Antibody Response of Infants to Cell Surface-Exposed Outer Membrane Proteins of *Haemophilus influenzae* Type b After Systemic *Haemophilus* Disease

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Received 23 November 1981/Accepted 2 March 1982

The immune response of nine infants with *Haemophilus influenzae* type b meningitis was examined by using a radioimmunoprecipitation procedure designed to detect antibodies directed against cell surface-exposed outer membrane proteins of this pathogen. Using intrinsically or extrinsically radiolabeled intact *H. influenzae* type b cells with acute- and convalescent-phase human sera in this radioimmunoprecipitation system, we found that all of the infants produced an antibody response directed against several different *H. influenzae* type b outer membrane proteins. Anti-*H. influenzae* type b outer membrane protein antibodies present in convalescent sera, but not found in acute sera, were directed against cell surface-exposed *H. influenzae* type b outer membrane proteins. In contrast, both acute and convalescent sera contained antibody activity directed against numerous *H. influenzae* type b outer membrane proteins whose antigenic determinants were apparently inaccessible to antibody on intact *H. influenzae* type b cells. The ability of infants to develop an antibody response to cell surface-exposed, antibody-accessible *H. influenzae* type b outer membrane proteins indicates that these proteins may have vaccinogenic potential.

*Haemophilus influenzae* type b is the leading cause of endemic bacterial meningitis in infants (5). The currently available *H. influenzae* type b vaccine consisting of purified capsular carbohydrate (phosphoribosylribitol phosphate) is incapable of eliciting protective levels of antibody in infants and young children at highest risk for systemic *Haemophilus* disease (17). Accordingly, there is considerable interest in identifying alternative noncapsular *H. influenzae* type b vaccine candidates (8). That antibodies directed against noncapsular cell surface antigens of an encapsulated pathogen can be protective against disease caused by that organism has been well established by studies with *Neisseria meningitidis* and *Streptococcus pneumoniae* (4, 7). Several different laboratories have determined that serum antibodies directed against noncapsular *H. influenzae* type b cell surface antigens can protect against experimental *H. influenzae* infection in animals (9, 15, 20) and facilitate opsonization (2) and complement-mediated lysis of *H. influenzae* type b (2, 20, 21) in vitro. The exact identities of all these antigens have not been determined, although a recent study from this laboratory showed that monoclonal antibody directed against an *H. influenzae* type b cell surface-exposed outer membrane protein can protect against systemic *Haemophilus* disease in an animal model system (12).

Identification of *H. influenzae* type b cell surface antigens which are both immunogenic in infants and accessible to antibodies would permit a rational approach to selection of alternative experimental vaccinogens. The finding that monoclonal antibody directed against an *H. influenzae* type b outer membrane protein is protective against experimental *Haemophilus* disease (12) suggested that the outer membrane proteins have potential for development as vaccine candidates. That proteinaceous vaccines are efficacious in infants has been amply demonstrated by the success of the diphtheria-pertussis-tetanus vaccine (24), and it is known that infants convalescing from meningococcal disease produce an antibody response directed against outer membrane proteins of the infecting meningococcus (6). Previous studies from this laboratory have shown that *H. influenzae* type b outer membrane proteins are immunogenic in infant (11) and adult (10) rats convalescing from *H. influenzae* type b infection. In the former study, radiolabeled outer membranes were incu-
bated with control and convalescent sera in a sensitive radioimmunoprecipitation (RIP) procedure (11). These experiments established that several H. influenzae type b outer membrane proteins were consistently immunogenic in infant animals. Recently, we developed a different RIP procedure to specifically identify immunogenic H. influenzae type b outer membrane proteins which are both exposed on the cell surface and accessible to antibodies (10). In this paper we report the use of this new RIP system to study the immune response to H. influenzae type b outer membrane proteins in human infants with H. influenzae type b meningitis. An antibody response directed against cell surface-exposed and antibody-accessible proteins occurred in all nine infants studied by this method.

MATERIALS AND METHODS

Bacterial strains and culture media. Clinical isolates of H. influenzae type b were obtained from the cerebral spinal fluid or blood of infants with H. influenzae type b meningitis. Isolates were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with Levinthal base (1) (BHIs) as a source of hemin and NAD. Solidified BHIs medium was prepared by incorporating 1.5% (wt/vol) agar (Difco) into BHIs. Stock cultures were stored at −70°C in BHIs containing 30% (vol/vol) glycerol.

Sera. Acute- and convalescent-phase sera were obtained from infants with H. influenzae type b meningitis after informed written consent of the infants' parents had been obtained. These infants were patients at Children's Medical Center, Dallas, Texas, and ranged in age from 3 to 15 months. Acute-phase serum was prepared from blood drawn immediately after diagnosis of H. influenzae type b meningitis and initiation of appropriate therapeutic measures. Convalescent-phase serum was obtained from 1 to 4 months later. All sera were stored at −70°C.

Preparation and analysis of outer membrane vesicles. A modification of the LiCl extraction method of McDade and Johnston (19) was used to prepare outer membrane vesicles from intact H. influenzae type b cells for determination of outer membrane protein content. A small quantity (0.4 g, wet weight) of cells harvested in the late logarithmic phase of growth was suspended in 15 ml of LiCl extraction buffer (200 mM LiCl, 100 mM lithium acetate, pH 6.0) in a 50-ml flask containing approximately 25 glass beads (6-mm diameter). The beads and cells were vigorously agitated on a rotary shaker for 2 h at 45°C. The cell suspension was decanted, and the beads were washed with two 5-ml volumes of LiCl extraction buffer. The washings were added to the original suspension and subjected to centrifugation at 12,000 × g for 15 min. The resultant supernatant was then subjected to centrifugation at 25,000 × g for 15 min to remove any remaining intact cells. The final supernatant was subjected to centrifugation at 45,000 × g for 1 h to collect outer membrane vesicles. The supernatant was discarded, and the vesicles were suspended in 2 ml of distilled water, lyophilized, and stored at −20°C. The protein content of these vesicles was quantitated by the method of Markwell et al. (18) and was qualitatively examined by using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Hansen et al. (11).

Radiolabeling of outer membrane proteins. The protein content of membrane proteins of intact H. influenzae cells were extrinsically radiolabeled with 125Iodine by the lactoperoxidase-catalyzed method of Hansen et al. (11). Intrinsic radiolabeling of Haemophilus proteins with [35S]methionine was accomplished by growing cells to late logarithmic phase in 3 ml of BHIs broth supplemented with 0.5 mM of [35S]methionine (New England Nuclear Corp., Boston, Mass.; specific activity, 500 to 700 Ci/mmol).

WC-RIP. The whole cell RIP (WC-RIP) was performed as described by Hansen et al. (10). Briefly, 125I- or 35S-labeled intact cells were washed three times in phosphate-buffered saline (PBS), pH 7.2. Equal amounts (75 to 325 μl) of acute serum, convalescent serum, or PBS were added to separate 1-ml portions of radiolabeled cells containing 107 cpm (specific activity, 0.005 to 0.01 cpm per colony-forming unit), and these mixtures were rocked at 4°C for 1.5 h. Unattached antibodies were removed from the cell suspension by washing the cells once with PBS. Antibody-antigen complexes were extracted from these cells by incubating the cells in solubilization buffer consisting of 10 mM Tris-hydrochloride (pH 7.5) containing 150 mM NaCl, 10 mM EDTA, 1% (vol/vol) Triton X-100, 0.2% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) SDS for 1 h at 37°C. The resultant preparation was subjected to centrifugation at 45,000 × g for 1 h at room temperature to remove insoluble material. A small amount (200 μl) of a 10% (vol/vol) Formalin-fixed suspension of Staphylococcus aureus bearing protein A was added to bind soluble antibody-antigen complexes in which the antibody is of the immunoglobulin G class (14). The S. aureus antibody-antigen complexes were washed five times in solubilization buffer and dissociated by boiling for 5 min in digestion buffer (0.0625 M Tris-hydrochloride [pH 6.8], containing 2% [wt/vol] SDS, 10% [wt/vol] glycerol, and 0.005% [vol/vol] pyronin Y tracking dye [11]). The S. aureus was removed by centrifugation (12,000 × g for 2 min), and the immune precipitates were resolved by SDS-polyacrylamide gel electrophoresis followed by autoradiography or fluorography to identify radiolabeled outer membrane proteins present in the immune precipitates (10).

PS-RIP. The presolubilized RIP (PS-RIP) was performed in exactly the same manner as the WC-RIP, except that the washed, radiolabeled cells were first solubilized in solubilization buffer before incubation with sera or PBS by the method of Hansen et al. (10).

RESULTS

Outer membrane protein profile of clinical isolates of H. influenzae type b. The nine strains and the accompanying homologous sera used in this investigation were obtained from infants with H. influenzae type b meningitis over a 1-year period. To ensure that different strains of H. influenzae type b were studied in this investigation, the LiCl extraction method (19) was used to prepare outer membrane vesicles from H. influenzae cells, and the protein content of these vesicles

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FIG. 1. Outer membrane protein profile of the *H. influenzae* type b strains employed in this study. Outer membrane vesicles were extracted from intact cells of each strain by the LiCl extraction method (19) and were collected by differential centrifugation as described in the text. Proteins present in these outer membrane vesicles were resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lanes 1 through 9 contain the proteins present in the outer membrane vesicles prepared from the *H. influenzae* type b strains isolated from patients C6, D6, E5, J4, O2, B7, A3, F8, and G1, respectively. Coelectrophoresis of purified myosin (molecular weight, 200,000), phosphorylase B (molecular weight, 92,000), ovalbumin (molecular weight, 45,000), and carbonic anhydrase (molecular weight, 29,000) was used to determine the positions of the molecular weight reference markers shown on the left side of this figure.

was examined by SDS-polyacrylamide gel electrophoresis. Outer membrane protein profiles of clinical isolates have previously been used by Loeb and Smith (16) and Barenkamp et al. (3) to differentiate strains of *H. influenzae* type b in epidemiological studies. Quantitative as well as qualitative variations were observed among the outer membrane protein profiles of each strain (Fig. 1). Therefore, these nine clinical isolates represent several different strains, as determined by outer membrane protein content.

Identification of immunogenic cell surface-exposed outer membrane proteins. *H. influenzae* type b cells intrinsically radiolabeled by growth in BHIs containing [35S]methionine were used initially as the source of antigen in the WC-RIP to identify outer membrane proteins of the infecting *H. influenzae* type b strain which were exposed on the cell surface, accessible to antibody, and immunogenic in that infant. Intact radiolabeled cells of each strain were incubated with the homologous acute and convalescent sera to allow antibodies directed against cell surface-exposed proteins to attach to their respective antigens. The use of equal amounts of acute and convalescent sera and the identical processing of these samples in the WC-RIP procedure allowed accurate comparison of the relative antibody content of these matched pairs of sera.

An antibody response directed against cell surface-exposed outer membrane proteins of *H. influenzae* was detected in the convalescent sera of the seven infants studied with the WC-RIP system, using 35S-labeled cells. Several different radiolabeled proteins were present only in the immune precipitates generated with convalescent sera and not in the immune precipitates obtained with the homologous acute sera (Fig. 2). Specifically, a group of high-molecular-weight outer membrane proteins with apparent molecular weights above 100,000 was immunoprecipitated by all of the infants' convalescent sera. In addition, another protein with an apparent molecular weight of 30,000 was immunoprecipitated by several of the infants' convalescent sera. Nonspecific binding of *H. influenzae* type b proteins to the *S. aureus* immunoadsorbent resulted in the appearance of only very faint protein bands when PBS was used as a negative control in the RIP procedure in place of acute or convalescent serum (Fig. 2, lane 1). Another protein with an apparent molecular weight of 60,000 was detected in all acute and convalescent sera. This same protein was also immunoprecipitated when both immune and normal rat sera were used in the WC-RIP (10). This particular protein may represent either a cross-reacting antigen which reacts with antibodies found in all sera tested by using [35S]methionine-labeled cells or a protein that reacts with some nonimmunoglobulin factor in serum that allows this protein to bind to *S. aureus*

Infant immune response to the total protein
The WC-RIP membrane.

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FIG. 2. RIP of 35S-labeled H. influenzae type b cell surface-exposed outer membrane proteins with infant acute and convalescent sera. Cells were intrinsically radiolabeled with [35S]methionine by growth in BHI containing 167 μCi of [35S]methionine per ml. RIP analysis was performed as described in the text. Briefly, equal volumes (75 to 325 μl) of acute and convalescent sera were employed in each assay. Matched sera were mixed with radiolabeled cells of the homologous infecting strain, and then immunogenic, cell surface-exposed proteins together with any adherent antibodies were solubilized out of the cells by treatment with solubilization buffer. Antigen-antibody complexes were isolated by adsorption to S. aureus and then processed by SDS-polyacrylamide gel electrophoresis followed by fluorography to detect radiolabeled outer membrane proteins. Lane 1 contains the immune precipitate obtained when PBS was used in place of human serum in the RIP system with radiolabeled cells of the H. influenzae type b strain obtained from patient J4. This control lane contains those proteins of this strain which adhere to S. aureus protein A in the absence of serum antibody. The seven pairs of lanes contain the immune precipitates obtained when acute (A) and convalescent (C) sera from each infant were employed in the RIP system with radiolabeled cells of the homologous infecting strain. The letter-number combination centered above each pair of gel lanes is the patient (infant) number. The volumes of matched acute and convalescent sera employed in each RIP were as follows: J4, 300 μl; C6, 200 μl; G1, 75 μl; B7, 200 μl; E5, 125 μl; F8, 100 μl; G2, 325 μl. Molecular weight markers are provided on the left side of each gel.

complement of the H. influenzae type b outer membrane. The WC-RIP procedure specifically detected antibodies directed against cell surface-exposed outer membrane proteins (10). To evaluate the immune response of human infants to all proteins present in the outer membrane, two additional strains isolated from patients A3 and D6 were extrinsically radiolabeled with [125I]iodine. These radioiodinated cells were solubilized before the addition of antisera to the PS-RIP system. The use of the PS-RIP system permitted immunoprecipitation of those immunogenic outer membrane proteins which were not exposed on the cell surface or antibody-accessible in their native state in intact cells, and which did not lose their antigenic reactivity after detergent treatment. Although this PS-RIP procedure theoretically allows any H. influenzae type b cell protein to react with its corresponding antibody, Hansen et al. (11) have established that radiolabeling intact H. influenzae type b cells by the lactoperoxidase-catalyzed method results in radioiodination of only outer membrane proteins, which can subsequently be identified in immune precipitates resolved on SDS gels by autoradiography. That essentially all H. influenzae type b outer membrane proteins are solubilized and can be immunoprecipitated by this method has been previously demonstrated by Hansen et al. (10) using immune rat serum.

The use of the 125I-labeled H. influenzae type b cells in the PS-RIP system showed that both the homologous acute and convalescent sera contained antibody activity directed against numerous outer membrane proteins (Fig. 3a and b, lanes 5, 6, and 7). Negative control experiments with PBS in place of serum showed that nonspecific binding of radiolabeled outer membrane proteins to the S. aureus immunoadsorbent did not occur in this system. Quantitative increases in some specific immunoprecipitated proteins between the acute and convalescent serum samples within matched pairs indicated that additional antibodies were produced against certain outer membrane proteins as a direct result of infection.

These same two strains labeled with 125I were also used in the WC-RIP procedure, and several high- and intermediate-molecular-weight outer membrane proteins, similar to those detected previously by using the 35S-labeled cells of other strains (Fig. 2), were immunoprecipitated in this system (Fig. 3a and b, lanes 2, 3, and 4). These cell surface-exposed, antibody-accessible outer
membrane proteins represent a subset of those detected with the PS-RIP procedure (Fig. 3a and b, lanes 5, 6, and 7), and include a major outer membrane protein with an apparent molecular weight of 39,000. Therefore, it appears that most of those anti-H. influenzae type b outer membrane protein antibodies present in both the acute and convalescent sera from these two infants were directed against outer membrane proteins whose antigenic determinants are either not exposed on the cell surface of intact cells or are otherwise inaccessible to their corresponding antibodies.

DISCUSSION

The necessity of identifying alternative H. influenzae type b vaccinogen candidates has led to the examination of the proteins present in the outer membrane of this pathogen for their immunogenicity in both animal model systems and human infants. H. influenzae type b outer membrane proteins have previously been shown to be immunogenic in infant rats convalescing from systemic Haemophilus disease (11). The data presented in this report indicate that H. influenzae type b outer membrane proteins are also immunogenic in human infants recovering from systemic Haemophilus disease. All nine infants studied in this investigation developed antibodies directed against cell surface-exposed outer membrane proteins after Haemophilus disease. The antibody response of these infants to the phosphoribosylribitol phosphate capsular antigen was not measured because the limited quantities (75 to 325 µl) of available sera were completely consumed in the RIP experiments. However, Käyhly et al. (13) have established that most infants convalescing from systemic Haemophilus disease produce little or no antibody response directed against phosphoribosylribitol phosphate. It is unlikely that the nine infants involved in our study were uniquely immunocompetent, because they were randomly chosen from those infants with Haemophilus disease who were 15 months of age or less and whose parents consented to their childrens’ participation in this study. Therefore, it can be concluded that normal infants can develop an antibody response directed against H. influenzae type b cell surface-exposed outer membrane proteins. However, the ability of this antibody response to be protective against systemic Haemophilus disease and the potential immunogenicity of purified H. influenzae type b outer membrane proteins in infants remain to be determined by future experiments.

It must be noted that antibodies directed against a number of different H. influenzae type b outer membrane proteins were found in both acute and convalescent sera when solubilized
cells were employed as the source of antigen in the PS-RIP system (Fig. 3). In contrast, antibodies directed against cell surface-exposed, antibody-accessible outer membrane proteins represented a smaller repertoire and were detected only or predominantly in the convalescent sera (Fig. 3). These data suggest that children susceptible to systemic Haemophilus disease may already possess relatively high levels of antibodies directed against H. influenzae type b outer membrane protein antigenic determinants which are concealed in the outer membrane. Alternatively, these particular antigenic determinants may be exposed on the cell surface of intact cells, but are inaccessible to their corresponding antibodies present in serum. In the latter case, steric hindrance of antibody binding to cell surface-exposed protein antigenic determinants could be effected by other cell surface structures (e.g., lipopolysaccharide) or by a relative excess of serum antibodies directed against these other cell surface antigens. The phosphoribosylribitol phosphate capsule itself apparently does not affect the antibody accessibility of cell surface-exposed proteins, since identical RIP patterns were obtained when both a wild-type strain and its unencapsulated variant were employed in the WC-RIP system (E. J. Hansen and C. F. Frisch, unpublished data).

The data obtained from the experiments depicted in Fig. 3 also suggest that other systems which might be used to measure anti-H. influenzae type b outer membrane protein antibody levels and which employ disrupted or solubilized outer membrane preparations as the source of antigen (e.g., enzyme-linked immunosorbent assay, radioimmunoassay, or Western blot analysis (22)) may detect not only antibodies against cell surface-exposed antigens, but also antibodies directed against these protein antigenic determinants which are inaccessible to antibody in intact cells. Accordingly, we have shown that an enzyme-linked immunosorbent assay which employs an outer membrane preparation as antigen detects monoclonal antibodies directed against outer membrane protein antigenic determinants which, in their native state in intact cells, are inaccessible to antibody (23).

The source of the antigens which induced the synthesis of the anti-H. influenzae type b outer membrane protein antibodies present in the acute sera is not known at this time. One possible explanation might involve exposure to, or infection with, other bacteria (20) or Haemophilus species bearing cross-reactive antigens. Alternatively, previous subclinical infection with H. influenzae type b or prolonged nasopharyngeal colonization with the infecting strain may account for the presence of these antibodies in the acute sera. Finally, in very young infants it is possible that residual maternal antibody may be the source of this antibody activity.

Data obtained from the use of the WC-RIP system together with convalescent infant sera and both 35S- and 125I-labeled H. influenzae type b cells showed that the immunogenic, cell surface-exposed, antibody-accessible outer membrane proteins present in these strains could be grouped into high-molecular-weight proteins, with apparent molecular weights greater than 100,000, and intermediate-molecular-weight proteins, with apparent molecular weights ranging from 30,000 to 39,000. The predominance of high-molecular-weight proteins detected in immune precipitates obtained from the use of the WC-RIP system with 125I-labeled H. influenzae type b cells is apparently not due to disproportionate radiolabeling of these proteins by the lactoperoxidase-catalyzed radioiodination system, as evidenced by the relative paucity of radiolabeled high-molecular-weight proteins visible in outer membrane preparations derived from these cells (Fig. 3). Similarly, outer membrane preparations derived from 35S-labeled cells showed that these high-molecular-weight proteins were not preferentially labeled with [35S]methionine relative to other outer membrane proteins (data not shown). We believe that these high-molecular-weight proteins, together with the major outer membrane protein with an apparent molecular weight of 39,000, are either more immunogenic or more accessible to antibody relative to other outer membrane proteins. We have also shown that these high-molecular-weight proteins and the major outer membrane protein with an apparent molecular weight of 39,000 were the predominant proteins found in immune precipitates when the WC-RIP system was employed to analyze convalescent serum obtained from weaning rats which experienced systemic Haemophilus disease induced at 6 days of age (P. A. Gulig and E. J. Hansen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B48, p 26.).

These data demonstrating the immunogenicity of H. influenzae type b outer membrane proteins in infants, together with the recent finding that monoclonal antibodies directed against a cell surface-exposed outer membrane protein of H. influenzae type b can protect against experimental Haemophilus disease (12), indicate that cell surface-exposed, antibody-accessible proteins present in the outer membrane have considerable potential for vaccine development. However, the fact that the immunogenic, cell surface-exposed proteins from the different H. influenzae type b strains involved in this study have apparently similar or identical molecular weights does not necessarily indicate that these proteins possess the same antigenic determi-
nants. The possibility that antigenic cross-reactivity may exist among these particular proteins is currently under investigation in this laboratory. If one or more of these cell surface-exposed, antibody-accessible outer membrane proteins can be shown to be antigenically common to or cross-reactive with most _H. influenzae_ type b strains, this particular protein(s) would represent a prime candidate for an experimental _H. influenzae_ type b vaccinogen.

**ACKNOWLEDGMENTS**

We thank Elizabeth Haanes and Dale Chrane for their superb technical assistance and Leon Eidsel and Jonathan Uhr for their comments concerning this manuscript.

P.A.G. was supported by National Science Foundation predoctoral fellowship SPI 81-66383.

**LITERATURE CITED**


