INFECTION AND IMMUNITY, Nov. 1989, p. 3270–3275
0019-9567/89/113270-06$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 57, No. 11

Structural and Antigenic Conservation of the P2 Porin Protein among Strains of *Haemophilus influenzae* Type b

ERIC J. HANSEN,[1*] SHARON E. PELZEL,[1] KIM ORTH,[2†] CAROLYN R. MOOMAW,[2] JUSTIN D. RADOLF,[1,3] AND CLIVE A. SLAUGHTER[2]

Department of Microbiology,[1] Howard Hughes Medical Institute,[2] and Department of Internal Medicine,[3] University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235

Received 24 April 1989/Accepted 23 July 1989

The P2 porin protein is the most abundant protein in the outer membrane of *Haemophilus influenzae* type b (Hib). Biochemical and immunochemical techniques were used to characterize the P2 proteins from a number of different Hib strains. P2 proteins from Hib outer membrane vesicles were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose for in situ tryptic digestion. Solid-phase tryptic digests of P2 from eight Hib strains were resolved by high-pressure liquid chromatography and shown to be similar if not identical. Radioimmunoprecipitation analysis involving Hib cells (containing intrinsically radiolabeled proteins or lipooligosaccharide) and Western blot (immunoblot) analysis were used to identify two P2-specific murine monoclonal antibodies (MAbs). These MAbs were shown to be reactive with 120 Hib strains tested in a colony blot radioimmunoassay. One of these MAbs bound to a surface-exposed P2 epitope that was antibody accessible on all Hib strains tested; the other MAb was directed against a P2 epitope that either was not exposed on the cell surface or was otherwise inaccessible to antibody.

Haemophilus influenzae type b (Hib) is a frequent cause of meningitis in the United States (6). Efforts to develop an efficacious vaccine to prevent systemic Hib disease in infants and young children have been focused primarily on the type b capsular polysaccharide, and new vaccines composed of Hib capsular polysaccharide covalently coupled to protein carrier molecules have produced encouraging results in recent field trials (5, 41). In addition, noncapsular surface antigens of this pathogen are being investigated for their vaccine potential, and at least four different outer membrane proteins of this organism have been shown to be targets for antibodies protective against experimental Hib disease (10, 12, 13, 24, 25, 32, 33).

The P2 major outer membrane protein of Hib represents one of the four protein antigens described above. This surface-exposed protein functions as a porin in Hib (36, 37) and also represents the most abundant protein in the outer membrane of this pathogen, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Hib outer membrane preparations (2, 26, 33, 38). Purified P2 has been shown to induce the synthesis of antibodies protective against experimental Hib disease (33); this earlier study also indicated that some degree of antigenic heterogeneity existed among the P2 proteins of different Hib strains. The gene encoding this porin protein has been cloned from two different isolates of this pathogen (21, 31). Nucleotide sequence analysis has revealed that the P2 structural genes from both strains are identical (22, 31). The Hib P2 protein has an unusually high content of strongly basic amino acids relative to the OmpF porin of *Escherichia coli* (30) and the P.I porin of *Neisseria gonorrhoeae* (4, 11), although its hydrophilicity profile is very similar to those of these other porins (4).

If the P2 protein is to be a practical Hib vaccine candidate, then at least some of the surface-exposed epitopes of this protein must be common to most if not all strains of Hib. The purpose of this study was to investigate the biochemical and antigenic properties of P2 from different Hib strains.

MATERIALS AND METHODS

Bacterial strains and culture media. The 120 Hib strains used in this study have been described previously (15, 17). Bacterial culture media (brain heart infusion [Difco Laboratories, Detroit, Mich.] supplemented with Levinthal base [20]) and growth conditions have been described previously (20).

SDS-PAGE. Outer membrane vesicles were used as the source of P2 for all biochemical analyses and were extracted from Hib cells as described elsewhere (16, 29). Outer membrane vesicles were solubilized in digestion buffer (20), and the solubilized proteins were subjected to SDS-PAGE as described elsewhere (20) for subsequent staining with Coomassie blue or for Western blot (immunoblot) analysis.

N-terminal amino acid sequence analysis of P2. Samples of Hib outer membrane vesicles were subjected to SDS-PAGE by the method of Hunkapiller et al. (23). Approximately 200 pmol of P2 protein was resolved by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane by the method of Matsudaira (28). N-terminal amino acid sequencing was performed on an amino acid sequencer (model 470A; Applied Biosystems, Foster City, Calif.) coupled to an on-line high-pressure liquid chromatography (HPLC) (model 120A; Applied Biosystems) system.

Solid-phase tryptic mapping of P2. Approximately 300 pmol of outer membrane vesicle-derived P2 protein was transferred from a 10% (wt/vol) polyacrylamide separating gel to nitrocellulose paper for in situ tryptic digestion on the paper by the method of Abersold et al. (1). For each Hib P2 protein, 50% of the peptide mixture released during digestion was separated by reverse-phase HPLC (model 130A; Applied Biosystems) with a Brownlee RP300 C_8 column (2.1 by 100 mm). Separation was performed in 0.1% trifluoroacetic acid by using a gradient of 0 to 50% acetonitrile over a period of 120 min at a flow rate of 50 ml/min. Similarity of

* Corresponding author.
† Present address: Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235.

chromatographic profiles was verified by performing experiments in which 25% of each P2 digest was mixed separately with 25% of a digest of Hib DL42 P2 prepared as described above; the P2 digest from DL42 was used as an internal mobility standard in these experiments.

MAbs. The monoclonal antibodies (MAbs) used in this study were obtained by fusing spleen cells from appropriately immunized mice with SP2/0-Ag14 plasmacytoma cells, as described elsewhere (17). MAb 3E9 was derived from a mouse immunized by intraperitoneal injection with viable cells of Hib DL42. MAb 9F5 was derived from a mouse immunized by intraperitoneal injection with P2 protein purified from Hib DL42 by the method of Munson et al. (33). MAb 4C4 is directed against an epitope in the oligosaccharide region of Hib lipooligosaccharide (LOS) (17). MAbs in the form of tissue culture supernatant fluids were used unless otherwise specified.

Western blot analysis. Western blotting was performed as described elsewhere (24) by using tissue culture supernatant fluid containing MAb 9F5.

Radioimmunoprecipitation analysis. The presolubilized radioimmunoprecipitation system (14, 15, 19) was used to determine the antigenic specificity of certain MAbs. Hib DL42 cells in the logarithmic phase of growth were intrinsically radiolabeled by inoculation into brain heart infusion-Levinthal base containing 500 μCi of [^{3}H]leucine (Amersham Corp., Arlington Heights, Ill.) or 250 μCi of [^{3}H]galactose (Amersham) per ml followed by incubation for 3 h at 37°C with agitation. The cells were harvested at 4°C by centrifugation at 8,000 × g for 10 min and washed four times with cold phosphate-buffered saline (pH 7.2). The cells were then used in the presolubilized radioimmunoprecipitation system as described above, with MAbs 3E9, 9F5, and 4C4 as precipitating antibodies. Briefly, the radiolabeled Hib cells were solubilized in solubilization buffer (16, 19), and insoluble debris was removed by centrifugation. The resultant supernatant fluid containing solubilized outer membrane antigens was incubated with the individual MAb probes for 1 h. The resultant antigen-antibody complexes were precipitated by the addition of protein A-bearing staphylococci. These immune precipitates were resolved by SDS-PAGE and analyzed by fluorography (16).

Colony blot RIA. The colony blot radioimmunoassay (RIA) was performed as described elsewhere (14, 15, 17). The antigens used were the 120 Hib strains described above; the primary antibody probes were MAbs 3E9 and 9F5.

Immunoelectron microscopy. The protein A–colloidal-gold method of Geohegan and Ackerman (9) for visualizing antibodies bound to cell surfaces, as modified by Gonzales et al. (10), was used for immunoelectron microscopy.

Antibody accessibility RIA. The indirect antibody accessibility RIA was performed as described by Kimura et al. (24) by using MAbs 3E9 and 9F5. Briefly, whole Hib cells were incubated with MAbs, washed, and probed with radioiodinated goat anti-mouse immunoglobulin to detect MAbs bound to the Hib cell surface.

RESULTS

Selection of prototypic Hib strains for P2 analysis. Eight Hib strains were chosen for detailed biochemical and antigenic analyses of their P2 proteins. These eight strains were isolated in seven different cities in the United States and included all three antigenic types of Hib LOS (Table 1). These strains also express both basic types of P2 protein, as determined by the apparent molecular weight of P2 in SDS-PAGE (14, 15). Strains DL42, CH100, OA104, DV102, H234, and Madigan synthesized a P2 protein with an apparent molecular weight of approximately 37,000, whereas strains DL26 and SL103 expressed a P2 protein with an apparent molecular weight of approximately 38,000 (Fig. 1).

TABLE 1. Characteristics of Hib strains chosen for analysis of their P2 proteins

Bacterial strain	Geographic source	LOS antigenic group	Apparent mol wt of P2 protein
DL42	Dallas, Tex.	2	37,000
CH100	Chicago, Ill.	2	37,000
DL26	Dallas, Tex.	1	38,000
DV102	Denver, Colo.	1	37,000
H234	Huntsville, Ala.	2	37,000
Madigan	Boston, Mass.	3	37,000
OA104	Oakland, Calif.	3	37,000
SL103	St. Louis, Mo.	2	38,000

N-terminal amino acid sequence analysis. The sequence of the first 22 amino acids in the P2 protein of each strain was determined as described in Materials and Methods. The P2 proteins from all eight strains had the same N-terminal amino acid sequence: Ala-Val-Val-Tyr-Asn-Asn-Glu-Gly-Thr-Asn-Val-Glu-Leu-Gly-Gly-Arg-Leu-Ser-Ile-Ile-Ala-Glu. This amino acid sequence was identical to that deduced from the nucleotide sequence of the Hib gene encoding P2 (22, 31).

Solid-phase tryptic mapping. Structural comparisons of P2 proteins from the eight Hib strains were performed by peptide mapping with HPLC. At least three independent tryptic digests of each P2 protein were compared by reverse-phase chromatography. Figure 2 is a comparison of the tryptic maps of the P2 proteins from strains DL42 and DL26. The major features of the two chromatograms are identical. Experiments in which digests of the two proteins were mixed together and then subjected to chromatography further verified that the major peaks had indistinguishable retention times (data not shown). Small quantitative differences in the relative sizes of some peaks, most notably peaks B and C, can be seen in Fig. 2. However, similar quantitative varia-

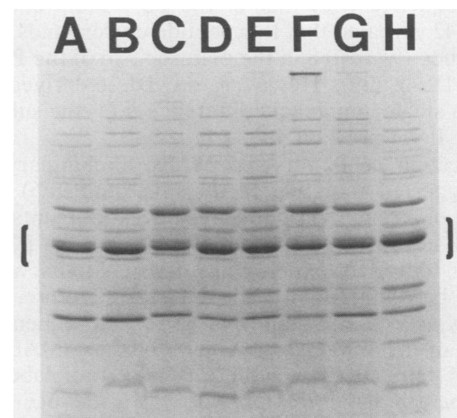

FIG. 1. Analysis of the protein content of outer membrane vesicles from different Hib strains by SDS-PAGE and Coomassie blue staining. Each lane contains 40 μg of protein; samples were not reduced before SDS-PAGE. Lanes: A, strain DL42; B, CH100; C, DL26; D, DV102; E, H234; F, Madigan; G, OA104; H, SL103. Brackets indicate the region containing the P2 protein, which is the most abundant protein in the outer membrane.

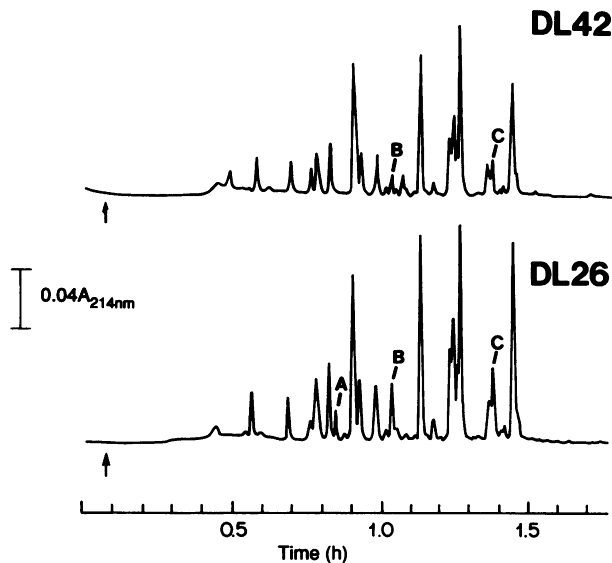

FIG. 2. Tryptic HPLC maps of peptides from the P2 proteins of Hib DL42 and DL26. Peaks B and C represent strain-independent quantitative variations between individual digests, whereas peak A represents a strain-specific peptide peculiar to Hib DL26. Arrows indicate the times of injection of the samples into the HPLC system.

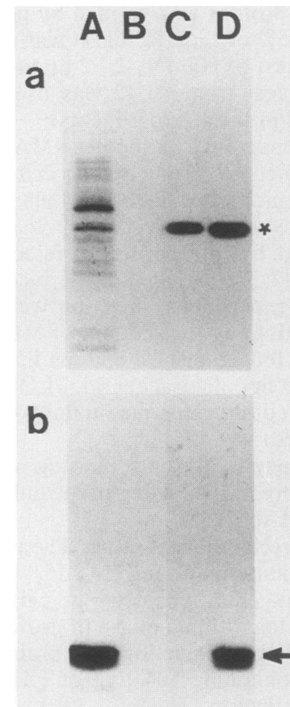

FIG. 3. Radioimmunoprecipitation analysis of the specificity of MAbs 3E9 and 9F5. Cells of Hib DL42 were intrinsically radiolabeled with [^{3}H]leucine (a) or [^{3}H]galactose (b). The radiolabeled cells were solubilized and used in the presolubilized radioimmunoprecipitation system with MAb 9F5 (lane B), MAb 3E9 (lane C), and MAb 4C4 (lane D). Lane A contains the solubilized, radiolabeled Hib cells used as a standard. All samples were resolved by SDS-PAGE and analyzed by fluorography. The asterisk indicates the position of the P2 protein; the arrow indicates the position of Hib LOS in this SDS-PAGE system.

tion between replicate digests of the two proteins was also observed and therefore provided no evidence for amino acid sequence differences. The tryptic peptide maps of the P2 proteins of the other six Hib strains were identical to that of Hib DL42 (Fig. 2).

Only one example of a qualitative strain-specific difference in chromatographic patterns was identified (Fig. 2, peak A). Peak A was invariably present in digests of P2 from strain DL26 but was absent from the P2 digests from the other seven strains. This peptide was isolated by reverse-phase chromatography under conditions for separation identical to those employed for peptide mapping; the peptide was then subjected to automated N-terminal sequencing. This sequence was determined to be Ile-Gly-Glu-Val-Asn-Asn-Gly-Ile-Gln-Val-Gly-Ala-Lys. A comparison of this strain DL26-derived sequence with that of the P2 protein encoded by strain DL42 revealed that this peptide corresponds to amino acid residues 175 to 188 in the mature form of the P2 protein of strain DL42 (22). However, the DL26-derived peptide contains a single amino acid difference, i.e., the substitution of Val for Ile at position 4.

Identification of P2-directed MAbs. Preliminary experiments suggested that two MAbs (3E9 and 9F5) obtained from two different hybridoma fusion experiments yielding nearly 400 new MAbs were directed against the P2 protein. In colony blot RIA experiments, these MAbs were both shown to be reactive with Hib DL42. Therefore, this Hib strain was used as the source of P2 for subsequent characterization of the antigenic specificity of these MAbs.

Cells of Hib DL42 were intrinsically radiolabeled with [^{3}H]leucine (to label P2) or with [^{3}H]galactose (to label LOS). These radiolabeled cells were then solubilized and used in the presolubilized radioimmunoprecipitation system together with MAbs 9F5 and 3E9. The cells radiolabeled with [^{3}H]leucine contained many different labeled proteins (Fig. 3a, lane A), whereas the cells grown in the presence of [^{3}H]galactose contained radiolabeled LOS (Fig. 3b, lane A). MAb 4C4, directed against an epitope in Hib DL42 LOS, was included in this radioimmunoprecipitation experiment as a positive control for immunoprecipitation of both P2 and LOS (Fig. 3, lane D). MAbs to Hib LOS will immunoprecipitate P2 from solubilized cells because P2 avidly binds to the Hib LOS molecule (15). MAb 3E9 precipitated radiolabeled P2 protein (Fig. 3a, lane C), whereas MAb 9F5 did not precipitate any detectable P2 protein (Fig. 3a, lane B). Radiolabeled P2 was also immunoprecipitated by MAb 4C4 (Fig. 3a, lane D). In contrast, only MAb 4C4 immunoprecipitated radiolabeled LOS (Fig. 3b, lane D); neither of the other MAbs precipitated detectable amounts of LOS.

Isotype analysis revealed that MAb 3E9 was an immunoglobulin G3 antibody, while MAb 9F5 was found to be an immunoglobulin G1 antibody. Accordingly, the negative result obtained with MAb 9F5 in the presolubilized radioimmunoprecipitation system was apparently due to the inability of this latter subclass of murine antibody to bind readily to the protein A immunoadsorbent used in the presolubilized radioimmunoprecipitation system (8). Therefore, Western blot analysis was used to confirm the specificity of MAb 9F5 for the Hib P2 protein. Proteins in outer membrane vesicles of the eight prototypic Hib strains were solubilized, resolved by SDS-PAGE, and transferred to nitrocellulose. MAb 9F5 bound to the P2 proteins of all eight Hib strains in Western blot analysis, whereas MAb 3E9 failed to bind to P2 in Western blot analysis (data not shown).

Cell surface exposure of P2 epitopes. The P2-directed MAbs 3E9 and 9F5 were used in the indirect antibody

accessibility RIA to determine whether these MAbs were directed against epitopes exposed on the Hib cell surface. Each MAb was used with all eight prototypic Hib strains in this assay. MAb 3E9 bound to the surface of all eight Hib strains (1,523 to 6,372 cpm of radioiodinated goat anti-mouse immunoglobulin bound per 10^8 Hib cells), whereas MAb 9F5 did not bind to the surface of any strain (62 to 97 cpm of radioiodinated goat anti-mouse immunoglobulin bound per 10^8 Hib cells).

The specificity of Mab 3E9 for a surface-exposed epitope of the P2 protein was confirmed by the use of immunoelectron microscopy. Whole cells of Hib DL42 were incubated with MAbs 3E9, 9F5, and 4C4, followed by the addition of protein A–colloidal-gold particles to detect immunoglobulin G antibodies bound to the Hib cell surface. MAb 3E9 bound to the surface of this Hib strain (Fig. 4A), as did the LOS-specific MAb 4C4 (Fig. 4B). As expected because of data obtained in the antibody accessibility RIA, MAB 9F5 did not bind to the surface of this Hib strain (Fig. 4C).

Bactericidal activity of MAb 3E9. The surface-directed MAb 3E9 was purified (8) and tested for its bactericidal activity against the homologous parent strain DL42 by using infant rat serum as the source of complement (17). This MAb did not exhibit any detectable killing of Hib DL42 in this assay system.

Strain distribution of MAb-defined P2 determinants. Colony blot RIA analysis was used to determine the distribution among Hib strains of the P2 epitopes defined by their reactivity with MAbs 3E9 and 9F5. A total of 120 Hib isolates representing 13 different geographic locations, including both the United States and Europe, were used as test antigens. Both MAbs bound to all 120 Hib strains.

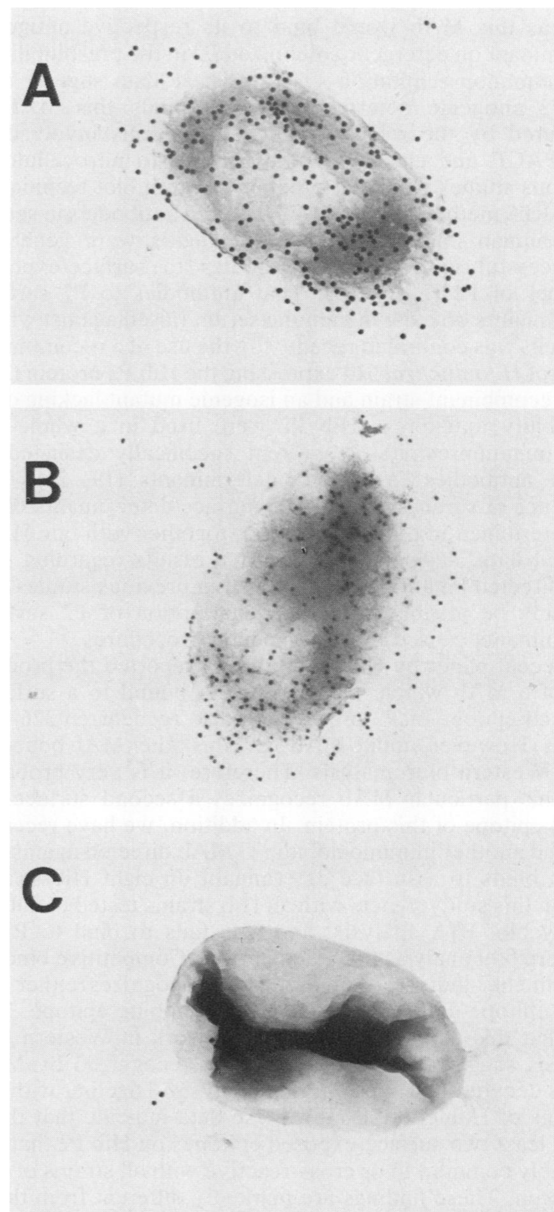

FIG. 4. Electron microscopic visualization of immune complexes on the bacterial cell surface. Cells of Hib DL42 were incubated with the P2-specific MAb 3E9 (A), the LOS-specific MAb 4C4 (as a positive control) (B), and the P2-specific MAb 9F5 (C). The resultant immune complexes on the bacterial cell surface were visualized by using protein A–colloidal-gold particles and electron microscopy as described in Materials and Methods.

DISCUSSION

One important criterion that must be met so that an Hib outer membrane protein can be a successful vaccine is that the protein must possess surface-exposed and antibody-accessible antigenic determinants that are common to most if not all strains of this pathogen. At least three Hib outer membrane proteins appear to satisfy this requirement. MAb data concerning the P6 protein indicate that this protein has at least one surface epitope that is common to all strains of this pathogen (35). Similarly, we have identified a surface epitope of P1 that is present in all 120 Hib strains tested to date (E. J. Hansen, et al., manuscript in preparation). The ability of polyclonal antisera raised against P1 or P6 to confer immunoprotection in animal models against multiple different Hib strains provides further biological evidence that one or more surface epitopes of each of these proteins is probably common to all Hib strains (13, 25). Finally, at least one surface-exposed epitope of P2 has been reported to be present in essentially all Hib strains (18).

Biochemical analysis of P2 from eight Hib strains established that, in addition to the antigenic conservation described above, the P2 proteins of these strains have conserved structures according to the tests employed in this study. The tryptic maps of these P2 proteins were indistinguishable, with the exception of one peptide in the P2 protein of strain DL26. The fact that the P2 proteins of these eight strains had the same N-terminal amino acid sequence, coupled with the nucleotide sequence data described above (22, 31), strongly suggests that there are only a few minor structural differences among P2 proteins of different Hib strains. In addition, it was recently reported that the nucleotide and derived amino acid sequences of the P2 genes of several different Hib strains were at least 95% identical (R. S. Munson, Jr., and C. Bailey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, D-82, p. 96).

The data obtained in the current study also indicate that there are at least two P2 epitopes that are common to all strains of this pathogen. The binding site for MAb 3E9 on this protein is exposed on the cell surface of the eight Hib strains tested in this study, whereas the epitope defined by its reactivity with MAb 9F5 either is not exposed on the surface of the Hib cell or is otherwise inaccessible to antibody in its native state in whole Hib cells. It is interesting that MAb 3E9 did not bind to P2 in Western blot analysis,

whereas this MAb would bind to its respective antigenic determinant on detergent-solubilized P2 in the presolubilized radioimmunoprecipitation system. These data suggest that the P2 antigenic determinant which binds this MAb is denatured by the relatively harsh conditions involved in SDS-PAGE and electrophoretic transfer to nitrocellulose. Previous studies which used either Western blot techniques or gel RIA methods to detect P2-directed antibodies in serum from human infants with Hib meningitis were generally unsuccessful in detecting antibodies to surface-exposed epitopes of P2 (7, 27, 39). That antibodies to P2 surface determinants do exist in immune serum raised against whole Hib cells was confirmed recently by the use of a recombinant strain of *H. influenzae* Rd expressing the Hib P2 protein (21). This recombinant strain and an isogenic mutant lacking only the ability to express Hib P2 were used in a whole-cell radioimmunoprecipitation system specifically designed to detect antibodies to surface determinants (19, 21). The existence of serum antibodies to surface determinants of P2 (as determined in this latter assay), together with our MAb-derived data, suggests that negative results regarding surface-directed P2 antibodies obtained in previous studies can probably be attributed to the denaturation of P2 surface determinants caused by experimental procedures.

A recent paper by Hamel et al. (18) reported the production of a MAb which, like MAb 3E9, bound to a surface-exposed epitope of P2 protein and also recognized 326 Hib strains. However, unlike MAb 3E9, this other MAb bound to P2 in Western blot analysis. Therefore, it is very probable that this particular MAb recognizes a second surface-exposed epitope of this protein. In addition, we have recently isolated another immunoglobulin G MAb directed against P2 which binds to a surface determinant on eight Hib strains used in this study, reacts with all Hib strains tested to date in colony blot RIA analysis, and also fails to bind to P2 in Western blot analysis (data not shown). Competitive binding experiments indicate that this MAb recognizes either the same epitope as MAb 3E9 or an overlapping epitope. The fact that this new MAb also fails to work in Western blot analysis suggests that the P2 epitope recognized by MAb 3E9 is denatured in Western blot analysis. Together with the findings of Hamel et al. (18), these data indicate that there are at least two surface-exposed epitopes on Hib P2 that are probably common to or cross-reactive with all strains of this pathogen. These findings are markedly different from those involving other mucosal pathogens like *N. gonorrhoeae*, the porin protein of which displays a significant degree of antigenic heterogeneity with regard to antibody-accessible surface epitopes (3). Similarly, the P2 protein of nontypeable *H. influenzae* has been reported to exhibit at least some antigenic heterogeneity among strains of this unencapsulated pathogen (34).

In contrast to the MAb-derived data regarding P2 surface epitopes described above, immunoprotection data obtained with polyclonal immune serum raised against purified Hib P2 protein suggest that some degree of antigenic heterogeneity exists among surface determinants of this protein (33). Infant rats passively immunized with this immune serum were protected against challenge with the homologous Hib strain but not against challenge with a heterologous Hib strain (33). There are several different ways in which these results could be explained. It is possible that the original detergent-based purification procedure for this protein (33) denatured those surface determinants which are common to all strains of this pathogen, while other nondenatured, strain-specific P2 surface determinants induced the synthesis of the observed protective antibodies. Alternatively, these results may indicate the existence of true antigenic heterogeneity involving immunodominant epitopes not reactive with the MAbs used in this and a previous study (18).

The use of MAb 3E9 in bactericidal assays showed that this P2 antibody did not kill Hib, even though this MAb bound to Hib cells at high levels in the antibody accessibility RIA. It has been reported that the presence of the Hib capsule prevents complement-mediated killing of Hib by antibodies to P1 (protein *a*) but does not prevent attachment of these P1-directed antibodies to Hib (40). The inability of MAb 3E9 to kill Hib may be related to the presence of the Hib capsule or to the specific epitope recognized by this MAb, and the immunoprotective ability of the polyclonal P2 antiserum (33) may have been due to antibodies with specificities for different P2 surface epitopes. Investigation of the antigenic and immunogenic properties of P2, including epitope mapping, will be required to determine the true potential of this protein for vaccine development.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-17621 from the National Institute of Allergy and Infectious Diseases to E.J.H.

We thank Porter Anderson for Hib Madigan and H234. We also thank Leslie Cope and Robert Munford for their comments concerning the manuscript, which was typed expertly by Cindy Baselski.

LITERATURE CITED

1. **Abersold, R. H., J. Leavitt, R. A. Saavedra, L. E. Hood, and S. B. H. Kent.** 1987. Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis by *in situ* protease digestion on nitrocellulose. Proc. Natl. Acad. Sci. USA **84:**6970–6974.
2. **Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff.** 1981. Subtyping isolates of *Haemophilus influenzae* type b by outer membrane protein profiles. J. Infect. Dis. **143:**668–676.
3. **Buchanan, T. M., and J. F. Hildebrandt.** 1981. Antigen-specific serotyping of *Neisseria gonorrhoeae*: characterization based upon principal outer membrane protein. Infect. Immun. **32:** 985–994.
4. **Carbonetti, N. H., and P. F. Sparling.** 1987. Molecular cloning and characterization of the structural gene for protein I, the major outer membrane protein in *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. USA **84:**9084–9088.
5. **Claesson, B. A., R. Schneerson, J. B. Robbins, J. Johansson, T. Lagergard, J. Taranger, D. Bryla, L. Levi, T. Cramton, and B. Trollfors.** 1989. Protective levels of serum antibodies stimulated in infants by two injections of *Haemophilus influenzae* type b capsular polysaccharide-tetanus toxoid conjugate. J. Pediatr. **114:**97–100.
6. **Cochi, S. L., and C. V. Broome.** 1986. Vaccine prevention of *Haemophilus influenzae* type b disease: past, present, and future. Pediatr. Infect. Dis. J. **5:**12–19.
7. **Erwin, A. L., G. E. Kenny, A. L. Smith, and T. L. Stull.** 1988. Human antibody response to outer membrane proteins and fimbriae of *Haemophilus influenzae* type b. Can. J. Microbiol. **34:**723–729.
8. **Ey, P. L., S. J. Prowse, and C. R. Jenkin.** 1978. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry **15:**429–437.
9. **Geohegan, W. D., and G. A. Ackerman.** 1977. Adsorption of horseradish peroxidase, ovomucoid and immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level: a new method, theory and application. J. Histochem. Cytochem. **25:** 1187–1200.
10. **Gonzales, F. R., S. Leachman, M. V. Norgard, J. D. Radolf, G. H. McCracken, Jr., C. Evans, and E. J. Hansen.** 1987.

Cloning and expression in *Escherichia coli* of the gene encoding the heat-modifiable major outer membrane protein of *Haemophilus influenzae* type b. Infect. Immun. 55:2993–3000.

11. **Gotschlich, E. L., M. E. Seiff, M. S. Blake, and M. Koomey.** 1987. Porin protein of *Neisseria gonorrhoeae*: cloning and gene structure. Proc. Natl. Acad. Sci. USA **84**:8135–8139.
12. **Granoff, D. M., and R. S. Munson, Jr.** 1986. Prospects for prevention of *Haemophilus influenzae* type b disease by immunization. J. Infect. Dis. **153**:448–461.
13. **Green, B. A., T. Quinn-Dey, and G. W. Zlotnick.** 1987. Biological activities of antibody to a peptidoglycan-associated lipoprotein of *Haemophilus influenzae* against multiple clinical isolates of *H. influenzae* type b. Infect. Immun. **55**:2878–2883.
14. **Gulig, P. A., C. F. Frisch, and E. J. Hansen.** 1983. 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. Infect. Immun. **42**:516–524.
15. **Gulig, P. A., and E. J. Hansen.** 1985. Coprecipitation of lipopolysaccharide and the 39,000-molecular-weight major outer membrane protein of *Haemophilus influenzae* type b by lipopolysaccharide-directed monoclonal antibody. Infect. Immun. **49**:819–827.
16. **Gulig, P. A., G. H. McCracken, Jr., C. F. Frisch, K. H. Johnston, and E. J. Hansen.** 1982. Antibody response of infants to cell surface-exposed outer membrane proteins of *Haemophilus influenzae* type b after systemic *Haemophilus* disease. Infect. Immun. **37**:82–88.
17. **Gulig, P. A., C. C. Patrick, L. Hermanstorfer, G. H. McCracken, Jr., and E. J. Hansen.** 1987. Conservation of epitopes in the oligosaccharide portion of the lipooligosaccharide of *Haemophilus influenzae* type b. Infect. Immun. **55**:513–520.
18. **Hamel, J., B. R. Brodeur, Y. Larose, P. S. Tsang, A. Belmaaza, and S. Montplaisir.** 1987. A monoclonal antibody directed against a serotype-specific, outer membrane protein of *Haemophilus influenzae* type b. J. Med. Microbiol. **23**:163–170.
19. **Hansen, E. J., C. F. Frisch, and K. H. Johnston.** 1981. Detection of antibody-accessible proteins on the cell surface of *Haemophilus influenzae* type b. Infect. Immun. **33**:950–953.
20. **Hansen, E. J., C. F. Frisch, R. L. McDade, Jr., and K. H. Johnston.** 1981. Identification of immunogenic outer membrane proteins of *Haemophilus influenzae* type b in the infant rat model system. Infect. Immun. **32**:1084–1092.
21. **Hansen, E. J., F. R. Gonzales, N. R. Chamberlain, M. V. Norgard, E. E. Miller, L. D. Cope, S. E. Pelzel, B. Gaddy, and A. Clausell.** 1988. Cloning of the gene encoding the major outer membrane protein of *Haemophilus influenzae* type b. Infect. Immun. **56**:2709–2716.
22. **Hansen, E. J., C. Hasemann, A. Clausell, J. D. Capra, K. Orth, C. R. Moomaw, C. A. Slaughter, J. L. Latimer, and E. E. Miller.** 1989. Primary structure of the porin protein of *Haemophilus influenzae* type b as determined by nucleotide sequence analysis. Infect. Immun. **57**:1100–1107.
23. **Hunkapiller, M. W., E. Lujan, F. Ostrander, and L. E. Hood.** 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. **91**:227–236.
24. **Kimura, A., P. A. Gulig, G. H. McCracken, Jr., T. A. Loftus, and E. J. Hansen.** 1985. A minor high-molecular-weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. Infect. Immun. **47**:253–259.
25. **Loeb, M. R.** 1987. Protection of infant rats from *Haemophilus influenzae* type b infection by antiserum to purified outer membrane protein *a*. Infect. Immun. **55**:2612–2618.
26. **Loeb, M. R., and D. H. Smith.** 1980. Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. Infect. Immun. **30**:709–717.
27. **Loeb, M. R., and D. H. Smith.** 1982. Human antibody response to individual outer membrane proteins of *Haemophilus influenzae* type b. Infect. Immun. **37**:1032–1036.
28. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**:10035–10038.
29. **McDade, R. L., Jr., and K. H. Johnston.** 1980. Characterization of serologically dominant outer membrane proteins of *Neisseria gonorrhoeae*. J. Bacteriol. **141**:1183–1191.
30. **Mizuno, T., M.-Y. Chou, and M. Inouye.** 1983. A comparative study on the genes for three porins of the *Escherichia coli* outer membrane. DNA sequence of the osmoregulated ompC gene. J. Biol. Chem. **258**:6932–6940.
31. **Munson, R., Jr., and R. W. Tolan, Jr.** 1989. Molecular cloning, expression, and primary sequence of outer membrane protein P2 of *Haemophilus influenzae* type b. Infect. Immun. **57**:88–94.
32. **Munson, R. S., Jr., and D. M. Granoff.** 1985. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. Infect. Immun. **49**:544–549.
33. **Munson, R. S., Jr., J. L. Shenep, S. J. Barenkamp, and D. M. Granoff.** 1983. Purification and comparison of outer membrane protein P2 from *Haemophilus influenzae* type b isolates. J. Clin. Invest. **72**:677–684.
34. **Murphy, T. F., and L. C. Bartos.** 1988. Purification and analysis with monoclonal antibodies of P2, the major outer membrane protein of nontypable *Haemophilus influenzae*. Infect. Immun. **56**:1084–1089.
35. **Murphy, T. F., M. B. Nelson, K. C. Dudas, J. M. Mylotte, and M. A. Apicella.** 1985. Identification of a specific epitope of *Haemophilus influenzae* on a 16,000 dalton outer membrane protein. J. Infect. Dis. **152**:1300–1307.
36. **Vachon, V., R. Laprade, and J. W. Coulton.** 1986. Properties of the porin of *Haemophilus influenzae* type b in planar lipid bilayer membranes. Biochim. Biophys. Acta **861**:74–82.
37. **Vachon, V., D. J. Lyen, and J. W. Coulton.** 1985. Transmembrane permeability channels across the outer membrane of *Haemophilus influenzae* type b. J. Bacteriol. **162**:918–924.
38. **Van Alphen, L., T. Klemens, J. Poolman, and H. C. Zanen.** 1983. Characteristics of major outer membrane proteins of *Haemophilus influenzae*. J. Bacteriol. **155**:878–885.
39. **Van Alphen, L., T. Riemens, and H. C. Zanen.** 1983. Antibody response against outer membrane components of *Haemophilus influenzae* type b strains in patients with meningitis. FEMS Microbiol. Lett. **18**:189–195.
40. **Van Alphen, L., T. Riemens, and H. C. Zanen.** 1986. The capsular polysaccharide of *Haemophilus influenzae* type b prevents killing by complement and antibodies against outer membrane protein *a*. FEMS Microbiol. Lett. **34**:273–276.
41. **Weinberg, G. A., and D. M. Granoff.** 1988. Polysaccharide-protein conjugate vaccines for the prevention of *Haemophilus influenzae* type b disease. J. Pediatr. **113**:621–631.