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Antigenic Heterogeneity of Lipid A of *Haemophilus influenzae*

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The chemical structure and biologic function of the lipid A portion of lipopolysaccharide are not identical among gram-negative bacteria. This study indicates that antigenically heterogeneous lipid A exists among strains of *Haemophilus influenzae*. An immunoglobulin G3 murine monoclonal antibody, 3D2, produced against a nontypable *H. influenzae* strain 3524 has specificity for a site on the lipid A portion of the *H. influenzae* lipopolysaccharide. With the Western blot and immunodot assay, 3D2 recognized this lipid A determinant on 14 of 24 (58%) of strains of nontypable *H. influenzae* and in 51 of 95 (54%) strains of *H. influenzae* type b. This lipid A epitope has a high degree of specificity for *H. influenzae*, since it is not present on the lipid A of 39 gram-negative strains from 14 non-*Haemophilus* species. In addition, studies of 36 strains of six *Haemophilus* species other than *H. influenzae* and 8 strains of 4 species of *Actinobacillus* did not contain the 3D2 epitope. Enzyme-linked immunosorbent assay analysis with a kinetic assay and enzyme-linked immunosorbent assay inhibition confirmed the antigenic heterogeneity of *H. influenzae* lipid A. Thin-layer chromatography demonstrated that the 3D2 epitope is associated with a chloroform-soluble lipid moiety in the lipid A. Fluorescent antibody analysis of *H. influenzae* indicated that the epitope is on the cell surface. The monoclonal antibody was not bactericidal for strain 3524, and it did not inhibit the bactericidal action of normal human serum against the same strain. These studies demonstrate that the lipid As of *H. influenzae* are antigenically heterogeneous.

Since 1963, the concept that the lipid A component of the lipopolysaccharide (LPS) of gram-negative bacteria represents a heterogeneous group of structures has gained acceptance (21). Chang and Nowotny have shown that the lipid A portions of the LPS structure are not identical in structure or function (4). Other investigators demonstrated that fractionation of a single lipid A preparation resulted in the identification of eight fractions which had a range of antigen expression (3, 16, 17). Five of these fractions could react with anti-lipid A antiserum, whereas the three with the greatest number of esterfied fatty acids failed to react. Thus it would appear that antigenic variability among lipid A's should be anticipated as more extensive studies specifically with monoclonal antibodies are developed.

We have had the opportunity to study a number of nontypable *Haemophilus influenzae* LPS preparations with a monoclonal antibody developed to a nontypable *H. influenzae* strain, 3524. This monoclonal antibody, designated 3D2, has been shown to react with a determinant present on the lipid A portion of the LPS. The investigations presented in this paper indicate that the determinant recognized by this monoclonal antibody is not present on the lipid A of all *H. influenzae* strains. Studies of other *Haemophilus* species and a diverse group of gram-negative and gram-positive bacteria indicate that this antigenic determinant is highly specific for *H. influenzae*.

MATERIALS AND METHODS

Bacteria. Prototype strains of nontypable *H. influenzae* representative of the eight sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) types described by Murphy et al. (19) were obtained from our own collection. The identity of each isolated strain had been confirmed previously as *H. influenzae* by colonial morphology and growth requirement for X and V factors. Counterimmunoelectrophoresis (20) had been used to determine serotype with antiserum and reference strains obtained from the Centers for Disease Control, Atlanta, Ga. Nontypable *H. influenzae* 3524 was isolated from the sputum of a patient with chronic bronchitis at the Erie County Medical Center, Buffalo, N.Y. It has been demonstrated to be piliated (2) and has been shown to have a type 2 outer membrane protein pattern on SDS-PAGE (19). Copies of *Haemophilus paraphrophilus*, ATCC 29240, 29241, and 29242, *H. segnis* ATCC 10977, *H. aphrophilus* ATCC 13252 and 19415, and NCTC 5906, 5907, and 5908, *H. parainfluenzae* ATCC 7901 and 9276, *H. aegypticus* ATCC 11116, *H. parahaemolyticus* VATCC 10014, nontypable *H. influenzae* ATCC 19418, *H. paraphrohaemolyticus* ATCC 29237, *Actinobacillus actinomycetemcomitans* ATCC 29522, 29523, and 29524 and NCTC 9707 and 9710, *A. equili* ATCC 19392, *A. seminis* ATCC 15768, and *A. suis* ATCC 15557 were provided by J. Zambon, State University of New York at Buffalo, School of Dentistry. Isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella* species, *Pseudomonas aeruginosa*, *Pseudomonas* species, *Enterobacter cloacae*, *Proteus vulgaris*, *Proteus mirabilis*, *Morganella morganii* *Serratia marcescens*, *H. parainfluenzae*, *H. parahaemolyticus*, *H. haemolyticus*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *N. gonorrhoeae*, *Streptococcus viridans*, and *Staphylococcus* species were provided by the clinical microbiology laboratory at the Erie County Medical Center. Strains were stored in Mueller-Hinton medium containing 10% glycerol at −70°C until studied and were reconstituted on appropriate media for sample preparation.

Development of hybridomas. (i) Immunization of spleen cell donor mice. BALB/c mice were immunized intraperitoneally with 0.1 ml of 10^9 nontypable *H. influenzae* 3524 cells on

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days 0 and 28. On day 32 after the initial immunization, selected animals were sacrificed with chloroform, their spleens were removed, and splenic lymphocytes were harvested by perfusion of splenic pulp with minimum essential medium (MEM).

(ii) Fusion of donor spleen cells to the NS-1 nonsecreting clone of the P3x63Ag8 BALB/c plasmacytoma. To achieve fusion of the donor spleen cells to the NS-1 (nonsecreting variant of the immunoglobulin G1 [IgG1] BALB/c plasmacytoma P3x63Ag8) plasmacytoma cells (obtained from the Salk Institute for Biology under National Cancer Institute contract NO1-CB-23886), 35% polyethylene glycol was used in a modification of the procedure of Kennett (13). Briefly, 10^7 spleen cells were combined with 10^6 NS-1 cells in MEM with serum. The cells were centrifuged at 170 × *g* for 10 min at 25°C. All of the supernatant was removed, and the pellet was tapped to loosen. A 0.2-ml sample of 35% polyethylene glycol 1000 (Sigma Chemical Co., St. Louis, Mo.) in MEM without serum was added, the mixture was stirred lightly and left at 25°C, and timing was started for 8 min. The cells were pelleted at 500 × *g* for 3 min. At the end of the original 8 min, 5 ml of MEM with serum was added and gently pipetted once to resuspend the pellet. The mixture was centrifuged at 250 × *g* for 5 min at room temperature. All of the supernatant was removed. A 5-ml sample of complete growth medium (MEM with high glucose and 20% bovine fetal serum) was added to resuspend the pellet. The mixture was transferred to a 25 ml Erlenmeyer flask containing the appropriate amount of complete MEM to obtain 3×10^5 plasmacytoma cells per ml. The cells were stirred lightly and distributed in 0.05-ml samples into microtiter wells. At 24 h after the polyethylene glycol fusion, 0.05 ml of hypoxanthine-aminopterin-thymidine medium was added to each well. The microtiter plates were placed in a tissue culture incubator at 85% humidity under 5% carbon dioxide. Fresh hypoxanthine-aminopterin-thymidine medium was added at day 7, and plates were checked for macroscopic plaques after day 10. The supernatant from all wells was tested for the presence of antibody by using an enzyme-linked immunosorbent assay (ELISA) system in which 10 μg of strain 3524 outer membranes per ml was coated to the microtiter plates. The peroxidase-conjugated rabbit anti-murine IgG or protein A was used to detect the presence of antibody binding to the outer membranes. Wells which contained hybridomas producing antibody to outer membranes were retested against microtiter plates coated with strain 3524 LPS to identify those clones producing antibody to LPS. These clones were propagated by subsequent transfer to larger tissue culture wells. Large quantities of antibody were produced in tissue culture and by the intraperitoneal injection of 10^5 cells from the LPS antibody-producing clone into 2,6,10,14-tetramethylpentadecane (pristane)-primed BALB/c mice. The resulting ascitic fluid was harvested in 3 to 4 weeks, and the ascitic fluid was tested for specificity of the respective antibody. The IgG3 antibody was removed from the ascitic fluid by passage over a protein A-Sepharose column. Similar peritoneal tumors were established by using NS-1 cells. The resultant fluid was used as a control.

The monoclonal antibody produced in tissue culture fluid was purified by affinity chromatography over a murine IgG affinity column. This antibody was used to confirm the results obtained with the ascitic fluid-derived antibody.

Preparation of LPS, lipid A and outer membranes. *H. influenzae* LPS was prepared by a modification of the phenol-water method of Perry et al. (22). Lipid A was prepared by heating 20 mg of the LPS preparation in 1.0 M acetic acid in a boiling water bath for 1 h. The lipid A precipitate was removed by centrifugation at 15,000 × *g* for 30 min. The precipitate was lyophilized after it was washed twice by suspension in distilled water followed by centrifugation. The acetic acid and wash supernatants were pooled and lyophilized. This contained the saccharide portion of the LPS. The lipid A fraction was further fractionated by the method of Chang and Nowotny (4). This involves sequential extractions with ethyl acetate and chloroform. Three fractions are recovered, an ethyl acetate-insoluble fraction, a chloroform-soluble fraction, and a chloroform-insoluble fraction. Outer membranes were prepared by the method of Murphy et al. (19).

Thin-layer chromatography. Thin-layer chromatography was performed by a modification of the method of Banerji and Alving with a chloroform-acetone (85:15) solvent system with silica gel 60 plates (EM Laboratories, Elmsford, N.Y.) (3). Lipid spots were identified on the dried chromatograms by using iodine vapors and confirmed with bromothymol blue spray, phosphate-containing residues were identified with molybdenum blue reagent, carbohydrates were identified with orcinol (0.25% orcinol in 75% sulfuric acid), and amino acid-containing compounds were identified with ninhydrin spray (0.3% in butanol-acetic acid) (26).

Western blot analysis. Analysis of the specificity of the 3D2 monoclonal antibody was performed by Western blot methodology (14) with a Trans-blot apparatus (Bio-Rad Laboratories, Richmond, Calif). Briefly, samples were subjected to SDS-PAGE by the Laemmli method (15). At the end of this electrophoresis, the gel was removed and placed in the Trans-blot apparatus with a 10- by 20-cm sheet of nitrocellulose over 1.5 h at 50 V in 25 mM Tris hydrochloride (pH 8.3)–192 mM glycine–20% methanol buffer. After transfer, the nitrocellulose sheet was placed in 3% gelatin and then treated with 3D2 monoclonal antibody at room temperature overnight, washed with buffer A (0.9% [wt/vol] NaCl, 10 mM Tris hydrochloride [pH 7.4]) and then treated with protein A-peroxidase conjugate (Zymed, South San Francisco, Calif.). The sheet was then washed with buffer A and treated with HRP reagent (Bio-Rad) for 45 min, and the resulting bands were observed on a light box.

Immunodot assay. A modified dot assay utilizing nitrocellulose paper was described previously (9). In this assay, the sample to be screened for the presence of antigen is solubilized in SDS-PAGE sample buffer at 1 mg/ml, and 10 μl is placed in a 1.5-cm square on the paper. The sample is allowed to dry, and the sheet is then treated in a fashion identical to that described above for the Western blot technique.

Kinetic ELISA. The quantitative, single-tube, kinetic-dependent ELISA (herein referred to as the kinetic ELISA) uses enzyme rate kinetics to quantitate bound enzyme and assay ligand (either antigen or antibody). This assay was performed by the method of Tsang and co-workers (27). Individual Ultra-UV disposable polystyrene cuvettes (Fisher Scientific Co., Pittsburgh, Pa.) were coated with LPS from nontypable *H. influenzae* strains in concentrations of 0.1, 1, and 5 μg/ml. The LPSs were suspended in sensitization buffer (0.05 M Tris, 2.0 mM EDTA, 0.3 M KCl [pH 8.0]) by sonication. Cuvettes were sensitized for 1 h a 37°C and overnight at 4°C. Protein A-purified monoclonal antibody 3D2 was washed with phosphate-buffered saline–0.3% Tween 20 and reacted at concentrations of 0.1, 1, and 10 μg/ml with the LPS-coated cuvettes for 1 h at 37°C. The cuvettes were washed with phosphate-buffer saline–Tween 20, and protein A-peroxidase (1:3,000) was reacted with the

cuvettes for 1 h at 37°C. The cuvettes were washed with phosphate-buffered saline–Tween 20. After the removal of the wash fluid, the cuvettes were placed in a Varian 634 spectrophotometer with the analog output connected through an A-D converter to an Apple IIe computer which had been programmed to calculate the rate of change of absorbance at 460 nm per minute. One milliliter of freshly prepared *O*-dianisidine dihydrochloride (Sigma) in 0.05 M sodium acetate (pH 5.0) was added to the cuvette. The change in absorbance at 460 nm was measured over the first 3 min of each reaction, and the rate of change of absorbance at 460 nm per minute for each antiserum and LPS concentration was calculated.

ELISA inhibition studies. ELISA inhibition studies were performed by the method of Apicella and Gagliardi (1).

Fluorescent antibody analysis. Indirect fluorescent antibody studies were performed to determine whether 3D2 recognized a surface-exposed epitope. Briefly, *H. influenzae* organisms were fixed to glass slides in acetone and exposed to affinity-purified 3D2 antibody derived from the tissue culture supernatant. The fluorescent conjugate used was an Fc-specific fluorescein conjugate obtained from Cappel Laboratories, Cochranville, Pa. Studies were performed with an A-O macro-star microscope with epifluorescence.

Bactericidal assay. Studies to determine whether 3D2 had bactericidal activity against *H. influenzae* strains were performed by the method of Rice and Kasper (24). Studies to determine whether 3D2 was capable of inhibiting the bactericidal response of normal human serum were also performed (25).

Other immunological studies. Immunodiffusion studies were performed on 10-fold-concentrated tissue culture fluid to determine the immunoglobulin class of the monoclonal antibody. For these studies, antisera specific for murine IgM, IgG1, IgG2a, IgG2b, and IgG3 were obtained from Bionetics, Kensington, Md.

RESULTS

Utilizing splenocytes from a BALB/c mouse immunized with nontypable *H. influenzae* 3524, a series of hybridomas were developed by fusion of NS-1 plasmacytoma cells. Among the clones producing antibody to the *H. influenzae* strain, one designated 3D2 was found by ELISA screening to be producing antibody to LPS isolated from this strain. This monoclonal antibody was determined by immunodiffusion to be of the IgG3 variety.

Analysis of the specificity of 3D2. To determine the nature of the antigenic specificity of 3D2, Western blot and immunodot blot analyses were utilized. Figure 1A shows a Western blot of LPS (lane a), lipid A (lane b), and oligosaccharides (lane c) from strain 3524. The antibody recognized a determinant of lipid A moiety. Because of the possibility that the saccharide portion of the LPS did not migrate into the SDS-PAGE system, immunodot assays were performed which confirmed the absence of reactivity of 3D2 with the saccharide portion of the 3524 LPS and the recognition of an epitope on the lipid A. In Fig. 1B, LPS from strains 3524 (lane a), 7502 (lane b), and 2019 (lane c) demonstrate the heterogeneity of the 3D2 epitope among the lipid As of *H. influenzae*. Monoclonal 3D2 did not recognize this determinant on strain 7502 or 2019 LPS. Immunodot assays of SDS extracts of the strains confirmed the presence of the epitope in strain 3524 and the absence of this epitope in strains 7502 and 2019, indicating that the loss was not secondary to preparation of the LPS or the lipid A (Fig. 2A).

To obtain additional information about the nature of the

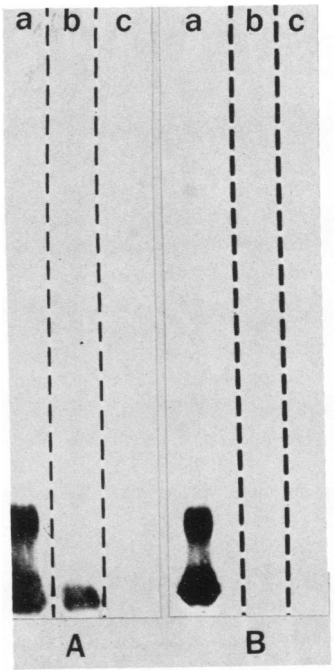

FIG. 1. (A) Western blot analysis demonstrating the reactivity of monoclonal antibody 3D2 with strain 3524 LPS (a) and lipid A (b) and failure to react with the saccharide portion of 3524 LPS (c). (B) Western blot analysis of LPS from strains 3524 (a), 2019 (b), and 7502 (c).

epitope recognized by 3D2 and to assure that the monoclonal is not recognizing saccharides which might be residual contaminants or on unhydrolyzed LPS in the lipid A, strain 3524 lipid A was subjected to sequential ethyl acetate-chloroform extraction. Monoclonal 3D2 immunodot assay of the three fractions obtained by this extraction indicated that strong reactivity was present in the chloroform-soluble fraction (Fig. 2B, lanes 2 and 3). Definite but weaker reactivity occurred in the chloroform-insoluble fraction. The ethyl acetate fraction did not react with 3D2 (Fig. 2B, lane 1). Analysis of these fractions with iodine vapors and chromatography sprays indicated that the chloroform-soluble and -insoluble fractions stained with iodine vapor and bromothymol blue, whereas the ethyl acetate fraction failed to stain with either reagent. Ninhydrin stained the chloroform-insoluble fraction strongly and the chloroform-soluble fraction weakly; the ethyl acetate fraction failed to stain with ninhydrin. Molybdenum reagent stained the chloroform-soluble fraction strongly and the chloroform-insoluble stained weakly; the ethyl acetate fraction did not stain. Orcinol analysis indicated that the ethyl acetate and the chloroform-insoluble fractions stained weakly, whereas the chloroform-soluble fraction failed to stain. This would indicate that the chloroform-soluble fraction, which monoclonal 3D2 recognized, was primarily lipid and contained both amino compounds and phosphates. It appeared to be free of significant amounts of unsubstituted carbohydrates. Thin-layer chromatographic analysis of this fraction on silica gel in the chloroform-acetone solvent system (85:15) resulted in three spots: one at the origin, a second with an R_f value of 0.38, and the third with an R_f value of 0.85. These spots were identified with iodine vapors and scraped from the plate and eluted with chloroform from the silica. Immunodot assay of this extract material indicated that 3D2 recognized the epitope in the eluate from each spot (Fig. 2C). These studies

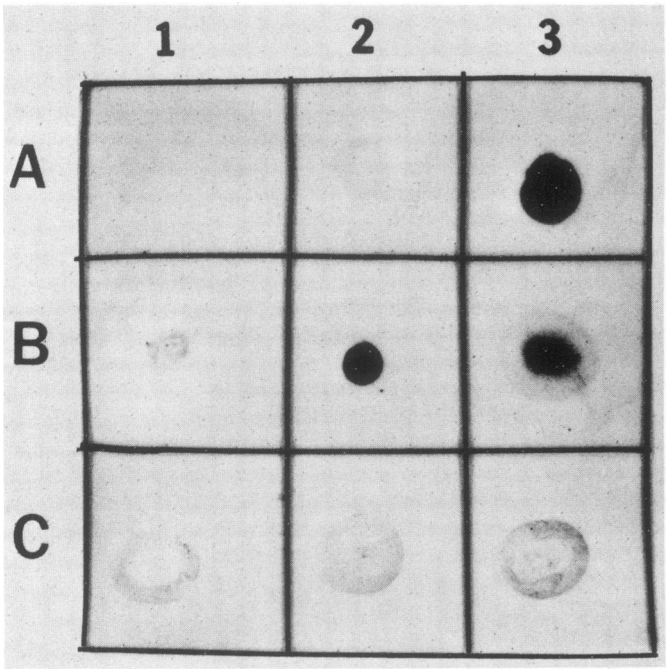

FIG. 2. Immunodot assay demonstrating reactivity of 3D2 with various lipid A extracts. Row A contains a phosphate-buffered saline control (lane 1) an SDS extract of strain 7502 (lane 2) and an SDS extract of strain 3524 (lane 3). Row B contains the ethyl acetate extract (lane 1), the chloroform-soluble extract (lane 2), and the chloroform-insoluble portion (lane 3) of 3524 lipid A. Row C contains extracts eluted from a thin-layer chromatographic plate containing the chloroform-soluble extract of the lipid A of strain 3524. Lane 1 contains extracts at the origin, lane 2 contains extracts at R_f 0.38, and lane 3 contains extracts at R_f 0.85.

would indicate that the 3D2 epitope is not associated with the oligosaccharide portion of the LPS and that a lipid moiety free of saccharides reacts with this monoclonal antibody.

Kinetic ELISA studies and ELISA inhibition. To determine whether the relationship of the epitope seen by 3D2 was similar on a variety of solid-phase surfaces in addition to nitrocellulose and whether antigen in solution was recognized by 3D2, kinetic ELISA and ELISA inhibition studies were performed. Kinetic ELISA analysis (Fig. 3) demonstrates the specificity of 3D2 for the epitope in the LPS from strain 3524 and the relative absence of this epitope on the LPS from strains 2019. ELISA inhibition studies were performed with LPS from strains 3524, 3198, 2019, and 7502. The concentration of strain 3524 or 3198 LPS necessary to achieve 50% inhibition of the 3D2-3524 ELISA was 100 times less than that necessary for inhibition of strain 2019 LPS and at least 1,000 times less than that required by strain 7502 LPS (Table 1). In addition to confirming the 3D2 reactivity of LPS from strains 3524 and 3198, these results also suggest that a portion of the 3D2 epitope may be present on strain 2019 LPS.

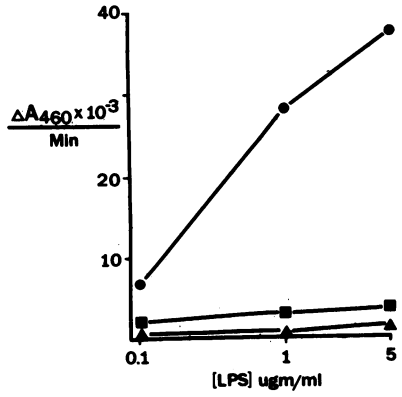

FIG. 3. Kinetic ELISA analysis of LPS from strains 3524 (●), 2019 (■), and 7502 (▲). Monoclonal antibody 3D2 concentration was 1 μg/ml. A_{460}, absorbancy at 460 nm.

Fluorescent antibody and bacterial studies. Fluorescent antibody studies with 3D2 indicated that it identified a surface exposed epitope on strain 3524. Bactericidal studies against strain 3524 with affinity-purified 3D2 from tissue culture supernatants with hypogammaglobulinemic serum as a complement source indicated that concentrations of antibody as high as 500 μg/ml failed to have any bactericidal effect against strain 3524. Additionally, pretreated strain 3524 with 3D2 did not inhibit the bactericidal effect of normal human serum on 3524.

Specificity and prevalence studies. Twenty-four nontypable *H. influenzae* clinical strains were studied for the 3D2 lipid A determinant in the immunodot assay. Fourteen strains (58%) were positive. Fifty-one of 95 *H. influenzae* type b strains were positive. One strain each of *H. influenzae* types a, c, d, and e were positive, and a single type f strain was negative. Twenty-four *H. parainfluenzae* strains, four strains of *H. paraphrophilus*, four strains of *H. aphrophilus*, two strains of *H. parahaemolyticus*, one strain of *H. aegyptis*, and one strain of *H. segnis* were negative for the 3D2 lipid A determinant. A variety of non-*Haemophilus* species were tested and were negative for this determinant. These include 10 *E. coli* strains, 6 *P. mirabilis* strains, 1 *P. vulgaris* strain, 3 *S marscescens* strains, 1 *M. morganii* strain, 1 *E. cloacae* strain, 1 *K. pneumoniae* strain, 1 *Klebsiella* species, 3 *P. aeruginosa* strains, 1 *Pseudomonas* species, 2 *Neisseria* species, 6 *N. gonorrhoeae* strains, 1 *Serratia* species, 8 *Actinobacillus* species, 5 *S. aureus* strains, 2 *Staphylococcus* species, 1 diphtheroid, and 2 streptococcal strains which were obtained from clinical isolates. Among the nontypable *H. influenzae* strains the 3D2 determinant was distributed randomly among the various outer membrane protein patterns which constituted the Murphy et al. subtyping system.

DISCUSSION

Lipid A is a component of the LPS of gram-negative bacteria. It has a basic structure which is highly conserved among a wide range of gram-negative species and which has

TABLE 1. 3D2 ELISA inhibition[a]

Inhibitor LPS	3D2 Immunodot	Concn of LPS (μg/ml) causing:	
		50% inhibition	100% inhibition
3524	+	0.041 (0.017–0.096)[b]	2.04 (0.56–7.41)
3198	+	0.123 (0.05–0.291)	11.64 (3.61–37.6)
2019	–	205 (35.9–>1,000)	>1,000
7502	–	>1,000	>1,000

[a] Microtiter wells were coated with 3524 LPS at a concentration of 5 μg/ml. Monoclonal 3D2 concentration was 1 μg/ml.

[b] Shown within parentheses are 95% confidence limits based on analysis of experiments run in triplicate.

been shown to be associated with the toxic activity ascribed to LPS. Lipid A is not a single macromolecular structure, but appears to be a complex array of a variety of lipids (3, 11, 12, 16, 18). Variation in the lipid A of the same strain has been demonstrated (16). This has been shown to be based on variation of the composition of the ester-linked fatty acids and substitution of the phosphate groups (16). The chemical structure of lipid A from enteric bacteria has been elaborated and has been shown to consist of a (1'-6)-linked diglucosamine oligosaccharide which contains two phosphate groups (8). This backbone structure is substituted by a variety of hydrophilic and hydrophobic substances, including amide-linked and ester-linked fatty acids. Lipid A also appears to have a highly conserved antigenic structure among a wide range of gram-negative bacteria. Immunological studies of lipid A from enteric bacteria have demonstrated a high degree of cross-reactivity which extends to the non-*Enterobacteriaceae* (6, 16). The fundamental antigenic component which will react with polyclonal antisera to lipid A has been shown to be the diglucosamine backbone substituted with a single amide-linked myristic acid (6). Lipid A as a portion of the intact LPS has been shown to be a poor immunogen. This can be enhanced by coating lipid A to acid-treated bacterial cells or to Re mutants of *Enterobacteriaceae* (7).

The lipid A of *H. influenzae* has not been completely studied. Studies by Raichvarg and associates have indicated that *H. influenzae* type a lipid A contained phosphorus, glucosamine, myristic, palmitic, and oleic acids and three hydroxymyristic acids. In addition, two other long chain fatty acids were found, but not identified (23). Of interest, the free lipid A was not toxic in Swiss mice at doses up to 50 mg/kg. No comments were made about the immunological characteristics of this lipid A preparation or antigenic differences in *H. influenzae* type a lipid A. However, it was concluded that this lipid A preparation did not exhibit all of the classical biological activities attributed to enterobacterial lipid A. Flesher and Insel (5) studied LPS from *H. influenzae* types a through f. Chemical analysis was completed only on the type b serotype and indicated that it contained 30% total carbohydrate and 29.3% lipid. Analysis of the sugars in the carbohydrate revealed glucose, galactose, glucosamine, and heptose, whereas the lipid component contained lauric, myristic, and palmitic acid. Biologic studies with these LPS preparations indicated that the preparations were toxic for mice and could be used to stimulate murine spleen cells. This latter effect could be blocked by polymyxin and was considered secondary to the lipid A component. Immunological studies with immunodiffusion and hemagglutination analysis suggested immunological heterogeneity among *H. influenzae* strains and indicated the presence of at least three antigenically distinct factors associated with these LPS. The source of these antigenic differences was considered to be the oligosaccharide portion of the LPS moiety; however, chemical analysis indicated that the carbohydrates were composed of identical sugars. It was assumed the differences were based on differences in sequence or the fine structure of the sugars. Recently, Insana demonstrated electrophoretic heterogeneity of *H. influenzae* by using microextracts of LPS on SDS-PAGE gels and silver staining (10); 50 strains were studied, and 33 could be classified into 11 subtypes. Nontypable strains appeared to demonstrate less LPS electrophoretic variation than that seen among type b strains. The basis for this heterogeneity has not been elucidated.

In this study, we have demonstrated that there are at least two antigenically distinct lipid A moieties among typable and nontypable *H. influenzae* strains. The epitope responsible for this differentiation is a lipid moiety which can be recovered in the chloroform-soluble portion of the lipid A. Analysis of non-*Haemophilus* species indicates that the epitope was absent among the strains studied. The epitope does not appear to be a bactericidal target but appears to be surface exposed based on fluorescent antibody analysis. These results are similar to those obtained by Mattsby-Baltzer and Kaliser, who showed the limited ability to demonstrate surface exposure of lipid A determinants on *Enterobacteriaceae* with polyclonal antisera (18). ELISA inhibition and kinetic ELISA analysis indicated that 3D2 recognizes the determinant on free lipid A and lipid A bound to polystyrene plastic surfaces. The ELISA inhibition studies would also indicate that this epitope may be present in low concentrations on strains which lack the determinant in Western blot analysis.

These studies demonstrate the antigenic heterogeneity of the lipid A of *H. influenzae*. Future efforts will be directed at determining the association of this determinant with the virulence of *H. influenzae* and in determining the precise nature of the structure responsible for the epitope recognized by monoclonal antibody 3D2.

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