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Strain-Specific and Immunodominant Surface Epitopes of the P2 Porin Protein of Nontypeable *Haemophilus influenzae*

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The P2 porin protein is the major outer membrane protein of nontypeable *Haemophilus influenzae*. Five monoclonal antibodies to P2 of four strains of nontypeable *H. influenzae* were developed by immunizing mice with whole bacterial cells. All five antibodies recognized epitopes on P2 in immunoblot assays of whole organism lysates, purified outer membrane, and purified P2. Competitive enzyme-linked immunosorbent assays and immunoblot assays of cyanogen bromide-digested P2 showed that two antibodies to the P2 protein of strain 1479 recognized different epitopes on the molecule. Immunofluorescence and immunoelectron microscopy demonstrated that each of the five antibodies recognized epitopes that were abundantly expressed on the bacterial surface. Analysis of 120 *H. influenzae* strains indicated that three of the five antibodies were reactive exclusively with the homologous strain. The remaining two antibodies were reactive with less than 3% of the strains. These studies indicate that the P2 protein expresses a highly strain-specific and immunodominant epitope on the bacterial surface. The expression of strain-specific and immunodominant epitopes on the bacterial surface may represent a mechanism by which the bacterium induces antibodies that will protect against recurrent infection by the homologous strain but will not protect against infection by heterologous strains.

Nontypeable *Haemophilus influenzae* is an important cause of infection in children and adults (19). In children, this bacterium is a common cause of otitis media and sinusitis (4, 5, 10, 33, 38). Nontypeable *H. influenzae* is also an important cause of respiratory tract infections particularly in the elderly, in patients with chronic bronchitis, and in children in the developing world (3, 26, 32, 37, 41). Finally, the organism occasionally causes invasive infections such as meningitis and bacteremia, especially in neonates and postpartum women (19, 39, 40).

In view of the importance of nontypeable *H. influenzae* as a human pathogen, recent work has focused on the potential role of outer membrane structures in pathogenesis and as targets of the human immune response. The major outer membrane protein (OMP) of *H. influenzae* has a molecular mass that varies from 36,000 to 42,000 Da among strains (1, 14, 24). This OMP has been called P2 and b/c (14, 17). Molecular mass differences in P2 are the basis of a subtyping system for nontypeable *H. influenzae* (23, 24).

P2 makes up a relatively large proportion of the protein content of the outer membrane. It functions as a porin protein and is the sole porin protein in *H. influenzae* (6, 36). Like many porins, P2 exists as a trimer in the outer membrane (12, 35). Polyclonal antibody to P2 of a type b strain is protective against the homologous strain in the infant rat model of *H. influenzae* infections (17). The protein is a target for human serum bactericidal antibody (20). Taken together, these observations indicate that P2 is the major protein antigen on the outer membrane of *H. influenzae*.

The goal of the present study is to analyze P2 antigenic determinants that are expressed on the bacterial surface of nontypeable strains of *H. influenzae*. In previous work, we described a monoclonal antibody to a surface epitope of P2 of one strain (21). This antibody is bactericidal for strains that express the epitope. In the present study, we extend the studies on this antibody and describe a total of five monoclonal antibodies developed to separate epitopes on P2 of four different strains. These antibodies were used to analyze surface-exposed determinants of P2, with particular emphasis on the strain specificity of these determinants among 120 *H. influenzae* strains of diverse clinical and geographic origins.

MATERIALS AND METHODS

Bacteria. All strains were grown on chocolate agar at 35°C in 5% CO_2 for 18 to 24 h. The identities of *H. influenzae* strains were confirmed by the growth requirement for hemin (X factor) and nicotinamide adenine dinucleotide (V factor). Capsular serotypes were determined by counterimmunoelectrophoresis with reference strains and antisera obtained from the Centers for Disease Control, Atlanta, Ga. (24). A total of 120 *H. influenzae* strains of diverse geographic and clinical origins were studied. These included 94 nontypeable, 24 type b, and one each of type e and type f strains. The 24 type b strains represent a diversity of electrophoretic types (27). The clinical sources of the 94 nontypeable strains are shown in Table 1. These isolates were recovered in seven American cities, including Buffalo, N.Y. (28 strains), Houston, Tex. (20 strains), Seattle, Wash. (13 strains), Boston, Mass. (11 strains), Dallas, Tex. (11 strains), St. Louis, Mo. (10 strains), and Rochester, N.Y. (1 strain).

Purification of P2 protein. Total bacterial membranes were isolated by a modification of a procedure previously described by Osborn et al. for enteric bacilli (28, 31). Briefly, bacteria were harvested from chocolate plates and washed in phosphate buffer. Pellets were suspended in hypertonic

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TABLE 1. Clinical sources of isolates of nontypeable *H. influenzae*

Source	No. of isolates
Sputum (expectorated)	32
Middle ear fluid	25
Blood	16
Nasopharynx	10
Cerebrospinal fluid	4
Tracheal aspirate	4
Eye	2
Pericardium	1

sucrose prior to the addition of lysozyme (200 μg/ml) and EDTA to produce spheroplasts. After the spheroplasts were concentrated by centrifugation, pellets were suspended in sucrose solution, sonicated, and centrifuged at a low speed. The supernatant was centrifuged for 3 h at 200,000 × *g* at 4°C. The pellet was resuspended in sucrose solution and centrifuged again.

The P2 protein was selectively solubilized in buffer Z (0.05% Zwittergent 3-14, 0.05% Tris, 0.01 M EDTA, pH 8.0) by subjecting the pellet obtained above to a differential detergent solubility method as previously described by Murphy and Bartos (21) with a slight modification. The final pellet was extracted five times in buffer Z, each time with incubation for 1 h at 37°C prior to centrifugation. The supernatant fluids were pooled, and the protein concentration was determined by optical density at 280 nm (OD_{280}) by using a bovine serum albumin standard curve. The protein yield from 60 100-mm (diameter) plates of strain 1479 was approximately 63 mg.

P2 was further purified from OMPs by anion-exchange chromatography as previously described (21). The yield of P2 from the entire Zwittergent extract was approximately 14 mg. P2 along with a small amount of lipooligosaccharide (LOS) was present in these preparations on silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Immunodot assays. Whole organism lysates were prepared by suspending cells from chocolate agar plates in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and immunodot assays were performed as previously described (21).

SDS-PAGE and immunoblot assays. Whole cell lysates, the outer membrane complex, and purified P2 were solubilized and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 13% separating gels as previously described (13, 21). Proteins on gels were stained with Coomassie blue (24) or silver (18, 34) or were electrophoretically transferred to nitrocellulose for immunoblot assays (21).

Immunofluorescence. Indirect immunofluorescence by using fluorescein-labeled goat anti-mouse immunoglobulin G was used to determine whether the epitopes recognized by the monoclonal antibodies on the homologous bacterial strain were surface exposed. *H. influenzae* cells from a chocolate agar plate were suspended in phosphate-buffered saline (PBS) and applied to glass slides. After air drying, the slides were fixed by briefly passing them through a flame. Undiluted tissue culture supernatant was applied to the appropriate well of the slide, incubated for 30 min, and washed prior to the addition of conjugate. The slides were examined for fluorescence of an individual bacterium on an American Optical epifluorescence microscope under oil at 1,000× magnification.

Immunoelectron microscopy. To determine whether the epitopes recognized by the monoclonal antibodies were surface exposed, immunoelectron microscopy by using goat anti-mouse immunoglobulin G conjugated to colloidal gold particles was performed as previously described (21).

Development of monoclonal antibodies. Monoclonal antibodies were raised to four strains of nontypeable *H. influenzae*, including 1479, 5657, 2019, and 7502. All four isolates were recovered from the sputum of adults in Buffalo, N.Y. The four strains represent different subtypes and serotypes based on differences in the P2 molecule (18, 23, 24).

BALB/c mice were immunized intraperitoneally with a suspension of approximately 10^8 to 10^9 organisms per ml in PBS on days 0 and 28. On day 32 after the initial immunization, mouse splenocytes were fused with SP2/0-Ag14 plasmacytoma cells to obtain antibody-producing hybridomas as previously described (11, 25). Antibodies reactive to the P2 protein were initially selected by screening hybridoma supernatants in an immunodot assay by using whole cell lysates of the homologous strain. The specificity of the antibody reactivity was verified by immunodot and immunoblot assays of both the whole organism and purified outer membranes. Hybridomas were cloned by limiting dilutions and were inoculated intraperitoneally into pristane-primed BALB/c mice to produce ascitic fluid.

Antibodies from four separate fusions are described in this study. Two of the fusions (strains 7502 and 2019) each yielded less than five antibody-producing hybridomas. Antibodies 1B10 and 7B11 were the only antibodies to P2 from these fusions. The remaining two fusions (5657 and 1479) each resulted in approximately 25 antibody-producing clones. Of the clones that were studied, all 12 of the antibodies to P2 recognized epitopes that were highly strain specific.

Competitive ELISA. To determine whether the two antibodies to P2 of 1479 recognized the same or different epitopes, a competitive enzyme-linked immunosorbent assay (ELISA) was performed. Biotinylated purified P2 protein and monoclonal antibodies from ascitic fluid purified by elution from a protein A column were used in all competitive ELISAs. Various modifications were tested to determine the optimal conditions under which to perform the assay. Before each assay, a checkerboard titration was done to determine the optimal concentrations for the coating antibody, biotinylated P2, and avidin peroxidase.

To perform the assay, one of the antibodies was used to coat the wells of a 96-well microtiter plate. Wells were blocked with 3% gelatin for 1 h at 37°C. Serial dilutions of antibody or SP2 ascitic fluid were preincubated with biotinylated P2 from strain 1479 for 1 h at room temperature. This mixture was added to the appropriate coated wells and incubated overnight at 4°C. After the wells were washed, avidin peroxidase conjugate was added to each well and incubated for 1 h at room temperature. Ortho-phenylenediamine substrate in citric-phosphate buffer (pH 5.0) plus 0.003% H_2O_2 was added, and the color was developed for 30 min at room temperature before being stopped with 5 N H_2SO_4. The OD_{490} of each well on a BIO-TEK EL-307 ELISA reader was determined.

Controls for these experiments included wells which were coated with carbonate buffer (no coating antibody) followed by biotinylated P2, conjugate, and substrate. Other wells contained coating antibody and diluting buffer (no biotinylated P2 or competing antibody) followed by conjugate and substrate. These wells showed minimal background. In addition, two other antibodies were used as competing antibodies in negative antibody controls. These included

TABLE 2. Strain specificity of monoclonal antibodies that recognize the P2 OMPs of *H. influenzae*

Antibody	Homologous strain	Specificity (no. positive/total)[a]
2E6	5657	3
7B11	2019	1
1B10	7502	2
6G3	1479	1
5F2	1479	1

[a] Number positive of 120 strains tested. All reactive isolates were nontypeable.

antibody 1G9, which recognizes an epitope on the LOS of strain 1479, and antibody 2E6, which recognizes an epitope on P2 of strain 5657 but not 1479. As a positive control for each assay, biotinylated P2 without a competing antibody was added to antibody-coated wells and developed with conjugate and substrate. The values from wells with SP2 as the competing antibody were averaged and used as the background mouse antibody control. The SP2 average value was then used to standardize the assay results. Each dilution was plated in duplicate for both the control and test antibodies. Each assay was repeated at least twice.

The percent inhibition was calculated to determine the extent to which a competing monoclonal antibody inhibited the binding of the coating antibody to biotinylated P2. The result was calculated relative to the SP2 control values by using the following formula: [(OD of SP2 − OD of antibody)(100)]/OD of SP2. The concentration of competing antibody (milligrams per milliliter) resulting in 50% inhibition for each competing antibody was determined.

RESULTS

Antigenic specificity of antibodies. To determine the antigen that the monoclonal antibodies recognized, tissue culture supernatants from hybridomas that contained antibody to a bacterial lysate of the homologous strain in the immunodot assay were tested in an immunoblot assay by using whole organism lysate. Five antibodies that bound to bands corresponding to the molecular weight of P2 for each of the homologous strains were identified. These five antibodies and their homologous strains are listed in Table 2. Antibodies 6G3 and 5F2 were from the same fusion and recognized epitopes on the P2 protein of strain 1479.

To further assess the protein band recognized by the antibodies, outer membranes of the homologous strains were purified (23). These were subjected to SDS-PAGE and an immunoblot assay. All five antibodies recognized the P2 band in purified outer membrane (Fig. 1). Each antibody was reactive with P2 in whole organism lysates, purified outer membrane, and purified P2 preparations.

Epitope specificity of antibodies 6G3 and 5F2. To determine whether antibodies 6G3 and 5F2 recognized the same or different epitopes on the P2 protein of strain 1479, competitive ELISAs were performed. These were designed to assess the ability of each of the antibodies to compete for binding to P2; separate experiments in which each of the two antibodies was assayed in both the solid phase and the soluble phase were performed.

Figure 2A shows the results when antibody 5F2 was used as the coating antibody. The homologous antibody showed 73.0 to 98.3% inhibition over a concentration range of 5 to 80 μg/ml, while control antibodies showed minimal (less than or equal to 23%) inhibition over the entire concentration range. Antibody 6G3 showed approximately 50% inhibition at 80 μg/ml. In this experiment, the average variation between duplicate wells was 0.95%. When 6G3 was used as the coating antibody, 5F2 demonstrated significant inhibition (Fig. 2B). Indeed, antibody 5F2 showed more inhibition than the homologous antibody at each concentration studied. Control wells again showed minimal inhibition. In this experiment, the average variation between duplicate wells was 0.90%. Collectively, the results of the competitive ELISAs suggested that antibodies 5F2 and 6G3 recognized different epitopes on the P2 protein.

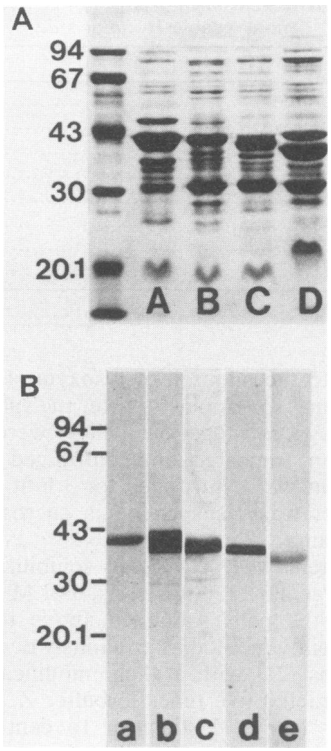

FIG. 1. (A) SDS-polyacrylamide gel stained with Coomassie blue. Lanes contain purified outer membranes of the following strains: 1479 (lane A), 2019 (lane B), 7502 (lane C), and 5657 (lane D). (B) Immunoblot assay in which monoclonal antibodies were assayed against purified outer membranes of the homologous strains. Lanes contain the following antibodies: 5F2 (lane a), 6G3 (lane b), 7B11 (lane c), 1B10 (lane d), and 2E6 (lane e). Immunoblot assays were incubated with protein A-peroxidase followed by horseradish peroxidase color developer. Molecular mass standards in both panels are noted on the left in daltons (thousands).

To further assess whether antibodies 6G3 and 5F2 recognized the same or different epitopes, purified P2 was treated with cyanogen bromide as previously described (21). SDS-PAGE determined that cleavage of the P2 protein of strain 1479 resulted in two fragments with approximate molecular masses of 32,000 and 10,000 Da (Fig. 3A). The immunoblot assay revealed that antibody 6G3 reacted with the 32,000-Da band while antibody 5F2 recognized the 10,000-Da band (Fig. 3B). This indicated that 5F2 recognized an epitope on a different part of the P2 molecule compared with 6G3.

Surface exposure. To determine whether each of the five antibodies recognized an epitope that is expressed on the bacterial surface, immunoelectron microscopy and immunofluorescence were performed. In control experiments, each

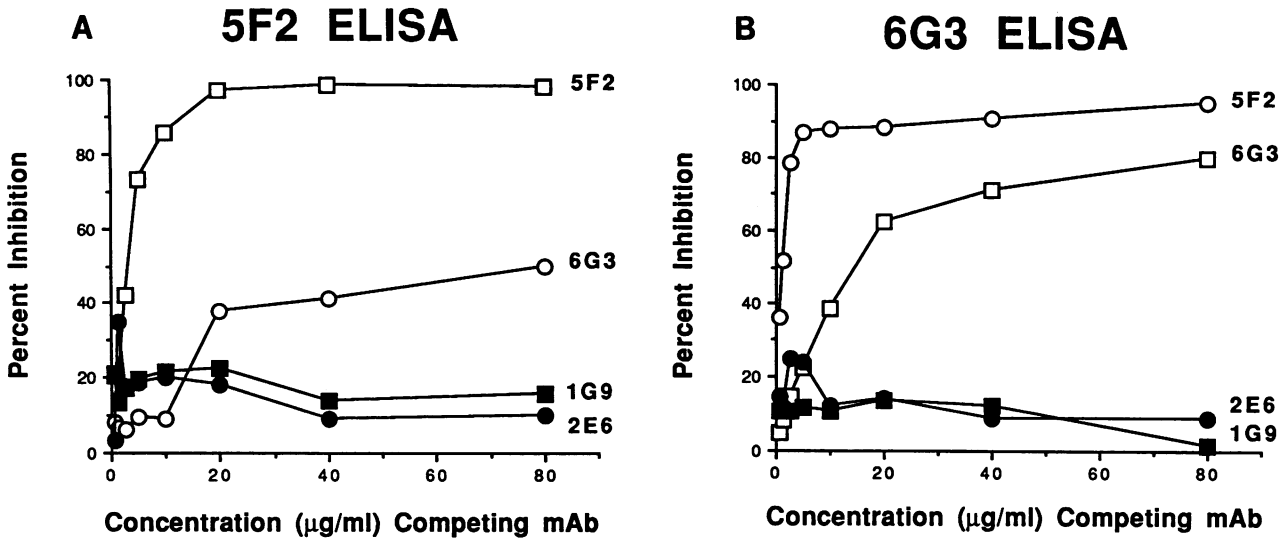

FIG. 2. (A) Competitive ELISA with antibody 5F2 as coating antibody (*y* axis, percent inhibition of binding of biotinylated P2; *x* axis, concentration of competing monoclonal antibodies) (5F2 and 6G3 [anti-P2, strain 1479] and controls 1G9 [anti-LOS, strain 1479] and 2E6 [anti-P2, strain 5657]). (B) Competitive ELISA with 6G3 as the coating antibody. Axes and antibodies are as described for panel A. mAb, Monoclonal antibody.

antibody was nonreactive with a strain that lacked the corresponding epitope. Irrelevant monoclonal antibodies and SP2 tissue culture supernatants were incubated in place of the test antibody as negative controls. These were consistently negative. All five of the monoclonal antibodies yielded positive results in immunoelectron microscopy and immunofluorescence when studied with strains that were reactive in immunodot assays (Fig. 4). All five showed prominent staining of the bacterial cells, indicating that each antibody recognized a determinant that is abundantly expressed on the bacterial surface.

Strain specificity. The strain specificity of a determinant recognized by a monoclonal antibody was determined by using immunodot assays of whole organism lysates (Table 2). Antibodies 7B11, 6G3, and 5F2 recognized only the homologous strain and none of the remaining 119 strains. Antibody 2E6 recognized epitopes on 3 of the 120 strains. These included the homologous strain 5657 (sputum isolate; Buffalo, N.Y.), SL1328 (blood isolate; St. Louis, Mo.), and H11 (sputum isolate; Houston, Tex.). Antibody 1B10 recognized epitopes on the homologous strain and one additional strain, H17 (sputum isolate; Houston). An immunoblot assay demonstrated that antibodies 2E6 and 1B10 recognized a determinant on P2 in the heterologous strains. All reactive isolates were nontypeable. Immunoelectron microscopy and immunofluorescence showed that the epitope was expressed on the bacterial surface in all strains that were reactive in the immunodot assay.

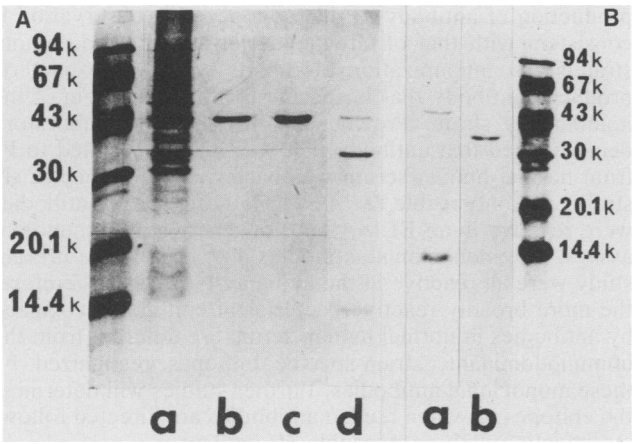

FIG. 3. (A) Cyanogen bromide cleavage of purified P2. Coomassie blue-stained SDS-PAGE gel. Nontypeable *H. influenzae* 1479 in all lanes. Lanes: a, whole cell lysate; b, purified P2; c, purified P2 incubated in 70% formic acid (control); d, purified P2 incubated with cyanogen bromide in 70% formic acid. (B) Immunoblot of cyanogen bromide-treated P2 incubated with antibodies 5F2 (lane a) and 6G3 (lane b). Molecular mass markers are noted in daltons (thousands).

DISCUSSION

The data reported in this study indicate that the P2 porin protein of nontypeable *H. influenzae* expresses a highly strain-specific epitope on the surface of the intact bacterium. All five of the antibodies described in this study were developed from splenocytes of mice that were immunized with whole organisms. The strain-specific epitopes on P2 are abundantly expressed on the bacterial surface on the basis of the strikingly positive immunoelectron microscopic and immunofluorescence assays.

Both 5F2 and 6G3 reacted to P2 in immunoblot assays of whole organism lysate, purified outer membrane, and purified P2 of strain 1479. Antibody 5F2 was distinctly less reactive in immunoblot assays compared with 6G3, although the concentration of these antibodies was comparable on the basis of titers in the immunodot assays and the extinction coefficient at OD_{280}. Further evidence of a difference between these antibodies was provided by the competitive ELISA. Apparently, the 5F2 epitope is sufficiently close to the 6G3 epitope, enabling antibody 5F2 to inhibit P2 binding to both antibodies. However, the 6G3 epitope is situated such that the binding of P2 by antibody 6G3 does not

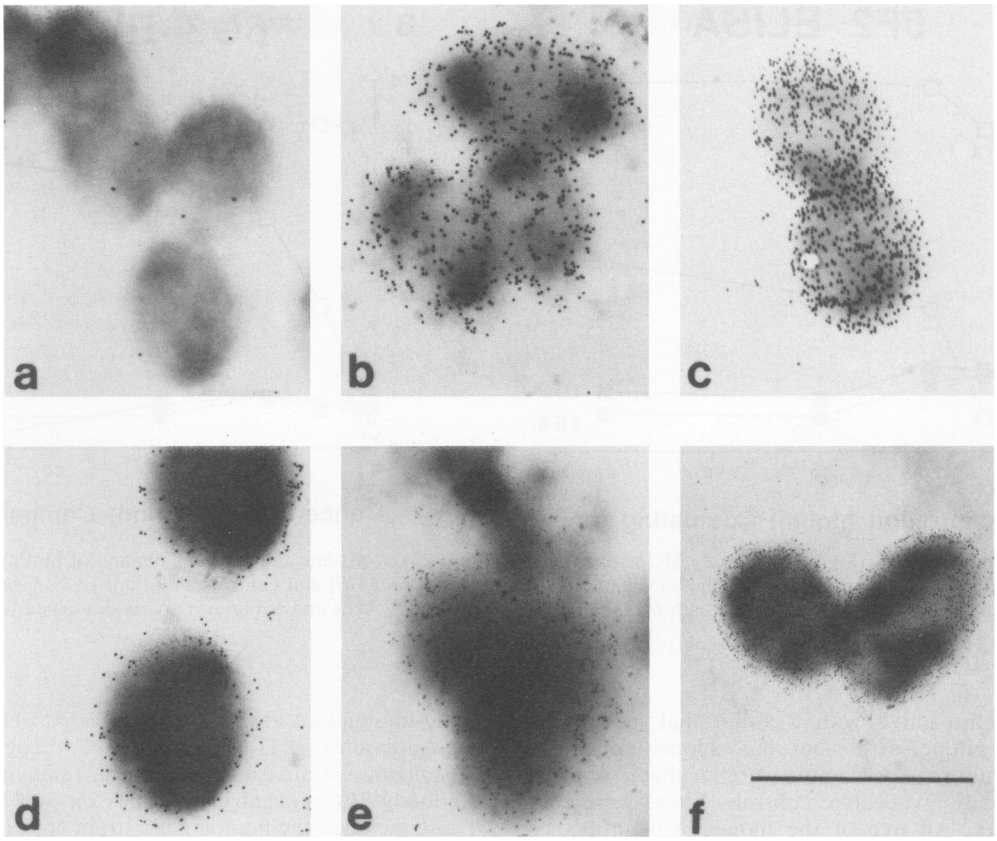

FIG. 4. Immunoelectron micrographs. Nontypeable *H. influenzae* whole organisms incubated with antibody followed by goat anti-mouse immunoglobulin G conjugated to colloidal gold particles. The strain, antibody, and size of the gold spheres, respectively, for each panel are as follows: strain 1479, SP2 as a negative control, 15 nm (panel a); strain 1479, antibody 5F2, 15 nm (panel b); strain 1479, antibody 6G3, 15 nm (panel c); strain 2019, antibody 7B11, 10 nm (panel d); strain 7502, antibody 1B10, 10 nm (panel e); and strain 5657, antibody 2E6, 5 nm (panel f). Bar = 1 μm.

efficiently inhibit the binding of 5F2 to its epitope. This may be the result of shared portions of linear epitopes or the presence of conformational epitopes. These data indicate that 5F2 recognized a different epitope compared with 6G3. Analysis of cyanogen bromide cleavage of P2 by the immunoblot assay confirmed this conclusion. Antibody 5F2 recognized an epitope on the 10,000-Da cyanogen bromide cleavage fragment, while antibody 6G3 recognized an epitope on the 32,000-Da fragment.

The P2 proteins of nontypeable strains of *H. influenzae* differ from the P2 proteins of type b strains with regard to antigenic heterogeneity. The P2 proteins of nontypeable strains show substantially more heterogeneity compared with type b strains in OMP profiles in SDS-PAGE (1, 2, 14, 24). Analysis of nucleotide sequences and restriction fragment length polymorphisms of P2 genes have demonstrated that the P2 proteins of type b strains are conserved among strains (9, 15, 16). By contrast, the P2 proteins of nontypeable strains express epitopes that are highly strain specific. Each of the antibodies in this study was reactive with less than 3% of the strains tested; indeed, three of the five antibodies were reactive exclusively with the homologous strain. These observations are consistent with genetic population studies that have shown that type b strains are basically clonal whereas nontypeable strains are genetically diverse (27, 29, 30).

The strain-specific epitopes on the P2 proteins of nontypeable strains appear to be immunodominant epitopes. All of our monoclonal antibodies to P2 were strain specific, suggesting that immunization with whole organisms selects for production of antibody to this epitope. This observation is consistent with that of Groeneveld et al. (8), who demonstrated that immunization of rabbits with whole bacteria produced antibody that is specific for the P2 protein of the homologous strain. Previous studies from our laboratory demonstrated that antibody that was immunopurified to P2 from normal human serum was bactericidal for four of six strains of nontypeable *H. influenzae* (20). These antibodies were reactive in an ELISA but not reactive in immunoblot assays. The monoclonal antibodies described in the present study were all reactive in the immunoblot assay. Therefore, the more broadly reactive bactericidal epitopes recognized by antibodies in normal human serum are different from the immunodominant, strain-specific epitopes recognized by these monoclonal antibodies. Further studies will determine the epitopes to which human antibodies are directed following infection with nontypeable *H. influenzae*.

The expression of a highly strain-specific and immunodominant antigenic determinant on the bacterial surface may represent a mechanism of host evasion by nontypeable *H. influenzae*, allowing the organism to cause recurrent disease. Infants and children generate strain-specific bactericidal antibody following otitis media due to nontypeable *H. influenzae* (7). Recurrent otitis media with nontypeable strains

occurs in spite of the presence of antigenically conserved structures, such as the P6 protein, on the bacterial surface (22). However, P6 makes up only a small percentage of the outer membrane of the bacterium. We hypothesize that the abundant expression of an immunodominant determinant on the bacterial surface induces a strain-specific immune response and hides conserved determinants that might induce antibodies that would protect from infection by other strains.

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