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Conservation of Epitopes in the Oligosaccharide Portion of the Lipooligosaccharide of *Haemophilus influenzae* Type b

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The antigenic characteristics of the lipooligosaccharide (LOS) of *Haemophilus influenzae* type b (Hib) were examined in strains obtained over an extended period of time. These Hib strains were isolated from patients with systemic Hib disease in Dallas, Tex., over a 20-year period and in New York City between 1941 and 1956. The antigenic characteristics of the LOS of these Hib strains were examined by using a set of four murine monoclonal antibodies directed against epitopes present in the oligosaccharide portion of the LOS molecule. The same basic set of LOS antigenic determinants that is expressed by recent Hib isolates was also found to be present in this collection of Hib strains spanning a 40-year period. Some variation with time was detected in the distribution of the systemic disease isolates among four Hib LOS antigenic groups; however, only 2 of 188 Hib isolates failed to react with a set of two LOS-specific monoclonal antibodies. Therefore, little variation has occurred among Hib strains with regard to the LOS epitopes defined by these monoclonal antibodies over a considerable period of time.

As the most important cause of bacterial meningitis in this country, *Haemophilus influenzae* type b (Hib) is the subject of considerable research activity which is focused on the surface antigens of this organism (13). The polysaccharide capsule which surrounds the Hib cell has been shown to be both the primary virulence factor of this organism and a protective antigen (11, 35, 36, 42, 43, 53) and it forms the basis for the new Hib vaccine (6). Similarly, a considerable quantity of data concerning Hib outer membrane proteins has been accumulated over the past few years (4, 7, 8, 14–23, 30, 32–34, 37–39, 45, 49–52), and genes encoding some of the outer membrane proteins of Hib have recently been cloned in *Escherichia coli* (25, 46).

Only recently, however, has the lipopolysaccharide (LPS) or lipooligosaccharide (LOS) present in the Hib outer membrane been studied with regard to its chemical, antigenic, and biologic properties (9, 26–29, 40). The LOS of Hib, like the LOS of *Neisseria gonorrhoeae* (44), *N. meningitidis* (47), and *Bordetella pertussis* (41) differs from the classic enteric LPS molecule in that Hib LOS lacks any detectable O-antigen repeat unit and apparently consists of a lipid A moiety covalently coupled to a structure equivalent to the core oligosaccharide of enteric LPS molecules (9, 29).

In contrast to the wide antigenic diversity present in the cell surface-exposed antigenic determinants (O antigens) of the LPS of enteric organisms such as *E. coli* and *Salmonella typhimurium*, the antigenic determinants of Hib LOS appear to exhibit relatively little antigenic heterogeneity. Flesher and Insel (9) found that polyclonal rabbit serum raised against purified Hib LOS reacted with several different serotypes of *H. influenzae*, whereas Anderson et al. (2) showed that antibodies to the LOS of Hib strain Eagan were bactericidal for several different strains of Hib and that one particular LOS antigenic determinant was present in 10 of 13 Hib strains. Van Alphen et al. (50) reported that Hib isolates from the Netherlands could be divided into essentially four different LOS serotypes and that 93% of their Hib strains had the same LOS serotype. Apicella et al. (3) showed that approximately 50% of Hib strains share at least one epitope in the lipid A region of Hib LOS, as determined by reactivity with a Hib lipid A-directed monoclonal antibody. We have already shown that a set of just two murine monoclonal antibodies directed against Hib LOS surface determinants collectively recognizes essentially all of 126 recently isolated strains of this pathogen and can be used to subdivide Hib strains into four different LOS antigenic groups (16). It must be emphasized, however, that both this latter study and most of the earlier studies used only relatively recent isolates of Hib. The results obtained in the present study indicate that multiple antigenic determinants in the oligosaccharide moiety of Hib LOS have been highly conserved among Hib strains over a considerable number of years.

MATERIALS AND METHODS

Bacterial strains and culture media. Hib strain DL26 is the prototypic LOS antigenic group 1 strain, Hib strain DL42 is the prototypic LOS antigenic group 2 strain, whereas Hib strain DL41 is the prototypic LOS antigenic group 3 strain; all three of these strains have been described previously (15, 16). One large set of Hib strains isolated at Children's Medical Center in Dallas, Tex., was obtained from the Department of Pediatrics, Southwestern Medical School, Dallas, Tex. These strains were all cerebrospinal fluid or blood isolates from children with systemic Hib disease and were isolated during several time periods: 1963 to 1965, 1971 to 1972, 1975 to 1977, and 1980 to 1982. An additional set of Hib strains isolated from children with systemic Hib disease in New York City between 1941 and 1956 by Hattie Alexander and Grace Leidy was obtained from Katheryn Sprunt and Arnold Smith. All strains were verified as being Hib by standard methods (21). Bacterial culture media and conditions have been described previously (21).

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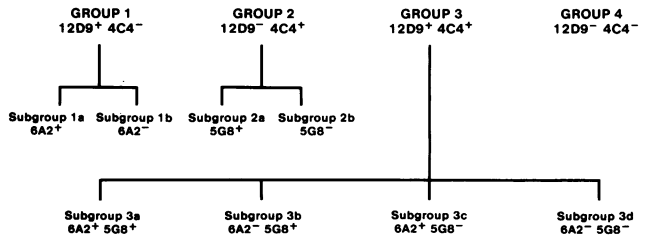

FIG. 1. Antigenic grouping of Hib strains by reactivity with LOS-specific monoclonal antibodies. Antigenic group determination was based on the reactivity of Hib strains with monoclonal antibodies 12D9 and 4C4. Antigenic subgrouping was based on the reactivity of Hib strains with monoclonal antibodies 6A2 and 5G8.

Purification of Hib LOS and its subunits. LOS was purified from unencapsulated variants of Hib strains DL26 and DL42 by the hot phenol-water method of Westphal and Jann (54). Oligosaccharide and lipid A were prepared from these purified LOS preparations by the method of Apicella et al. (3). Oligosaccharide preparations were neutralized with 0.1 N sodium hydroxide and further centrifuged at 200,000 × *g* for 14 h to precipitate any unhydrolyzed LOS or lipid A. The resultant supernatant was lyophilized and then dissolved in a minimal (1 to 2 ml) volume of distilled water.

Whole-cell lysates for LOS analysis. Proteinase K-digested whole-cell lysates of Hib were used as the source of LOS for Western blot analysis involving polyclonal rat antibody to Hib LOS. These lysates were prepared as described by Kimura and Hansen (31) by using a modification of the method of Hitchcock and Brown (24).

Monoclonal antibodies. The LOS-specific monoclonal antibodies 12D9, 4C4, 6A2, and 5G8 have been described (15, 16). Hybridoma culture supernatant fluid was used as the source of these antibodies for all experiments.

Polyclonal antiserum to Hib LOS. Adult Sprague-Dawley rats were immunized with 50 μg of the purified major outer membrane with an apparent molecular weight of 39,000 (39K) from Hib strains DL26 and DL42 as described previously (16). This quantity of 39K protein contained 5 to 10 μg of Hib LOS as determined by comparison with LOS purified from these Hib strains in sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis (SDS-PAGGE) followed by silver staining. The anti-LOS response to the protein-LOS complex was much greater than that obtained by immunization with purified Hib LOS alone (P. A. Gulig, A. Kimura, and E. J. Hansen, unpublished data), and therefore these antisera were used as the source of polyclonal antibody to Hib LOS.

SDS-PAGGE. SDS-PAGGE was performed as described previously (31), and the silver stain method of Tsai and Frasch (48) was used for nonimmunological detection of LOS in gels.

Western blot analysis. Western blot analyses with murine monoclonal antibodies were performed as described (31), while experiments involving polyclonal rat sera used hoseradish peroxidase-conjugated goat anti-rat immunoglobulin G (heavy and light chain specific; Cooper Biomedical, Malvern, Pa.) in place of goat anti-mouse immunoglobulin G.

AA-RIA for use in binding inhibition studies. The indirect antibody-accessibility radioimmunoassay (AA-RIA) was used to determine whether specific monoclonal antibodies were directed against epitopes present in the oligosaccharide portion of Hib LOS. The quantity of each monoclonal antibody used in these experiments was adjusted by titration in the AA-RIA to be the limiting factor in this system. A 500-μl volume of each monoclonal antibody diluted in phosphate-bufferedsaline (pH 7.2) containing 10% (vol/vol) fetal bovine serum was incubated with increasing quantities of purified Hib LOS or purified oligosaccharide for 1 h at room temperature with gentle agitation. Then, cells of the appropriate Hib strain were added, and the AA-RIA was performed exactly as described by Kimura and Hansen (30, 31).

Colony blot-RIA. The colony blot-RIA was performed as described by Gulig et al. (15). Briefly, Hib strains were grown overnight on solid medium and the resultant bacterial colony growth was placed in an array pattern on filter paper. The filters were blocked by incubation with phosphate-buffered saline (pH 7.2) containing 1% (vol/vol) normal rabbit serum to prevent nonspecific protein binding. These filters were then incubated with a 10 to 20% (vol/vol) solution of hybridoma culture supernatant fluid containing a LOS-specific monoclonal antibody. Antibody-antigen complexes were detected by incubation of the filters with ^{125}I-labeled goat anti-mouse immunoglobulin. Hib strains reacting with particular LOS-specific monoclonal antibodies were detected as dark spots on X-ray film after autoradiographic analysis of the filters. The antigenic grouping system of Gulig and Hansen (16; Fig. 1) was used to classify the Hib strains according to their reactivity with the LOS-specific monoclonal antibodies.

Immunoelectron microscopy. The protein A-colloidal gold method of Geohegan and Ackerman (10) for visualizing antibodies bound to cell surfaces, as modified by Holmans et al. (25), was used for immunoelectron microscopy.

RESULTS

Characterization of antibody specificity. We have previously shown that a set of just two murine monoclonal antibodies reactive with Hib LOS recognizes essentially all recent isolates of this pathogen from patients with systemic Hib disease and permits the classification of Hib strains into four different LOS antigenic groups (16). In addition, the use of two additional LOS-directed monoclonal antibodies further allowed these four LOS antigenic groups to be divided into eight subgroups (15, 16; Fig. 1). A whole-cell radioimmunoprecipitation system was originally employed to determine whether these four monoclonal antibodies bound to epitopes exposed on the surface of Hib (15). We have now confirmed that all of these antibodies bind to cell surface-exposed epitopes on the homologous Hib strains through the use of immunoelectron microscopy. Each of the four monoclonal antibodies was incubated with whole Hib cells from the different LOS antigenic groups, and then protein A-colloidal gold was added to permit detection of antibodies bound to the Hib cell surface (Fig. 2). Monoclonal antibody 12D9 bound to both Hib strains DL26 (LOS antigenic group 1) and DL41 (LOS antigenic group 3) but did not bind to strain DL42 (antigenic group 1). Monoclonal antibody 4C4 bound to strains DL42 (antigenic group 2) and DL41 (antigenic group 3) but did not bind to strain DL26 (antigenic group 1). Thus, the reactivity of these two monoclonal antibodies with their homologous strains of Hib involves LOS epitopes exposed on the surface of these cells. Monoclonal antibodies 6A2 and 5G8 also bound to surface-exposed LOS epitopes on their homologous Hib strains as determined by immunoelectron microscopy (data not shown).

While all four of these monoclonal antibodies have been shown to bind to LOS epitopes exposed on the surface of the

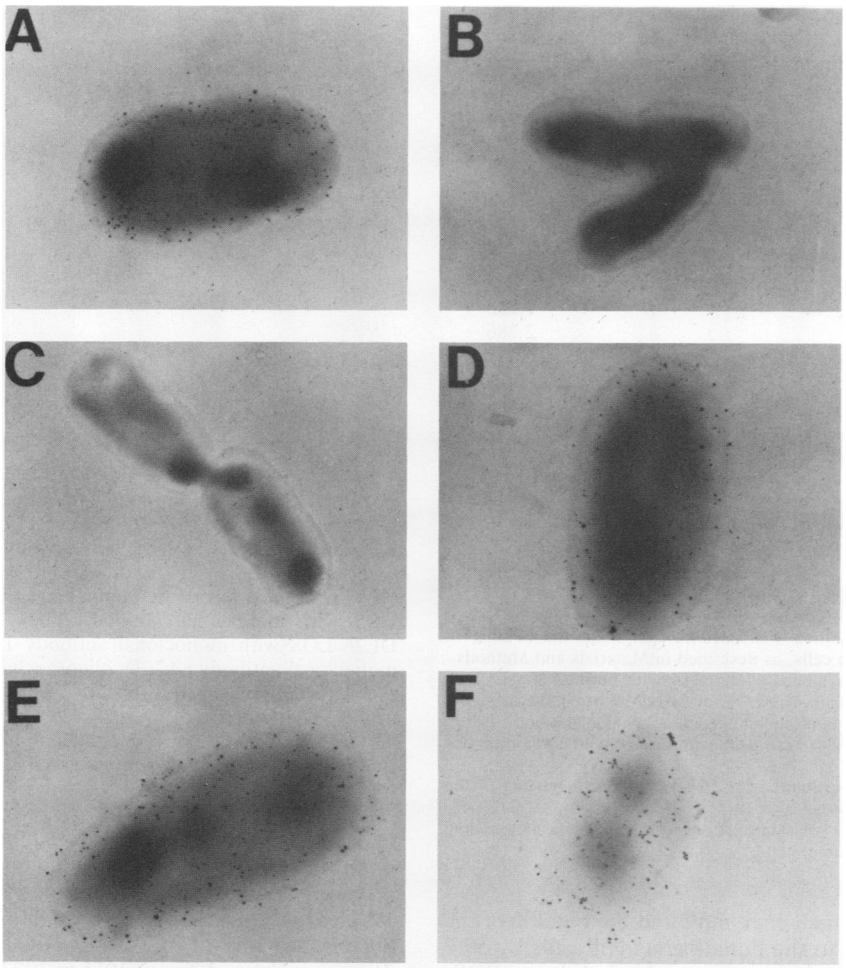

FIG. 2. Reactivity of LOS-specific monoclonal antibodies with surface-exposed epitopes on Hib cells. Whole cells of Hib strains DL26 (LOS antigenic group 1), DL42 (LOS antigenic group 2), and DL41 (LOS antigenic group 3) were incubated with monoclonal antibodies 12D9 and 4C4, and the resultant immune complexes on the bacterial cell surface were visualized through the use of protein A-colloidal gold particles and electron microscopy as described in Materials and Methods. (A) Strain DL26 with monoclonal antibody 12D9; (B) strain DL26 with monoclonal antibody 4C4; (C) strain DL42 with monoclonal antibody 12D9; (D) strain DL42 with monoclonal antibody 4C4; (E) strain DL41 with monoclonal antibody 12D9; (F) strain DL41 with monoclonal antibody 4C4. The individual magnifications used for photography ranged from ×25,000 to ×45,000.

homologous Hib strains, the specificity of these antibodies for the oligosaccharide or lipid A portions of the Hib LOS molecule was not known. In view of the fact that Apicella et al. (3) have recently reported that a monoclonal antibody to Hib lipid A binds to the surface of whole Hib cells, it was imperative to elucidate the true antigenic specificity of these four monoclonal antibodies.

LOS purified from unencapsulated variants of strains DL26 (antigenic group 1) and DL42 (antigenic group 2) were subjected to acid hydrolyis at 100°C, and oligosaccharide and lipid A were prepared from each of these two different LOS molecules. Various amounts of each purified LOS and the homologous oligosaccharide preparation were incubated individually with each of the four monoclonal antibodies for 1 h to permit antibody binding to the oligosaccharide. Whole Hib cells of the Hib strain reactive with the monoclonal antibody were then added to this mixture, and the indirect AA-RIA was used to determine whether these oligosaccharides had bound the monoclonal antibodies, an event which in turn would prevent the binding of these antibodies to the Hib cells. These titration curves were used to determine the quantity of Hib LOS or oligosaccharide required to produce 50% inhibition of antibody binding to the Hib cells (Table 1).

The data presented in Table 1 indicate that the monoclonal antibodies 12D9 and 6A2 both bound the oligosaccharide prepared from strain DL26 LOS, whereas monoclonal antibodies 4C4 and 5G8 both bound the oligosaccharide derived from strain DL42 LOS. In addition, the binding of monoclonal antibodies 12D9 and 6A2 to strain DL26 cells was not inhibited by strain DL42 oligosaccharide, whereas the binding of monoclonal antibodies 4C4 and 5G8 to strain DL42 was not inhibited by strain DL26 oligosaccharide. These latter results confirm the antigenic specificity of these inhibition tests and eliminate the possibility that the positive inhibition tests resulted from some type of nonspecific effect of the oligosaccharide preparations.

We next performed Western blot analysis of these oligosaccharide preparations to confirm that these oligosaccharides were free of contaminating intact LOS molecules or lipid A which could have yielded the positive inhibition results. Quantities of the purified Hib LOS and the oligosac-

TABLE 1. Inhibition of monoclonal antibody binding to cells by purified Hib LOS and LOS-derived oligosaccharide[a]

Reaction mixture[b]	Binding (cpm)[c]
12D9	5,080
12D9 + 2 μg DL26 LOS[d]	2,482
12D9 + 20 μl DL26 oligosaccharide[e]	2,318
6A2	1,209
6A2 + 0.1 μg DL26 LOS[d]	641
6A2 + 60 μl DL26 oligosaccharide[e]	590
4C4	4,818
4C4 + 3 μg DL42 LOS[d]	2,286
4C4 + 5 μl DL42 oligosaccharide[e]	2,312
5G8	6,134
5G8 + 1 μg DL42 LOS[d]	3,460
5G8 + 10 μl DL42 oligosaccharide[e]	3,015
12D9	6,017
12D9 + 20 μl DL42 oligosaccharide	6,136
4C4	3,833
4C4 + 80 μl DL26 oligosaccharide	3,603

[a] The indirect AA-RIA method was used to quantitate the binding of monoclonal antibodies to Hib cells, as described in Materials and Methods.
[b] Monoclonal antibody was incubated alone or with purified Hib LOS or with oligosaccharide derived from Hib LOS for 1 h before use in the AA-RIA.
[c] DL26 cells were used to quantitate binding of monoclonal antibodies 12D9 and 6A2 in AA-RIA. DL42 cells were used with monoclonal antibodies 4C4 and 5G8 in AA-RIA.
[d] Quantity of purified LOS required for 50% inhibition of binding of the specified antibody to target cells.
[e] Volume of oligosaccharide solution required for 50% inhibition of binding of the specified antibody to target cells.

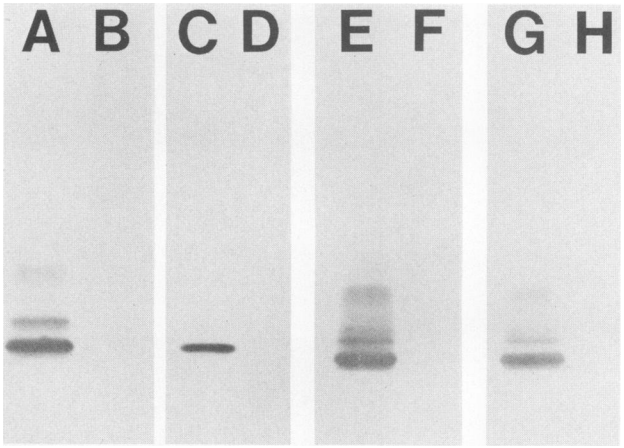

FIG. 3. Western blot analysis of purified LOS and oligosaccharide preparations. The amounts of purified LOS and oligosaccharide which produced at least 50% inhibition of binding of the specified monoclonal antibodies to the homologous Hib strains (Table 2) were resolved by SDS-PAGGE, transferred to nitrocellulose, and incubated with the appropriate monoclonal antibodies. Lanes: A, DL26 LOS with monoclonal antibody 12D9; B, oligosaccharide from DL26 LOS with monoclonal antibody 12D9; C, DL26 LOS with monoclonal antibody 6A2; D, oligosaccharide from DL26 LOS with monoclonal antibody 6A2; E, DL42 LOS with monoclonal antibody 4C4; F, oligosaccharide from DL42 LOS with monoclonal antibody 4C4; G, DL42 LOS with monoclonal antibody 5G8; H, oligosaccharide from DL42 LOS with monoclonal antibody 5G8.

charide preparations which each inhibited the binding of the monoclonal antibodies to the homologous Hib cells by 50% in the preceding experiments were loaded onto SDS-polyacrylamide gradient gels and were subjected to electrophoresis followed by electrophoretic transfer onto nitrocellulose membranes. Western blot analysis was performed by using the homologous monoclonal antibodies.

It can be seen that the quantities of purified LOS which caused 50% inhibition of monoclonal antibody binding to whole Hib cells in the AA-RIA (Table 1) were readily detectable in these Western blot analyses (Fig. 3, lanes a, c, e, and g). In contrast, there was no reactivity observed in the lanes into which were loaded the corresponding oligosaccharide samples (Fig. 3, lanes b, d, f, and h). These findings are consistent with those of Apicella et al. (3), who showed that the acid hydrolysis-derived oligosaccharide moiety of nontypable *H. influenzae* LOS did not migrate into the separating gel during SDS-PAGGE, whereas both purified LOS and lipid A did enter the separating gel and migrated to approximately the same position. The lack of contaminating LOS or lipid A in these oligosaccharide preparations is confirmed by the absence of an immunoreactive band migrating at the level of the purified LOS. In addition, Western blot analysis of lipid A prepared from the LOS of strains DL26 and DL42 indicated that these monoclonal antibodies did not bind to lipid A (data not shown), thus confirming the specificity of these antibodies for epitopes in the oligosaccharide region of Hib LOS.

Retrospective analysis of LOS antigenic characteristics. We proceeded to use these LOS-directed monoclonal antibodies as highly specific immunochemical probes to study the antigenic composition of the LOS of Hib strains isolated over an extended period of time. One set of Hib strains was obtained from infants and young children with systemic Hib disease in Dallas, Tex., during the period from 1963 through 1982. This collection consisted of 19 Hib strains isolated during 1963 to 1965, 12 strains isolated during 1971 to 1972, 21 strains isolated during 1975 to 1977, and 54 strains isolated during 1980 to 1982. These Hib strains were examined for reactivity with the four LOS-specific monoclonal antibodies by using the colony blot-RIA and were subsequently classified into antigenic groups and subgroups as previously described (16). The antigenic grouping data are summarized in Table 2. Also included in Table 2 are the antigenic characteristics of 72 other recent Hib isolates obtained from other pediatric research centers across the United States.

All of the 106 Hib strains isolated in Dallas as early as 1963 were recognized by our set of LOS-specific monoclonal

TABLE 2. Antigenic characteristics of the LOS of Hib isolates obtained over a 40-year period[a]

Source	Time of isolation (yr)	No. (%) of strains in antigenic group:			
		1	2	3	4
Dallas, Tex.	1963–1965	0 (0)	9 (47)	10 (53)	0 (0)
Dallas, Tex.	1971–1972	3 (25)	6 (50)	3 (25)	0 (0)
Dallas, Tex.	1975–1977	4 (19)	13 (62)	4 (19)	0 (0)
Dallas, Tex.	1980–1982	10 (19)	31 (57)	13 (24)	0 (0)
United States (excluding Dallas)[b]	Recent (1980–1982)	7 (10)	47 (65)	17 (24)	1 (1)
New York City	1945–1956	0 (0)	8 (80)	1 (10)	1 (10)

[a] Hib strains were classified into antigenic groups by using the reactivity of the strains with a set of monoclonal antibodies directed against epitopes in the oligosaccharide portion of Hib LOS (16; Fig. 1).
[b] These strains have been previously described (15).

antibodies and thus they could be classified into LOS antigenic groups 1, 2, or 3. In addition, the subgroup distribution of these strains was similar to that of our nationally based culture collection in that most of the older Dallas isolates were LOS antigenic subgroup 1a, 2a, 3a, or 3c (data not shown). These data indicate that the antigenic determinants of Hib LOS defined by this set of two monoclonal antibodies have been conserved in Hib strains in Dallas for at least 20 years.

None of the 19 Dallas strains from the earliest sampling period (1963 to 1965) was classified as LOS antigenic group 1. Instead, there were approximately equal numbers of LOS antigenic group 2 and group 3 strains (47 and 53%, respectively). The first LOS antigenic group 1 strains from Dallas were identified in the set of strains isolated during the 1971 to 1972 time period. This set of strains had an equal distribution of group 1 and group 3 strains (25% each), whereas the largest number of strains from this time period belonged to group 2 (50%). This distribution of LOS antigenic determinants was similar to that found in both the 1975 to 1977 and 1980 to 1982 isolates.

With minor variations, the antigenic characteristics of the recent (1980 to 1982) Dallas strains were very similar to those of the recent non-Dallas strains (Table 2). However, to eliminate any possible geographical bias in this study and to obtain a set of Hib strains isolated before 1960, we also examined Hib strains isolated in New York City between 1941 and 1956 (Table 2). It is important to note that only 1 of these 10 isolates from New York City failed to react with the set of two LOS-directed monoclonal antibodies. The distribution of LOS antigenic determinants among these very early Hib isolates was similar to that found with the earliest Dallas isolates in that no group 1 strains were present in either sample. Unlike the 1963 to 1965 Dallas strains, however, the New York City strains were composed primarily of group 2 strains (80%).

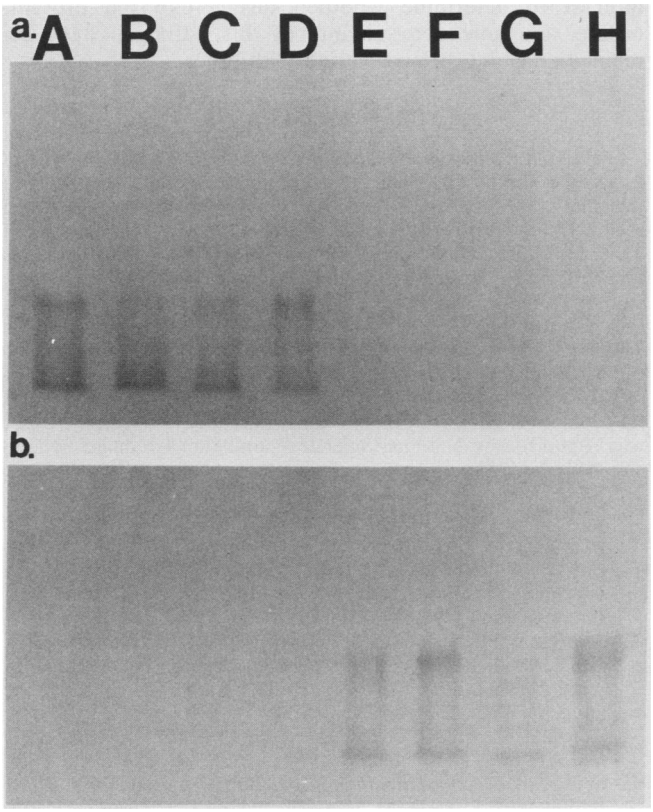

FIG. 4. Western blot analysis of the reactivity of polyclonal antiserum to Hib LOS with different Hib strains. Proteinase K-derived cell extracts of Hib strains from LOS antigenic groups 1 and 2 were used as antigen and were resolved by SDS-PAGGE before electrophoretic transfer to nitrocellulose. LOS antigenic group 1 strains DL26, DL63, DL90, and CH102 are in lanes A, B, C, and D, respectively. LOS antigenic group 2 strains DL42, DL88, DL119, and DL136 are in lanes E, F, G, and H, respectively. The extracts in panel a were probed with rat serum raised against DL26 LOS; the extracts in panel b were probed with rat serum raised against DL42 LOS.

Studies with polyclonal antiserum. Each monoclonal antibody used in this study recognizes a specific epitope in the oligosaccharide of the homologous LOS molecule. It is possible that additional discriminating epitopes may be present in these oligosaccharides which would permit further differentiation of, or cross-reactivity between, these monoclonal antibody-defined LOS antigenic groups. We therefore raised polyclonal rat antisera against DL26 LOS and DL42 LOS and used these antisera in Western blot analysis together with LOS preparations derived from additional different strains of Hib by proteinase K treatment. Polyclonal antiserum to the LOS of strain DL26 (LOS antigenic group 1) reacted with the LOS of four LOS antigenic group 1 strains (DL26, DL63, DL90, and CH102), but not with the LOS of any of four LOS antigenic group 2 strains (DL42, DL88, DL119, and DL136) (Fig. 4a). Similarly, antiserum to the LOS of DL42 (LOS antigenic group 2) reacted with the LOS preparations from all four of the group 2 strains but not with the LOS of the group 1 strains (Fig. 4b). These data indicate that other discriminating epitopes besides those defined by monoclonal antibodies 12D9, 4C4, 6A2, and 5G8 are either not present in the oligosaccharides of the LOS of Hib strains DL26 and DL42 or are present but not immunodominant and thus are not capable of inducing a readily detectable polyclonal antibody response directed against themselves.

DISCUSSION

There is currently much interest in the LOS of Hib. This amphipathic molecule, which is a major constituent of the Hib outer membrane, has been implicated, secondary to the type b capsule, in the expression of virulence by this pathogen (12, 31, 55, 56). In addition, although it has been shown that epitopes of both the lipid A (3) and oligosaccharide portions of this macromolecule are exposed on the cell surface of Hib, polyclonal antibody to surface determinants of Hib LOS is apparently not protective against systemic Hib disease in experimental animals (27, 45). Definition of the exact role played by LOS in the pathogenesis of systemic Hib disease and elucidation of the basis for the inability of most Hib LOS-directed antibodies to protect against Hib disease requires detailed information about the antigenic characteristics of Hib LOS. For these reasons, we have chosen to use monoclonal antibody technology in the study of Hib LOS.

Earlier observations by other groups using polyclonal antisera to Hib LOS suggested that only limited antigenic heterogeneity might exist among the various epitopes of Hib LOS (2, 9, 50). We have confirmed the findings of these workers and extended their findings to develop a serogrouping system for Hib based on the reactivity of Hib strains with monoclonal antibodies specific for epitopes in the oligosaccharide region of the Hib LOS molecule (Fig. 1). Character-

ization of 126 strains of Hib collected from pediatric research centers across the country between 1980 and 1982 showed that two epitopes in the oligosaccharide of Hib LOS were collectively common to essentially all of these recent isolates of Hib (16).

More importantly, alterations in the oligosaccharide moiety of Hib LOS have been shown to affect the ability of this pathogen to produce systemic disease (31). This result suggests that the oligosaccharide region of the LOS in whole Hib cells may interact directly with certain host defense mechanisms or that changes in this region of the Hib LOS molecule may affect other outer membrane constituents with a concomitant alteration of virulence potential. In view of these possibilities and because of the striking degree of conservation of the oligosaccharide epitopes defined by our LOS-directed monoclonal antibodies among recent isolates of Hib, we chose to use these monoclonal antibodies to perform a retrospective antigenic analysis of Hib strains collected over a period of 40 years to determine whether one or more of these epitopes had been conserved with respect to time. The data summarized in Table 2 indicate that even the invasive Hib strains isolated 40 years ago collectively contain some of the same oligosaccharide epitopes as recent Hib isolates from children with systemic disease.

It is apparent that the distribution of systemic disease isolates among the LOS antigenic groups has varied somewhat with time, as evidenced by the absence of group 1 strains among the New York City strains and the oldest Dallas isolates (Table 2). In fact, LOS antigenic group 1 strains seem to have appeared in Dallas for the first time during the interval between 1965 and 1971. The molecular basis for the emergence of these LOS antigenic group 1 strains is unknown. It should be noted, however, that group 3 strains collectively possess the LOS antigenic determinants found in group 1 strains (as defined by monoclonal antibodies 12D9 and 6A2) and group 2 strains (as defined by monoclonal antibodies 4C4 and 5G8). Therefore, the possibility exists that group 1 strains arose from group 3 strains as a result of the elimination or inactivation (by mutation) of the genetic material encoding the antigenic determinants recognized by monoclonal antibodies 4C4 and 5G8. In this regard, it is interesting to note that the appearance of group 1 strains in 1971 to 1972 coincided with a relative decrease in the percentage of group 3 strains isolated during the same time period. However, it must be emphasized that the same basic set of LOS antigenic determinants, as defined by reactivity with monoclonal antibodies 12D9 and 4C4, has been present throughout this 40-year period. If these particular antigenic determinants had been lost with time or replaced with new determinants, then this occurrence would have resulted in an increase in the number of group 4 strains among the more recent isolates.

It must also be noted that both the single group 4 strain among these New York City strains and the group 4 strain among the non-Dallas strains have recently been shown to possess the genetic information necessary to synthesize the LOS antigenic group 2 determinants, as defined by reactivity with monoclonal antibodies 4C4 and 5G8. These two strains (COL10 and NO100) spontaneously give rise to variants with LOS antigenic group 2 characteristics (31). This finding again emphasizes the fact that the antigenic determinants of Hib LOS have been highly conserved with time. In view of the fact that Hib LOS is associated with the virulence of this organism (12, 31, 55, 56), this striking degree of antigenic conservation may be related to a possible requirement for the expression of certain LOS phenotypes in the production of systemic disease. All of the Hib strains characterized in this study were systemic disease isolates, however, and this fact raises the possibility that there may exist Hib strains with different LOS antigens which can colonize the nasopharynx but which are not capable of causing invasive disease.

With the exception of the continuing surveillance of *Salmonellla* species by the Centers for Disease Control (5) and the recent studies of Achtman et al. (1) with *E. coli*, we are not aware of any other studies which have examined the stability of noncapsular surface antigens of gram-negative bacterial pathogens isolated over an extended period of time. In a recent report, Hampton et al. (19) noted that the distribution of outer membrane protein subtypes among Hib strains obtained in St. Louis, Mo., over a 5-year period had varied with time. These researchers suggested that changes in the outer membrane protein subtypes could indicate a change in the antigenic characteristics of the outer membrane proteins. No information concerning possible changes in the antigenic composition of Hib outer membrane proteins over an extended period of time has been published to date. The data contained in the present report show that certain antigenic determinants in the oligosaccharide of the LOS of Hib strains isolated 40 years ago are the same as those of recent Hib isolates. Therefore, it will be of considerable interest to determine whether surface-exposed proteinaceous antigenic determinants of this pathogen have remained constant or have varied with time.

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