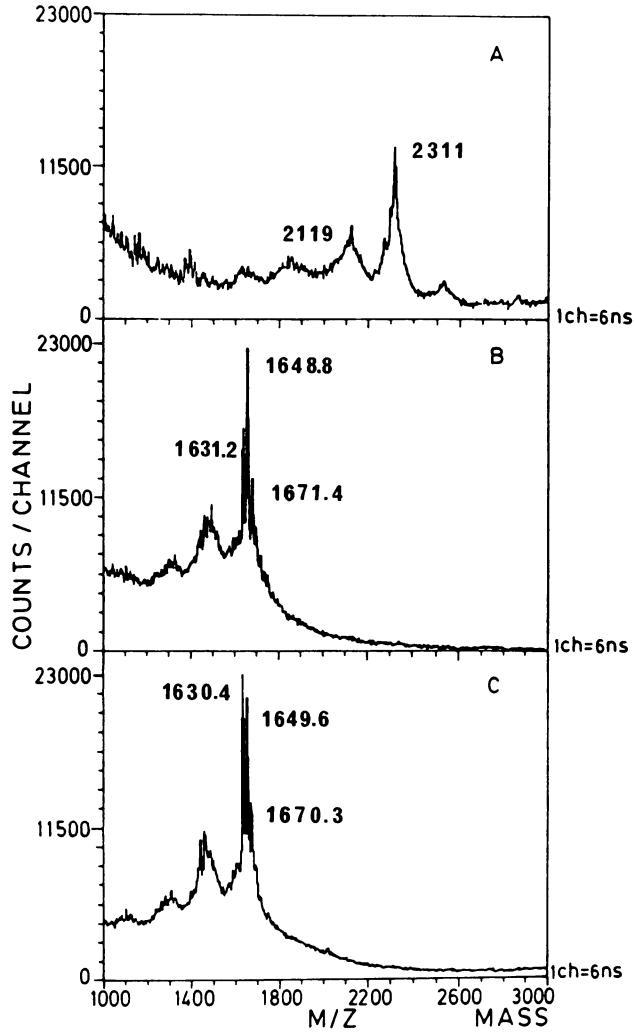

FIG. 5. Mass spectra produced by ^{252}Cf plasma desorption of the underivatized glycose fragments PS-1414 (A), PS-A100 (B), and PS-134 (C) obtained from the corresponding endotoxin preparations after dephosphorylation and mild acid hydrolysis.

endotoxins to rabbit anti-*B. pertussis* antibodies were obtained by coating the wells with various amounts of the endotoxins and plotting, for each endotoxin, the values (ELISA) of the binding to rabbit anti-*B. pertussis* antibodies (*y* axis) as a function of the binding to human anti-lipid A antibodies (*x* axis). The results (Fig. 6) show that endotoxin 1414 gave the strongest reaction with the rabbit anti-*B. pertussis* serum. The antigenicity of endotoxin A100 was considerably lower than this, but was still significantly higher than those of endotoxin 134 and nad-LPS. As expected, the endotoxin of the deep rough mutant *S. minnesota* Re-595 did not react with the rabbit serum; i.e., the level of anti-lipid A antibodies raised in the rabbit during immunization with heat-killed *B. pertussis* cells was negligible.

DISCUSSION

Major progress in our knowledge of the structure of LPSs resulted from the isolation of a series of mutants of enteric bacteria in which the glycose chains of the LPSs were

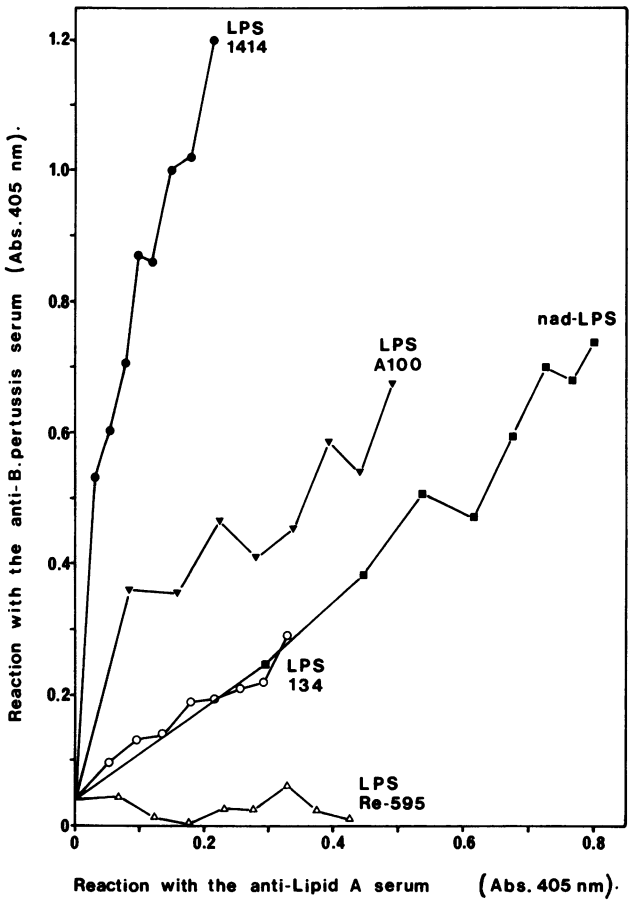

FIG. 6. Plot of the amount of rabbit anti-*B. pertussis* antibodies bound to different endotoxins versus the amount of human anti-lipid A antibodies bound to the same endotoxins, as measured by ELISA. Endotoxins: *B. pertussis* 1414 (●), A100 (▼), and 134 (○); *S. minnesota* Re-595 (△); nad-LPS (■).

shorter than those of the wild-type representative strains (20, 47). The occurrence of long O polysaccharide chains made up of repeating oligosaccharide units and linked to the core region of the LPS is not as frequent in other microorganisms as it is in enterobacteria. Although often present in LPSs isolated from bacteria belonging to the genera *Vibrio* (49) and *Pseudomonas* (30), long chains of repeating units of oligosaccharides attached to a core region are absent from the endotoxins of many important pathogens belonging to the genera *Acinetobacter* (40), *Bacteroides* (27), *Bordetella* (10), *Chlamydia* (39), *Haemophilus* (1), *Neisseria* (55), and *Pasteurella* (19). For this reason, some of these LPSs have been designated lipooligosaccharides in recent publications (37, 54). However, in agreement with the recommendations of Hitchcock et al. (25), we shall avoid the use of this nomenclature, since the term oligosaccharide (less than 10 sugars) is inadequate for the glycose chain of *B. pertussis* endotoxin (which contains at least 12 sugar residues) and because the general architecture and biological activities of this endotoxin are not fundamentally different from those of typical LPSs (4).

Endotoxin preparations, irrespective of the bacteria from which they originate, are heterogeneous with respect to all three regions (O chains, core, and lipid A) of their constituent LPSs. Even in LPSs of the nonenteric pathogens mentioned above, which lack the O chains, heterogeneity has been revealed (10, 29, 54). Precise cognizance of differences in the structures of LPSs of pathogenic organisms is probably quite important: structurally because the LPS is interconnected with other constituents of the bacterial membrane and functionally because the LPS interacts with the cells of the host. Thus, it has been shown that alterations in the glycose chain of the LPS of *Haemophilus influenzae* type b affect the ability of this pathogen to cause systemic disease (29). As regards the endotoxin of *B. pertussis*, because of its electrophoretic pattern it has been suggested (23) that it might contain an O chain consisting of a monosaccharide repeating unit, resembling those found in *Vibrio cholerae* (49) and *Brucella abortus* (7). Such structural features are not present, however, in the endotoxin of the common phase I *B. pertussis* 1414 (10).

Although LPS constituents of endotoxin preparations have often been separated by SDS-PAGE, recovery of sizable amounts of the separated entities is not easy, and yields are poor. Accordingly, conditions have been elaborated in which components A and B (43, 46) of the *B. pertussis* endotoxin were well separated by TLC and milligram quantities of each component were thus obtained. It was established that, on a weight basis, in the endotoxin of strain 1414 the slow-migrating component A predominates (91%). The minor, fast-migrating component, B (8%), moved faster than A, but less so than the endotoxin of the *S. minnesota* Re-595 mutant, whose glycose chain consists of only two 2-keto-3-deoxyoctulosonic acid units. This was taken to indicate that component B was a smaller molecule than component A. The higher rate of movement on SDS-PAGE of component B than component A is in agreement with both this conclusion and previous experience with enterobacterial endotoxins (44).

The amounts of material made available by preparative TLC did not enable us to perform a detailed analysis of LPS-B. However, upon large-scale preparation of the glycose chains of endotoxin 1414, a small amount (10%) of an oligosaccharide with the same molecular mass as PS-A100 was separated from the major polysaccharide by chromatography on Sephadex G-50. Furthermore, the endotoxin extracted from *B. pertussis* A100 cells gave one major band on both SDS-PAGE and TLC; its migration was identical to that of LPS-B in the endotoxin 1414 preparation. Similar results were obtained with endotoxin 134. These observations suggest that LPS-134 and LPS-A100 are identical to the LPS-B component of endotoxin 1414.

The difference of 662 atomic mass units between the molecular masses of PS-1414 and PS-A100 detected by mass spectrometry confirmed that PS-A100 was smaller than PS-1414. The NMR spectra revealed the identity of the missing sugars as *N*-acetylglucosamine (GlcNAc), 2,3-di-*N*-acetyl-2,3-dideoxyhexuronic acid (DAA), and *N*-methyl-*N*-acetylfucosamine (FucNMeAc), known (Caroff et al., XIVth International Carbohydrate Symposium, 1988) to be present in the distal region of the carbohydrate chain of PS-1414. As a corollary, the difference between the measured molecular masses is exactly accounted for by the sum of the molecular masses of the three monosaccharides mentioned.

Upon specific cleavage of the glycose chains by nitrous acid, a pentasaccharide (containing heptose, 2,5-anhydromannose, FucNMeAc, DAA and GlcNAc) was released from endotoxin 1414, whereas a disaccharide (consisting of heptose and 2,5-anhydromannose) was produced from endotoxin A100. Based on these results, the partial structures I and II are proposed as nonreducing termini present in

PS-1414 and PS-A100, respectively:

Structure I (in PS-1414): GlcNAc-DAA-FucNMeAc-$GlcNH_2$- . . .
↑
Hept

Structure II (in PS-A100): $GlcNH_2$- . . .
↑
Hept

As regards the lipid regions of endotoxins 1414 and A100, they appeared to be very similar (Fig. 3), except that in A100 an additional fatty acid, hexadecanoic acid, was detected (Fig. 3B). Small and variable amounts of this acid have also been detected in some preparations of LPS-1414; it may not be a component characteristic of LPS-A100.

The structural differences between endotoxins 1414 and A100 raised the question of the antigenic characteristics of these LPSs. According to Aprile and Wardlaw (2), five distinct antigenic determinants (A, B, C, E, and F) are present in endotoxins that can be isolated from conventional, phase I strains of *B. pertussis* such as strain 18334, whereas in strain 134, also in phase I, only two determinants (B and D) have been detected. In endotoxin 134, the absence of at least one antigenic determinant detectable by a monoclonal antibody was also reported by Ming Li et al. (37). Our results, obtained by ELISA, clearly establish that the antigenicity of endotoxin A100 is lower than that of endotoxin 1414, but higher than that of nad-LPS. This indicates that both the distal trisaccharide (absent from LPS-A100) and the carbohydrate region proximal to lipid A (present in endotoxins 1414 and A100 and in nad-LPS) are antigenic and that significant antigenicity is carried by a third carbohydrate region which is present in endotoxins 1414 and A100 but absent from nad-LPS. The antigenicity of endotoxin 134 is as weak as that of nad-LPS.

The absence of a block of three sugars in the endotoxin of the A100 strain and the fact that the amino groups of these sugars are *N*-acetylated whereas those of the other amino sugars are not support the hypothesis that the distal trisaccharide of the endotoxin of a common *B. pertussis* strain (like endotoxin 1414) is, in fact, a single O antigen unit. Endotoxins produced by cells of *Salmonella* serogroups B and E, in which the O chains consist of a single repeating unit and which are called semirough (SR), are well known (58). According to our hypothesis, the LPS-A constituent of *B. pertussis* endotoxin should be classified as SR, whereas the LPS-B constituent and endotoxins A100 and 134 might represent rough (R) chemotypes. The presence of a majority of SR-LPSs in *B. pertussis* could arise from differences in the kinetics of the three main steps of endotoxin biosynthesis: O unit assembly (51), polymerization (50), and transfer to the core (58). In *B. pertussis* organisms the rate of the last reaction would be much higher than those of the first two. If this is true, the presence of small amounts of R endotoxins (LPS-B component in strain 1414) can be easily explained, and the presence of some LPSs of the smooth chemotype can be predicted.

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