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Antigenic and Phenotypic Variations of *Haemophilus influenzae* Type b Lipopolysaccharide and Their Relationship to Virulence

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Haemophilus influenzae **type b (Hib) strains NO100 and COL10 were found to produce bacteremia in infant rats at a much lower frequency than other Hib strains previously tested. These relatively avirulent strains were the only Hib strains among 200 clinical isolates examined to date which failed to react with two Hib lipopolysaccharide (LPS)-specific monoclonal antibodies (MAbs). LPS analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that strains NO100 and COL10 possessed LPS which migrated faster than the LPS of Hib strains that reacted with one of the two or with both of these MAbs. These observations suggested that the relative lack of virulence of strains NO100 and COL10 might be related to their unusual LPS phenotype. To determine whether alteration of LPS structure would affect the virulence of these strains, we identified and isolated isogenic LPS antigenic variants of strains NO100 and COL10 using the LPS-specific MAbs 4C4 and 5G8 in a colony blot radioimmunoassay. Antigenic variation of LPS was found to occur spontaneously in these two strains at a relatively high frequency in terms of both acquisition and loss of MAb reactivity (ca. 0.2 to 16.7%). LPS antigenic variants of strains NO100 and COL10 reactive with both MAbs 4C4 and 5G8 ($4C4^+$ $5G8^+$) were more virulent in the infant rat model than their respective $4C4^-$ $5G8^-$ parental strains ($P < 0.01$). An antigenic variant of COL10 reactive with only MAb 4C4 ($4C4^+$ $5G8^-$) was also significantly more virulent than its $4C4^-$ $5G8^-$ parent. These LPS antigenic variants with increased virulence synthesized altered LPS molecules which possessed apparent molecular weights higher than those of the LPS of the parental strains. Increased resistance of strain NO100 to the bactericidal activity of normal infant rat serum was associated with changes in LPS structure, while strain COL10 and its LPS variants were all uniformly resistant to serum bactericidal activity. Our results demonstrate that (i) spontaneous antigenic and phenotypic variation of LPS occurs at a relatively high frequency in some strains of Hib; (ii) the higher-molecular-weight type of LPS is associated with the full expression of Hib virulence; (iii) LPS phenotype may not correlate with Hib serum resistance; and (iv) serum resistance of Hib is not an accurate indicator of virulence.**

Haemophilus influenzae type b (Hib) remains the leading cause of bacterial meningitis in infants and young children in the United States (3). The essential role of the type b capsule in the virulence of Hib has been well established (27, 32, 41). Much less attention, however, has been given to the possible involvement of noncapsular cell surface components in the virulence of this organism.

Numerous studies have shown that lipopolysaccharide (LPS) is an important factor in the expression of virulence by other gram-negative bacteria (5, 20, 22, 29). Hib LPS is known to elicit many of the biological effects of endotoxin, and it has been suggested that alterations in Hib LPS may reduce the virulence of this organism (43). The first biochemical characterization of Hib LPS suggested that, like meningococcal and gonococcal LPS molecules (34), Hib LPS lacks an O-antigen-like repeat unit and consists of lipid A and a structure analogous to the core oligosaccharide of enteric LPS molecules (6). This early work was recently confirmed and expanded by Inzana et al. (17), who performed detailed chemical and antigenic analyses of the Hib core oligosaccharide. Characterization of Hib LPS by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) revealed microheterogeneity among the LPS molecules of different Hib strains, which permitted the development of a system for subtyping Hib strains based on their LPS profile (14). This classification system has proven useful in epidemiologic studies of Hib disease (16).

Antigenic analysis has revealed a remarkable lack of heterogeneity among the cell surface-exposed antigenic determinants of Hib LPS. Flesher and Insel (6) and Anderson et al. (2) have independently identified several different Hib LPS antigenic determinants using polyclonal antiserum reactive with Hib LPS, while van Alphen et al. (40) have found four different LPS serotypes among 80 strains of Hib isolated in The Netherlands.

In our laboratory, characterization of the LPS of 126 Hib strains using monoclonal antibodies (MAbs) directed against cell surface-exposed antigenic determinants of Hib LPS confirmed that limited antigenic heterogeneity exists among the LPS determinants exposed on the cell surface (8, 9). These Hib strains were divided into four different antigenic groups based on their reactivities with two MAbs (Table 1). The availability of these MAbs specific for Hib LPS allowed us to identify spontaneously arising and isogenic LPS phenotypic variants of Hib which were used to evaluate the role of Hib LPS in the virulence of this organism. The experiments reported here indicate that LPS may be an effector of Hib virulence.

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MATERIALS AND METHODS

Bacterial strains. Hib strains NO100 and COL10 were used in most of these experiments. Strain NO100 was an ear fluid isolate provided by Robert Daum, New Orleans, La. Strain COL10 was supplied by Katherine Sprunt, New York, N.Y., and was originally isolated from cerebrospinal fluid by HattieAlexander. Additional strains of Hib representative of LPS antigenic groups 1, 2, and 3 were all isolated from cerebrospinal fluid (8). Bacterial culture media and growth conditions have been described previously (12).

Whole cell lysates for LPS analysis. Proteinase K-digested whole cell lysates of Hib were used as the source of LPS for analysis by SDS-PAGE. These lysates were prepared by a modification of the method described by Hitchcock and Brown (13). Bacteria were grown in broth culture to a density of 10^9 CFU/ml. A 10-ml quantity of this exponential-phase culture was centrifuged at 7,000 × *g* for 20 min, washed once with cold phosphate-buffered saline (PBS; pH 7.2), and then suspended in 1 ml of PBS. The bacteria were solubilized by adding 0.5 ml of concentrated digestion buffer composed of 0.1875 M Tris hydrochloride (pH 6.8), 6% (wt/vol) SDS, 30% (vol/vol) glycerol, and 0.015% (wt/vol) pyronin Y tracking dye, followed by heating at 100°C for 5 min. Multiple portions of each lysate were stored at −20°C. For LPS analysis, a 10-μl portion of the lysate (equivalent to ca. 7×10^7 cells) was diluted with 35 μl of LPS digestion buffer (0.0625 M Tris hydrochloride [pH 6.8], 0.1% [wt/vol] SDS, 10% [vol/vol] glycerol, and 0.005% [wt/vol] pyronin Y) and treated with 5 μg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 37°C. These preparations were heated at 100°C for 5 min prior to electrophoresis.

SDS-PAGE of LPS. LPS was resolved by discontinuous SDS-PAGE (1) using a 4% (wt/vol) polyacrylamide stacking gel and a separating gel (16 cm in length) consisting of a linear 10 to 15% (wt/vol) polyacrylamide gradient containing a linear 0 to 37.5% (vol/vol) glycerol gradient. Electrophoresis was carried out at 4°C and 20 mA constant current until the associated voltage increased to 400 V, at which time the current was reduced to 12 mA. Electrophoresis was terminated when the tracking dye reached a point 1 cm above the bottom of the separating gel. LPS was visualized by the silver stain method of Tsai and Frasch (39).

MAbs. MAbs 4C4 and 5G8 are both murine subclass immunoglobulin G3 (IgG3) antibodies and are directed against cell surface-exposed antigenic determinants of antigenic group 2 Hib LPS (8, 9). These MAbs are specific for epitopes present in the core oligosaccharide of group 2 Hib LPS (A. Kimura and E. J. Hansen, manuscript in preparation). Hybridoma culture supernatant fluids were used as the source of these antibodies for all experiments.

Western blot procedure. Immunoblotting of LPS was accomplished by a modification of a procedure described previously (18). Briefly, proteinase K-digested whole cell lysates of Hib (10 μl containing ca. 7×10^7 cells) were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes by the method of Towbin et al. (37). The SDS-PAGE-resolved LPS preparations were transferred overnight at 180 mA constant current in a Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, Calif.). The resultant electrophoretic blots were incubated for 1 h in PBS containing 2.5% (wt/vol) bovine serum albumin to saturate nonspecific protein binding sites. The blots were then incubated with a mixture of 35 ml of hybridoma culture supernatant fluid containing either MAb 4C4 or MAb 5G8 and 35 ml of PBS containing 2.5% (wt/vol) bovine serum albumin for 6 to 8 h at room temperature. After three 20-min washes in PBS, MAb bound to LPS was detected by incubating the blots in a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (heavy and light chain specific; Cappel Laboratories, Cochranville, Pa.) in PBS containing 2.5% (wt/vol) bovine serum albumin at 4°C overnight. The blots were then washed three times with PBS and developed in PBS containing 0.5 mg of 4-chloro-1-naphthol (Sigma) per ml and 0.01% hydrogen peroxide.

Colony blot radioimmunoassay. Colonies of Hib reactive with MAbs 4C4 and 5G8 were detected by using the colony blot radioimmunoassay (RIA) method described by Gulig et al. (8).

Isolation of LPS antigenic variants. The two group 4 Hib strains (NO100 and COL10) were purified by repeated single colony isolations. Exponential-phase broth cultures of the group 4 strains grown to a density of 10^9 CFU/ml were diluted and plated on solid media. Following overnight incubation at 37°C in a candle extinction jar, the resultant colonies were screened for reactivity independently with MAbs 4C4 and 5G8 in the colony blot RIA system. A colony of each group 4 strain that was reactive with only MAb 4C4 was identified and purified by repeated single colony isolations. These primary variants ($4C4^+$ $5G8^-$) were grown in broth, diluted, and plated on solid media. The resultant colonies were again screened with MAbs 4C4 and 5G8 independently, and a secondary variant of each primary variant that was reactive with both of these MAbs ($4C4^+$ $5G8^+$) was identified and purified.

Antibody accessibility RIA. An indirect antibody accessibility (AA) RIA was used to assess cell surface exposure of the LPS determinants recognized by MAbs 4C4 and 5G8 (18). Organisms grown overnight on solid media were suspended in cold PBS to a concentration of 10^8 CFU/ml. A 100-μl volume of this suspension was reacted with 500 μl of either MAb 4C4 or MAb 5G8 hybridoma culture supernatant fluid for 2 h at 4°C with gentle rocking. After centrifugation at 12,000 × *g* for 5 min, the pellet was washed once with PBS containing 10% (vol/vol) heat-inactivated fetal calf serum and then suspended in 1 ml of this same buffer. To detect MAbs specifically bound to the cell surface, the bacterial suspension was incubated with 10^6 cpm of affinity-purified and radioiodinated goat anti-mouse immunoglobulin (specific activity, 10^8 cpm per μg of protein) for 1 h at 4°C. A carrier organism (ca. 10^8 cells of Hib strain NO100 lacking the determinants recognized by MAbs 4C4 and 5G8) was added to the suspension which was then washed three times with 1-ml quantities of PBS containing 10% (vol/vol) fetal calf serum. The final pellet was suspended in 500 μl of solubilization buffer (12), and the amount of radioactivity (counts per minute) bound to the cells was determined.

Animal model. The relative virulence of Hib strains was determined with an infant rat model (25). Infant rats, 5 to 6 days of age, were challenged either by the intraperitoneal route with ca. 10^2 CFU (35) or by the intranasal route with ca. 2×10^8 to 3×10^8 CFU (26). The intranasal challenge was accomplished by carefully dropping the 10-μl inoculum onto the rat nares, as described by Moxon and Vaughn (27). Bacteremia was quantitated 24 h after challenge by culturing a 10-μl sample of tail vein blood on solid media. Results were analyzed for statistical significance by chi-square analysis.

Capsular polysaccharide quantitation. Cell-associated capsular polysaccharide was quantitated by rocket immunoelectrophoresis as described by Sutton et al. (36). Quantitation was performed on sonicated preparations of Hib cells suspended in 0.025 M Veronal buffer (pH 8.6; Winthrop Labo-

ratories, Div. Sterling Drug Co., New York, N.Y.). Burro 132 anti-type b antiserum was used as the immunoprecipitating antibody (kindly provided by John B. Robbins, National Institutes of Health, Bethesda, Md.). Purified type b capsular polysaccharide (kindly provided by Joseph Kuo, Lederle Laboratories, Pearl River, N.Y.) was used as the standard.

Outer membrane protein preparations. Outer membrane vesicles were prepared by a modification (10) of the LiCl-based extraction procedure of McDade and Johnston (21). The proteins in the vesicles were resolved by SDS-PAGE and visualized by staining with Coomassie blue (12).

Serum bactericidal assay. Exponential-phase broth cultures of Hib were centrifuged at 7,000 × *g* for 10 min and then suspended and diluted to a concentration of 10^5 CFU/ml in cold Veronal-buffered saline (pH 7.4) containing 0.1% gelatin. Each assay mixture contained 10 μl of the bacterial suspension (ca. 1,000 organisms), 10 μl of Veronal-buffered saline containing 5 mM $MgCl_2$ and 1.5 mM $CaCl_2$, and 20 to 80 μl of infant rat serum. Veronal-buffered saline was added to make a total volume of 100 μl. All of these components were kept in crushed ice until they were mixed in a 0.5-ml microcentrifuge tube which was then incubated at 37°C in a stationary water bath. At time zero and at various intervals thereafter, 10-μl samples were removed and plated in duplicate on solid media. Hib strains were always tested in duplicate.

Infant rat serum was prepared from the blood of 40 normal 8-day-old Sprague-Dawley rats. Blood was obtained by cardiac puncture and was allowed to clot for 1 h at room temperature. The blood was then placed in ice for 1 to 2 h and centrifuged, and the serum was harvested and pooled. The serum was frozen in an ethanol-dry ice bath and stored at −70°C. When necessary, complement activity in this serum was destroyed by heating at 56°C for 30 min.

RESULTS

Relative virulence of Hib LPS antigenic groups. During the course of earlier work which established the immunoprotective ability of a Hib outer membrane protein-directed MAb (18), a single Hib strain was found to be unable to produce bacteremia consistently in infant rats after intraperitoneal injection of 100 CFU. In contrast, all other Hib strains tested in this previous study produced bacteremia in essentially all animals challenged with the same size of inoculum. This relatively avirulent Hib strain, NO100, was also the only Hib strain of 126 strains tested in another study that failed to react with the LPS-specific MAbs 12D9 and 4C4 and which was classified as a LPS antigenic group 4 strain (Table 1; 8, 9). More recently, a second Hib strain (COL10) which is nonreactive with these two MAbs was identified by colony blot RIA analysis.

Preliminary experiments involving SDS-PAGE analysis of the LPS synthesized by a large number of representative strains of Hib LPS antigenic groups 1 through 3 (Table 1) revealed the microheterogeneity pattern in LPS profiles first reported by Inzana (14). LPS from these clinical isolates was found to migrate in these gels to approximately the same position as did LPS from a Ra mutant of *Salmonella typhimurium* (9). The only consistent difference between the LPS profiles of the Hib strains from antigenic groups 1, 2, and 3 and those of the two group 4 strains was that the group 4 LPS molecules (Fig. 1, lanes 4 and 8) migrated in a single band and faster in the gel than the other LPS molecules (Fig. 1). The only exception was the group 2 Hib strain SL103 (Fig. 1, lane 6) which possessed multiple LPS bands which migrated at various rates. Thus, the lack of reactivity of group 4 Hib LPS with MAbs 12D9 and 4C4 was correlated with increased electrophoretic mobility of this LPS in SDS-PAGE. This result suggests that the lack of reactivity of the group 4 LPS with these MAbs may be due to the physical absence of antigenic determinants reactive with MAbs 4C4 and 12D9, which in turn results in a smaller LPS molecule with greater electrophoretic mobility.

TABLE 1. Antigenic analysis and grouping of 126 Hib strains with LPS-specific MAbs[a]

Antigenic classification (phenotype)[b]	No. of strains (%)
Group 1 ($12D9^+$ $4C4^-$)[c]	17 (13.5)
Group 2 ($12D9^-$ $4C4^+$)	78 (61.9)
Group 3 ($12D9^+$ $4C4^+$)	30 (23.8)
Group 4 ($12D9^-$ $4C4^-$)	1 (0.8)

[a] These data are summarized from a previous report (8).
[b] Reactivity with MAbs was determined by using the colony blot RIA.
[c] Group classification was based on reactivity with MAbs 12D9 and 4C4.

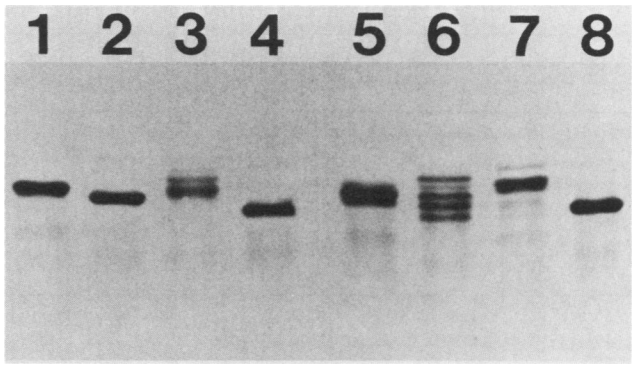

FIG. 1. LPS of representative Hib strains from each of the four LPS antigenic groups. The LPS in proteinase K-treated whole cell digests of Hib was resolved by SDS-PAGE (as described in the text) and visualized by the silver stain method of Tsai and Frasch (39). Group 1: lane 1, strain DL26; lane 5, strain CH102. Group 2: lane 2, strain DL42; lane 6, strain SL103. Group 3: lane 3, strain DL41; lane 7, strain NO101. Group 4: lane 4, strain NO100; lane 8, strain COL10. Only the relevant portion of the gel is shown.

The preceding observations raised the possibility that the relative lack of virulence of the group 4 strain NO100 might be related to its unusual LPS phenotype. To determine whether alteration of the LPS of group 4 Hib would affect the virulence of this strain, strain NO100 was transformed with DNA from a group 2 Hib strain ($4C4^+$), and the resultant transformants were screened with MAb 4C4 (A. Kimura et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B21, p. 21). MAb 4C4-reactive colonies obtained from these transformation experiments were markedly more virulent than the original NO100 strain in an infant rat model and synthesized an altered LPS molecule which migrated similarly to group 2 Hib LPS in SDS-PAGE. However, it was also subsequently found that NO100 cells which had not been incubated with the group 2 Hib DNA spontaneously gave rise to colonies which bound MAb 4C4.

Spontaneous antigenic variation of group 4 Hib LPS. To characterize further this apparent spontaneous variation in the antigenic characteristics of group 4 Hib LPS, both strain NO100 and strain COL10 were used in a series of experiments together with MAbs 4C4 and 5G8. MAb 5G8 previously has been shown to react with 74 of 78 group 2 Hib

TABLE 2. Reactivity of Hib strains NO100 and COL10 and their LPS antigenic variants with MAbs 4C4 and 5G8[a]

Strain[b]	LPS antigenic phenotype	No. of colonies screened	No. of colonies (%) with the following antigenic phenotypes		
			4C4$^-$ 5G8$^-$	4C4$^+$ 5G8$^-$	4C4$^+$ 5G8$^+$
NO100	4C4$^-$ 5G8$^-$	1,986	1,982 (99.8)	4 (0.2)	0 (0)
NO100 (p.v.)	4C4$^+$ 5G8$^-$	1,088	6 (0.5)	1,078 (99.1)	4 (0.4)
NO100 (s.v.)	4C4$^+$ 5G8$^+$	968	3 (0.3)	0 (0)	965 (99.7)
COL10	4C4$^-$ 5G8$^-$	2,248	2,234 (99.3)	13 (0.6)	1 (<0.1)
COL10 (p.v.)	4C4$^+$ 5G8$^-$	2,677	173 (6.5)	2,473 (92.4)	31 (1.1)
COL10 (s.v.)	4C4$^+$ 5G8$^+$	3,678	613 (16.7)	8 (0.2)	3,057 (83.1)

[a] Reactivity of these Hib strains with MAbs was assessed in the colony blot RIA system.
[b] p.v., primary variant; s.v., secondary variant.

strains, and it recognizes a different LPS antigenic determinant in the core oligosaccharide from that which binds MAb 4C4 (8, 9).

Screening of individual colonies of strains NO100 and COL10 by colony blot RIA revealed that both group 4 strains were capable of giving rise to colonies expressing antigenic determinants reactive with MAb 4C4. These primary antigenic variants (4C4$^+$ 5G8$^-$) were isolated and then grown in broth, plated on solid media, and screened for reactivity with MAb 5G8. Secondary variants (4C4$^+$ 5G8$^+$) of these primary variants (4C4$^+$ 5G8$^-$) that were recognized by both MAbs were identified in this manner. The results of these experiments are summarized in Table 2. The original group 4 strain NO100 and its primary (4C4$^+$ 5G8$^-$) and secondary (4C4$^+$ 5G8$^+$) variants each yielded populations which were relatively homogenous in that greater than 99% of the colonies of each strain retained their respective antigenic characteristics. However, 0.2% of the NO100 colonies were reactive with MAb 4C4, while no MAb 5G8-reactive colonies were detected. Similarly, 0.5% of the colonies derived from the primary variant (4C4$^+$ 5G8$^-$) of NO100 lost reactivity with MAb 4C4, while 0.4% of the total number of colonies exhibited reactivity with both MAb 4C4 and MAb 5G8. When colonies of the secondary variant (4C4$^+$ 5G8$^+$) were screened with these MAbs, 0.3% of the colonies reacted with neither MAb. Colonies expressing a 4C4$^-$ 5G8$^+$ phenotype were never detected in these experiments.

Similar results were obtained with the other group 4 Hib strain COL10 (Table 2). Antigenic variation among the LPS phenotypes occurred, however, at somewhat higher frequencies than those observed with strain NO100. It is interesting that, again, expression of the determinant recognized by MAb 5G8 occurred exclusively among the population of COL10 cells already expressing the 4C4 determinant. Furthermore, as with strain NO100, the loss of reactivity with MAb 5G8 by the secondary variant (4C4$^+$ 5G8$^+$) was almost always associated with the simultaneous loss of reactivity with MAb 4C4.

These data demonstrate that the group 4 Hib strains NO100 and COL10 possess the genetic information encoding the group 2 LPS determinants, as defined by reactivity with MAbs 4C4 and 5G8, and that the expression of these genes is not constitutive but is variable. The data also indicate that the synthesis of the two different group 2 LPS determinants are closely related: the presence of the 4C4 determinant appears to be a prerequisite for the acquisition of the 5G8 determinant, and while the loss of the 5G8 determinant could occur independently, its loss is almost always associated with the simultaneous loss of the 4C4 determinant.

LPS antigenic variants of strains NO100 and COL10: analysis by SDS-PAGE and Western blotting. The LPS of the antigenic variants of the group 4 Hib strains NO100 and COL10 were characterized by SDS-PAGE and silver staining (Fig. 2A). As shown previously in Fig. 1, the group 4 Hib strains NO100 and COL10 exhibited a single fast-migrating LPS band (Fig. 2A, lanes 1 and 4). A comigrating band was present in the LPS of the NO100 primary variant (4C4$^+$ 5G8$^-$) which also possessed a slightly slower migrating band that stained more intensely (Fig. 2A, lane 2). The primary variant (4C4$^+$ 5G8$^-$) of COL10 (Fig. 2A, lane 5), however, possessed a single LPS band which migrated markedly slower than the LPS molecules of both its 4C4$^-$ 5G8$^-$ parental strain (Fig. 2A, lane 4) and the NO100 variant with the identical phenotype (Fig. 2A, lane 2). The acquisition of MAb 5G8 reactivity in both NO100 and COL10 resulted in a substantial alteration of the LPS profiles. The LPS profile of the NO100 secondary variant (4C4$^+$ 5G8$^+$) (Fig. 2A, lane 3)

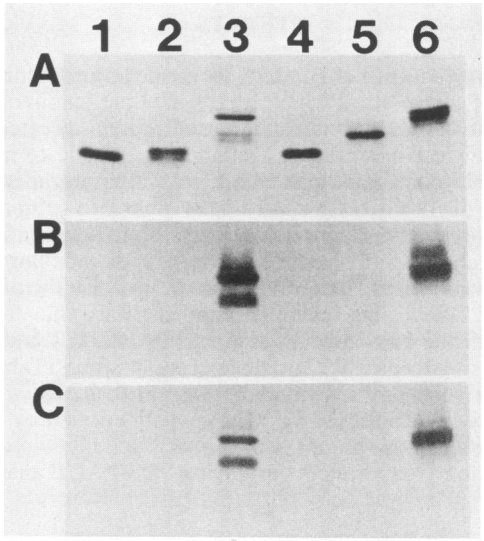

FIG. 2. LPS of group 4 Hib strains NO100 and COL10 and their LPS antigenic variants: analysis by SDS-PAGE and Western blotting. The LPS in proteinase K-treated whole cell digests was resolved by SDS-PAGE as described in the text. The resultant gels were either silver stained (A) or immunoblotted with MAb 4C4 (B) or MAb 5G8 (C). Lane 1, NO100 (4C4$^-$ 5G8$^-$); lane 2, NO100 primary variant (4C4$^+$ 5G8$^-$); lane 3, NO100 secondary variant (4C4$^+$ 5G8$^+$); lane 4, COL10 (4C4$^-$ 5G8$^-$); lane 5, COL10 primary variant (4C4$^+$ 5G8$^-$); lane 6, COL10 secondary variant (4C4$^+$ 5G8$^+$). Only the relevant portions of the silver-stained polyacrylamide gel and the Western blots are shown.

exhibited three LPS bands, all of which migrated much more slowly through the gel than the LPS bands of both the primary variant ($4C4^+$ $5G8^-$) (Fig. 2A, lane 2) and the original $4C4^-$ $5G8^-$ NO100 strain (Fig. 2A, lane 1). Similarly, the LPS of the secondary variant ($4C4^+$ $5G8^+$) of COL10 exhibited two major LPS bands (Fig. 2A, lane 6) which migrated considerably more slowly than the LPS bands of the other COL10 phenotypes (Fig. 2A, lanes 4 and 5). These differences in the mobility of the LPS molecules of the different variants in SDS-PAGE probably reflected differences in the apparent molecular weight and chemical composition of the LPS (13, 28, 30, 34, 38). Because our MAbs recognized antigenic determinants in the Hib core oligosaccharide, the differences between the LPS of the phenotypic variants were most likely due to alterations in this region of the LPS molecule. These alterations also probably involved the addition of new core oligosaccharide components, as indicated by the slower electrophoretic migration rates of the LPS molecules of the variants. These differences in electrophoretic migration rates, however, reflect only relatively minor changes in the apparent molecular weights of these LPS molecules.

The LPS molecules synthesized by these strains were also extracted from whole Hib cells by using the phenol extraction method described by Inzana (14) to eliminate the possibility that other macromolecules present in the proteinase K-treated samples might have affected the electrophoretic mobility of the LPS molecules or might have been stained with the silver stain. SDS-PAGE analysis of these phenol-extracted LPS molecules yielded profiles (Fig. 3) identical to those obtained with the proteinase K-treated samples (Fig. 2A).

Western blot analysis demonstrated that the LPS molecules of the secondary variants ($4C4^+$ $5G8^+$) of NO100 and COL10 were reactive with MAbs 4C4 and 5G8 (Fig. 2B and C, lanes 3 and 6). The original parental strains ($4C4^-$ $5G8^-$) did not react with either MAb (Fig. 2B and C, lanes 1 and 4). As expected, the LPS of the primary variants ($4C4^+$ $5G8^-$) did not react with MAb 5G8 (Fig. 2C, lanes 2 and 5). Surprisingly, however, the LPS of the primary variants ($4C4^+$ $5G8^-$) did not react with MAb 4C4 either (Fig. 2B, lanes 2 and 5). The reason for this discrepancy is not clear because MAb 4C4 is capable of binding to the LPS of the secondary variants ($4C4^+$ $5G8^+$) in this same Western blot system (Fig. 2B, lanes 3 and 6).

Cell surface exposure of the LPS determinants recognized by MAbs 4C4 and 5G8. An indirect AA RIA was used to determine whether the group 2 LPS determinants (as defined by reactivity with MAbs 4C4 and 5G8) expressed by the variants of NO100 and COL10 were exposed on the cell surface, as they are in group 2 clinical isolates of Hib (8, 9). These data were of particular interest since the bacterial cell surface plays an important role in host-parasite interactions. Cell surface exposure of a given antigenic determinant was defined here by accessibility to antibody in the AA RIA method as described previously (18).

The data in Table 3 show that the group 2 LPS determinants expressed by the NO100 and COL10 antigenic variants are exposed on the surface of these cells. It is also interesting that MAb 4C4 reactivity with both NO100 and COL10 increases dramatically with the acquisition of MAb 5G8 reactivity. This finding suggests that the presence of the 5G8 determinant somehow stabilizes or enhances the binding of MAb 4C4 to its respective determinant and offers a possible explanation for the aforementioned discrepancy observed in Western blot analysis in which MAb 4C4 preferentially reacts with LPS from the secondary variants ($4C4^+$ $5G8^+$) and not with LPS from the primary variants ($4C4^+$ $5G8^-$) (Fig. 2B, compare lanes 3 and 6 with lanes 2 and 5).

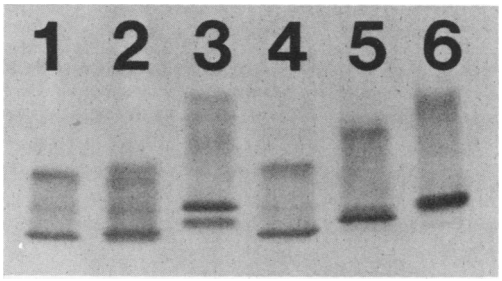

FIG. 3. Phenol-extracted LPS of group 4 Hib strains NO100 and COL10 and their LPS antigenic variants. LPS was isolated by a rapid micromethod based on phenol-water extraction (14). The LPS was resolved by SDS-PAGE and visualized by silver staining. Lane 1, NO100 ($4C4^-$ $5G8^-$); lane 2, NO100 primary variant ($4C4^+$ $5G8^-$); lane 3, NO100 secondary variant ($4C4^+$ $5G8^+$); lane 4, COL10 ($4C4^-$ $5G8^-$); lane 5, COL10 primary variant ($4C4^+$ $5G8^-$); lane 6, COL10 secondary variant ($4C4^+$ $5G8^+$). Only the relevant bottom portion of the gel is shown.

Virulence studies. Results of preliminary experiments revealed that the group 4 strains NO100 and COL10 are relatively avirulent in the infant rat model when compared with other isolates of Hib belonging to LPS antigenic groups 1, 2, and 3. The identification and isolation of isogenic LPS phenotypic variants of NO100 and COL10 provide a means of assessing whether changes in the cell surface-exposed antigenic determinants of Hib LPS would alter the virulence of the group 4 strains.

Infection via the intraperitoneal route with 10^2 CFU of strain NO100 ($4C4^-$ $5G8^-$) produced a detectable bacteremia in only 3 of the 20 (15%) infant rats that were challenged (Table 4). Similarly, only 1 of 18 (6%) animals developed bacteremia when infant rats were challenged with the same strain by the intranasal route. The primary variant ($4C4^+$ $5G8^-$) of NO100 was also comparatively avirulent. In contrast, acquisition of the LPS determinant recognized by MAb 5G8 and the simultaneous dramatic alteration of the LPS profile of the $4C4^+$ $5G8^+$ secondary variant (Fig. 2A,

TABLE 3. Cell surface exposure of the LPS antigenic determinants of Hib strains NO100 and COL10 and their LPS antigenic variants[a]

Strain[b]	LPS antigenic phenotype	Reactivity of the following in AA RIA (cpm)[c]	
		MAb 4C4	MAb 5G8
NO100	$4C4^-$ $5G8^-$	1,535	1,616
NO100 (p.v.)	$4C4^+$ $5G8^-$	7,256	1,478
NO100 (s.v.)	$4C4^+$ $5G8^+$	40,443	10,860
COL10	$4C4^-$ $5G8^-$	2,060	1,456
COL10 (p.v.)	$4C4^+$ $5G8^-$	3,826	1,451
COL10 (s.v.)	$4C4^+$ $5G8^+$	29,684	10,225

[a] The AA RIA system was used to assess the cell surface exposure of the antigenic determinants. A negative control, involving incubation of the NO100 secondary variant with a *Treponema pallidum*-specific MAb, bound 1,962 cpm.

[b] p.v., primary variant; s.v., secondary variant.

[c] Counts per minute of radioiodinated goat anti-mouse immunoglobulin bound to Hib cells incubated with the designated MAb. Data represent the average of duplicate samples.

TABLE 4. Relative virulence of Hib strains NO100 and COL10 and their LPS antigenic variants[a]

Strain[b]	LPS antigenic phenotype	Challenge route[c]	No. of animals bacteremic/total no. of animals (%)[d]	CFU/ml of blood (geometric mean)
NO100	4C4$^-$ 5G8$^-$	IP	3/20 (15)	11,599
NO100 (p.v.)	4C4$^+$ 5G8$^-$	IP	8/20 (40)	816
NO100 (s.v.)	4C4$^+$ 5G8$^+$	IP	19/20 (95)[e]	6,782
NO100	4C4$^-$ 5G8$^-$	IN	1/18 (6)	1,500
NO100 (p.v.)	4C4$^+$ 5G8$^-$	IN	1/19 (5)	6,200
NO100 (s.v.)	4C4$^+$ 5G8$^+$	IN	15/19 (79)[e]	2,840
COL10	4C4$^-$ 5G8$^-$	IN	2/20 (10)	1,265
COL10 (p.v.)	4C4$^+$ 5G8$^-$	IN	13/19 (68)[e]	7,664
COL10 (s.v.)	4C4$^+$ 5G8$^+$	IN	14/21 (67)[e]	8,490

[a] Virulence was assessed using the infant rat model as described in the text.
[b] p.v., primary variant; s.v., secondary variant.
[c] Infant rats were challenged intraperitoneally (IP) with ca. 100 CFU of the designated Hib variant. Infant rats were challenged intranasally (IN) with ca. 2×10^8 to 3×10^8 CFU of the designated Hib variant.
[d] Pooled data from two separate experiments.
[e] $P < 0.01$ compared with the 4C4$^-$ 5G8$^-$ parental strain by chi-square analysis.

lane 3) was associated with a significant increase in virulence as assessed by both routes of challenge (intraperitoneal route, 95% bacteremic; intranasal route, 79% bacteremic).

Since intranasal challenge is the more relevant route of infection for Hib (26), we chose to test strain COL10 and its LPS phenotypic variants using only this route. The results obtained with the parental strain (4C4$^-$ 5G8$^-$) and the secondary variant (4C4$^+$ 5G8$^+$) were essentially identical to those obtained with NO100 and its secondary variant (4C4$^+$ 5G8$^+$) in that a significant enhancement in virulence was observed with the expression of both the 4C4 and 5G8 determinants. However, unlike the primary variant (4C4$^+$ 5G8$^-$) of NO100, the corresponding primary variant of COL10 was fully virulent relative to the secondary variant (4C4$^+$ 5G8$^+$). It should also be noted, however, that this COL10 primary variant (4C4$^+$ 5G8$^-$) synthesized an LPS molecule which migrated significantly more slowly in SDS-PAGE than the LPS of the COL10 parent strain (4C4$^-$ 5G8$^-$) (Fig. 2A, compare lane 4 with lane 5).

Capsular quantitation. It was important to consider virulence factors other than LPS in the characterization of these phenotypic variants of the group 4 Hib strains. Because the capsular polysaccharide has been well established as the primary virulence factor of Hib (27, 32, 41), differences in the quantity of cell-associated capsular polysaccharide could account for the observed differences in virulence among these LPS variants. Therefore, the cell-associated capsular polysaccharide of these strains was quantitated by rocket immunoelectrophoresis, using burro anticapsular serum as the precipitating antibody (Table 5). No significant differences between the capsular content of strains NO100 and COL10 and that of their respective LPS phenotypic variants were detected. Thus, the enhanced virulence of certain of the LPS phenotypic variants was not related to quantitative increases in their content of capsular polysaccharide.

TABLE 5. Capsular polysaccharide content[a] of group 4 Hib strains and their LPS antigenic variants

Strain[b]	LPS antigenic phenotype	μg of capsular polysaccharide/10^9 CFU (mean ± SD)[c]
NO100	4C4$^-$ 5G8$^-$	38.6 ± 3.2
NO100 (p.v.)	4C4$^+$ 5G8$^-$	34.3 ± 5.1[d]
NO100 (s.v.)	4C4$^+$ 5G8$^+$	35.7 ± 5.8[d]
COL10	4C4$^-$ 5G8$^-$	43.9 ± 12.5
COL10 (p.v.)	4C4$^+$ 5G8$^-$	36.2 ± 6.4[d]
COL10 (s.v.)	4C4$^+$ 5G8$^+$	35.1 ± 6.0[d]

[a] Capsular polysaccharide content was measured by rocket immunoelectrophoresis as described in the text.
[b] p.v., primary variant; s.v., secondary variant.
[c] These data represent the mean of three independent electrophoretic analyses.
[d] Not statistically significant compared with the 4C4$^-$ 5G8$^-$ parental strain by Student's t test.

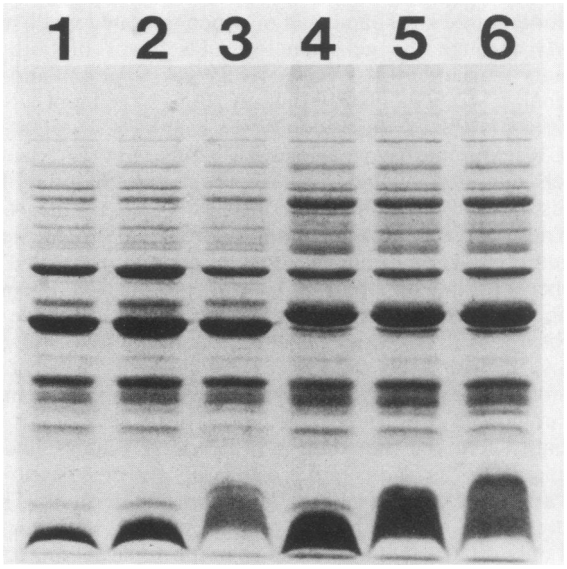

FIG. 4. Outer membrane protein profiles of the group 4 Hib strains NO100 and COL10 and their LPS antigenic variants. Outer membrane vesicles were extracted by the LiCl method described previously (10, 21). The proteins present in these preparations were resolved by SDS-PAGE and stained with Coomassie blue (12). Lane 1, NO100 (4C4$^-$ 5G8$^-$); lane 2, NO100 primary variant (4C4$^+$ 5G8$^-$); lane 3, NO100 secondary variant (4C4$^+$ 5G8$^+$); lane 4, COL10 (4C4$^-$ 5G8$^-$); lane 5, COL10 primary variant (4C4$^+$ 5G8$^-$); lane 6, COL10 secondary variant (4C4$^+$ 5G8$^+$).

Outer membrane protein profiles. Outer membrane protein profiles of strains NO100 and COL10 and their LPS variants are shown in Fig. 4. The phenotypic variants possessed protein profiles virtually identical to those of their respective group 4 parental strains (Fig. 4; NO100 and its variants, lanes 1 through 3; COL10 and its variants, lanes 4 through 6). The altered profiles of the variants seen near the bottom of the gel were due to their LPS differences (P. A. Gulig, A. Kimura, and E. J. Hansen, unpublished data).

Selection of LPS variants in vivo. The increased virulence of the secondary variants (4C4$^+$ 5G8$^+$) led us to perform additional experiments in the animal model system to determine whether growth in vivo would select for the more virulent LPS antigenic variants of NO100 and COL10. Hib isolated from the blood of infant rats challenged with strains NO100 and COL10 and their LPS antigenic variants were screened with MAbs 4C4 and 5G8 by colony blot RIA. The majority of Hib cultured from three of six infant rats which developed bacteremia after challenge with the original NO100 strain (4C4$^-$ 5G8$^-$) expressed the virulence-associated 4C4$^+$ 5G8$^+$ LPS phenotype (Table 6). A 4C4$^+$

TABLE 6. LPS antigenic phenotype of Hib strains cultured from the blood of infant rats challenged with strains NO100 and COL10[a]

Challenge strain	LPS antigenic phenotype	Rat no.	No. of colonies cultured from blood and screened	No. of colonies (%) with the following antigenic phenotypes		
				4C4⁻ 5G8⁻	4C4⁺ 5G8⁻	4C4⁺ 5G8⁺
NO100	4C4⁻ 5G8⁻	1	180	28 (15.6)	0 (0)	152 (84.4)
		2	3	3 (100)	0 (0)	0 (0)
		3	688	0 (0)	0 (0)	688 (100)
		4	27	0 (0)	0 (0)	27 (100)
		5	12	0 (0)	12 (100)	0 (0)
		6	21	21 (100)	0 (0)	0 (0)
NO100 (p.v.)[b]	4C4⁺ 5G8⁻	1	24	0 (0)	0 (0)	24 (100)
		2	31	0 (0)	1 (3.2)	30 (96.8)
		3	56	2 (3.6)	25 (44.6)	29 (51.8)
		4	16	0 (0)	2 (12.5)	14 (87.5)
COL10	4C4⁻ 5G8⁻	1	189	29 (15.3)	160 (84.7)	0 (0)
		2	367	56 (15.3)	311 (84.7)	0 (0)
		3	141	20 (14.2)	121 (85.8)	0 (0)
		4	8	8 (100)	0 (0)	0 (0)

[a] Infant rats were challenged with the designated Hib strain by either the intraperitoneal route with 100 CFU or by the intranasal route with approximately 2×10^8 to 3×10^8 CFU. Blood isolates were obtained by culturing tail vein blood 24 h after challenge. These colonies were screened for reactivity with MAbs 4C4 and 5G8 by using the colony blot RIA procedure.

[b] p.v., primary variant.

5G8⁺ blood isolate of strain NO100 was found to be fully virulent in the infant rat model system (data not shown). The 4C4⁺ 5G8⁺ LPS phenotype was also expressed by most Hib isolated from four bacteremic animals that had originally been challenged with the primary variant (4C4⁺ 5G8⁻) of NO100. Similarly, isolates from the blood of three of four bacteremic animals originally challenged with the parental COL10 strain (4C4⁻ 5G8⁻) possessed LPS reactive with MAb 4C4 (Table 6), which in Hib strain COL10 was associated with full virulence (Table 4). SDS-PAGE analysis of the LPS synthesized by these blood isolates revealed that these molecules migrated more slowly (Fig. 5, lanes 4 and 5 and lanes 9 and 10) than the LPS of their respective (4C4⁻ 5G8⁻) parent strains (Fig. 5, lanes 1 and 6). It is of interest that the LPS profiles of the blood isolates of strain NO100 were not identical to that of the corresponding fully virulent variant (4C4⁺ 5G8⁺) generated in vitro (Fig. 5, lane 3). These latter data indicate that the spontaneous alterations in LPS phenotype which result from the synthesis of the antigenic determinants recognized by MAbs 4C4 and 5G8 in strain NO100 and that recognized by MAb 4C4 in strain COL10 are associated with the increased ability of these organisms to survive and multiply in vivo.

Serum bactericidal studies. The serum sensitivity of the group 4 Hib strains and their LPS antigenic variants was evaluated to determine whether the alterations in LPS phenotype and virulence could be associated with changes in susceptibility to the bactericidal activity of normal serum. Because virulence was assessed in infant rats, we felt that rats of the same age would be the most relevant source of serum for these studies. Table 7 contains data from an experiment in which the concentration- and time-dependent bactericidal activity of infant rat serum against Hib was investigated. Hib strain DL42, which is a standard group 2 LPS strain possessing full virulence, increased in number in every serum concentration during the 2-h incubation period. In contrast, the group 4 strain NO100 (4C4⁻ 5G8⁻) was rapidly killed in 30 min by as little as 20% serum. Bactericidal activity against NO100 was completely eliminated by heating the serum. The virulent secondary variant (4C4⁺ 5G8⁺) of strain NO100, however, was fully resistant to the bactericidal activity of serum and multiplied in the various serum mixtures.

Based on the data presented above, a concentration of 50% serum and a test period of 60 min was chosen to evaluate further the serum sensitivity of the LPS variants (Fig. 6). Greater than 90% of strain NO100 (4C4⁻ 5G8⁻) and its primary variant (4C4⁺ 5G8⁻) were killed during the 60-min incubation period; heat treatment of serum destroyed all of this bactericidal activity. The secondary variant (4C4⁺ 5G8⁺) of strain NO100 was again found to be serum resistant and capable of growing in serum. Unlike strain NO100, however, strain COL10 and its LPS variants displayed no differences in susceptibility to killing by serum. Hib strain COL10 and its LPS variants were serum resistant to a degree comparable to that exhibited by strain DL42, a fully virulent

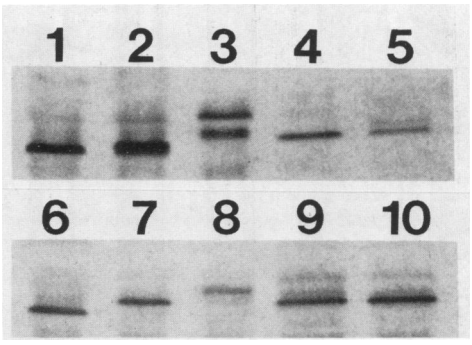

FIG. 5. LPS profiles of Hib strains cultured from the blood of infant rats challenged with strains NO100 and COL10. The LPS in proteinase K-treated whole cell digests was resolved by SDS-PAGE and visualized by silver staining. Lane 1, NO100 (4C4⁻ 5G8⁻); lane 2 NO100 primary variant (4C4⁺ 5G8⁻); lane 3, NO100 secondary variant (4C4⁺ 5G8⁺); lane 4, NO100 blood isolate from rat no. 1 (4C4⁺ 5G8⁺; Table 6); lane 5, NO100 blood isolate from rat no. 3 (4C4⁺ 5G8⁺; Table 6); lane 6, COL10 (4C4⁻ 5G8⁻); lane 7, COL10 primary variant (4C4⁺ 5G8⁻; lane 8, COL10 secondary variant (4C4⁺ 5G8⁺); lane 9, COL10 blood isolate from rat no. 1 (4C4⁺ 5G8⁻; Table 6); lane 10, COL10 blood isolate from rat no. 2 (4C4⁺ 5G8⁻; Table 6). Only the relevant bottom portions of the gels are shown.

TABLE 7. Concentration- and time-dependent bactericidal activity of infant rat serum against Hib strains DL42, NO100, and the secondary variant (4C4$^+$ 5G8$^+$) of NO100[a]

Hib strain	% Serum	CFU/10 μl at the following times (min):			
		0	30	60	120
DL42	20	88	207	305	1,016
	40	95	199	500	1,108
	60	76	225	336	764
	60 (heat-inactivated)	102	231	407	800
NO100 (4C4$^-$ 5G8$^-$)	20	96	0	0	0
	40	75	1	5	6
	60	97	4	0	0
	60 (heat-inactivated)	106	135	217	207
NO100 (4C4$^+$ 5G8$^+$) (s.v.)[b]	20	82	175	308	844
	40	74	170	242	375
	60	93	148	244	246
	60 (heat-inactivated)	92	176	204	200

[a] The bactericidal assay was performed as described in the text.
[b] s.v., secondary variant.

clinical isolate (Table 7 and Fig. 6). These three COL10 strains were found to be capable of multiplying in assay mixtures containing up to 80% serum (data not shown).

These results demonstrate that in Hib strain NO100, acquisition of the higher-molecular-weight type of LPS and expression of full virulence are closely associated with the acquisition of serum resistance. In strain COL10, however, alterations in LPS and virulence do not correlate with any detectable changes in serum resistance.

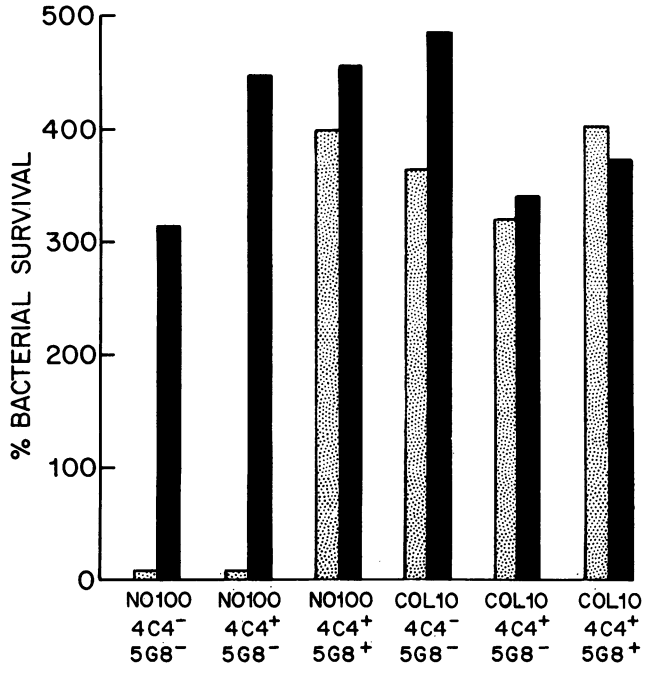

FIG. 6. Serum sensitivity of Hib strains NO100 and COL10 and their LPS antigenic variants. Hib strains tested in duplicate were incubated in 50% infant rat serum at 37°C. Samples of the mixture were removed at time zero and at 60 min and were plated in duplicate on solid media, as described in the text. Symbols: □, 50% serum; ■, 50% heat-inactivated serum.

DISCUSSION

MAbs directed against cell surface-exposed determinants of Hib LPS were used to identify and isolate spontaneously occurring LPS phenotypic variants of Hib strains NO100 and COL10. The phenotypic properties of these strains are summarized in Fig. 7. These phenotypic variants occurred at a relatively high frequency (Table 2) and possessed LPS molecules which were physically and antigenically distinct from those of the parental strains. Phenotypic variation of Hib LPS was not associated with alterations in either capsular polysaccharide quantity (Table 5) or outer membrane protein profiles (Fig. 4). The results obtained from the use of these LPS phenotypic variants as isogenic challenge strains in an animal model for systemic Hib disease indicate that Hib LPS may be an effector of virulence. Parental strains NO100 and COL10, which possess low-molecular-weight LPS, were relatively avirulent in the infant rat; phenotypic variants of these strains that express a higher-molecular-weight LPS were, by comparison, much more virulent. (We use the descriptive terms low, intermediate, and higher molecular weight to refer to the LPS molecules synthesized by the parental strains and their LPS variants. These are operational definitions only and are intended to simplify discussion. The use of these terms does not imply that there are large differences in apparent molecular weight among these molecules.) These results indicate that the higher-molecular-weight type of LPS is associated with the expression of full virulence by Hib. In strain NO100, acquisition of the higher-molecular-weight LPS and enhanced virulence is directly associated with the acquisition of serum resistance. Similar alterations in LPS and virulence, however, were not

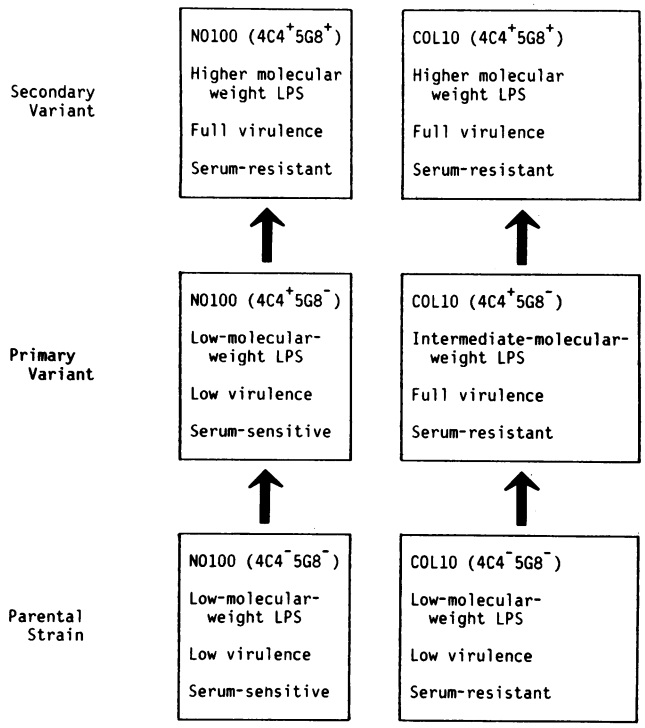

FIG. 7. Summary of the phenotypic characteristics of Hib strains NO100 and COL10 and their isogenic LPS variants.

associated with differences in serum sensitivity of strain COL10.

Spontaneous phenotypic variation of LPS occurred at a relatively high frequency in Hib strains NO100 and COL10. Among other gram-negative bacteria possessing rough LPS, phenotypic variation of LPS has been shown to occur in *Neisseria gonorrhoeae* (11) and *Bordetella pertussis* (31). The frequency of variation reported for the gonococcus was 10^{-6}, which is several orders of magnitude lower than those observed for Hib LPS in this study. Other bacterial cell surface structures which can exhibit varied antigenic characteristics at a high frequency among cells of a single culture include the flagella of *Salmonella typhimurium* (42), the pili of *N. gonorrhoeae* (23), and the O side chains of *Salmonella* LPS (19). The genetic mechanism underlying the phenotypic variation of Hib LPS remains to be determined, but it could possibly involve chromosomal rearrangements similar to those affecting the expression of the flagella of *S. typhimurium* and that of gonococcal pili.

The fact that strains NO100 and COL10 are the only group 4 strains identified among 200 Hib isolates tested in the past several years in this laboratory suggests that these strains may be unusual with regard to LPS determinants. In fact, all other systemic disease isolates examined to date exhibit reactivity with MAb 4C4 or MAb 12D9 (Table 1) and synthesize the higher-molecular-weight LPS. We have observed, however, a loss of reactivity with MAbs 4C4 and 5G8 in some clinical isolates of Hib after passage in vitro (data not shown). This observation suggests that LPS phenotypic variation may be a more generalized phenomenon among Hib strains. Moreover, these data also raise the possibility that the group 4 Hib strains NO100 and COL10 may represent artifacts of in vitro passage. Without selective pressure, these two strains may have stopped expressing the virulence-associated higher-molecular-weight LPS. This latter consideration points out the necessity for the characterization of LPS in fresh clinical isolates of Hib.

The association of changes in Hib LPS with alterations in the virulence of this pathogen was evidenced by significant differences in the relative virulence of the LPS phenotypic variants in an animal model system. The secondary antigenic variants of strains NO100 and COL10 that reacted with both MAbs 4C4 and 5G8 ($4C4^+$ $5G8^+$) possessed a higher-molecular-weight LPS and were fully virulent (Fig. 7). In contrast, this association of LPS structure and virulence with MAb reactivity was less clear in the primary variants ($4C4^+$ $5G8^-$). The primary variant ($4C4^+$ $5G8^-$) of strain NO100 was relatively avirulent and possessed an LPS profile only slightly different from that of its $4C4^-$ $5G8^-$ parental strain (Fig. 2A). The primary variant ($4C4^+$ $5G8^-$) of strain COL10, however, produced bacteremia in the majority of animals challenged with this strain and had an LPS molecule which migrated markedly slower in SDS-PAGE than those of both its parental strain ($4C4^-$ $5G8^-$) and the NO100 strain with the identical antigenic phenotype ($4C4^+$ $5G8^-$) (Fig. 2A). These findings demonstrate that acquisition of the 4C4 antigenic determinant results from the synthesis of two different LPS molecules in strains NO100 and COL10. Furthermore, these data suggest that overall LPS core oligosaccharide structure (as determined by changes in the electrophoretic mobility of LPS molecules in SDS-PAGE) may be more important than specific antigenic determinants (as defined by MAb reactivity) in the expression of Hib virulence.

The specific mechanism by which Hib LPS can affect the expression of virulence by this organism remains unclear at this time. However, it must be noted that the majority of Hib cells recovered from the blood of animals challenged with NO100 or COL10 expressed the LPS antigenic phenotype ($4C4^+$ $5G8^+$, NO100; $4C4^+$ $5G8^-$, COL10; Table 6) associated with the higher-molecular-weight LPS (Table 6). This finding strongly suggests that this form of Hib LPS confers an enhanced ability of these organisms to survive and multiply in vivo. It should be noted that blood isolates of the group 4 strains NO100 and COL10 possessed LPS patterns dissimilar to those of the in vitro-derived LPS variants of the same phenotype (Fig. 5). This finding indicates that two strains derived from the same parent and which possess apparently identical antigenic phenotypes can possess LPS molecules which yield different SDS-PAGE profiles.

There are few reports in the literature that have dealt with virulence-related properties of LPS in gram-negative bacteria possessing rough LPS. Guymon et al. (11) and Morse and Apicella (24) have shown that LPS mutants of *N. gonorrhoeae* which lack sugars in the core oligosaccharide are no longer resistant to killing by serum. A similar relationship between LPS structure and serum sensitivity was observed for Hib strain NO100 (Fig. 7). The relatively avirulent strain NO100 ($4C4^-$ $5G8^-$) possessing low-molecular-weight LPS was found to be extremely susceptible to the bactericidal action of serum (Fig. 6). Acquisition of the higher-molecular-weight LPS in this strain was accompanied by resistance to serum killing and, more importantly, enhanced virulence (Fig. 6 and Table 4). This finding suggests that the higher-molecular-weight LPS increases virulence by rendering these bacterial cells resistant to serum killing. The fact that strain NO100 and both its primary and secondary LPS variants possessed essentially identical quantities of cell-associated capsular polysaccharide indicates that differences in capsular quantity are not responsible for the varying degrees of serum sensitivity and virulence of these strains (4, 36).

This association between serum resistance and both LPS phenotypic variation and virulence of Hib, however, is not supported by results from experiments with strain COL10 (Fig. 7). Alterations in LPS and expression of virulence were not associated with changes in the serum sensitivity of this strain; COL10 and its LPS variants were uniformly resistant to killing by serum (Fig. 6). Thus, unlike strain NO100, the molecular weight of LPS in strain COL10 did not correlate with differences in susceptibility to killing by serum. Schneider et al. (33) have reported the same finding with isolates of gonococcus. The possibility exists that differences in the number of LPS molecules per cell between strains NO100 ($4C4^-$ $5G8^-$) and COL10 ($4C4^-$ $5G8^-$) may be responsible for the observed differences in the sensitivity of these strains to serum (7). SDS-PAGE analysis of lysate prepared from equal numbers of cells of these two strains, however, indicated that these strains possess very similar amounts of LPS (Fig. 2A, lanes 1 and 4). In light of these data, it is unclear as to what role LPS plays in determining Hib resistance to killing by serum. However, these data do indicate that alterations of LPS in strain COL10 and its variants correlate directly with increased virulence and that these changes do not involve resistance to killing by serum. Furthermore, it is important to note that the relative lack of virulence of the fully serum-resistant strain COL10 ($4C4^-$ $5G8^-$) implies that factors other than serum resistance are also critical in the expression of virulence by Hib. These data reinforce the multifactorial nature of the virulence of Hib.

Results of a recent study performed by Inzana and

Anderson (15) show that the ability of Hib strains to acquire increased resistance to the bactericidal activity of anti-LPS serum after incubation in serum or serum dialysates is related to a quantitative increase in the amount of LPS synthesized by the Hib cells. However, as determined by SDS-PAGE, the banding patterns of the LPS molecules from the anti-LPS serum-sensitive and the anti-LPS serum-resistant cells in their study were the same, suggesting that no different LPS molecules are involved in this phenomenon. Furthermore, LPS phenotypic variation did not occur when strain COL10 was incubated in infant rat serum (data not shown). Taken together, these data indicate that the serum-induced resistance phenomenon studied by Inzana and Anderson (15) is unrelated to the LPS phenotypic variation described in this report.

Results of this study also emphasize the usefulness of MAbs in the characterization of surface antigens involved in pathogenesis. The LPS-specific MAbs provided a rapid means of screening individual Hib colonies for their LPS phenotype. We plan to use these MAbs as reagents in the elucidation of both the biochemical structures and genetic systems involved in Hib LPS variation.

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