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Haemophilus influenzae Type b Lipooligosaccharide: Stability of Expression and Association with Virulence

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Spontaneous antigenic and phenotypic variations in the lipooligosaccharide (LOS) of two strains of *Haemophilus influenzae* type b (Hib) were previously shown to be associated with changes in virulence (A. Kimura and E. J. Hansen, Infect. Immun. 51:69–79, 1986). The goal of the present study was to define further the stability of LOS expression by this pathogen and the role of Hib LOS in virulence. Variation in LOS antigenic reactivity, as detected with LOS-specific monoclonal antibodies, was observed in 3 of 30 Hib strains after single-colony passage. When large numbers of individual colonies from seven other Hib strains were screened, however, spontaneous LOS antigenic variation was detected in all of the strains. Antigenic variation was not consistently associated with an altered LOS phenotype, as determined by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis and silver staining of LOS preparations. Changes in the LOS antigenic phenotype were correlated with altered virulence potential in two strains. In these strains, acquisition of reactivity with certain LOS-directed monoclonal antibodies was associated with the synthesis of a higher-molecular-weight LOS, enhanced virulence, and increased resistance to serum killing involving the classical complement pathway.

The vast majority of *Haemophilus influenzae* strains that cause systemic disease elaborate the type b polysaccharide capsule (4, 38). *H. influenzae* type b (Hib) remains the leading cause of bacterial meningitis in infants and young children in the United States (3). That the type b polysaccharide capsule is essential for the pathogenicity of Hib has been well established (31).

In contrast, the role of noncapsular cell surface components in the expression of virulence by Hib remains unclear. Recent studies from several laboratories have identified the lipooligosaccharide (LOS) component of the outer membrane of Hib as a possible effector of the virulence of this organism. Using monoclonal antibodies (MAbs) specific for epitopes in the oligosaccharide portion of Hib LOS, we demonstrated that Hib LOS can antigenically and phenotypically vary spontaneously in two Hib strains (21). From these experiments, LOS variants of each strain were isolated and were then used to evaluate the role of LOS in the virulence of Hib. A close association between electrophoretic mobility of LOS in sodium dodecyl sulfate (SDS)-polyacrylamide gradient gel electrophoresis (PAGGE) and virulence in the infant rat model was shown. More precisely, parental Hib strains possessing an electrophoretically fast migrating LOS were found to produce bacteremia at very low frequencies in infant rats, while their isogenic LOS antigenic variants, which possessed distinctly slower-migrating LOS, were, by comparison, significantly more virulent. This enhanced virulence was associated with increased serum resistance in one set of these Hib strains. Zwahlen and co-workers (39, 40), using transformants of Hib, have reported a similar association among LOS electrophoretic mobility in SDS-polyacrylamide gel electrophoresis, virulence in infant rats, and serum sensitivity, while Gilsdorf and Ferrieri (7) have reported that passage of Hib in infant rats yielded a strain which possessed increased resistance to killing by normal rat serum, as well as a phenotypically and antigenically altered LOS molecule.

We have continued our studies on the antigenic and phenotypic variations of Hib LOS and their association with alterations in the virulence potential of this pathogen. Information concerning the stability of expression of LOS by Hib is also important because LOS phenotypes, as assessed by SDS-PAGGE, are being used as strain markers in epidemiologic studies of Hib disease (14, 22). The goal of this study was to extend our previous observations to other systemic disease isolates of Hib to determine whether LOS phenotypes are stably expressed by most Hib strains and to define further the involvement of LOS in the expression of virulence by Hib.

MATERIALS AND METHODS

Bacterial strains. The Hib strains used in this study were obtained from pediatric research centers located throughout the United States and have been described previously (9). Bacterial media (brain heart infusion [BHI; Difco Laboratories, Detroit, Mich.] supplemented with Levinthal base [BHIs] [1]) and growth conditions have been described previously (13). Hib strains were stored at −70°C in a mixture of equal parts of glycerol and BHI.

MAbs. Production and characterization of the Hib LOS-specific murine MAbs 12D9, 4C4, 6A2, and 5G8 have been described elsewhere (9–11). In previous studies (10, 11) it has been shown that Hib strains can be grouped according to their reactivity with these four MAbs, all of which are directed against epitopes in the oligosaccharide region of Hib LOS (Table 1). By using MAbs 12D9 and 4C4, Hib isolates can be classified into four major groups. Further subdivision is accomplished by using strain reactivity with MAbs 6A2 and 5G8. This classification scheme represents the data base

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for the present investigation. Hybridoma culture supernatant fluids were used as the source of these antibodies for all experiments.

Colony blot radioimmunoassay. The colony blot radioimmunoassay (RIA) described by Gulig et al. (9) was used for the initial screening of Hib isolates for antigenic variation. In this method, cells of each strain were taken from an area of confluent growth on a BHIs agar plate and transferred onto filter paper (no. 40; Whatman, Inc., Clifton, N.J.) with a wooden applicator stick. The Hib cells on the filter paper were then tested for reactivity with the LOS-specific MAbs as described previously (9).

LOS antigenic variation of an Hib strain was further assessed by using a modification of the colony blot RIA method to screen large numbers of individual colonies (21). Briefly, a broth culture of the Hib strain grown to a density of 1.5×10^9 CFU/ml was diluted and spread onto 10 BHIs agar plates at 150 to 250 CFU per plate. Following overnight incubation at 37°C in an atmosphere containing 5% carbon dioxide, the colonies on five plates were blotted onto filter paper (diameter, 82.5 mm; no. 40; Whatman), dried for 1 h at 37°C, and then reacted with the LOS antigenic group 1-specific MAb 12D9 as described previously (21). The colonies on the same five agar plates were then incubated again at 37°C in a 5% CO_2 atmosphere for 3 to 5 h and were blotted onto a second set of filter pads. These new blots were reacted with the LOS antigenic group 1-specific MAb 6A2. The colonies were allowed to grow again for 2 h, and the plates were then stored at 4°C until LOS antigenic variants could be identified and isolated by using the resultant autoradiographs from the colony blot RIA. The other five plates were treated in the same manner but were reacted with the Hib LOS antigenic group 2-specific MAbs 4C4 and 5G8.

LOS analysis by SDS-PAGGE. Analysis of Hib LOS in proteinase K-treated whole-cell lysates by SDS-PAGGE has been described previously (21). All samples were prepared from BHIs agar plate-grown organisms.

Virulence studies. Assessment of the relative virulence of Hib strains by intranasal challenge of infant rats has been described elsewhere (21, 25). All litters were randomized prior to infection.

Serum bactericidal assay. The sensitivity of Hib strains and their LOS variants to killing by normal infant rat serum was determined by using a previously described method (21). Briefly, Veronal buffered saline (VBS; pH 7.3) containing 0.1% gelatin, 5.0 mM $MgCl_2$, and 1.5 mM $CaCl_2$ was employed as the standard bactericidal assay buffer. Infant rat serum, at a final concentration of 50% (vol/vol), was used together with 10^3 CFU of Hib in a 100-μl volume. The involvement of the classical and alternative pathways of complement activation in serum bactericidal activity against Hib was assessed by the use of chelating agents to selectively inhibit the two different pathways (30). EDTA has a high affinity for both calcium and magnesium and therefore blocks both the calcium- and magnesium-dependent classical pathway and the magnesium-dependent alternative pathway. Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) has a high affinity for calcium, but does not readily bind magnesium, thereby selectively blocking only the classical complement pathway. For these inhibition experiments, VBS-gelatin containing 5 mM EDTA and VBS-gelatin containing 10 mM $MgCl_2$ and 10 mM EGTA were incubated with normal infant rat serum on ice for 60 min prior to use in the bactericidal assay system. Normal infant rat serum in VBS-gelatin lacking supplemental calcium and magnesium was used as the positive control for bactericidal activity in these inhibition experiments. All assays were performed twice in duplicate. Results were analyzed by two-way analysis of variance by using the general linear models procedure of SAS.

TABLE 1. LOS antigenic grouping of the strains used in this study

Antigenic classification (MAb)[a]	No. of strains
Group 1 (12D9$^+$ 4C4$^-$)	
Subgroup 1a (6A2$^+$)	6
Subgroup 1b (6A2$^-$)	1
Group 2 (12D9$^-$ 4C4$^+$)	
Subgroup 2a (5G8$^+$)	11
Subgroup 2b (5G8$^-$)	3
Group 3 (12D9$^+$ 4C4$^+$)	
Subgroup 3a (6A2$^+$ 5G8$^+$)	2
Subgroup 3b (6A2$^-$ 5G8$^+$)	0
Subgroup 3c (6A2$^+$ 5G8$^-$)	4
Subgroup 3d (6A2$^-$ 5G8$^-$)	2
Group 4 (12D9$^-$ 4C4$^-$ 6A2$^-$ 5G8$^-$)	1

[a] Group classification was based on reactivity with MAbs 12D9 and 4C4 in the colony blot RIA; subgroup classification was based on reactivity with MAbs 6A2 and 5G8.

Capsular polysaccharide quantitation. Cell-associated Hib capsular polysaccharide was quantitated by rocket immunoelectrophoresis (34).

Detection of Hib-directed antibodies in infant rat serum. The indirect antibody accessibility RIA was used to detect infant rat serum antibodies to Hib surface antigens (21). Hib cells grown on BHIs agar plates were suspended to a final concentration of 1.5×10^9 CFU/ml in phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS). A 1-ml volume of these cells was incubated with a 100-μl volume of infant rat serum at 4°C for 90 min with gentle agitation. The cells were then centrifuged at 12,000 × *g* for 2 min, washed once with PBS-FCS to remove unattached antibody, and centrifuged again. This new cell pellet was suspended in 1 ml of PBS-FCS containing 10^6 cpm of affinity-purified and radioiodinated goat anti-mouse immunoglobulin (specific activity, 10^7 cpm/μg of protein), which is highly cross-reactive with rat immunoglobulin, and incubated with gentle agitation for 1 h at 4°C. The cells were then washed three times with PBS-FCS, and the radioactivity associated with the final cell pellet was determined by the use of a gamma counter (20).

RESULTS

LOS antigenic variation. The primary aim of our initial experiments was to determine the extent of LOS antigenic variation in Hib strains during routine in vitro passage and storage in the laboratory. Thirty isolates of Hib representing the four LOS antigenic groups were used in this study (Table 1). Colony blots of these strains prepared 1 year earlier and stored at −20°C, colony blots prepared from current stocks of these strains, and colony blots prepared from colonies obtained after three single-colony passages of the current stocks were reacted with LOS-specific MAbs in the colony blot RIA. Variation in LOS antigenic reactivity was observed in only three strains and occurred subsequent to single-colony passage in all three cases (data not shown). Strains DL38 (12D9$^-$ 4C4$^+$ 6A2$^-$ 5G8$^-$) and LA100 (12D9$^+$ 4C4$^+$ 6A2$^+$ 5G8$^-$) gained reactivity with MAb 5G8, whereas

TABLE 2. Frequency of LOS antigenic variation among individual colonies of Hib strains[a]

Strain	LOS antigenic group	LOS antigenic phenotype	No. of colonies screened	No. (%) of colonies with the following antigenic phenotypes:					
				12D9$^-$ 6A2$^-$	12D9$^+$ 6A2$^-$	12D9$^+$ 6A2$^+$	4C4$^-$ 5G8$^-$	4C4$^+$ 5G8$^-$	4C4$^+$ 5G8$^+$
DL26	1a	12D9$^+$ 4C4$^-$ 6A2$^+$ 5G8$^-$	978	0 (0)	0 (0)	978 (100)			
			1,012				1,011 (99.9)	1 (0.1)	0 (0)
DV102	1b	12D9$^+$ 4C4$^-$ 6A2$^-$ 5G8$^-$	1,030	12 (1.2)	1,017 (98.7)	1 (0.1)			
			1,098				1,098 (100)	0 (0)	0 (0)
SL103	2a	12D9$^-$ 4C4$^+$ 6A2$^-$ 5G8$^+$	691	691 (100)	0 (0)	0 (0)			
			622				0 (0)	2 (0.3)	620 (99.7)
NO103	2b	12D9$^-$ 4C4$^+$ 6A2$^-$ 5G8$^-$	722	722 (100)	0 (0)	0 (0)			
			663				3 (0.4)	609 (91.9)	51 (7.7)
NA105	2b	12D9$^-$ 4C4$^\pm$ 6A2$^-$ 5G8$^-$ [b]	1,079	1,079 (100)	0 (0)	0 (0)			
			859				14 (1.6)[c]	14 (1.6)	3 (0.4)
DL67	3a	12D9$^+$ 4C4$^+$ 6A2$^+$ 5G8$^+$	552	0 (0)	0 (0)	552 (100)			
			509				0 (0)	40 (7.9)	469 (92.1)
LA103	3d	12D9$^+$ 4C4$^+$ 6A2$^-$ 5G8$^-$	1,154	0 (0)	1,142 (99)	12 (1)			
			1,156				27 (2.3)	1,125 (97.3)	4 (0.4)

[a] Individual colonies were screened for MAb reactivity by the colony blot RIA, as described in the text.
[b] Weak reactivity with MAb 4C4.
[c] A total of 828 (96.4%) of the colonies had the same reactivity as that of the parent (12D9$^-$ 4C4$^\pm$ 6A2$^-$ 5G8$^-$).

strain DV102 (12D9$^+$ 4C4$^-$ 6A2$^-$ 5G8$^-$) lost extensive reactivity with MAb 12D9. No LOS antigenic variation was detected between the blots prepared 1 year earlier and the ones prepared from the current stocks of these three strains.

LOS antigenic variation was not observed in the group 4 strain NO100 in this set of experiments. However, this same strain was shown in our previous study (21) to antigenically vary its LOS when large numbers of individual colonies were screened. This result suggested that LOS antigenic variation could be occurring in the other 27 Hib strains but at a frequency that could be detected only by the screening of large populations. Accordingly, 500 to 1,100 individual colonies of each of 7 representative Hib strains from the original 30 strains were screened for MAb reactivity in the colony blot RIA. LOS antigenic variation was detected in all seven strains that were analyzed by this more stringent method (Table 2). Both loss and acquisition of MAb reactivity occurred independently in strains such as DV102 and NO103, which reacted originally with only the group-specific MAbs (either 12D9 or 4C4). In strains SL103 and DL67, which possess both the group and subgroup MAb reactivities of LOS groups 2a and 3a, respectively, a loss of reactivity with MAb 5G8 was observed. Among the LOS antigenic group 1 and group 2 strains, only DL26 (LOS antigenic group 1) was found to be capable of expressing the determinants of the other group; it gave rise to a variant belonging to LOS antigenic group 3.

SDS-PAGGE analysis of LOS antigenic variants. In our previous report (21), LOS antigenic variation of the group 2 LOS determinants in strains NO100 and COL10 was associated with altered LOS profiles in SDS-PAGGE. Reactivity with MAbs 4C4 and 5G8 was associated with an electrophoretically slow migrating LOS, while lack of reactivity with these MAbs was associated with a faster-migrating LOS. To determine if this finding was consistent for other strains of this pathogen, the LOS of the antigenic variants of six of the Hib strains in Table 2 were analyzed by SDS-PAGGE.

The loss of MAb 12D9 reactivity in strain DV102 (12D9$^+$ 4C4$^-$ 6A2$^-$ 5G8$^-$) did not affect the LOS profile of this strain (Fig. 1, lanes A1 and A2); however, the acquisition of MAb 6A2 reactivity resulted in a slower migrating LOS band (Fig. 1, lane A3). Surprisingly, neither loss of reactivity with MAb 4C4 nor acquisition of reactivity with MAb 5G8 affected the LOS profile of strain NO103 (12D9$^-$ 4C4$^+$ 6A2$^-$ 5G8$^-$) (Fig. 1, lanes B1, B2, and B3). In the LOS group 3 strain LA103 (12D9$^+$ 4C4$^+$ 6A2$^-$ 5G8$^-$), the LOS profile of a variant that acquired MAb 6A2 reactivity in conjunction with a loss of MAb 4C4 reactivity was not detectably altered (Fig. 1, compare lanes C1 and C2). Another variant of strain LA103 that acquired reactivity with both MAb 6A2 and MAb 5G8, however, possessed a dramatically altered LOS profile that consisted of two slower-migrating bands (Fig. 1, lane C3). The LOS profile of NA105 (12D9$^-$ 4C4$^\pm$ 6A2$^-$ 5G8$^-$) was similar to those of its LOS antigenic variants that had either increased or decreased reactivity with MAb 4C4 (Fig. 1, lanes D1, D2, and D3). The acquisition of MAb 5G8 reactivity, together with increased reactivity with MAb 4C4 in strain NA105, resulted in a LOS with greatly decreased electrophoretic mobility (Fig. 1, lane D4). In both SL103 (12D9$^-$ 4C4$^+$ 6A2$^-$ 5G8$^+$) and DL67 (12D9$^+$ 4C4$^+$ 6A2$^+$ 5G8$^+$), a LOS group 2a strain and a LOS group 3a strain, respectively, the loss of MAb 5G8 reactivity resulted in a LOS with an altered banding pattern and a faster migration rate than the LOS of the respective parental strain (Fig. 1, compare lane E1 with lane E2 and lane F1 with lane F2).

Virulence studies. In our previous studies (21), we have demonstrated that there is an association among the lack of reactivity with the LOS-specific MAbs 4C4 and 5G8, electrophoretically fast migrating LOS, and a relative lack of virulence. To determine whether Hib strains which lack reactivity with these particular LOS-specific MAbs are inherently less virulent, we tested the virulence of strains belonging to groups 1b, 2b, and 3d. These strains lack reactivity with either one or both of the LOS-specific MAbs used for subgrouping (6A2 and 5G8). Strains DV102 (12D9$^+$ 4C4$^-$ 6A2$^-$ 5G8$^-$) and NO103 (12D9$^-$ 4C4$^+$ 6A2$^-$ 5G8$^-$)

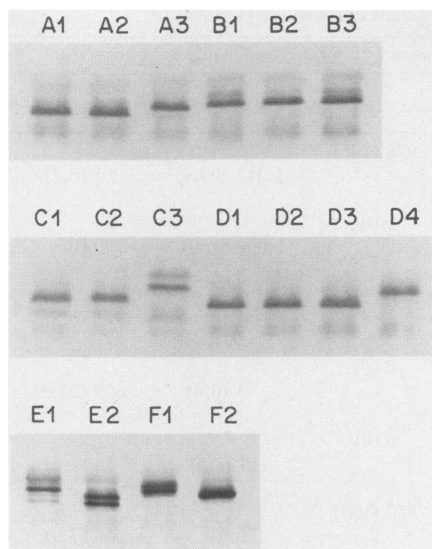

FIG. 1. LOS profiles of Hib strains and their LOS antigenic variants. The LOS in proteinase K-treated whole-cell lysates was prepared from plate-grown organisms and was resolved by SDS-PAGGE as described previously (21). The LOS was visualized by the silver stain method described by Tsai and Frasch (37). Lane A1, DV102 parental strain (12D9^{+} 4C4^{-} 6A2^{-} 5G8^{-}); lane A2, DV102 variant (12D9^{-} 4C4^{-} 6A2^{-} 5G8^{-}); lane A3, DV102 variant (12D9^{+} 4C4^{-} 6A2^{+} 5G8^{-}); lane B1, NO103 parental strain (12D9^{-} 4C4^{+} 6A2^{-} 5G8^{-}); lane B2, NO103 variant (12D9^{-} 4C4^{-} 6A2^{-} 5G8^{-}); lane B3, NO103 variant (12D9^{-} 4C4^{+} 6A2^{-} 5G8^{+}); lane C1, LA103 parental strain (12D9^{+} 4C4^{+} 6A2^{-} 5G8^{-}); lane C2, LA103 variant (12D9^{+} 4C4^{-} 6A2^{+} 5G8^{-}); lane C3, LA103 variant (12D9^{+} 4C4^{+} 6A2^{+} 5G8^{+}); lane D1, NA105 parental strain (12D9^{-} 4C4$^{\pm}$ 6A2^{-} 5G8^{-}); lane D2, NA105 variant (12D9^{-} 4C4^{-} 6A2^{-} 5G8^{-}); lane D3, NA105 variant (12D9^{-} 4C4^{+} 6A2^{-} 5G8^{-}); lane D4, NA105 variant (12D9^{-} 4C4^{+} 6A2^{-} 5G8^{+}); lane E1, SL103 parental strain (12D9^{-} 4C4^{+} 6A2^{-} 5G8^{+}); lane E2, SL103 variant (12D9^{-} 4C4^{+} 6A2^{-} 5G8^{-}); lane F1, DL67 parental strain (12D9^{+} 4C4^{+} 6A2^{+} 5G8^{+}); lane F2, DL67 variant (12D9^{+} 4C4^{+} 6A2^{+} 5G8^{-}).

readily produced systemic disease in the infant rat after intranasal challenge (Table 3). Strains NA105 (12D9^{-} 4C4$^{\pm}$ 6A2^{-} 5G8^{-}) and LA103 (12D9^{+} 4C4^{+} 6A2^{-} 5G8^{-}), however, were found to be much less virulent than the preceding two Hib strains. Strain NA105 failed to produce bacteremia in any of the infant rats challenged, while only low levels of bacteremia occurred in 4 of 10 animals challenged with strain LA103. In contrast, the LOS antigenic variants of these two strains that expressed electrophoretically slower migrating LOS molecules were significantly more virulent than their respective parental strains. The 12D9^{-} 4C4^{+} 6A2^{-} 5G8^{+} variant of strain NA105 produced bacteremia in 7 of 10 infant rats. Although the number of animals with bacteremia caused by the LOS variant (12D9^{+} 4C4^{+} 6A2^{+} 5G8^{+}) of LA103 was not significantly different from that produced by the parental strain, the level of bacteremia was significantly higher. Cell-associated capsular polysaccharide was similar between the parental strains NA105 and LA103 and their LOS antigenic variants (NA105 parental strain, 31.9 ± 5.1 μg/10^9 CFU [n = 3]; NA105 LOS variant, 29.5 ± 2.9 μg/10^9 CFU; LA103 parental strain, 22.9 ± 3.4 μg/10^9 CFU; LA103 LOS variant, 17.5 ± 3.7 μg/10^9 CFU), indicating that the observed differences in virulence were not due to large differences in cell-associated capsular polysaccharide (34).

Serum bactericidal assay. The serum sensitivities of strains

TABLE 3. Comparative virulence studies in the infant rat[a]

Strain	LOS antigenic phenotype	No. of bacteremic animals/total no. of animals (%)	CFU/ml of blood (geometric mean)
DV102 parental strain	12D9^{+} 4C4^{-} 6A2^{-} 5G8^{-}	10/10 (100)	5,888
NO103 parental strain	12D9^{-} 4C4^{+} 6A2^{-} 5G8^{-}	8/11 (73)	1,778
NA105 parental strain	12D9^{-} 4C4$^{\pm}$ 6A2^{-} 5G8^{-}	0/10 (0)	0
NA105 LOS variant	12D9^{-} 4C4^{+} 6A2^{-} 5G8^{+}	7/10 (70)[b]	2,512
LA103 parental strain	12D9^{+} 4C4^{+} 6A2^{-} 5G8^{-}	4/10 (40)	513
LA103 LOS variant	12D9^{+} 4C4^{+} 6A2^{+} 5G8^{+}	7/10 (70)	5,495[c]

[a] Relative virulence was determined in 5- to 6-day-old infant rats that were challenged by the intranasal route, as described previously (21).
[b] $P < 0.01$ compared with the parental strain by chi-square analysis.
[c] $P < 0.05$ compared with the parental strain by Student's t test.

NA105 and LA103 and their LOS antigenic variants are presented in Table 4. At a 50% concentration, infant rat serum was highly bactericidal for the parental NA105 strain; heating of the serum at 56°C for 30 min completely eliminated this activity. In contrast, the LOS antigenic variant of NA105 was resistant to the bactericidal activity of the serum and multiplied readily. Although the parental LA103 strain appeared to be serum resistant, it was not capable of multiplying to the same extent in the normal infant rat serum as its LOS antigenic variant with enhanced virulence.

Identification of the complement activation pathway involved in bactericidal activity. The chelating agents EDTA and EGTA were used to selectively inhibit the classical and alternative pathways of complement activation in this bactericidal assay system (30). The inclusion in the assay system of only EDTA, which inactivates both the classical and alternative pathways, prevented killing of the serum-sensitive strains of NO100 and NA105 (Table 5). The incubation of the normal infant rat serum with Mg^{2+}-EGTA prior to its use in this bactericidal assay also prevented killing of these serum-sensitive strains. This latter chelating compound selectively inhibits the classical pathway by binding calcium but does not affect the activity of the magnesium-

TABLE 4. Serum sensitivity of parental strains NA105 and LA103 and their LOS antigenic variants[a]

Strain	LOS antigenic phenotype	CFU/10 μl (mean ± SEM) at the following times (min): 0	60
NA105 parental strain	12D9^{-} 4C4$^{\pm}$ 6A2^{-} 5G8^{-}	74 ± 5	1 ± 0.8
		70 ± 15	269 ± 20[b]
NA105 LOS variant	12D9^{-} 4C4^{+} 6A2^{-} 5G8^{+}	113 ± 9	299 ± 38[c]
		116 ± 16	307 ± 46[b]
LA103 parental strain	12D9^{+} 4C4^{+} 6A2^{-} 5G8^{-}	97 ± 3	103 ± 10
		125 ± 10	247 ± 31[b]
LA103 LOS variant	12D9^{+} 4C4^{+} 6A2^{+} 5G8^{+}	91 ± 8	168 ± 17[d]
		101 ± 12	182 ± 17[b]

[a] Serum sensitivity was assessed as described in the text.
[b] Negative control with heat-inactivated serum; normal infant rat serum was heat inactivated at 56°C for 30 min to inactivate complement.
[c] Significantly different from parental strain ($P < 0.001$).
[d] Significantly different from parental strain ($P < 0.008$).

TABLE 5. Effect of EDTA and Mg^{2+}-EGTA on bactericidal activity of normal infant rat serum[a]

Hib strain	Reaction mixture[b]	CFU/10 μl at the following times (min): 0	60
NO100 ($4C4^-$ $5G8^-$)[c]	Serum	83	1
Parental strain	Heat-inactivated serum	91	163
	Serum + EDTA	88	208
	Heat-inactivated serum + EDTA	83	165
	Serum + Mg^{2+}-EGTA	73	271
	Heat-inactivated serum + Mg^{2+}-EGTA	83	242
NO100 ($4C4^+$ $5G8^+$)[d]	Serum	82	236
LOS variant	Heat-inactivated serum	104	248
NA105 ($4C4^{\pm}$ $5G8^-$)[e]	Serum	94	0
Parental strain	Heat-inactivated serum	88	248
	Serum + EDTA	91	301
	Serum + Mg^{2+}-EGTA	99	292
NA105 ($4C4^+$ $5G8^+$)[f]	Serum	78	241
LOS variant	Heat-inactivated serum	91	304

[a] The bactericidal assay was performed as described in the text.
[b] A 50% (vol/vol) normal infant rat serum sample in VBS-gelatin was used in the bactericidal assay. For negative controls, this same serum sample was heat inactivated for 30 min at 56°C to inactivate complement.
[c] Serum-sensitive strain of NO100.
[d] Serum-resistant strain of NO100.
[e] Serum-sensitive strain of NA105.
[f] Serum-resistant strain of NA105.

dependent alternative pathway. The absence of bactericidal activity against the serum-sensitive strains in this latter experiment in which the alternative pathway was functional but classical pathway activity was absent strongly suggests that the classical pathway of complement activation is involved in the bactericidal activity of normal infant rat serum against the serum-sensitive NO100 and NA105 parental strains.

The use of this infant rat serum in Western blot analysis with whole-cell lysates from both the serum-sensitive and serum-resistant strains of NO100 and NA105 did not detect any differences in serum antibody to these four strains (data not shown). Proteinase K-derived LOS preparations from these same strains also failed to exhibit differential binding of normal infant rat serum antibodies in Western blot analyses (data not shown).

An indirect antibody accessibility assay was also used to detect antibody directed against surface-exposed antigenic determinants on these strains. Use of the serum-resistant NO100 parental strain and the serum-resistant LOS antigenic variant of this strain showed that small quantities of antibody present in the infant rat serum did bind to both of these Hib strains, although the serum-sensitive strain did not bind any more antibody than did the serum-resistant strain (Table 6). Immune serum obtained from 30-day-old rats that were systemically infected with Hib strain DL42 at the age of 5 days contained readily detectable quantities of antibodies directed against surface antigens of both the homologous DL42 strain and the other two Hib strains (Table 6), thus confirming that specific rat antibody to Hib surface antigens can be detected by this RIA method.

DISCUSSION

The well-known transition of enteric bacteria from smooth-colony-forming strains to rough-colony-forming strains is directly related to changes in lipopolysaccharide structure and is usually associated with a decrease in the relative virulence of the strain. The biochemical basis for this transition normally involves either the lack of synthesis of the O-antigen repeat unit or the lack of the enzyme responsible for attaching the polymerized O-antigen chain to the core oligosaccharide. In contrast, bacteria which synthesize the relatively short LOS molecule instead of lipopolysaccharide have only six to nine saccharide moieties in their oligosaccharide portion and no O-antigen repeat unit but are still fully virulent and able to produce systemic disease (5, 15, 28). Alterations in even this relatively simple LOS molecule, however, can profoundly affect the properties of these organisms that are related to pathogenesis (12, 24).

In view of the fact that changes in LOS phenotype have been shown to be associated with changes in the relative virulence of Hib (21, 39), it was important to determine whether this organism stably expresses LOS phenotypes in vitro under normal laboratory conditions. Storage of 30 strains for 1 year at −70°C did not result in any detectable changes in LOS antigenic phenotype. Single-colony passage of these 30 Hib strains resulted in the identification of 3 strains which gave rise to variants with antigenically different LOS characteristics. These data are similar to those of Tolan et al. (36), who recently reported that three of six Hib isolates from an outbreak of Hib disease in an Amish community yielded variants with altered LOS profiles, as determined by SDS-polyacrylamide gel electrophoresis, after in vitro passage. In contrast, four other Hib strains used routinely by these workers did not yield any LOS variants after 21 passages in vitro (36). These data, together with those obtained from the present study, suggest that the stability of expression of the LOS phenotype by Hib is a strain-specific phenomenon, with some Hib strains giving rise to LOS phenotypic variants at much higher rates than others.

More detailed analysis of LOS antigenic variation involving immunologic examination of large numbers of individual colonies of selected Hib strains revealed that variants ex-

TABLE 6. Measurement of Hib-directed antibody in normal and immune infant rat serum in the indirect antibody accessibility RIA[a]

Bacterial strain	Serum type	cpm of ^{125}I-labeled probe bound[b]
NO100 parental strain	None[c]	67
NO100 LOS variant	None	62
NO100 parental strain	Normal infant	393
NO100 LOS variant	Normal infant	320
NO100 parental strain	Immune infant	2,293
NO100 LOS variant	Immune infant	2,097
DL42[d]	Immune infant	2,896

[a] The indirect antibody accessibility RIA was used to measure binding of rat immunoglobulin to Hib, as described in the text.
[b] Counts per minute of radioiodinated goat anti-mouse immunoglobulin G bound to rat antibody on Hib cells.
[c] Negative control to assess nonspecific binding of the radioiodinated probe to Hib cells.
[d] Positive control for detection of Hib-directed rat antibodies.

pressing antigenically altered LOS molecules could be detected in all seven strains tested in the colony blot RIA (Table 2). It should be emphasized that six of the seven strains tested in this manner did not yield LOS antigenic variants in the initial experiment involving single-colony passage. Thus, LOS antigenic variation occurs in these strains but at frequencies which can only be detected by the screening of large populations. The LOS antigenic group 1 epitopes recognized by MAbs 12D9 and 6A2 appeared to be more stably expressed by Hib strains than did the LOS antigenic group 2 determinants that were reactive with MAbs 4C4 and 5G8. The determinant recognized by MAb 5G8 was least stable in expression, and alterations involving this antigenic determinant were associated with the most striking changes in the LOS phenotype, as assessed by SDS-PAGGE (Fig. 1).

Our findings concerning the stability of expression of LOS by Hib have potentially important implications for two different areas of research involving Hib. As assessed by SDS-PAGGE, the LOS phenotype has been used by several laboratories studying the epidemiologic aspects of Hib disease (14, 22, 36). While our data indicate that not every LOS antigenic variant has an altered LOS profile in SDS-PAGGE relative to the respective parental strain (Fig. 1), all of the Hib strains used in our study exhibited detectable frequencies of LOS antigenic variation (Table 2). These data and those of Tolan et al. (36) suggest that the LOS phenotype may not be a reliable strain marker for epidemiologic studies. Perhaps more importantly, investigation of the genetic control of Hib LOS synthesis involving genetic transformation experiments must now take into account the occurrence of spontaneous LOS variation. The frequency of LOS variation observed with certain Hib strains in our study (Table 2) is much greater than the normal frequency of genetic transformation obtained with *H. influenzae* (19). Spontaneous LOS variation in Hib obviously has the potential to confound or at least confuse analyses based on transformation of genes involved in Hib LOS biosynthesis.

While the chemical composition and structure of the LOS antigenic determinants recognized by our four LOS-specific MAbs remain undefined, it has recently been found that MAb 4C4 recognizes an epitope containing an α-linked galactose residue in the oligosaccharide of the serum-resistant ($12D9^-$ $4C4^+$ $6A2^-$ $5G8^+$) LOS variant of strain NO100 (Suzanne Zamze and E. Richard Moxon, personal communication). The LOS of the $12D9^-$ $4C4^-$ $6A2^-$ $5G8^-$ NO100 parental strain also has been shown to contain less galactose by chemical analysis (Zamze and Moxon, personal communication), a finding that is consistent with a shorter oligosaccharide in this LOS molecule, which exhibits a faster migration rate in SDS-PAGGE (21) than does the LOS of the $12D9^-$ $4C4^+$ $6A2^-$ $5G8^+$ variant.

Strain DL26, a group 1 strain, was found to be capable of expressing the group 2 determinant recognized by MAb 4C4. Thus, this LOS variant of strain DL26 can be classified as a group 3 strain. This finding also raises the interesting question of whether all Hib strains possess the genetic material to code for both sets of LOS determinants and that the LOS antigenic diversity present among these strains, although very limited (11), is due to differences in expression. Our recent finding that some strains of Hib apparently synthesize two different LOS molecules with antigenically distinct oligosaccharide regions (C. C. Patrick, C. Hall, E. H. Fritz, P. A. Gulig, G. H. McCracken, Jr., and E. J. Hansen, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 863, 1986) is consistent with this hypothesis, which must ultimately be tested with cloned genes involved in Hib LOS synthesis.

In our previous report (21), the lack of reactivity with MAbs 4C4 and 5G8 in one Hib strain and the lack of reactivity with MAb 5G8 in another strain was associated with attenuated virulence. The lack of reactivity with the subgrouping MAbs 6A2 and 5G8 was inconsistently associated with decreased virulence in the Hib strains in the present study. DV102 and NO103, which are strains that lack MAb 6A2 and 5G8 reactivity, respectively, were readily capable of producing systemic disease in the infant rat. In contrast, other strains (NA105 and LA103) possessing similar reactivities with these two MAbs were much less virulent. The virulence of strains NA105 and LA103 was significantly enhanced in LOS antigenic variants that had acquired reactivity with one or both of these MAbs. Taken together, these data indicate that specific LOS antigenic phenotypes are not reliable indicators of the virulence potential of a given Hib strain. When virulence was associated with LOS antigenic variation in the present study, however, it was also consistently associated with changes in serum resistance. Thus, these data strongly suggest that changes in the LOS phenotype can affect Hib virulence by mediating changes in serum resistance. Similar results for Hib have been reported by Zwahlen et al. (39), and an association between LOS or lipopolysaccharide structure and serum resistance has also been demonstrated in *Haemophilus ducreyi* (27), as well as in many other gram-negative bacteria (8, 12, 17, 24, 26, 29, 32). It must also be noted, however, that there is at least one other mechanism by which changes in the LOS phenotype are associated with changes in the virulence of Hib, and this second mechanism is apparently unrelated to serum resistance (21).

The mechanism(s) by which changes in Hib LOS phenotype are associated with changes in serum resistance is unclear. Hib has been shown to activate the alternative complement pathway in the absence of specific antibody, but it is apparently not susceptible to its bactericidal activity under these conditions (33, 36). Conversely, the serum-sensitive strains involved in the present study and in a previous study (7) were killed by normal serum bactericidal activity involving the classical pathway of complement activation (Table 5). The normal infant rat serum involved in the present study did contain very low levels of antibody to Hib but did not have increased levels of antibody to a serum-sensitive Hib strain relative to its serum-resistant LOS variant. Accordingly, it is possible that a membrane attack complex resulting from classical complement pathway activation by small amounts of serum antibody binding to Hib inserts more stably into the outer membranes of the serum-sensitive strains than into those of the serum-resistant strains (16–18). Alternatively, the classical pathway could be activated by direct, antibody-independent interaction of the C1 complement component with Hib LOS or outer membrane proteins (2, 6), and it is possible that the faster-migrating LOS (in SDS-PAGGE) of the serum-sensitive strains might activate this pathway more effectively than does the LOS of the serum-resistant strains (23). Previous experience with other bacteria suggests, however, that both the serum-sensitive and the serum-resistant strains probably activate the complement cascade to the same extent, but the faster-migrating (in SDS-PAGGE) and presumably smaller LOS molecules produced by the serum-sensitive strains may render these strains more susceptible to the bactericidal action of the membrane attack complex (16). In fact, we observed a consistent correlation between electrophoreti-

cally fast migrating LOS and serum sensitivity in this study. Precise elucidation of the association between Hib LOS and serum resistance will require detailed analyses of the type performed previously with *Salmonella typhimurium* (8, 16–18) and *Neisseria gonorrhoeae* (16).

The relationship between the LOS antigenic phenotype and virulence remains to be defined, because there is no obvious correlation between reactivity with selected LOS-directed MAbs and expression of virulence by Hib. Final resolution of these apparent anomalies and of the relationship between Hib LOS and serum resistance may necessarily have to await detailed chemical and structural analyses of Hib LOS molecules.

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