INFECTION AND IMMUNITY, Apr. 1987, p. 882–887
0019-9567/87/040882-06$02.00/0

Vol. 55, No. 4

Antigenic Diversity of Lipooligosaccharides of Nontypable *Haemophilus influenzae*

ANTHONY A. CAMPAGNARI,* MALA R. GUPTA, KATHLEEN C. DUDAS, TIMOTHY F. MURPHY, AND MICHAEL A. APICELLA

Division of Infectious Diseases, Department of Medicine, and the Department of Microbiology, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14215

Received 14 July 1986/Accepted 16 December 1986

The lipooligosaccharides (LOS) of nontypable *Haemophilus influenzae* are an antigenically heterogeneous group of macromolecules. Immunodiffusion and enzyme-linked immunosorbent assay inhibition studies with phenol-water-extracted LOS and absorbed antisera specific for the oligosaccharide portion of the LOS identified six LOS strain-specific antigens. To facilitate screening large numbers of strains to search for LOS antigenic heterogeneity, a system utilizing proteinase K whole cell digests in Western blots was developed. Seventy-two nontypable *H. influenzae* LOS extracts were analyzed in this Western blot assay. Thirty-seven of these extracts could be segregated into 10 antigenically distinct LOS groups based on immunologic recognition by one or more of the rabbit antisera. Thirty-five of the strains did not contain these LOS antigens. These results demonstrate that antigenic differences exist among the LOS of nontypable *H. influenzae* strains, and this heterogeneity has the potential to be used to establish an LOS-based serogrouping system.

For many years, *Haemophilus influenzae* type b has been recognized as a human pathogen. In contrast, nontypable *H. influenzae* only recently has been considered as being the etiologic agent in human diseases. It is now well established that nontypable *H. influenzae* causes pneumonia, bacteremia, meningitis, postpartum sepsis, and acute tracheobronchitis in adults (4, 6, 19, 28–30). Studies with *H. influenzae* type b have indicated that the lipooligosaccharide (LOS) is an important virulence factor that may contribute significantly to the organisms pathogenicity (32, 33). Nontypable *H. influenzae* organisms are known to contain LOS in their cell membranes, and this LOS is capable of inducing toxicity in organ culture systems (5, 21). There is little information about the antigenic structure of the LOS of nontypable *H. influenzae*. Flesher and Insel in their study of the LOS of *H. influenzae* type b antigenic diversity suggested that the LOSs of nontypable *H. influenzae* strains were not antigenically diverse (7). Our own studies have recently demonstrated that the lipid A component of the LOS of *H. influenzae* is antigenically heterogeneous (2). In this paper we describe studies with rabbit antisera to nontypable *H. influenzae* strains which indicate that the oligosaccharide portion of the LOS of nontypable *H. influenzae* is also antigenically heterogeneous.

MATERIALS AND METHODS

Bacteria. Prototype strains of nontypable *H. influenzae* which represent the sodium dodecyl sulfate-polyacrylamide gel electrophoresis types described by Murphy et al. (18) were obtained from our own collection. Other nontypable strains were obtained from the clinical laboratories of the Buffalo Veterans Administration Medical Center, the Erie County Medical Center, and Children's Hospital of Buffalo. The identity of each strain had been confirmed previously as *H. influenzae* by colony 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.

Preparation of LOS. *H. influenzae* LOS was prepared by three methods. The phenol-water extraction method of Westphal and Jann (31) was used to prepare LOS used in enzyme-linked immunosorbent assay (ELISA) inhibition, and immunodot assay. A microadaption of the above method described by Inzana (11) was also used for Western blot assays. LOS extracts were also prepared by the method of Hitchcock and Brown (10) for Western blot assays. Lipid A was prepared as previously described (2).

Antisera. New Zealand White rabbits were prebled and then immunized with heated, acetone-dried nontypable *H. influenzae* organisms by the schedule of Rappuoli (24). This rabbit antiserum to nontypable *H. influenzae* was heat inactivated at 60°C for 1 h and then absorbed with other strains of nontypable *H. influenzae*. The absorption was based on results of immunodiffusion assays with unabsorbed sera reacting with LOS from the homologous strain and other nontypable *H. influenzae* strains. To absorb the antisera, 1-ml samples of antisera were incubated with 10^9 heat-killed nontypable *H. influenzae* cells at 4°C overnight with gentle agitation. These suspensions were centrifuged at 1,500 × g for 5 min, and the supernatants were removed and stored at −70°C.

Immunodiffusion. Ouchterlony analysis was performed in 1.5% Noble agar with 0.1 M sodium barbital buffer, pH 8.6 (22).

ELISA and ELISA inhibition. The ELISA and ELISA inhibition studies with purified LOS were performed by the method of Apicella and Gagliardi (3).

Immunodot assay. A modified dot assay utilizing nitrocellulose paper was performed as previously described (9). For this assay, the LOS were solubilized in phosphate-buffered saline and then sonicated at 50 W for 10 to 15 s. Samples of approximately 1 μg of these preparations were dotted onto the nitrocellulose and allowed to air dry. The dots were then blocked with 3% gelatin for 1 h. The individual antisera were mixed with these dots and allowed to incubate overnight. The dots were washed with 10 mM Tris hydrochloride (pH

* Corresponding author.

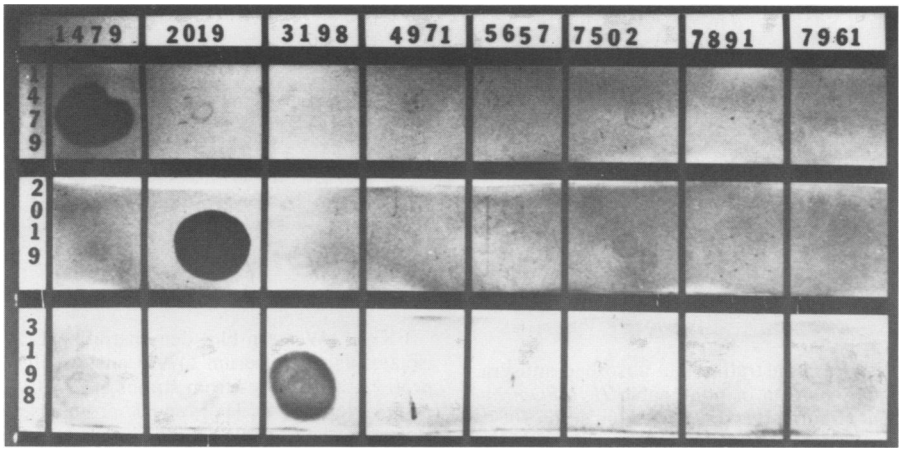

FIG. 1. Immunodot assay demonstrating the specificity of the homologous LOS preparation for its antiserum. In the vertical lanes are the respective LOS preparations. In the horizontal lanes are the respective antisera. Note that each antiserum only recognizes its homologous LOS preparation.

7.4) containing 0.9% NaCl (buffer A). Either peroxidase-conjugated protein A or goat anti-rabbit immunoglobulin G were diluted 1:3,000 in buffer A, and this was incubated with the dots for 1 h. Both of these conjugates gave similar results. The dots were washed, and the substrate, HRP color developer (Bio-Rad Laboratories, Richmond, Calif.), was added for 1 h in the dark.

Western blot analysis. The Western blot assay was performed as previously described (15). Briefly, the proteinase K LOS lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 14% gel by a modified version of the Laemmli method (16). At the end of the electrophoretic run, the gel was placed into the Transblot chamber (Bio-Rad) with a sheet of nitrocellulose paper, and the transfer was run for 1.5 h at 0.5 A, constant current. After the transfer was completed, the nitrocellulose sheet was treated as described above for the immunodot assay.

RESULTS

To investigate the antigenic spectrum of the LOS of nontypable *H. influenzae*, a series of immunologic studies including immunodiffusion, ELISA, immunodot, and Western blot were performed. Initially, LOS was isolated from each of the outer membrane protein prototype strains of Murphy et al. (18), and antisera were made to these strains.

With these reagents, immunodiffusion analyses were the initial studies conducted. These provided a direct examination for differences among LOS isolated from these strains. In these studies, evidence of antigenic differences between the LOS of some of these strains became apparent. At least four and possibly five different LOS strain-specific antigens were detected as well as LOS antigens which appeared to be shared by a number of the strains. Using these immunodiffusion studies as a basis, the rabbit antisera to nontypable *H. influenzae* were absorbed with heat-killed nontypable *H. influenzae* strains which lacked the homologous LOS antigens. These absorbed antisera were then used to establish ELISA systems specific for each LOS (data not shown).

ELISA inhibition studies. ELISA inhibition assays were then developed for each of these sets of LOS determinants (Table 1). A high degree of specificity existed for each homologous LOS, with 50% inhibition occurring at concentrations 20- to 1,000-fold less than that obtained with heterologous LOS. These data confirmed the immunodiffusion observations that a series of unique LOS determinants analogous to LPS serotype antigens exists among nontypable *H. influenzae* strains. The inhibition results in Table 1 also indicate that strains 1479 and 4971 share determinants, and subsequent Western blot studies confirmed that they contained the same LOS-specific antigens. An investigation was then undertaken to determine whether these differences in LOS antigenicity were strain specific or whether they represented a series of determinants which could be used to describe the LOS antigenic characteristics of a number of strains. Attempts were made to inhibit different ELISA systems with whole organisms, but extensive cross-reactivity with surface antigens other than LOS made the results difficult to interpret.

Immunodot assay. With absorbed antisera and phenol-water-extracted LOS isolated by the Westphal procedure (31), an immunodot assay could be established which was specific for each group of LOS determinants (Fig. 1). Since

TABLE 1. Nontypable *H. influenzae* LOS ELISA inhibition[a]

Antiserum[b]	LOS concn (μg/ml) causing 50% inhibition with antiserum:					
	1479	4971	3198	5657	2019	7502
1479	0.03	1.67	1,000	239	1,000	350
4971	19.8	38.6	1,000	1,000	237	1,000
3198	1,000	1,000	1.00	1,000	1,000	1,000
5657	1,000	1,000	1,000	3.56	1,000	181
7502	1,000	1,000	1,000	1,000	1,000	1.00

[a] Samples of 5 μg of LOS were coated in each well.
[b] Absorbed rabbit antisera to the respective NTHI strains.

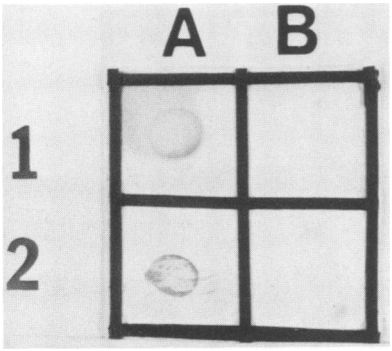

FIG. 2. Immunodot assay demonstrating that absorbed antisera do not recognize the lipid A portion of nontypable *H. influenzae* LOS. Dots: A-1, LOS from strain 2019; A-2, LOS from strain 5657; B-1, lipid A from strain 2019; B-2 contains lipid A from strain 5657. Row 1 was developed with absorbed 2019 antiserum, and row 2 was developed with absorbed 5657 antiserum.

isolation of LOS by this method is laborious, it was impractical to apply this method to prepare LOS to screen large numbers of strains. Proteinase K lysates were prepared and used in the immunodot assay. However, partially digested protein fragments were present in these preparations, causing non-LOS antibodies in the absorbed antisera to react. Similarly, LOS prepared by the microphenol method (11) had sufficient protein contamination to give background staining which obscured interpretation of the assay. To determine the nature of the LOS antigen responsible for the antigenic specificity we were studying, an immunodot assay was performed with LOS from strains 2019 and 5657 along with the purified lipid A from each of these strains. The absorbed antiserum to each of these strains reacted with its homologous LOS but failed to recognize the lipid A from the homologous and heterologous strain (Fig. 2). This would indicate that the determinants responsible for the LOS antigenic heterogeneity demonstrated by this absorbed antiserum are derived from the oligosaccharide portion of the macromolecule.

Western blot analyses. As an alternative method, the proteinase K lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot analysis. This procedure demonstrated specificity similar to that seen in the initial immunodot assay with purified LOS (Fig. 3). The microphenol water preparations were also tested and gave comparable results, but because of easier preparation proteinase K lysates were used to screen nontypable *H. influenzae* strains in the following studies. Proteinase K lysates of 72 nontypable *H. influenzae* strains were prepared, and these samples were analyzed in Western blots by using five absorbed antisera; these represented distinct LOS antigen groups based on immunodiffusion, ELISA inhibition, and dot assay with purified LOS. Fifteen samples were electrophoresed on each polyacrylamide gel. Each gel contained a lysate from a homologous and heterologous prototype strain. Figures 4 and 5 show typical Western blots performed during this screening process. The absorbed antiserum to strain 1479 was employed in Fig. 4, and the antiserum to strain 5657 was used in Fig. 5. In each case, the antibodies recognize their homologous LOS and fail to react to any of the LOS preparations from the four other prototypes used in these studies. Table 2 summarizes the results obtained from the analysis of the proteinase K lysates from these 72 nontypable *H. influenzae* strains with the five antisera. The LOS from 28 strains were recognized by a single antiserum, whereas the LOS from four strains were recognized by two antisera. As mentioned above, strain 1479 typed in the same group as strain 4971, confirming the ELISA inhibition data that the LOS of these two strains share antigenic composition. Thirty-eight nontypable *H. influenzae* strains were untypable with these sera. Given the limited number of strains serotyped, it was not possible to correlate the site of isolation with the particular LOS antigen group.

Subsequently, one of the untypable strains, 2780, was

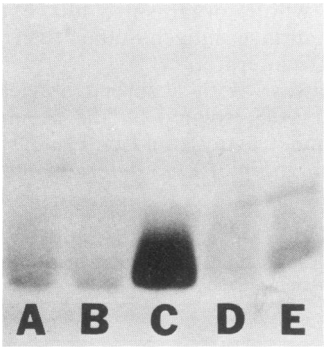

FIG. 3. Western blot assay demonstrating specificity of strain 3198 antiserum with the LOS in the proteinase lysate from strain 3198. Lanes: A, proteinase K lysate 1479; B, proteinase K lysate 2019; C, proteinase K lysate 3198; D, proteinase lysate 5657; and E, proteinase K lysate 7502. Antiserum to strain 3198 absorbed with the heterologous strains and diluted 1:500 was used to develop the blot.

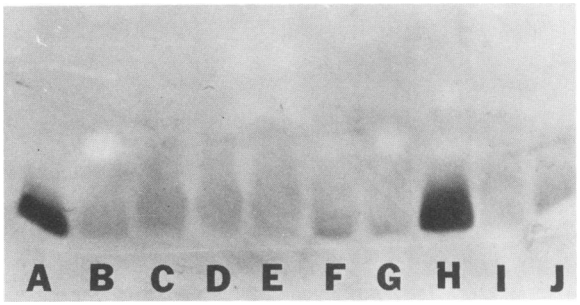

FIG. 4. Western blot demonstrating LOS serotyping of clinical isolates using antiserum 1479. Lanes: A, B, C, D, and E, prototype proteinase K lysates from strains 1479, 2019, 3198, 5657, and 7502, respectively; F, G, H, I, and J, proteinase K lysates from clinical isolates of nontypable *H. influenzae*; H, strain 4971, which has the same LOS represented by the 1479 prototype strain.

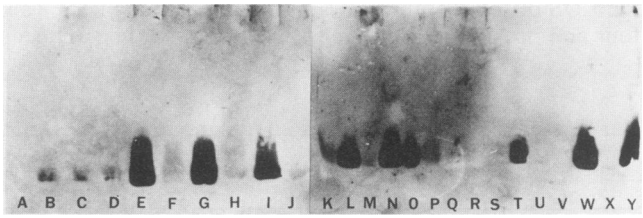

FIG. 5. Western blot analysis of PK lysates of clinical isolates of nontypable *H. influenzae* developed with antiserum to prototype strain 5657. Lanes: A through D, proteinase K lysates from prototype strains 1479, 2019, 3198, and 7502, respectively; Y, proteinase K lysate 5657; E, G, I, L, N, O, T, and W, strains 1808, 4505, 6539, 4554, 4556, 4555, and 5987, respectively, which were considered positive for this LOS group.

TABLE 2. Nontypable *H. influenzae* LOS groups as defined by Western blot analysis

LOS group	Strains
I	1479,[a] 6491, H22, 4971
II	2019,[a] H14, 4213, 1488
III	3198,[a] H15, 6416, C8057, 6522, 8802
IV	5657,[a] 1808, 4449, 4505, 4556, 4555, 4978, 1093, 6539, 4554, 3134, 5987
V	7502,[a] 8969
VI	1831
VII	SL1610
VIII	1285
IX	2030
X	2780,[a] 9290, C9607, C9623

[a] LOS group based on this strain.

selected, and an antiserum to this strain was prepared as previously described. The antiserum was absorbed with the five prototype strains used in this study. This antiserum was then used to analyze 28 LOS proteinase K lysates from the untypable group. This antiserum recognized the homologous LOS type and also the LOS from strains 9290, C9607, and C9623 (Fig. 6). The LOS from these strains appear to make up another antigen group.

Stability of the LOS patterns. Proteinase K lysates were made of prototype strains at different times in the growth cycle corresponding to early log, midlog, late log, and stationary phases to determine whether the phase of growth influenced LOS antigen expression. Individual nontypable strains were tested at different occasions over an 18-month period. The results of these tests were concordant for each strain each time it was tested. These studies were performed by the Western blot methods described above and indicated that phase of growth and passage or storage for long periods of time had no detectable effect on LOS antigen expression (data not shown).

DISCUSSION

The results of these studies demonstrated that strain-specific LOS antigens exist among nontypable *H. influenzae* strains. We have previously reported the existence of heterogeneity in the lipid A portion of the lipopolysaccharide of the *H. influenzae* (2). The antigenic heterogeneity we are describing in this study is based on the oligosaccharide portion of the LOS, since the absorbed polyclonal antisera used did not recognize the lipid A from the strains used for absorption. The immunodiffusion and ELISA inhibition data support the observation that antigenically distinct oligosaccharide determinants are present on the LOS of nontypable *H. influenzae* strains. Immunodot and Western blot analysis

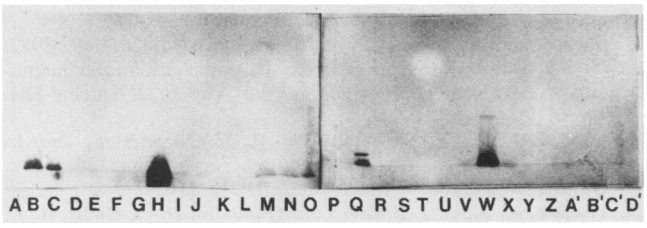

FIG. 6. Western blot analysis of proteinase K lysates of clinical isolates of nontypable *H. influenzae* strains previously untyped in Fig. 3 and 4. These blots were developed with antiserum to strain 2780. Lanes: H and W, homologous proteinase K lysate in the blot; B, C, and Q strains C9607, C9623, and 9290, respectively.

further substantiated these findings and demonstrated that strains could be segregated into groups based on differences in LOS antigenicity. Proteinase K lysates from 72 strains of nontypable *H. influenzae* were analyzed in this system. It was possible to group 36 of these strains into 10 antigenically distinct LOS antigen patterns with six different antisera.

Previous work on LOS of *H. influenzae* has focused primarily on serotype b strains. Flesher and Insel (7) defined three different antigenic factors associated with this LOS and, using thin-layer chromatography and gas-liquid chromatography, characterized the chemical composition of the lipid A and carbohydrate portion. In the latter, they demonstrated the presence of glucose, galactose, glucosamine, heptose, and 2-keto-3-deoxyoctulosonic acid. Parr and Bryan (23) studied the LOS of three *H. influenzae* type b strains and found similar sugars. More recently, Inzana and co-workers studied *H. influenzae* type b strain Eagan LOS by using gas chromatography-mass spectrometry, fast atom bombardment-mass spectrometry, amino acid analysis, and conventional colorimetric assays. They demonstrated the absence of O-repeating units, defined a lipid-free molecular weight of the oligosaccharide of 1,768, and indicated the presence of D-galactose in a terminal nonreducing residue linked to *N*-acetyl-D-galactosamine (14). Inzana has developed an electrophoretic typing system for *H. influenzae* type b with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11) and has demonstrated that this system has the potential to track strains during epidemics of *H. influenzae* type b infections (13).

Although the role of the LOS in infection due to nontypable *H. influenzae* is unclear, studies with *H. influenzae* type b indicate that the LOS is an important virulence factor. *H. influenzae* LOS is biologically active (5, 7, 21) and probably plays a major role in contributing to the pathogenicity of various serotypes (14, 32). Zwahlen and co-workers demonstrated that the elaboration of a unique capsular polysaccharide may not be sufficient explanation for the greater virulence of *H. influenzae* type b and that the LOS may contribute to the mediation of the differential pathogenicity of the various serotypes (33). More recently, Zwahlen and associates (32) transformed *H. influenzae* Rd^- with a recombinant phage (I-69) which contained a 10.2-kilobase fragment of DNA from a virulent strain of *H. influenzae* type b. The transformant had two defined cell wall alterations, one in LOS and one in the outer membrane protein. The transformant had a stable expression of type b capsular polysaccharide. In contrast to the Rd^-/b^+ strain, the Rd^-/b^+/I-69 transformant was serum sensitive in vitro and avirulent in vivo in rats. This work supported the previous observation of this group that the potential for *H. influenzae* type b to cause invasive disease can be substantially attenuated by altering expression of one or more genes that affect LOS or outer membrane protein expression. Studies to date indicate that antibodies to LOS can prevent mortality (17) but not disease (1, 26, 27). Shenep and co-workers (26) showed that anti-LOS antibodies eluted from a Sepharose affinity column did not protect against experimental bacteremia in infant rats. Studies of anti-LOS antibody in acute- and convalescent-phase sera from children recovering from *H. Influenzae* type b meningitis indicated that the presence of antibody to LOS in the acute-phase serum did not confer protection (27). The role of LOS as a bactericidal target in *H. influenzae* is unclear. Shaw and co-workers (25), Anderson and co-workers (1), and Inzana and Anderson (12) have shown that *H. influenzae* type b strain Eagan grown in broth is more sensitive to complement-mediated antibody killing than is *H.*

influenzae type b in bacteremic rats. This has been ascribed to the increased density of LOS in the sensitive phenotype, with decreased antibody binding to the LOS in the resistant phenotype. Recently, using biological selection by passage in rats, Gilsdorf and Ferrieri (8) recovered an *H. influenzae* type b variant which differed in colonial morphology, serum sensitivity, and LOS configuration from the parent strain. The LOS of this variant had different electrophoretic mobility and monoclonal and antibody-binding pattern when compared with the parent strain. The difference in susceptibility to complement-mediated bactericidal activity of normal rat serum displayed by these two strains is associated with their phenotypes, appears to be unrelated to differences in major outer membrane protein or in the amounts of capsular polysaccharides elaborated, and, according to these investigators, is associated with differences in surface LOS expression.

This study has demonstrated the existence of antigenic heterogeneity in the oligosaccharide portion of the LOS of nontypable *H. influenzae* strains. These antigens could potentially form the basis of a serogrouping system. Such a serogrouping system based on the LOS of nontypable *H. influenzae* could provide valuable information as to the epidemiology of the organisms and the potential virulence of some strains. Preliminary data in our laboratory indicate that monoclonal antibodies directed at the serogroup-specific antigens can be used to screen large numbers of strains rapidly. As this develops, it will allow us to use rapid and reliable screening procedures to study the epidemiology of the LOS serogroups of nontypable *H. influenzae*. Future studies will be directed at the development of a polyclonal antibody-based serogrouping system to include the majority of nontypable *H. influenzae* strains, to determine the relationship of *H. influenzae* type b strains to this system, and to develop a monoclonal antibody-based LOS serogrouping system for all *H. influenzae* strains.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI 19641 from the National Institute of Allergy and Infectious Diseases.

The secretarial assistance of Phyllis Rosenberg is greatly appreciated.

LITERATURE CITED

1. **Anderson, P., A. Flesher, S. Shaw, and A. L. Harding.** 1980. Phenotypic and genetic variation in the susceptibility of *Haemophilus influenzae* type b to antibodies to somatic antigens. J. Clin. Invest. **65**:885–891.
2. **Apicella, M. A., K. C. Dudas, A. Campagnari, P. Rice, J. M. Mylotte, and T. F. Murphy.** 1985. Antigenic heterogeneity of lipid A from *Haemophilus influenzae*. Infect. Immun. **50**:9–14.
3. **Apicella, M. A., and N. C. Gagliardi.** 1979. Antigenic heterogeneity of the non-serogroup antigen structure of *Neisseria gonorrhoeae* lipopolysaccharides. Infect. Immun. **26**:870–874.
4. **Berk, S. L., S. A. Moltsclaw, S. L. Wiener, and J. K. Smith.** 1982. Nontypable *Haemophilus influenzae* in the elderly. Arch. Intern. Med. **142**:537–537.
5. **Denny, F. W.** 1974. Effect of a toxin produced by *Haemophilus influenzae* on ciliated respiratory epithelium. J. Infect. Dis. **129**:93–100.
6. **Ellner, P. D., and A. Shahidi.** 1969. Postpartum bacteremia due to *Haemophilus influenzae*. Obstet. Gynecol. **34**:403–405.
7. **Flesher, A. R., and R. A. Insel.** 1978. Characterization of lipopolysaccharide of *Haemophilus influenzae*. J. Infect. Dis. **138**:719–730.
8. **Gilsdorf, J. R., and P. Ferrieri.** 1986. Susceptbility of phenotypic variants of *Haemophilus influenzae* type b to serum bactericidal activity: relation to surface lipopolysaccharide. J. Infect. Dis. **153**:223–231.
9. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
10. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
11. **Inzana, T. J.** 1983. Electrophoretic heterogeneity and interstain variation of the lipopolysaccharide of *Haemophilus influenzae*. J. Infect. Dis. **148**:492–299.
12. **Inzana, T. J., and P. Anderson.** 1985. Serum factor-dependent resistance of *Haemophilus influenzae* type b to antibody to lipopolysaccharide. J. Infect. Dis. **151**:869–877.
13. **Inzana, T. J., and M. E. Pichichero.** 1984. Lipopolysaccharide subtypes of *Haemophilus influenzae* type b from an outbreak of invasive disease. J. Clin. Microbiol. **20**:145–150.
14. **Inzana, T. J., W. E. Seifert, Jr., and R. P. Williams.** 1985. Composition and antigenic activity of the oligosaccharide moiety of *Haemophilus influenzae* type b lipooligosaccharide. Infect. Immun. **48**:324–330.
15. **Knecht, D. A., and R. L. Dimond.** 1984. Visualization of antigenic proteins on Western blots. Anal. Biochem. **136**:180–184.
16. **Laemmli, U. K.** 1970. Cleavage of the structural proteins during assembly of the head of the bacteriophage T4. Nature (London) **227**:680–685.
17. **Marks, M. I., E. J. Ziegler, H. Douglas, L. B. Corbell, and A. I. Braude.** 1982. Induction of immunity against lethal *Haemophilus influenzae* type b infection by *Escherichia coli* core lipopolysaccharide. J. Clin. Invest. **69**:742–749.
18. **Murphy, T. F., K. C. Dudas, J. M. Mylotte, and M. A. Apicella.** 1983. A subtyping system for nontypable *Haemophilus influenzae* based on outer membrane proteins. J. Infect. Dis. **147**:838–846.
19. **Musher, D. M., K. R. Kubitsher, J. Crennan, and R. E. Baughn.** 1983. Pneumonia and acute febrile tracheobronchitis due to *Haemophilus influenzae*. Ann. Intern. Med. **99**:444–450.
20. **Myre, E. B.** 1974. Typing of *Haemophilus influenzae* by counterimmunoelectrophoresis. Acta Pathol. Microbiol. Scand. Sect. B **82**:164–166.
21. **Mylotte, J. M., R. R. Stack, T. F. Murphy, J. Asirwathan, and M. A. Apicella.** 1985. Functional and ultrastructural affects of nontypable *Haemophilus influenzae* in a hamster trachea organ culture system. In Vitro **21**:575–582.
22. **Ouchterlony, O.** 1962. Diffusion-in-gel, methods for immunological analysis. Prog. Allergy **6**:30–54.
23. **Parr, T. R., Jr., and L. E. Bryan.** 1984. Lipopolysaccharide composition of three strains of *Haemophilus influenzae*. Can. J. Microbiol. **30**:1184–1187.
24. **Rappuoli, R.** 1982. Production and characterization of high titre rabbit antigonococcal R-type lipopolysaccharide serum. Microbiologica **5**:225.
25. **Shaw, S., A. L. Smith, P. Anderson, and D. H. Smith.** 1976. The paradox of *Haemophilus influenzae* type b bacteremia in the presence of serum bactericidal activity. J. Clin. Invest. **58**:1019–1029.
26. **Shenep, J. L., R. S. Munson, Jr., S. J. Barenkamp, and D. M. Granoff.** 1983. Further studies of the role of noncapsular antibody in protection against experimental *Haemophilus influenzae* type b bacteremia. Infect. Immun. **42**:257–263.
27. **Shenep, J. L., R. S. Munson, Jr., and D. M. Granoff.** 1982. Human antibody responses to lipopolysaccharide after meningitis due to *Haemophilus influenzae* type b. J. Infect. Dis. **145**:181–190.
28. **Simon, H. B., F. S. Southwick, R. C. Moellering, and E. Sherman.** 1980. *Haemophilus influenzae* in hospitalized adults: current perspectives. Am. J. Med. **69**:219–226.
29. **Wallace, R. J., C. J. Baker, F. J. Quinones, D. G. Hollis, R. E. Weaver, and K. Wiss.** 1983. Nontypable *Haemophilus influenzae* (Biotype 4) as a neonatal, maternal and genital pathogen. Rev. Infect. Dis. **5**:123–135.
30. **Wallace, R. J., D. M. Musher, E. J. Septimus, J. E. McGowan, F. J. Quinones, K. Wiss, P. H. Vance, and P. A. Trier.** 1981.

Haemophilus influenzae infections in adults: characterization of strains of serotypes, biotypes and beta-lactamase production. J. Infect. Dis. **144**:101–106.

31. **Westphal, O., and K. Jann.** 1954. Extraction with phenol-water and further applications of the procedure, p. 83–91. *In* Methods in carbohydrate chemistry. Academic Press, Inc., New York.
32. **Zwahlen, A., L. G. Rubin, C. J. Connelly, T. J. Inzana, and E. R. Moxon.** 1985. Alteration of the cell wall of *Haemophilus influenzae* type b by transformation with cloned DNA: association with attenuated virulence. J. Infect. Dis. **152**:485–492.
33. **Zwahlen, A., J. A. Winkelstein, and E. R. Moxon.** 1983. Surface determinants of *Haemophilus influenzae* pathogenicity: comparative virulence of capsular transformants in normal and complement-depleted rats. J. Infect. Dis. **148**:385–394.