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A Set of Two Monoclonal Antibodies Specific for the Cell Surface-Exposed 39K Major Outer Membrane Protein of *Haemophilus influenzae* Type b Defines All Strains of This Pathogen

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Six murine plasma cell hybridomas producing monoclonal antibodies (mabs) directed against the 39,000-molecular-weight (39K) major outer membrane protein of *Haemophilus influenzae* type b were employed in the antigenic analysis of the 39K protein. The initial characterization of the mabs by radioimmunoprecipitation analysis showed that four of these mabs reacted with antigenic determinants of the 39K protein that are exposed on the bacterial cell surface and accessible to antibody. The other two mabs reacted with antigenic determinants of the 39K protein that are either not exposed on the *H. influenzae* type b cell surface or not accessible to antibody (internal determinants). A total of 126 clinical isolates of *H. influenzae* type b obtained from pediatric research centers throughout the United States were examined for reactivity with the six mabs by using a solid-phase radioimmunoassay in which bacterial colony growth from agar plates was placed on filter paper and used as antigen. The reactivities of these strains with two of the mabs recognizing cell surface-exposed antigenic determinants of the 39K protein were used to divide the 126 strains into four different groups. Group 1 strains reacted with mab 12D9, group 2 strains reacted with mab 4C4, group 3 strains reacted with both mabs 12D9 and 4C4, and group 4 strains (only one was found) did not react with either mab. The reactivities of two other mabs recognizing cell surface-exposed antigenic determinants of the 39K protein were used to further divide the four groups into eight subgroups. A single mab recognizing an internal antigenic determinant of the 39K protein reacted with every *H. influenzae* type b strain examined in this study. These data indicate that only limited antigenic heterogeneity exists among the cell surface-exposed antigenic determinants of the 39K outer membrane proteins among *H. influenzae* type b strains and that at least one internal antigenic determinant of the 39K protein is universally present in all *H. influenzae* type b strains. Radioimmunoprecipitation analysis also demonstrated that *H. influenzae* type b strains which lacked a 39K major outer membrane protein possessed a 38K major outer membrane protein which reacted with the anti-39K mabs, indicating that the 38K and 39K outer membrane proteins of different *H. influenzae* type b strains are antigenically related.

Haemophilus influenzae type b is the leading cause of endemic bacterial meningitis in infants and young children in the United States (4). The polysaccharide capsule of this pathogen is composed of polyribosylribitol-phosphate and has been shown to be a necessary virulence factor for the production of disease (5, 20, 27). Furthermore, antibodies against the capsule have been shown to protect against systemic *H. influenzae* type b disease (6, 21, 28). However, a vaccine composed of the purified capsular polysaccharide is incapable of inducing the production of protective antibodies in that population at greatest risk for *H. influenzae* type b disease, namely, infants under 14 months of age (19). This lack of immunogenicity of the purified capsular polysaccharide in infants has resulted in two different approaches to identify alternative vaccine candidates: (i) increasing the immunogenicity of the capsular polysaccharide by covalently coupling it to protein carrier molecules (2, 23) and (ii) identifying noncapsular somatic antigens which have potential for vaccine development (7, 8, 10, 11, 18). Our research efforts have focused on evaluating the outer membrane proteins of *H. influenzae* type b in this capacity.

Previous work from this laboratory demonstrated that *H. influenzae* type b outer membrane proteins are immunogenic in both infant (11) and adult (9) rats. The latter study also included the finding that several of these outer membrane proteins are exposed on the cell surface of *H. influenzae* type b and accessible to antibody in this state. Subsequently, we showed that human infants convalescing from *H. influenzae* type b meningitis produced antibodies against cell surface-exposed outer membrane proteins of this pathogen (8). The production in this laboratory of monoclonal antibodies (mabs) directed against *H. influenzae* type b outer membrane proteins permitted a more precise examination of the potential of these proteins for vaccine development (22). A mab directed against a cell surface-exposed antigenic determinant of the 39,000-molecular-weight (39K) major outer membrane protein was shown to protect infant rats against experimental *H. influenzae* type b infection and was also able to eradicate established systemic *H. influenzae* type b disease (bacteremia) in these infant mammals (12). Most recently, we determined that *H. influenzae* type b growing in vivo in infected rats expresses most of the same outer membrane proteins synthesized by this bacterium growing in vitro in broth culture (P. A. Gulig and E. J. Hansen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, p. 62, D21). These studies were essential to determine which outer membrane proteins of *H. influenzae* type b are immunogenic in infant mammals, accessible on whole *H. influenzae* type b cells to potentially protective antibodies, and expressed by the bacterium growing in the infected host.

All of the preceding data suggested that the 39K outer membrane protein of *H. influenzae* type b may have considerable potential for vaccine development. However, for an immunogenic and cell surface-exposed protein to be considered as a vaccine candidate, the protein should be common to, or antigenically cross-reactive with, most or all strains of *H. influenzae* type b. Few data have been published concerning antigenic cross-reactivity among the outer membrane proteins of *H. influenzae* type b (18, 22). Preliminary experiments from this laboratory indicated that one mab directed against a single cell surface-exposed antigenic determinant of the 39K outer membrane protein reacted with four of six randomly selected *H. influenzae* type b strains (22). The availability of mabs specific for a single *H. influenzae* type b outer membrane protein made possible the precise analysis of antigenic cross-reactivity of the 39K outer membrane protein among many different strains of this pathogen.

In this report, we present data obtained from the use of these mabs to examine the antigenic characteristics of the 39K outer membrane proteins of 126 *H. influenzae* type b strains. These experiments established that a considerable amount of cross-reactivity exists among the cell surface-exposed antigenic determinants of the 39K outer membrane proteins and that at least one internal antigenic determinant of the 39K outer membrane protein is present in all *H. influenzae* type b strains.

MATERIALS AND METHODS

Bacterial strains. A total of 126 strains of *H. influenzae* type b were obtained from pediatric research centers throughout the United States. Fifty-four of these strains were obtained from the Dallas (Tex.) area and were kindly provided by George H. McCracken, Jr., Department of Pediatrics, Southwestern Medical School. The other 72 *H. influenzae* type b isolates, in groups of four to eight strains each, were generously supplied by the following individuals: Ram Yogev, Chicago, Ill.; Dan Granoff, St. Louis, Mo.; Barbara Robinson, Chapel Hill, N. C.: Robert Daum, New Orleans, La.; Michael Jacobs, Cleveland, Ohio; Mimi Glode, Denver, Colo.; William Feldman, Atlanta, Ga.; Melvin Marks, Oklahoma City, Okla.; Parvon Azimi, Oakland, Calif.; Kathryn Edwards, Nashville, Tenn.; and Harry Wright, Los Angeles, Calif.

The bacterial culture media (brain heart infusion broth supplemented with Levinthal's base [BHIs]) and growth conditions have been described elsewhere (11).

Preparation of outer membrane vesicles. Outer membrane vesicles were prepared by the treatment of whole *H. influenzae* type b cells with lithium chloride (LiCl) buffer as described elsewhere (8).

Immunization of mice for use in hybridoma production. Several different immunization protocols were used to produce immune spleen cells for three independent hybridization experiments. Mabs 6A2 and 12D9 were derived from immune spleen cells obtained from mice immunized with outer membrane vesicles of *H. influenzae* type b strain DL26 as described elsewhere (22). Mab 2F4 was derived from spleen cells obtained from a mouse immunized by the intraperitoneal injection of 10^7 CFU of strain DL43 suspended in pH 7.2 phosphate-buffered saline containing 0.1% (wt/vol) gelatin. Twenty-one days later, this mouse was given a second intraperitoneal injection of 10^7 CFU of strain DL43. Four days after the boost, the spleen was removed and processed by the standard hybridization protocol. Mabs 4C4, 5G8, and 11E4 were derived from a mouse which was immunized by the intraperitoneal injection of 10^7 CFU of *H. influenzae* type b strain DL42 as a primary immunization. This mouse was then injected intraperitoneally with 10^7 CFU at both 21 and 42 days after the primary immunization, and the spleen from this mouse was used in the hybridization procedure 4 days after the last injection of viable bacteria.

Construction and identification of hybridomas producing 39K outer membrane protein-specific mabs. The hybridomas were produced by fusing spleen cells from immunized mice with SP2/0 plasmacytoma cells exactly as described by Robertson et al. (22). The selection and growth of the resultant hybridomas were accom-

plished as described elsewhere (22). Hybridomas producing mabs directed against outer membrane antigens of the immunizing *H. influenzae* type b strain were identified by an enzyme-linked immunosorbent assay procedure (22). Culture supernatants from hybrid clones which scored as positive in the enzyme-linked immunosorbent assay were tested by a radioimmunoprecipitation (RIP) method for the presence of mabs directed against the 39K outer membrane protein as described elsewhere (22). The identification of those mabs directed against cell surface-exposed antigenic determinants of the 39K outer membrane protein was accomplished by published methods, as was the identification of antibody isotype (22).

RIP methods. The presolubilized (PS) RIP system used for detecting antibodies directed against both internal and cell surface-exposed antigenic determinants of *H. influenzae* type b outer membrane proteins has been described in detail elsewhere (8). The PS RIP system was also utilized in conjunction with antibody adsorption experiments designed to investigate the cell surface exposure of selected antigenic determinants of the 39K outer membrane protein. Hybridoma culture supernatants (500 μl) containing mabs were adsorbed twice at 4°C for 1.5 h with washed, packed cells from 10 ml of an overnight broth culture of the appropriate *H. influenzae* type b strain. The adsorbed hybridoma culture supernatants were then assayed for the presence or absence of mabs by means of the PS RIP system.

SDS-PAGE and autoradiography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of both protein samples and immune precipitates and autoradiography of the resultant gels were performed as described elsewhere (11).

Colony blot-RIA. The colony blot-radioimmunoassay (RIA) employed for the rapid screening of *H. influenzae* type b strains for the expression of an antigenic determinant recognized by an individual mab was a modification of the method developed by Henning et al. (14). *H. influenzae* type b strains were grown overnight on BHIs agar plates, and a small amount of colony material from each strain was transferred onto filter paper (Whatman no. 40) with wooden applicator sticks. The filter paper was then dried at 37°C for 1 h. All of the following operations were performed at 4°C with a gentle rocking motion unless otherwise specified. The filter paper was soaked in blotting buffer (BB; phosphate-buffered saline [pH 7.2] containing 1% [vol/vol] normal rabbit serum, 1 mM sodium iodide, and 0.02% [wt/vol] sodium azide) for 1 h to block nonspecific protein-binding sites. The filter paper was next soaked for 2 h in 20 ml of a 10% (vol/vol) solution of hybridoma culture supernatant in BB. During this incubation period, antibodies specific for antigens present in the bacterial colony paste attach to their respective antigens. Unattached mabs were then removed from the filter paper by four consecutive 30-min washes with 20 ml of BB. Mabs specifically bound to the *H. influenzae* type b colony material on the filter paper were detected by incubating the filter paper overnight in 20 ml of BB containing 10^6 cpm of affinity-purified rabbit anti-mouse immunoglobulin (specific activity, 2×10^7 cpm/μg protein) which had been radioiodinated by the chloramine-T method (16). The filter paper was then washed four times as before, dried, and exposed to X-ray film (Fuji RX safety film; Fuji Industries, Tokyo, Japan) for autoradiography. *H. influenzae* type b strains which reacted with a specific mab were readily identified by the resultant dark spots on the autoradiograph corresponding to the position of the colony material on the filter paper. Negative control experiments involving the use of SP2/0 culture supernatants or an irrelevant mab directed against *Haemophilus ducreyi* in place of *H. influenzae* type b-specific mabs resulted in no detectable binding of ^{125}I-labeled rabbit anti-mouse immunoglobulin to the *H. influenzae* type b colony material.

SDS-mediated extraction of *H. influenzae* type b cell proteins. The estimation of the apparent molecular weights of the major outer membrane proteins was accomplished by solubilizing *H. influenzae* type b cells in SDS and resolving the resultant protein solutions by SDS-PAGE. A *H. influenzae* type b colony was suspended in digestion buffer (0.0625 M Tris-hydrochloride [pH 6.8] containing 2% [wt/vol] SDS, 10% glycerol, and 5% [vol/vol] 2-mercaptoethanol) and heated at 100°C for 5 min. Insoluble debris was removed by centrifugation at 12,000 × *g* for 2 min, and the proteins present in the resultant supernatant were resolved by SDS-PAGE and visualized by staining with Coomassie brilliant blue.

RESULTS

Characterization of mabs specific for the 39K outer membrane protein. Lymphocyte hybridomas producing mabs 6A2 and 12D9, which are directed against cell surface-exposed antigenic determinants of the 39K outer membrane protein of strain DL26, were isolated from mice immunized with outer membranes of strain DL26 as described elsewhere (22). Preliminary experiments showed that mabs 6A2 and 12D9 did not react with two other *H. influenzae* type b strains, DL42 and DL43, which are clinical isolates routinely used in this laboratory. Therefore, lymphocyte hybridomas producing mabs directed against the 39K outer membrane proteins of these two strains were isolated by using spleen cells from mice immunized by multiple intraperitoneal injections with 10^7 CFU of these strains. Six different mabs were selected for use in the molecular antigenic analysis of the 39K outer membrane protein (see above). All six of these immunoglobulin G mabs reacted with the 39K outer membrane proteins of their homologous immunizing strains in the PS RIP system.

To determine whether a mab was directed against an antigenic determinant of the 39K outer membrane protein that was both exposed on the cell surface of intact *H. influenzae* type b cells and accessible to antibody, we exhaustively adsorbed each hybridoma culture supernatant with whole cells of the homologous *H. influenzae* type b strain before the use of the hybridoma supernatant in the PS RIP system. Simultaneously, the cross-reactivity of each mab was examined by testing unadsorbed hybridoma cul-

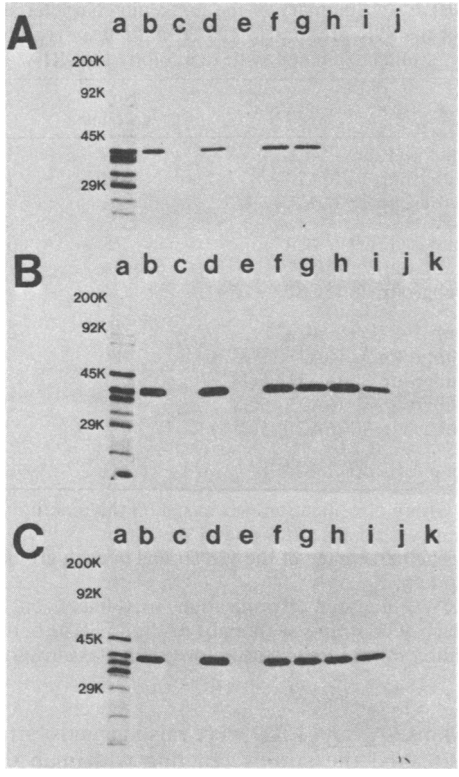

FIG. 1. PS RIP analysis of mabs specific for the 39K outer membrane protein. *H. influenzae* type b strains DL26, DL42, and DL43 were employed as antigen. To determine the relative cell surface exposure of an antigenic determinant, we adsorbed each hybridoma culture supernatant with intact bacteria of each *H. influenzae* type b strain to remove mab directed against a cell surface-exposed antigenic determinant before use of this hybridoma culture supernatant in the PS RIP system. Lane a of each figure does not contain an immune precipitate; instead, it contains a sample of the solubilized cell preparation containing radiolabeled outer membrane proteins that was employed as antigen in RIP analysis. (A) RIP analysis of mabs using strain DL26 as antigen. Lane b, mab 6A2; lane c, mab 6A2 (adsorbed); lane d, mab 12D9; lane e, mab 12D9 (adsorbed); lane f, mab 2F4; lane g, mab 2F4 (adsorbed); lane h, mab 5G8; lane i, mab 4C4; and lane j, mab 11E4. (B and C) RIP analysis of mabs using strains DL42 and DL43, respectively, as antigen. Lane b, mab 5G8; lane c, mab 5G8 (adsorbed); lane d, mab 4C4; lane e, mab 4C4 (adsorbed); lane f, mab 2F4; lane g, mab 2F4 (adsorbed); lane h, mab 11E4; lane i, mab 11E4 (adsorbed); lane j, mab 6A2; and lane k, mab 12D9.

ture supernatants in the PS RIP system with solubilized radioiodinated cells of all three immunizing strains.

Mabs 6A2 and 12D9 both reacted only with the 39K outer membrane protein of strain DL26 (Fig. 1). These two mabs were shown to be directed against cell surface-exposed antigenic determinant(s) of this protein, as evidenced by the complete adsorption of antibody activity from these two hybridoma culture supernatants by whole cells of strain DL26. Mabs 4C4, 5G8, and 11E4 reacted with the 39K outer membrane proteins from both strains DL42 and DL43, but not with the 39K protein from strain DL26 (Fig. 1). Mabs 4C4 and 5G8 were shown to be directed against cell surface-exposed determinant(s) of the 39K outer membrane proteins in strains DL42 and DL43. Mab 11E4, which was not adsorbed by whole cells of either strain DL42 or DL43, was apparently directed against an antigenic determinant of the 39K outer membrane protein that is not exposed on the cell surface or is otherwise inaccessible to antibody on whole cells. Finally, mab 2F4 reacted with all three *H. influenzae* type b strains and, like mab 11E4, was shown to be directed against an antigenic determinant of the 39K outer membrane protein that is not exposed on the cell surface of these strains or is otherwise inaccessible to antibody on whole cells (Fig. 1). Therefore, we will refer to the antigenic determinants recognized by mabs 2F4 and 11E4 as internal antigenic determinants to distinguish them from the cell surface-exposed antigenic determinants recognized by mabs 6A2, 12D9, 4C4, and 5G8. These data are summarized in Table 1.

Strain distribution of selected antigenic determinants of the 39K outer membrane protein. The identification of protein antigenic determinants which are common to, or cross-reactive with, most or all *H. influenzae* type b strains is fundamental to the selection of potential vaccine candidates from the cell surface-exposed proteins present in the outer membrane of this organism. The strain distribution of the antigenic determinants recognized by the six anti-39K mabs described above was determined by the use of the colony blot-RIA, together with 126 disease isolates of *H. influenzae* type b collected

TABLE 1. Cell surface exposure of the antigenic determinants defined by anti-39K mabs on the immunizing strains of *H. influenzae* type b

H. influenzae type b strain	Reactivity in RIP analysis of mabs:					
	12D9	6A2	4C4	5G8	11E4	2F4
DL26	CSE[a]	CSE	—	—	—	INT
DL42	—[b]	—	CSE	CSE	INT[c]	INT
DL43	—	—	CSE	CSE	INT	INT

[a] CSE, Positive reaction of a mab with a cell surface-exposed antigenic determinant of the 39K protein (mab was totally adsorbed by intact bacteria).

[b] –, Negative reaction (mab did not recognize the 39K protein of this strain).

[c] INT, Positive reaction of a mab with an internal antigenic determinant of the 39K protein (mab was not adsorbed by intact bacteria).

from 12 pediatric research centers throughout the United States (Fig. 2).

The reactivities of these strains with mabs 12D9 and 4C4, which both recognize cell surface-exposed antigenic determinants of the 39K outer membrane protein, allowed the division of the 126 *H. influenzae* type b strains into four basic antigenic groups (Table 2). Group 1 strains, as exemplified by strain DL26, reacted with mab 12D9 but not mab 4C4. Group 1 strains comprised 13.5% of the strains tested with the colony blot-RIA. Group 2 strains, as exemplified by strains DL42 and DL43, reacted with mab 4C4 but not mab 12D9. Group 2 strains were by far the most prevalent, comprising 61.9% of the strains tested. Group 3 strains reacted with both mabs 12D9 and 4C4 and represented 23.8% of the strains. Finally, group 4 strains, of which there was only one, did not react with either mab 12D9 or 4C4. This latter group comprised 0.8% of the strains tested with the colony blot-RIA.

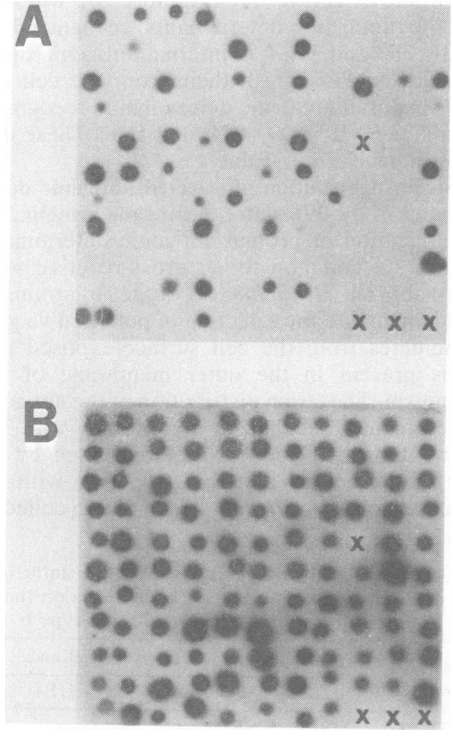

FIG. 2. Evaluation of the reactivities of two outer membrane protein-specific mabs with 117 *H. influenzae* type b strains using the colony blot-RIA procedure. Each of the two autoradiographs depicts an experiment in which 117 *H. influenzae* type b strains were examined for reactivity with a single mab. (A) Mab 12D9. (B) Mab 2F4. Locations on the filter pads that did not receive bacterial growth are labeled with a X. All of the readily visible colony spots on these autoradiographs were scored as positive reactions.

TABLE 2. Summary of the antigenic characteristics of the 39K proteins of 126 *H. influenzae* type b strains examined with the colony blot-RIA

Antigenic classification[a]	No. of strains	%[b]
Group 1 ($12D9^+$, $4C4^-$)	17	13.5
Subgroup 1a ($6A2^+$)[c]	14	11.1
Subgroup 1b ($6A2^-$)	3	2.4
Group 2 ($12D9^-$, $4C4^+$)	78	61.9
Subgroup 2a ($5G8^+$)	74	58.7
Subgroup 2b ($5G8^-$)	4	3.2
Group 3 ($12D9^+$, $4C4^+$)	30	23.8
Subgroup 3a ($6A2^+$, $5G8^+$)	11	8.7
Subgroup 3b ($6A2^-$, $5G8^+$)	4	3.2
Subgroup 3c ($6A2^+$, $5G8^-$)	13	10.3
Subgroup 3d ($6A2^-$, $5G8^-$)	2	1.6
Group 4 ($12D9^-$, $4C4^-$)	1	0.8

[a] Group classification was based on the reactivity of strains with mabs 12D9 and 4C4.

[b] The percentage of the 126 strains in each group or subgroup.

[c] The subgroup classification was based on the reactivity of strains with mabs 6A2 or 5G8 or both in addition to those mabs used for group classification.

Mabs 6A2 and 12D9 were raised against strain DL26, and the strains reacting with mab 6A2 were a subset of those which reacted with mab 12D9. Mabs 5G8, 4C4, and 11E4 were raised against strain DL42. The strains recognized by mab 5G8 were a subset of those reacting with mab 4C4. Mab 11E4, which recognizes an internal antigenic determinant of the 39K outer membrane protein in this strain, had a reactivity pattern virtually identical to that exhibited by mab 4C4, which recognizes a cell surface-exposed antigenic determinant of this protein.

Using the reactivities of these *H. influenzae* type b strains with the other two mabs that recognize cell surface-exposed antigenic determinants of the 39K outer membrane protein (mabs 6A2 and 5G8), we could further divide the four groups into smaller subgroups (Table 2). Group 1 was divided into subgroup 1a, which reacted with both mabs 12D9 and 6A2, and subgroup 1b, which reacted with only mab 12D9. Group 2 was similarly divided into subgroup 2a, which reacted with mabs 4C4 and 5G8, and subgroup 2b, which reacted with only mab 4C4. Because group 3 strains reacted with both mabs 12D9 and 4C4, its subgroup classification scheme is somewhat more complicated. Subgroup 3a strains reacted with all four mabs recognizing cell surface-exposed antigenic determinants of the 39K outer membrane protein (12D9, 4C4, 6A2, and 5G8). Subgroup 3b strains lack reactivity with mab 6A2, whereas subgroup 3c strains lack reactivity with mab 5G8, and

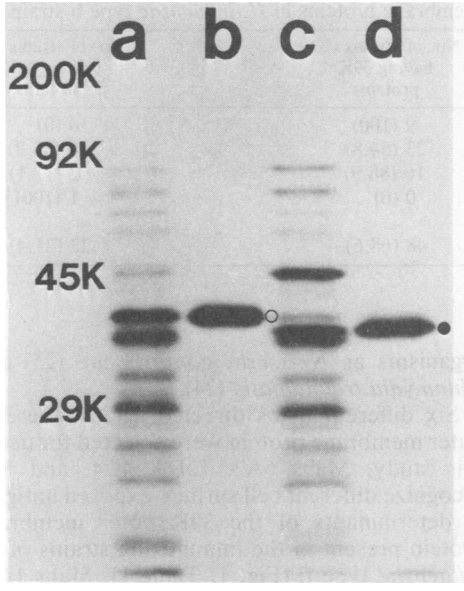

FIG. 3. PS RIP analysis demonstrating the presence of antigenically related 38K and 39K outer membrane proteins in different *H. influenzae* type b strains. Lanes a and c contain the solubilized preparations of radioiodinated cells of *H. influenzae* type b strains CL104 and NA105, respectively, that were employed as antigen in the PS RIP system. It can be seen that these preparations contain a number of different radioiodinated outer membrane proteins. Lanes b and d contain the radiolabeled outer membrane proteins present in the immune precipitates obtained by immunoprecipitating the solubilized preparations of strains CL104 and NA105, respectively, with mab 2F4. In lane b, mab 2F4 immunoprecipitated a 39K protein (○) from strain CL104, whereas in lane d, mab 2F4 immunoprecipitated a 38K protein (●) from strain NA105.

subgroup 3d strains lack reactivity with mabs 6A2 and 5G8.

Finally, mab 2F4, which was raised against strain DL43 and is also directed against an internal antigenic determinant of the 39K outer membrane protein of this strain, reacted with every *H. influenzae* type b strain tested in this study (Fig. 2). Similarly, mab 16C2, which has been described previously and recognizes an internal determinant of the 37K outer membrane protein of *H. influenzae* type b strain DL26 (21), also reacted with all 126 strains examined with the colony blot-RIA (data not shown).

Molecular weight variation in the 39K outer membrane protein. To confirm that the anti-39K mabs were reacting with their respective outer membrane protein antigens in the colony blot-RIA, nine different *H. influenzae* type b strains from the 126 strains examined with the colony blot-RIA were radioiodinated and used in the PS RIP system with each of the six mabs. These strains included both those which gave very dark spots and those which gave only light spots in the colony blot-RIA. Autoradiographic analysis of the resultant immune precipitates showed that mabs directed against the 39K outer membrane proteins of strains DL26, DL42, and DL43 immunoprecipitated a 38K and not a 39K outer membrane protein from certain *H. influenzae* type b strains (Fig. 3). However, in all nine strains, either a 39K or 38K outer membrane protein was always immunoprecipitated by those mabs which reacted in the colony blot-RIA. This result prompted a survey of several strains from each of the four antigenic groups defined above to determine the relative distributions of 38K and 39K outer membrane proteins. Initial experiments which employed LiCl extraction of outer membrane vesicles and SDS-PAGE analysis showed that each strain possessed either a 38K or a 39K major outer membrane protein. Subsequently, over half of the 126 *H. influenzae* type b strains examined in the colony blot-RIA were surveyed for their outer mem-

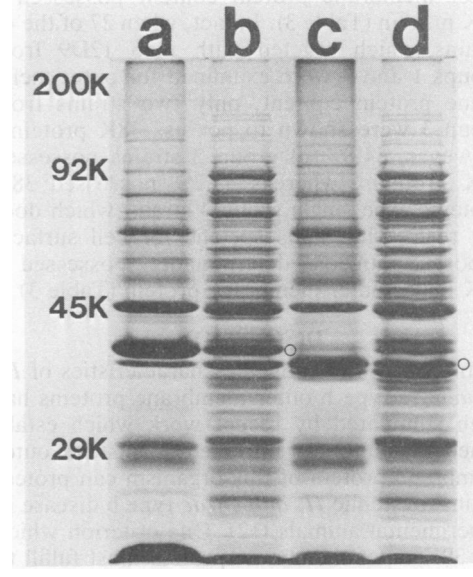

FIG. 4. Comparison by SDS-PAGE of the protein-containing preparations obtained from *H. influenzae* type b cells by the LiCl and SDS extraction procedures. Lanes a and c contain outer membrane proteins present in LiCl extracts of strains DL26 (possessing a 39K major outer membrane protein) and AT100 (possessing a 38K major outer membrane protein), respectively. Lanes b and d contain the proteins present in SDS extracts of strains DL26 and AT100, respectively. This figure shows that the SDS extraction procedure can be employed to characterize the molecular weights of the 38K/39K major outer membrane proteins (○ in lanes b and d).

TABLE 3. Distribution of 38K and 39K major outer membrane proteins in *H. influenzae* type b strains

Antigenic group	No. of strains examined	No. of strains (%) having 39K proteins	No. of strains (%) having 38K proteins
1	9	9 (100)	0 (0)
2	42	23 (54.8)	19 (45.2)
3	18	16 (88.9)	2 (11.1)
4	1	0 (0)	1 (100)
Total		48 (68.6)	22 (31.4)

brane protein content by analyzing SDS-solubilized extracts of whole bacterial cells by SDS-PAGE. Although this solubilization procedure obviously extracts many different proteins from the *H. influenzae* type b cell, the major outer membrane proteins in the molecular weight range of 38,000 to 39,000 could be readily identified after SDS-PAGE analysis of these extracts (Fig. 4). Of the strains examined with the SDS extraction procedure, 68.6% possessed a 39K major outer membrane protein, whereas 31.4% possessed a 38K major outer membrane protein.

All of the group 1 strains examined for major outer membrane protein content possessed a 39K protein (Table 3). In fact, when 27 of the 47 strains which reacted with mab 12D9 from groups 1 and 3 were examined for outer membrane protein content, only two strains from group 3 were shown to possess 38K proteins. However, 54.8% of group 2 strains possessed 39K proteins, whereas 45.2% possessed 38K proteins. The single group 4 strain, which does not react with mabs specific for cell surface-exposed antigenic determinants, possessed a 38K major outer membrane protein (Table 3).

DISCUSSION

Interest in the antigenic characteristics of *H. influenzae* type b outer membrane proteins has been stimulated by recent work which established that antibody directed against an outer membrane protein of this organism can protect against systemic *H. influenzae* type b disease in experimental animals (12). One criterion which the 39K outer membrane protein must fulfill to be considered as a vaccine candidate is that this protein must be common to, or antigenically cross-reactive with, most or all strains of *H. influenzae* type b. Analysis of the antigenic composition of the 39K outer membrane protein among many *H. influenzae* type b strains necessitated the use of murine mabs which were specific for this protein and nonreactive with other antigenic components of this organism. The utility of mabs in the antigenic classification of microorganisms has been recently demonstrated by other workers, using such diverse organisms as *Neisseria gonorrhoeae* (25) and *Chlamydia trachomatis* (24).

Six different mabs directed against the 39K outer membrane protein were selected for use in this study. Mabs 6A2, 12D9, 4C4, and 5G8 recognize different cell surface-exposed antigenic determinants of the 39K outer membrane protein present in the immunizing strains of *H. influenzae* type b (Fig. 1, Table 1). Mabs 11E4 and 2F4 react with antigenic determinants of this protein that are not accessible to these antibodies on intact bacteria. These latter internal antigenic determinants may actually be buried within the outer membrane, or they may be exposed on the cell surface in such a way as to be inaccessible to their corresponding mabs due to steric hindrance by other cell surface macromolecules (e.g., proteins or lipopolysaccharide). The solubilization of whole *H. influenzae* type b cells in a detergent-EDTA solution permitted mabs 11E4 and 2F4 to react readily with the 39K outer membrane protein (Fig. 1). The fact that these mabs recognizing internal antigenic determinants reacted in the colony blot-RIA indicates that either the colony material contains a significant number of lysed cells or that drying the bacterial growth on the filter pads results in membrane disruption.

The use of the colony blot-RIA facilitated the rapid screening of 126 *H. influenzae* type b strains for their reactivities with this panel of six different mabs. In view of the importance for vaccine development of identifying antigenically common or cross-reactive call surface-exposed proteins, the primary emphasis for classification purposes was placed on the reactivity of each strain with those mabs directed against cell surface-exposed antigenic determinants of the 39K outer membrane protein. Accordingly, this large number of *H. influenzae* type b strains collected from pediatric research centers throughout the country was divided into four basic groups based on the reactivity of each strain with mabs 12D9 and 4C4 (Table 2). Group 1 strains reacted only with mab 12D9, whereas group 2 strains reacted only with mab 4C4. Group 3 strains reacted with both mabs, and

group 4 strains, of which there was only one, reacted with neither of these mabs. Further subdivision of groups 1, 2, and 3 was accomplished by examining the reactivities of the members of each group with mabs 6A2 and 5G8. A total of eight different subgroups was identified in this manner.

We also found that 68.6% of the *H. influenzae* type b strains studied possessed 39K major outer membrane proteins, whereas 31.4% possessed 38K major outer membrane proteins. These data confirm previous observations on the variable molecular weight of this particular major outer membrane protein among different strains of this pathogen (17). Although no definite relationships were found between the molecular weight of the 38K/39K outer membrane protein and mab reactivity, one consistent finding was that none of the group 1 strains examined in our study possessed 38K proteins. In addition, preliminary results from this laboratory indicate that there is no strict correlation between the outer membrane protein subtyping scheme developed by Barenkamp et al. (3) and our antigenic grouping system. However, the use of the protein subtyping scheme in conjunction with these 39K protein-specific mabs may yield a very effective epidemiological classification system with increased sensitivity and specificity.

A limitation to the use of mabs in antigenic analysis is that only the antigenic determinants defined by the mabs can be analyzed, as opposed to those antigenic determinants recognized by conventional antisera, which may possess antibody activity against a more varied spectrum of antigenic determinants on a protein molecule. Therefore, the antigenic characterization presented in this report may represent the minimum degree of cross-reactivity between 39K proteins, because other antigenic determinants of the 39K outer membrane protein may exist that are cell surface exposed and more highly cross-reactive among *H. influenzae* type b strains than those defined by mabs 12D9 and 4C4. However, this is probably not the case, because essentially all of 30 different mabs derived from five different hybridization experiments that were characterized as reacting with cell surface-exposed antigenic determinants of the 39K outer membrane proteins of strains DL26 and DL42 had reactivity patterns identical to those of mabs 12D9, 6A2, 4C4, or 5G8 in preliminary experiments (data not shown).

The limited heterogeneity of cell surface-exposed antigenic determinants on the 39K outer membrane protein demonstrated in this report reinforces the validity of evaluating this protein as a vaccine candidate. Mab 4C4, characterized as recognizing a cell surface-exposed antigenic determinant on its homologous *H. influenzae* type b strain (DL42), reacted with 85.7% of the 126 strains tested in this study. Mab 12D9, which also was characterized as recognizing a cell surface-exposed antigenic determinant of the 39K outer membrane protein in a different strain (DL26), reacted with 37.3% of the strains tested. Only one strain was found not to react with either of these two cell surface-reactive mabs. These data indicate that a purified 39K protein(s) from one strain belonging to group 3 that possesses antigenic determinants which react with both of these mabs could possibly be sufficient to induce protective humoral immunity against essentially all *H. influenzae* type b strains.

That there appear to be two major sets of cell surface-exposed antigenic determinants on the 39K outer membrane protein (group 1 strains versus group 2 strains) and the fact that these two antigenic sets can be expressed simultaneously by group 3 strains raise an important question: are the group 1 and group 2 antigenic determinants in the group 3 strains expressed on the same molecule or on two different molecules? This question is currently under investigation by both immunochemical and genetic techniques.

Finally, it appears that at least one internal antigenic determinant of the 39K protein is highly conserved, because mab 2F4, which recognizes an internal antigenic determinant of this protein, is reactive with all *H. influenzae* type b strains examined to date. Whether the antigenic determinants defined by mab 2F4 and the other mabs used in this study cross-react with other *H. influenzae* serotypes or with other *Haemophilus* species is currently under investigation. It is of interest to note that a recent report of antigenic cross-reactivity between other non-39K outer membrane proteins of different *H. influenzae* type b strains was based on data derived from experimental techniques which did not differentiate between antibodies directed against internal antigenic determinants and antibodies specific for cell surface-exposed antigenic determinants of the same outer membrane protein (18). That internal segments of bacterial outer membrane proteins may be highly conserved within a given species has been indicated by peptide mapping studies involving outer membrane proteins of both *Neisseria gonorrhoeae* (13) and *Neisseria meningitidis* (26). Similarly, the high degree of antigenic cross-reactivity reported to exist among outer membrane proteins of the enteric bacteria may be due to shared internal antigenic determinants of these proteins (15). It must be emphasized that the precise evaluation of the antigenic characteristics of the cell surface-exposed proteins of *H.*

influenzae type b is a prerequisite for the selection of potential vaccine candidates from the outer membrane proteins of this pathogen.

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