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Mapping of Bactericidal Epitopes on 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* and is a potential target of a protective immune response. Nine monoclonal antibodies (MAbs) to P2 were developed by immunizing mice with nontypeable *H. influenzae* whole organisms. Each MAb reacted exclusively with the homologous strain in a whole-cell immunodot assay demonstrating exquisite strain specificity. All nine MAbs recognized abundantly expressed surface-exposed epitopes on the intact bacterium by immunofluorescence and immunoelectron microscopy. Each MAb was bactericidal to the homologous strain in an in vitro complement-mediated killing assay. Immunoblot assay of cyanogen bromide cleavage products of purified P2 indicated that MAb 5F2 recognized the 10-kDa fragment, and the other eight MAbs recognized the 32-kDa fragment. Competitive ELISAs confirmed that 5F2 recognized an epitope that is different from the other eight MAbs. To further localize epitopes, MAbs 5F2 and 6G3 were studied in protein footprinting by using reversed-phase high-performance liquid chromatography. Three potential epitope-containing peptides which were reactive in an enzyme-linked immunosorbent assay with both 5F2 and 6G3 were isolated. These peptides were identified by N-terminal amino acid sequence and localized to loops 5 and 8 of the proposed model for P2. Fusion proteins consisting of glutathione *S*-transferase fused with variable-length peptides from loops 5 and 8 were expressed in the pGEX-2T vector. Immunoblot assay of fusion peptides of loops 5 and 8 confirmed that 5F2 recognized an epitope within residues 338 to 354 of loop 8; 6G3 and the remaining MAbs recognized an epitope within residues 213 to 229 of loop 5. These studies indicate that nontypeable *H. influenzae* contains bactericidal epitopes which have been mapped to two different surface-exposed loops of the P2 molecule. These potentially protective epitopes are strain specific and abundantly expressed on the surface of the intact bacterium.

Since the recognition of nontypeable *Haemophilus influenzae* as a major pathogen in such diseases as otitis media, chronic sinusitis, and chronic obstructive pulmonary disease (COPD), among others (4, 21, 29, 50, 51), investigators have focused on identifying a potentially protective immune response to this organism (5, 10, 14, 16, 31, 32, 36). Several lines of evidence indicate that bactericidal antibodies are associated with protection from infection. Shurin et al. demonstrated that serum bactericidal antibodies develop during the course of otitis media resulting from nontypeable *H. influenzae* (40). They further demonstrated that susceptibility to *Haemophilus* otitis correlated with the absence of bactericidal antibody in acute-phase serum samples. More recently, in a prospective trial of children with otitis media, Faden et al. showed that bactericidal antibody to strains of nontypeable *H. influenzae* correlated with strain-specific protection from otitis media (9). Furthermore, the presence of serum bactericidal antibody was associated with a significant reduction in the concentration of bacteria in middle ear fluid (9). These data indicate that bactericidal antibody is associated with protection from infection by nontypeable *H. influenzae*.

One of the specific targets of bactericidal antibody to nontypeable *H. influenzae*, in both normal human sera and immune sera, is the heterogeneous P2 protein (2, 12, 31, 49). P2 is the predominant outer membrane protein, ranging in molecular mass from 36 to 42 kDa among strains (3, 24, 28, 41). As the sole porin for *H. influenzae*, it exists as a trimer and is closely associated with lipooligosaccharide (7, 13, 22, 25, 47, 48). Even in immune sera depleted of polyribosylribitol phosphate capsule, antibodies to the P2 protein were protective in the infant rat model of experimental meningitis resulting from *H. influenzae* type b (27); this demonstrates the potential importance of antibodies to P2 in the immune response not only to nontypeable strains but to typeable strains as well.

The goal of this study was to characterize monoclonal antibodies (MAbs) that recognize P2 of nontypeable *H. influenzae*, map the epitopes, and assess the bactericidal activity of the antibodies. Localization of surface-exposed bactericidal epitopes will expand our understanding of antigenic structure and identify critical portions of the P2 molecule in its interaction with the host.

MATERIALS AND METHODS

Bacterial strains. Four strains of nontypeable *H. influenzae* (strains 1479, 3198, 5657, and 7502) used in this study were recovered from the sputum of adults with chronic bronchitis in Buffalo, N.Y. An additional 97 nontypeable *H. influenzae* strains, 25 type b, and one each of types a, c, d, e, and f from diverse clinical sources and geographic areas were studied. Each strain required both hemin (X factor) and NAD (V factor) for growth. Capsular serotypes were determined by counterimmunoelectrophoresis or agglutination with specific antisera. There were 19 isolates of other *Haemophilus* species used in this study and 24 isolates of a variety of other

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gram-negative bacilli, including *Actinobacillus actinomycetemcomitans*, *Moraxella* (*Branhamella*) *catarrhalis*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Serratia* species, *Salmonella minnesota*, *Proteus mirabilis*, *Morganella morganii*, and *Pseudomonas aeruginosa*. Isolates were stored in Mueller-Hinton broth with 10% glycerol at −70°C.

Development of MAbs. Whole cells of strain 1479 were used to immunize BALB/c mice intraperitoneally on days 0 and 28. On day 32, the mice were sacrificed and splenic lymphocytes were fused with SP2/0-Ag14 plasmacytoma cells as described previously (19, 35). Nine clones which produced antibodies reactive to the strain 1479 P2 protein by immunodot and immunoblot assays were cloned by limiting dilution (15, 30). Hybridomas producing the selected antibodies were injected intraperitoneally into pristane-primed BALB/c mice. The resulting ascitic fluid was harvested 2 to 3 weeks later, tested for specificity, and stored at −70°C.

Antibody isotyping. The isotype of each MAb was determined by immunodiffusion using the Ouchterlony method. Plates were prepared by using 13 ml of 1% DNA-grade agarose (Bio-Rad, Hercules, Calif.) in phosphate-buffered saline (PBS)–0.1% NaN_3 per 100-mm plate. After the agarose solidified, 4-mm wells were punched into the agarose in a circular pattern. The center well contained goat anti-mouse subclass-specific antibody (Southern Biotechnologics Associates, Birmingham, Ala.). Outer wells were filled with either undiluted or, where necessary, five-times-concentrated tissue culture supernatant containing MAbs to be tested alternated with MAbs of known isotypes. After the plates were incubated for 24 to 96 h in a humid chamber at ambient temperature, they were examined for white lines of immunoprecipitation.

Immunofluorescence microscopy. Indirect immunofluorescence was used to determine whether the MAbs recognized surface epitopes on the homologous bacterial strain. A suspension of *H. influenzae* in PBS was air dried, heat fixed to a glass slide, and incubated with MAb in undiluted tissue culture supernatant. Reactivity was detected with fluorescein-labeled goat anti-mouse immunoglobulin G (IgG) as described previously (15).

Immunoelectron microscopy. Anti-mouse IgG conjugated to 15-nm colloidal gold particles was used to detect MAb reactivity to surface-exposed antigenic determinants by immunoelectron microscopy as described previously (30).

Bactericidal assay. Bacterial strains were grown on chocolate agar overnight in 5% CO_2 at 37°C. A turbid suspension was made in a small amount of growth medium (brain heart infusion broth supplemented with 10 μg of hemin per ml and fresh NAD at 10 μg/ml) and used to adjust a flask of growth medium to a starting optical density at 600 nm (OD_{600}) of 0.05. The bacteria were grown to the mid-logarithmic phase (OD_{600}, 0.2) at 37°C with agitation. After the culture was diluted to 5×10^4 CFU/ml in Gey's balanced salt solution (GIBCO Laboratories, Life Technologies, Inc., Grand Island, N.Y.)-10% bovine serum albumin (GBSS-BSA), a 25-μl aliquot was added to each reaction vial. This was the equivalent of ~2,000 CFU per 250-μl total reaction volume.

The complement source used in the bactericidal assays was serum from patients with hypogammaglobulinemia. Whole blood was allowed to clot on ice, and aliquots of the serum were frozen at −70°C. Each aliquot was thawed only once. The antibody source was MAbs from ascitic fluid which were protein A (IgG2a and IgG2b) or protein G (IgG1) affinity purified. Each MAb was used at a single concentration of 80 μg/ml per assay vial except for MAbs 5F2 and 6G3, which were diluted for titer determination. Heat-inactivated normal human serum containing bactericidal antibody plus the complement source was used as a positive control of complement activity. A negative control containing only bacteria and complement was included to ensure that complement was not killing in the absence of antibody. An irrelevant MAb (8E6) specific for *M. catarrhalis* was also used as a negative control. Each MAb was tested both with and without the addition of complement. Both nontypeable *H. influenzae* 1479 and 5657 were tested with each antibody.

The reaction vial consisted of 25 μl of bacteria, 20 μg of MAb or 25 μl of normal human serum, and 22 μl of complement (8.8% of the total volume), with the remainder of the 250-μl reaction mixture made up of GBSS. Complement was thawed and added to the vials last. Reaction vials were incubated in a rotary shaker at 37°C for 2 h. A 25-μl aliquot was inoculated onto chocolate agar plates in duplicate at times 0, 60, and 120 min. Colony counts were determined from duplicate plates after an overnight incubation in 5% CO_2 at 37°C. The average of the duplicate plates was calculated.

SDS-PAGE and immunoblot assays. Whole-cell preparations were solubilized in sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% gels under denaturing conditions (23, 30). Gels were stained with Coomassie blue (34) or silver stain (28, 46) or transferred to nitrocellulose for immunoblot assay (30).

Purification of P2. P2 was purified as described previously (30).

Competitive ELISA. To determine whether each of the nine MAbs recognized the same or different epitopes on the P2 of strain 1479, a competitive enzyme-linked immunosorbent assay (ELISA) using biotinylated purified P2 was performed as detailed by Haase et al. (15). Briefly, 5F2 and 6G3 were used to coat microtiter plates in separate experiments; nonspecific reactivity was blocked with 3% gelatin. Serial dilutions of each MAb were preincubated with biotinylated P2 from strain 1479 for 1 h at ambient temperature prior to being added to the coated microtiter plates. Avidin peroxidase conjugate was added; after the plates were incubated for 1 h at ambient temperature, color was developed with ortho-phenylenediamine substrate in citric-phosphate buffer (pH 5.0)–0.003% H_2O_2. After the plates were incubated for 30 min at ambient temperature, color development was stopped with the addition of 5 N H_2SO_4. The A_{490} was determined for each well on a BIO-TEK EL-307 ELISA reader.

Negative controls included MAb 1G9, which recognizes an epitope on the lipooligosaccharide of strain 1479, and an anti-P2 MAb (2E6), which does not react with strain 1479. In addition, affinity-purified SP2 hybridoma ascites was used to monitor nonspecific background mouse antibody.

Protein footprinting using rHPLC. The reversed-phase high-performance liquid chromatography (rHPLC) procedure was a modification of the one used by Jemmerson and Paterson to map epitopes on cytochrome *c* (17).

Trypsin digestion. To prepare for trypsin hydrolysis, the P2 and antibody samples were precipitated in ethanol and briefly air dried before being solubilized in digestion buffer (50 mM NH_4HCO_3 [pH 8.3]). P2 was combined with antibody in an equimolar ratio (0.56:1) where indicated. The P2, MAb, and P2-MAb samples, including the NH_4HCO_3 controls, were preincubated at 4°C for 4 h with end-over-end mixing. Trypsin (Sigma Chemical Co., St. Louis, Mo.) was reconstituted just before use in 0.01% trifluoroacetic acid (TFA). Trypsin (5% [wt/wt] P2) was added to the appropriate vials, and the vials were incubated at 37°C for 90 min in a shaking water bath. Trypsin digestion was stopped by adding 30 μl of 2.0 N acetic acid to the 150-μl total reaction volume. Each sample was

frozen in dry ice and ethanol before being lyophilized in a Speedvac (Savant) under low heat and stored at −20°C. Controls included NH_4HCO_3 without trypsin (0 h and 90 min), trypsin (5 μg at 0 h and at 90 min), and trypsin (15 μg at 0 h) and the oxidized beta chain of bovine insulin plus trypsin (5% [wt/wt] insulin). The controls identified NH_4HCO_3 breakdown, trypsin self-hydrolysis, and trypsin activity.

Separation of peptides by rHPLC. A Waters Deltapak C18 reversed-phase column (3.9 mm by 15 cm) with a 30-nm pore size and a 5-μm particle size was used in all HPLC separations. Prior to being loaded, lyophilized samples were reconstituted in 200 μl of 0.1% (wt/vol) TFA to which 25 μl of an internal protein standard was added. The samples were centrifuged for 1 min at 2,000 × *g*. A 200-μl sample was loaded onto the column in 100% solvent A (0.1% [wt/vol] TFA in H_2O) and eluted at a flow rate of 1.0 ml/min in a linear gradient of 0 to 72% solvent B (0.075% [wt/vol] TFA in 70% [wt/vol] acetonitrile) over 137 min at ambient temperature. Peak absorbance was monitored simultaneously at 214 and 280 nm. Peak fractions at 214 nm from the trypsin digestion of P2 were collected manually, lyophilized in a Speedvac, and stored at −20°C.

Purification of peptide fractions. Selected fractions from the tryptic digestion of P2 were reconstituted in 0.1% (wt/vol) TFA, pooled from a number of runs, and applied to the same column with solvents A and B as described above. Peptide fractions were separated at a flow rate of 1.0 ml/min in a linear gradient where the increase in solvent B was 10% over 50 min. The starting and ending concentrations of the elution buffer in the linear gradient were optimized for each selected fraction region. Peaks were monitored at 214 and 280 nm. Single peaks at 214 nm were manually collected, lyophilized in a Speedvac, and stored at −20°C.

ELISA. Lyophilized P2 tryptic fractions were each suspended in 50 μl of 50 mM carbonate buffer (pH 9.6) and coated onto 96-well flat-bottom microtiter plates, and the plates were incubated overnight at 4°C. Purified strain 1479 P2 (250 ng per well) was used as a positive control, and strain 3198 P2 and strain 7502 P2 at the same concentrations (i.e., 250 ng per well) were used as negative controls. Following a single wash in PBS, nonspecific binding was blocked with 3% BSA in PBS (300 μl per well), and the plates were incubated for 1 h at 37°C. After five washes with PBS–0.05% Tween 20 (PBS-Tween), MAb (6G3) appropriately diluted in assay diluent (PBS, 1% BSA, 0.05% Tween 20) was added to designated wells (50 μl per well), and the wells were incubated for 2 h at 37°C. The plates were washed in PBS-Tween as described above, and protein A-horseradish peroxidase (protein A-HRP) conjugate (Zymed) diluted in assay diluent was added to each well and incubated at ambient temperature for 1 h. Color was developed by using tetramethylbenzidine (Sigma)–0.015% H_2O_2 (50 μl per well) for 30 min at 37°C. The reaction was stopped by the addition of 4 N H_2SO_4. The A_{450} of each well was determined on a Dynatech MR600 Microplate Reader.

The use of MAb 5F2 (IgG1) as a primary antibody in the assay required a goat anti-mouse IgG-HRP conjugate (Kirkegaard & Perry Laboratories, Inc.) in place of the protein A-HRP. As in the protein footprinting assay, only MAbs 6G3 and 5F2 were used to screen for potential epitopes in the ELISA.

PCR. PCR was employed to obtain appropriate fragments of the P2 gene for subcloning and expression as fusion proteins. A plasmid which contains the 3′ half of the P2 gene of nontypeable *H. influenzae* 1479 was used as template DNA (41). Primers were designed with restriction sites for directional cloning into pGEX-2T. $MgCl_2$ was added to the reaction mixture to a final concentration of 3.7 mM. PCR was carried out as follows. The template was denatured at 95°C for 5 min; five cycles were run at 95°C for 1 min 30 s, 37°C for 2 min, and 50°C for 3 min. This was followed by twenty-five cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min (extension). Finally, 8 min of extension time at 72°C was included to complete the reaction.

Construction of plasmids. The pGEX-2T vector was used to express peptides as fusion proteins (43). The vector was digested with *Eco*RI and *Bam*HI for most of the constructions except for pTM208-A, which had two *Bam*HI sites. Since the primers were designed so that the PCR products had different restriction sites, directional subcloning was possible in most cases. Appropriate DNA bands were excised from 2.4% low-melting-point agarose (NuSieve; FMC Bioproducts, Rockland, Maine). Agarose was heated at 65°C with the same volume of TE buffer (10 mM Tris-HCl, 1mM EDTA [pH 8.0]) until it was melted, and the sample was extracted with phenol and precipitated with ethanol. Restriction digestion was performed on purified DNA fragments to acquire compatible ends with the pGEX-2T vector. Digested vector and insert DNAs were mixed at a 1:2 molar ratio. T4 DNA ligase was used as instructed by the manufacturer (Promega). The ligation mixture was incubated at 16°C overnight. The recombinant DNA molecule was electroporated into *E. coli* DH5α F′. The electroporation was performed at 25 μF, 200 Ω, and 1.80 kV for 4 to 5 ms. Transformed cells were plated on LB amp (Luria-Bertani medium plus 60 μg of ampicillin per ml). Transformants were screened by restriction digest analysis of plasmid DNA. Clones which contained the expected size insert were confirmed by DNA sequencing.

Purification of fusion protein. Fusion proteins were purified by the method of Smith and Johnson (43). Briefly, bacteria were grown overnight at 37°C in 80 ml of LB amp with vigorous shaking. The overnight culture was diluted with fresh medium to 800 ml. After 1 h of incubation, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.4 mM; 3 to 4 h of incubation followed. The cells were pelleted, resuspended in MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 [pH 7.3]), and lysed by moderate sonication on ice. After Triton X-100 (Fisher Scientific, Fair Lawn, N.J.) was added to 1%, the lysed cell suspension was centrifuged at 10,000 × *g* for 5 min at 4°C. The supernatant was mixed with 2 ml of 50% glutathione agarose beads (Sigma) with gentle shaking by inverting the tube for 2 min. After centrifugation at 500 × *g*, the mixture was washed with 50 ml of MTPBS three times. To elute the fusion protein from the beads, the same volume of 5 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0) was added to the mixture with gentle shaking by inverting the tube for 2 min to elute the fusion protein. The eluate was collected by centrifuging the beads at 500 × *g*. The eluting process was carried out twice for thorough harvest of fusion proteins.

RESULTS

Characterization of MAbs. (i) Antigenic specificity. Nine MAbs from a single fusion using splenocytes from mice immunized with whole cells of nontypeable *H. influenzae* 1479 were identified by reactivity in an immunodot assay using bacterial whole-cell lysates of the homologous strain. In immunoblot assays, each antibody bound to a 42-kDa protein which corresponds to the P2 protein in whole-cell lysates of strain 1479 (Fig. 1). Each MAb also recognized purified P2 in immunoblot assays and ELISAs. MAbs 5F2 and 6G3 were characterized previously (15).

(ii) MAb isotype. The isotyping results obtained for the

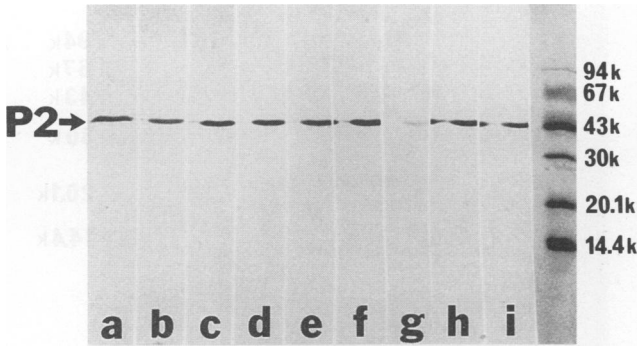

FIG. 1. Immunoblot assay of nontypeable *H. influenzae* 1479 whole-cell lysate (all lanes) incubated with MAbs and detected with protein A-peroxidase and 4-chloro-1-naphthol. Lanes were incubated with the following MAbs: a, 7B10; b, 6G3; c, 6F2; d, 6D2; e, 6C5; f, 6A11; g, 5F2; h, 3C10; i, 1B5. Molecular mass standards in kilodaltons (k) are shown on the right.

MAbs by Ouchterlony assay were as follows: 3C10, IgG2b; 1B5, 6A11, 6C5, 6D2, 6F2, 6G3, and 7B10, IgG2a; 5F2, IgG1.

(iii) Surface exposure. Immunoelectron microscopy and immunofluorescence were performed to determine whether the epitopes recognized by the MAbs on the homologous strain were exposed on the surface of the intact bacterial cell. Previously, it was shown that both MAbs 5F2 and 6G3 recognized surface-exposed epitopes on strain 1479 (15).

In this study, the other seven MAbs (1B5, 3C10, 6A11, 6C5, 6D2, 6F2, and 7B10) also reacted with an abundant surface-exposed antigenic determinant(s) on *H. influenzae* 1479 by immunoelectron microscopy as represented by MAb 3C10 in Fig. 2. Controls consisting of SP2 hybridoma supernatant and MAb to an irrelevant antigen used in place of the anti-P2 MAbs were consistently negative. These results were confirmed by indirect immunofluorescence microscopy which showed that the other seven MAbs were highly reactive. We conclude that all nine MAbs recognize epitopes which are abundantly expressed on the surface of strain 1479.

(iv) Strain and species specificity. To determine the strain and species specificity, MAb in undiluted tissue culture supernatant was incubated with a panel of immunodots consisting of whole-cell lysates of each of the 174 organisms listed in "Bacterial strains" above. Each of the nine MAbs reacted exclusively with the homologous strain, indicating that the epitopes were unique to this strain.

(v) Bactericidal activity of MAbs. In vitro assays were performed to determine if each of the MAbs was bactericidal for strain 1479. Table 1 shows that each of the nine anti-P2 MAbs was bactericidal, with a 0% survival at 2 h. The corresponding negative controls consisting of MAb in the absence of complement had a survival of greater than 100%, indicating that killing was complement mediated. Similarly, a

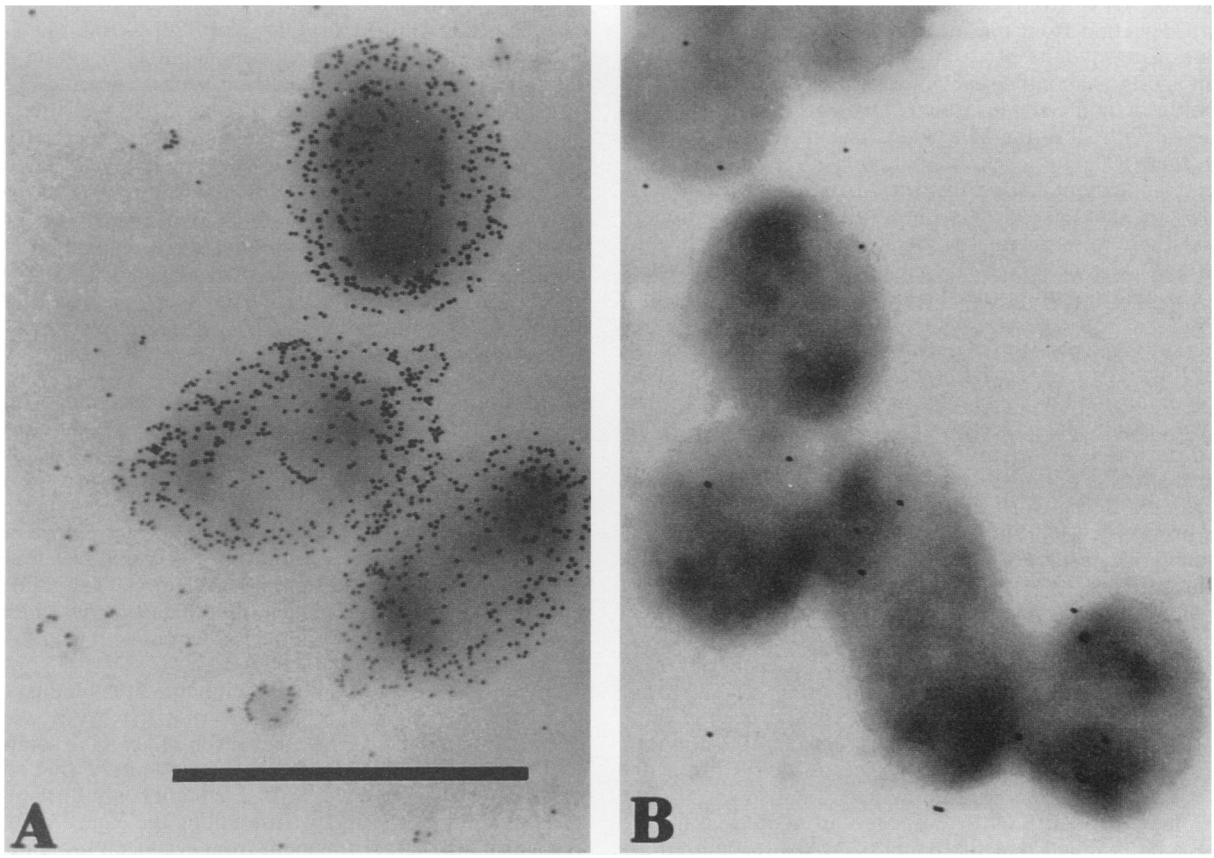

FIG. 2. Immunoelectron micrographs of nontypeable *H. influenzae* 1479 whole organisms incubated with MAb and then with goat anti-mouse IgG conjugated to 15-nm colloidal gold particles. (A) MAb 3C10 undiluted tissue culture supernatant; (B) SP2 undiluted tissue culture supernatant (negative control). Bar, 1 μm.

TABLE 1. Bactericidal activity of nine MAbs to the P2 protein of nontypeable *H. influenzae*

MAb	Concn (μg/ml)	% Survival at 120 min[a]	
		Strain 1479 with complement	Strain 5657 with complement[b]
1B5	80	0	126.0
3C10	80	0	164.9
5F2[c]	68	0	89.8
6A11	80	0	217.0
6C5	80	0	191.0
6D2	80	0	171.3
6F2	80	0	166.3
6G3[d]	50	0	97.2
7B10	80	0	152.1
Control	0	257.8	114.8

[a] % Survival = [(average CFU at $T_{2\ h}$)/(average CFU at $T_{0\ h}$)] × 100, where T is time.
[b] Standard deviation from the mean was 4.7 per 100 CFU per duplicate plate set.
[c] Titer, 40 μg/ml.
[d] Titer, 0.100 μg/ml.

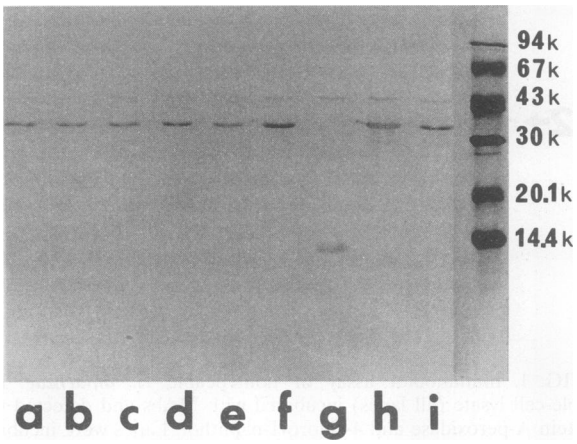

FIG. 3. Immunoblot assay of cyanogen bromide cleavage of purified P2 from *H. influenzae* 1479. Lanes were incubated with the following MAbs: a, 7B10; b, 6G3; c, 6F2; d, 6D2; e, 6C5; f, 6A11; g, 5F2; h, 3C10; i, 1B5. Molecular mass standards in kilodaltons (k) are shown on the right.

control containing only bacteria and complement had a survival of greater than 100%, indicating that the complement source did not contain bactericidal antibody to the specific strain. The complement activity was sufficient since the normal human serum with the addition of complement was bactericidal. At each sampling time, i.e., 0, 1, and 2 h, the number of CFU on duplicate plates was averaged. Analysis of plates with at least 100 CFU from 227 assay vials indicated that the standard deviation from the mean was 4.7 per 100 CFU per duplicate plate set.

As an additional control, each of the anti-P2 antibodies was tested with the nontypeable strain *H. influenzae* 5657, which is nonreactive with all of the antibodies. The survival at 2 h was greater than 89% for each MAb both with (Table 1) and without complement. An irrelevant MAb to *M. catarrhalis* (8E6) was included as a negative control. When this antibody was used, there was a survival of greater than 100% at 2 h with both strains 1479 and 5657. We conclude that each of the nine MAbs recognizes a strain-specific bactericidal epitope on the P2 protein.

Bactericidal activity was defined as less than or equal to 50% survival [(average CFU at 2 h)/(average CFU at 0 h) × 100] after 2 h of incubation. Upon diluting MAbs 5F2 and 6G3, the minimum bactericidal concentration for 5F2 was 40 μg/ml in comparison with 0.100 μg/ml for 6G3.

Identification of epitopes. (i) Cyanogen bromide cleavage of P2. Purified P2 was cleaved with cyanogen bromide as described previously (30). The P2 protein of strain 1479 contains one methionine residue and was cleaved into two fragments with approximate molecular masses of 32 and 10 kDa as determined by SDS-PAGE. The cleaved P2 was transferred onto nitrocellulose for the immunoblot assay. Previously, it was shown that MAb 5F2 reacted with the 10-kDa band and 6G3 reacted with the 32-kDa band (15). The other seven MAbs (1B5, 3C10, 6A11, 6C5, 6D2, 6F2, and 7B10) all reacted with the 32-kDa band as shown in Fig. 3. Therefore, 6G3 and the other seven MAbs recognize an epitope(s) which is distinctly different from the one recognized by 5F2.

(ii) Competitive ELISAs. By cyanogen bromide cleavage of P2, it was determined that the panel of MAbs recognized at least two epitopes on P2. Competitive ELISAs were designed to further define epitope differences by comparing the abilities of the MAbs to bind to P2 in the liquid phase, thereby blocking the ability of P2 to bind MAb immobilized on a microtiter plate. The percent inhibition was calculated to determine the extent to which a competing MAb inhibited the binding of the coating MAb to biotinylated P2 at each P2 concentration tested. The result, relative to purified SP2 hybridoma control ascites which contained nonspecific mouse background antibodies, was calculated by using the following formula: [(OD_{490} of SP2 − OD_{490} of MAb)/OD_{490} of SP2] × 100. The concentration of MAb (micrograms per milliliter) resulting in 50% inhibition was then determined for each competing MAb.

To compare the inhibition of each MAb with that of the coating MAb, the relative concentration was calculated as follows: concentration (micrograms per milliliter) of the heterologous MAb at 50% inhibition/concentration (micrograms per milliliter) of the homologous MAb at 50% inhibition. Figure 4 shows the results of competitive ELISAs when 5F2 and 6G3 were used as the coating antibodies. When MAb 5F2 was used as the coating antibody (Fig. 4A), the eight heterologous MAbs inhibited less than the homologous antibody (arrow), with relative concentrations greater than 1.0. The average variation between duplicate wells was 0.90%. In comparison, when 6G3 was used to coat the plates (Fig. 4B), seven of the heterologous MAbs were better inhibitors, with relative concentrations less than 1.0, and one MAb inhibited slightly less than the homologous antibody (arrow), with a relative concentration of 1.7. The average variation between duplicate wells was 0.98%. In each assay, the relative concentration of the negative control MAbs 1G9 and 2E6 was much greater than 1.0, indicating no inhibition. The differences between the homologous and the heterologous antibodies were considerably greater in the 5F2 experiment (Fig. 4A) than in the 6G3 experiment (Fig. 4B).

Previously, we showed that 5F2 inhibited the binding of 6G3 to P2 but that the reverse was not true (15). We now expand our findings to include the other seven MAbs. The competitive ELISAs confirm that 5F2 recognizes an epitope that is different from the other eight MAbs. Whether any further differences in epitope specificity exist among the eight MAbs which recognized the larger fragment from cyanogen bromide cleavage remains inconclusive from competitive ELISAs.

On the basis of present and previous results from cyanogen bromide cleavage and competitive ELISAs, MAbs 5F2 and

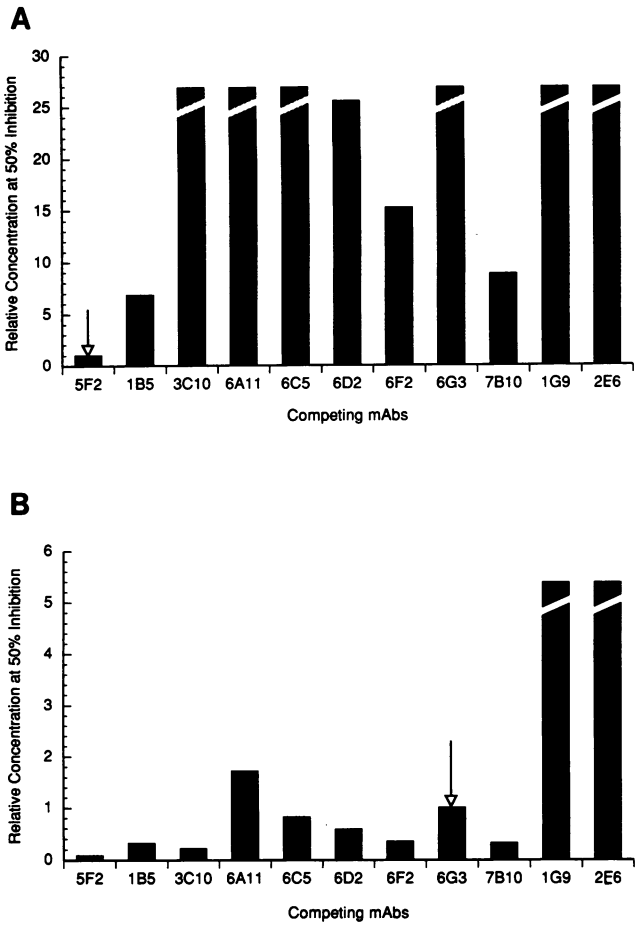

FIG. 4. Competitive ELISA. MAbs 5F2 and 6G3 were used to coat microtiter plates. Serially diluted MAbs were preincubated with biotinylated P2 prior to being added to the coated plates to determine if the soluble MAbs were able to inhibit the binding of the immobilized MAbs to P2. Each panel shows the comparison of the relative concentration of competing MAbs at 50% inhibition when 5F2 (A) or 6G3 (B) was used as the coating MAb. Relative concentration is the concentration (micrograms per milliliter) of heterologous MAb at 50% inhibition divided by the concentration (micrograms per milliliter) of homologous MAb at 50% inhibition. Coating MAb is the homologous MAb (⇩). Symbols: ▨, strain 1479 P2-specific MAbs; ■, negative controls. 1G9 is the strain 1479 lipooligosaccharide-specific MAb, and 2E6 is the strain 5657 P2-specific MAb.

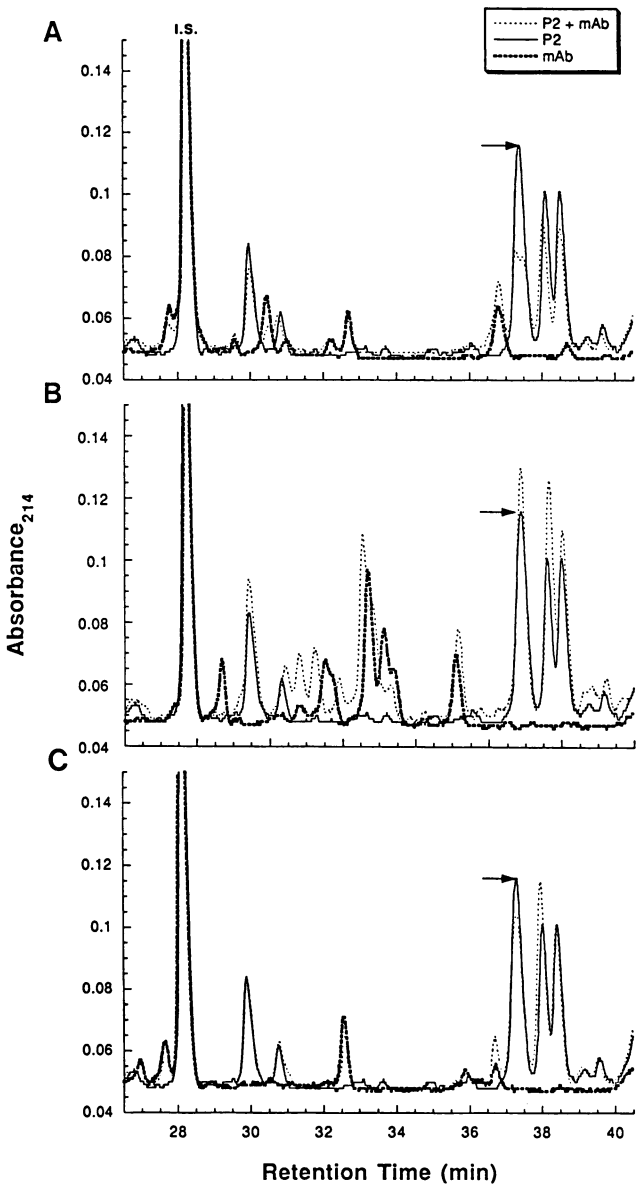

FIG. 5. Comparison of the 39.2 P2 peak (arrow) in the tryptic digests of P2 versus P2-MAb complex separated by rHPLC. Each panel consists of an overlay of chromatograms of P2 alone, MAb alone, and P2-MAb. (A) MAb 6G3; (B) MAb 2E6 (negative control); (C) MAb 5F2. I.S., internal standard. The column used was a Waters Deltapak C18 (3.9 mm by 15 cm) with a 30-nm pore size and a 5-μm particle size. The flow rate was 1.0 ml/min. The mobile phases were 0.1% (wt/vol) TFA in H_2O (solvent A) and 0.075% (wt/vol) TFA in 70% acetonitrile (solvent B). The eluent was 0 to 72% solvent B over 137 min. Monitoring was done at 214 nm.

6G3 were selected for protein footprinting to define the epitopes which they recognize.

(iii) Protein footprinting by rHPLC. (a) Strategy. Protein footprinting of the P2 protein by rHPLC was used to determine the epitopes recognized by MAbs 5F2 and 6G3 in a modification of a method previously described by Jemmerson and Paterson for horse cytochrome *c* (17). Protein footprinting is based on the concept that antibody binding to a protein will protect the protein from trypsin hydrolysis in the region of binding. Purified P2 from strain 1479 was preincubated with and without MAb before partial digestion with trypsin. MAb 2E6, which was reactive with the P2 of strain 5657 but not with strain 1479, was used as the negative control. The chromatogram of trypsin hydrolysis of the P2–P2-specific MAb complex was compared with that of P2 alone to determine where specific antibody binds to P2. The chromatograms were examined for the disappearance of peaks, appearance of new peaks, and changes in peak height of existing peaks. To confirm that selected peptide fractions from protein footprinting were specific for MAb and to probe for additional peptides that could not be picked out by footprinting because of multiple peak overlays, single peak fractions from the trypsin digestion of P2 were collected and tested in an ELISA and reactive peptides were identified by N-terminal amino acid sequencing.

(b) Analysis of peptides from trypsin digestion of P2. To

TABLE 2. Reactivity and identification of tryptic peptides with MAbs 6G3 and 5F2 in an ELISA

Peptide peak no.	Reactivity[a] (% of P2 control)		N-terminal sequence	Location in P2 molecule[b]
	6G3	5F2		
19.2	20.1	11.1	DDSNNHYQQ	Loop 5
39.2	24.4	0	DACTTTTIIYYP	Loop 5
43.5	19.2	35.4	EQAVLFGIDHK	Loops 7 and 8
55.2	26.3	48.3	ATHNLLGA	(?) Mixed[c]

[a] Reactivity was calculated as follows: (absorbance of peptide − background absorbance)/(absorbance of P2 − background absorbance), where background absorbance is the absorbance produced by the irrelevant tryptic peptide of P2.
[b] See Fig. 5.
[c] This sequence did not correspond to the P2 sequence, so it likely represents a mixed peptide.

achieve partial digestion of P2 with isolated peaks and good peak height and minimal digestion of the MAbs, the optimal digestion conditions of 90 min and 37°C were chosen. Proteolysis of purified P2 produced a tryptic map of approximately 49 peptides. MAbs 6G3 and 5F2 were digested into approximately 52 peptides on average, and control MAb 2E6 produced approximately 59 peptides. The Maxima 825 software program (Dynamic Solution, Division of Millipore, Ventura, Calif.) enabled the comparison of the trypsin digestion of P2, MAb, and the P2-MAb complex by overlaying chromatograms. The probable source of each peak of the P2-MAb chromatogram was identified as P2, MAb, trypsin, or a combination. Those areas where the P2-MAb peak overlapped a P2 peak were considered potential epitopes in protein footprinting. Suspected epitope peaks were those P2 peaks which had an overall decrease in peak height with the addition of antibody of greater than or equal to 50%. Some P2 peaks where a shoulder peak either appeared or disappeared with the addition of antibody were also considered suspicious epitope regions. In each assay, the tryptic map of P2 was compared with those of P2-6G3 and P2-5F2 and the negative control P2-2E6. In at least four assays, triads of peaks at 39 min (Fig. 5) and 55 min (data not shown) were identified as regions containing possible epitopes. However, a comparison of the areas of change in P2-6G3 and P2-5F2 were not consistently different and showed considerable overlap. As expected in the negative control, the P2 peaks in the P2-2E6 tryptic map remained relatively unchanged compared with those of P2 alone. Since peaks at 39 and 55 min could only be identified as being changed with the addition of strain 1479-specific MAb, it was necessary to try to clarify the 5F2-specific and 6G3-specific peptides by ELISA using the P2 tryptic peptides separated by rHPLC.

All peptide fractions from five tryptic digests of P2 were collected and tested with MAbs 5F2 and 6G3 in an ELISA. Each peptide fraction was lyophilized, reconstituted in carbonate buffer, and used to coat a microtiter plate. The optimal concentrations of purified P2 from strain 1479 for the positive control and of P2 from strains 7502 and 3198 for the negative controls were determined by checkerboard titration. Because of the limited sample, the optimal concentration of each peptide could not be determined. Furthermore, to achieve optimal peptide reactivity, different conjugates were required for 5F2 and 6G3 because of the difference in isotypes. This resulted in considerable background reactivity, which was subtracted by using the result of an apparently nonreactive peptide.

The results of these ELISAs along with the footprinting results noted above were used to direct further analysis of peptides. In addition to peaks in the 39- and 55-min regions identified by protein footprinting, peaks in the 19- and 43-min regions were also reactive in ELISAs. Peptide fractions containing these selected peaks were pooled from several runs and purified to single peaks by rHPLC using optimal linear gradients for each region. The reactivity of individual isolated peaks, i.e., peaks 19.2, 39.2, 43.5, and 55.2, with MAb 5F2 or 6G3 was verified by ELISA. Table 2 shows the results of ELISAs and N-terminal amino acid analysis of the four peptide peaks. The results of ELISAs were standardized by subtracting the result obtained from an apparently irrelevant peptide. Table 2 shows that peaks 19.2 and 39.2 produced reactivity with MAb 6G3 out of proportion to that with MAb 5F2. The N-terminal amino acid sequence of these two peptides indicated that they corresponded to the loop 5 region of the P2 molecule, suggesting that MAb 6G3 recognizes an epitope on loop 5.

Similarly, peaks 43.5 and 55.2 showed reactivity with MAb 5F2 out of proportion to that with MAb 6G3. Analysis of peak 55.2 indicated that it was a mixed peptide. The N-terminal sequence of peak 43.5 corresponded to the carboxy-terminal portion of loop 7 which is buried in the membrane. Since the actual length of this peptide is unknown and the N-terminal amino acid sequence identified only 11 residues, it is probable that the peptide extended into the surface-exposed portion of loop 8. The combination of these results with those of the cyanogen bromide cleavage and surface exposure studies suggested that MAb 5F2 recognizes an epitope on loop 8 of the P2 molecule.

In summary, analysis of the results of protein footprinting and ELISAs of tryptic peptides of the P2 protein suggested that MAb 6G3 recognized an epitope on loop 5 and MAb 5F2 recognized an epitope on loop 8. By using these results as a

TABLE 3. Oligonucleotides used for fusion protein construction

Clone	5′ Oligonucleotide	3′ Oligonucleotide	Amino acid residues of mature P2
pTM205-A	5′GCGTGGATCCACAAATTATAAAGACAG3′	5′CGATGAATTCCTAAACTAGCTAAAGC3′	203–248
pTM205-L	5′GCGTGGATCCACAAATTATAAAGACAG3′	5′CGATGAATTCGGTAAATTATGGTGGTG3′	203–232
pTM205-M	5′GCGTGGATCCACAAATTATAAAGACAG3′	5′CGATGAATTCGTTGGCTTTGGGGATTT3′	203–220
pTM205-S	5′GCGTGGATCCACAAATTATAAAGACAG3′	5′CGATGAATTCATAACTATGATTACTGT3′	203–212
pTM205-LI	5′GCGTGGATCCGACGCCGACACCGACAC3′	5′CGATGAATTCGGTAAATTATGGTGGTG3′	223–232
pTM205-LJ	5′GCGTGGATCCACGCAAAAAATCCCCAA3′	5′CGATGAATTCGGTGGTGTCGGTGTCGG3′	213–229
pTM208-A	5′GCGCGGATCCTATATTGAAGGTGC3′	5′GCGCGGATCCTACACCCACTGATT3′	331–364
pTM208-L	5′GCGTGGATCCTCTAGAACTAGAACAAC3′	5′CGATGAATTCTTTTTCTTTTTCAGTTTTTACT3′	338–360
pTM208-M	5′GCGTGGATCCTCTAGAACTAGAACAAC3′	5′CGATGAATTCTACTTTTGAAGCAACTT3′	338–354
pTM208-S	5′GCGTGGATCCTCTAGAACTAGAACAAC3′	5′CGATGAATTCACCTACAGAAGTTGTTC3′	338–346

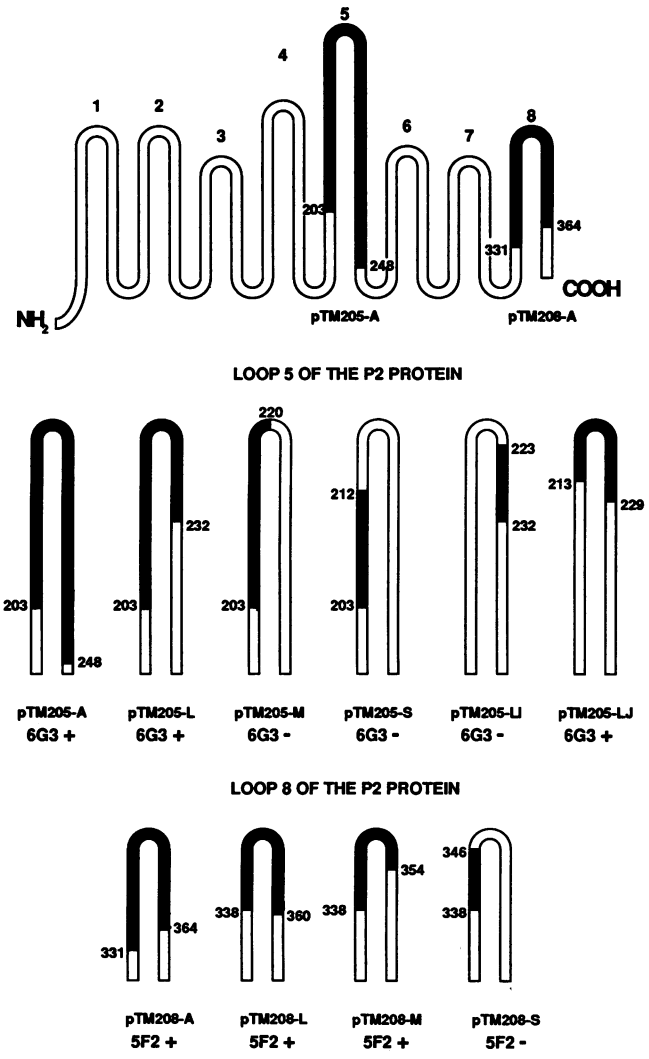

FIG. 6. Schematic diagram of the P2 molecule of nontypeable *H. influenzae* 1479 showing the regions of loops expressed as fusion proteins with GST. Expressed peptides are represented as filled bars. The numbers correspond to amino acid numbers of the mature protein (21). Loops 5 and 8 are illustrated to reveal the peptides expressed by each clone. The reactivity of the MAbs (positive or negative) is shown below the corresponding clone.

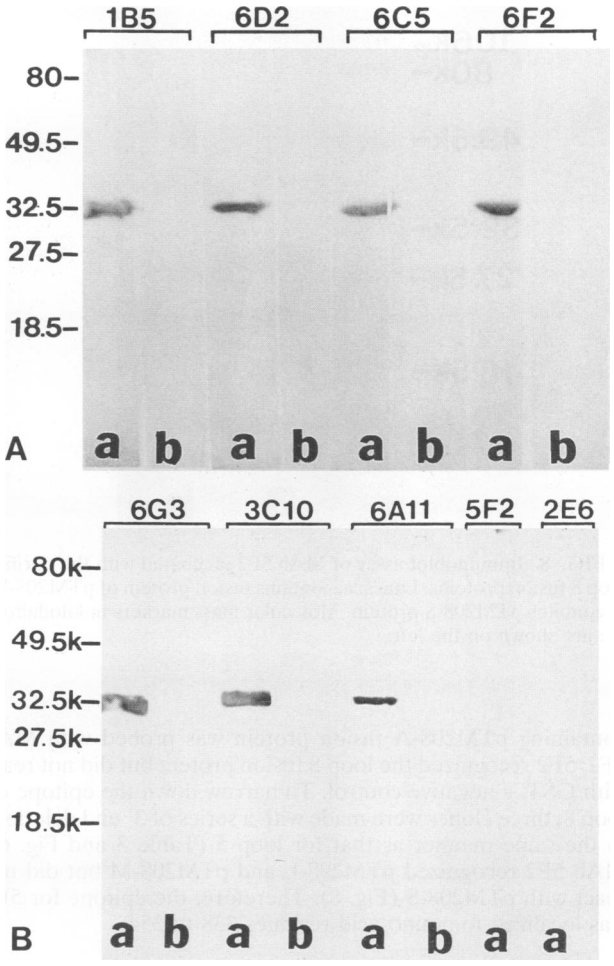

FIG. 7. Immunoblot assay of nine MAbs to the purified loop 5 fusion protein (pTM205-A). Lanes: a, contain loop 5 fusion protein; b, contain GST only. Molecular mass markers in kilodaltons (k) are shown on the left. Each MAb is labeled at the top. (A) MAbs 1B5, 6D2, 6C5, and 6F2 are specific for strain 1479. (B) MAbs 6G3, 3C10, 6A11, and 5F2 are specific for strain 1479; MAb 2E6 (negative control) is specific for the P2 of strain 5657 and is nonreactive with strain 1479.

guide, the next series of experiments was designed to specifically localize the epitopes recognized by these antibodies.

(iv) Expression and analysis of P2 fusion peptides. To more precisely characterize the epitopes, defined regions which correspond to loop 5 and loop 8 of the P2 gene were subcloned into the pGEX-2T vector. The clones expressed corresponding peptides as fusion proteins with glutathione *S*-transferase (GST).

pTM205-A, which included the entire loop 5, was constructed and expressed as a fusion protein with GST (Table 3 and Fig. 6). The purified fusion protein of pTM205-A was visualized by SDS-PAGE. The fusion protein shifted up slightly compared with GST, indicating that it indeed gained an extra peptide. After being transferred to nitrocellulose, the pTM205-A fusion peptide was probed with MAbs (Fig. 7). Eight MAbs recognized the pTM205-A peptide, while 5F2 and control MAb 2E6, which recognizes the P2 of strain 5657, did not recognize the fusion peptide. In an effort to further define the epitope, three clones of different lengths (pTM205-L, pTM205-M, and pTM205-S) were constructed by excluding amino acids from the C-terminal end of the loop 5 peptide (Table 3 and Fig. 6). Only pTM205-L fusion protein was reactive to the eight MAbs. Since this suggested that the epitope might be located within a unique region of pTM205-L, clone pTM205-LI was constructed (Table 3 and Fig. 6). None of the eight MAbs, however, recognized the pTM205-LI fusion protein. Another clone was made to encompass amino acid residues 213 to 229 (pTM205-LJ). The eight MAbs which recognized the pTM205-A fusion protein also recognized the pTM205-LJ fusion protein.

On the basis of previous data which showed that MAb 5F2 recognized the smaller fragment of CNBr-cleaved P2 protein (15) and on the basis of protein footprinting, it was suspected that 5F2 recognized an epitope on loop 8. Therefore, the clone pTM208-A which covered the entire loop 8 region was constructed (Table 3 and Fig. 6). The purified fusion protein of pTM208-A was visualized by SDS-PAGE. The immunoblot

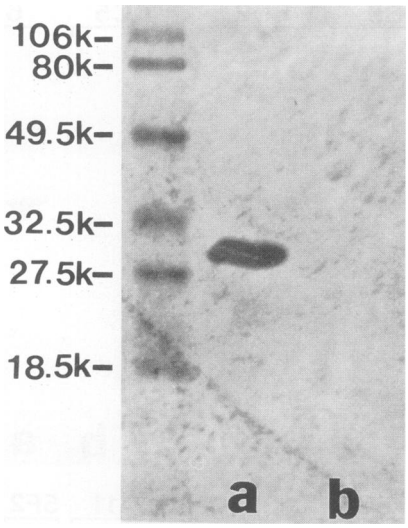

FIG. 8. Immunoblot assay of MAb 5F2 incubated with the purified loop 8 fusion proteins. Lanes: a, contains fusion protein of pTM208-M; b, contains pTM208-S protein. Molecular mass markers in kilodaltons (k) are shown on the left.

containing pTM208-A fusion protein was probed with MAb 5F2; 5F2 recognized the loop 8 fusion protein but did not react with GST, a negative control. To narrow down the epitope on loop 8, three clones were made with a series of 3′ end deletions in the same manner as that for loop 5 (Table 3 and Fig. 6). MAb 5F2 recognized pTM208-L and pTM208-M but did not react with pTM208-S (Fig. 8). Therefore, the epitope for 5F2 was localized to amino acid residues 338 to 354.

DISCUSSION

A panel of MAbs was generated to the P2 protein by immunizing mice with nontypeable *H. influenzae* whole organisms. Each MAb was exquisitely strain specific and recognized abundantly expressed epitopes on the surface of the bacterium. Additionally, all nine MAbs were bactericidal to the homologous strain in an in vitro complement-mediated killing assay. Immunoblot assays of the cyanogen bromide-cleaved P2 and competitive ELISAs indicated that MAb 5F2 recognized an epitope clearly different from the other MAbs and that the epitopes of the other eight MAbs may be identical or closely related. Therefore, MAbs 5F2 and 6G3, as representatives of the eight other MAbs, were chosen for further study.

Protein footprinting using rHPLC was a physical means that was used in an effort to further map the epitopes of the two MAbs. The basic premise of this method is that specific antibody will protect portions of the antigen from proteolysis (17, 39). The parts of the antibody in direct contact with antigen (epitope) and indirect contact (steric hindrance) will reduce the rate of release of the P2 peptides from these regions. Therefore, in the P2 tryptic map, those peaks containing suspected epitope peptides will be smaller when specific antibody is added. This strategy is useful in characterizing not only linear epitopes but conformational ones as well.

Peptides from four regions of the tryptic digest of P2 were identified by protein footprinting and ELISA as containing potential epitopes of MAbs 5F2 and 6G3. N-terminal amino acid sequencing of these rHPLC-purified peptides identified loop 5 and loop 8 by the molecular model of strain 1479 developed by Sikkema and Murphy (41).

On the basis of the results of protein footprinting and ELISAs of tryptic peptides of P2, loops 5 and 8 were chosen for the expression of peptides as fusion proteins. This approach is useful for defining linear epitopes. Since the panel of MAbs recognized epitopes when P2 was presented both in the whole organism and the purified form immobilized on nitrocellulose or coated onto a microtiter plate, the epitopes were most likely linear. Fusion proteins containing variable-length peptides from loops 5 and 8 were constructed and used in an immunoblot assay. MAb 5F2 mapped within residues 338 to 354 of loop 8. This is a proposed surface-exposed variable region. A similar region of the P2 of *H. influenzae* type b was recognized by two MAbs developed by Srikumar et al. (44, 45); however, these MAbs were neither bactericidal in vitro nor immunoprotective in the infant rat model.

MAb 6G3 and the other seven MAbs that reacted with the 32-kDa cyanogen bromide fragment of P2 recognized an epitope within residues 213 to 229 of loop 5. This region contains part of a variable region which is the largest among three nontypeable *H. influenzae* strains sequenced in our laboratory and produces the largest surface-exposed loop according to the topological model of this protein (41). Whether the epitopes of the MAbs that recognize loop 5 are identical or closely related was not resolved.

In ELISAs with each of the fusion proteins (208-M and 205-LJ) used to coat the plates, 5F2 reacted exclusively with the loop 8 peptide and 6G3 reacted exclusively with the loop 5 peptide, confirming the immunoblot results and the lack of cross-reactivity of the MAbs. The absence of differentiating protein footprinting results may have occurred because of technical details or possibly because 5F2 binding to loop 8 of P2 in a three-dimensional state also protects the 6G3 epitope on loop 5 by steric hindrance, reducing the rate of release of peptides from both the 5F2 and 6G3 epitopes. This hypothesis was supported by the competitive ELISA data in which 5F2 blocked binding of both 5F2 and 6G3, whereas 6G3 blocked binding only to itself. A similar situation was reported by Klebba et al. in competitive radioimmunoassay experiments of surface-reactive MAbs to the OmpF porin of *E. coli* (20). Antibodies recognizing site S1 blocked binding of antibodies that recognized site S2, but the reverse was not observed. The schematic model for P2 does not totally describe the surface topology of the molecule. Whether P2 is membrane bound or in purified form, loops 5 and 8 may be geometrically closer than is apparent in the model; steric hindrance by 5F2 could have been a factor in experiments where P2 was presented in the soluble phase. One additional explanation for such results is that conformational changes in tertiary porin structure induced by binding of antibody to one epitope caused a disruption of the other epitope (20).

Identification of variable regions in the P2 protein shows where potential immunodominant epitopes are located. In this study, after the immunization of mice with whole organisms, which simulates the natural form of presentation of the organism, strain-specific bactericidal MAbs which recognize surface-exposed epitopes on loops 5 and 8 of the P2 protein were developed. We hypothesize that loop 5 is an apparent immunodominant portion of the molecule. Epitope mapping using MAbs that recognize the P2 of other strains of nontypeable *H. influenzae* is needed to confirm this hypothesis. Also, depending on the conformation of P2 within the membrane, binding of P2 by antibodies such as 5F2 that bind loop 8 could conceivably block binding of antibodies to loop 5, decreasing the effective P2-specific antibody repertoire. These are two of

five hypervariable regions previously identified by Duim et al. by nucleotide sequencing nontypeable *H. influenzae* isolates from patients with COPD and otitis media (8). Duim et al. found that loop 6 is particularly variable only in isolates from patients with COPD with persistent infection and that antibody response to this loop may interfere with the immune response to other portions of the P2 molecule (8). In studying the P2 from *H. influenzae* type b, both Srikumar et al. and Martin et al. mapped the surface-exposed epitopes of a panel of MAbs to loops 4 and 8 (26, 44, 45).

In initial prevalence studies of normal adult human sera, bactericidal antibody to nontypeable strains of *H. influenzae* was present but less frequently than antibody to the type b strains (14, 16). These antibodies may have arisen from natural immunity induced by cross-reacting antigen on other non-*Haemophilus* bacteria colonizing the nasopharynx or gastrointestinal tract or by infection with *H. influenzae* (37). The strain-specific nature of the antibody response to infection with nontypeable *H. influenzae* has been noted in several studies. Animal studies using chinchillas for experimental induction of *Haemophilus* otitis media have shown the production of strain-specific serum antibodies to outer membrane proteins which provide passive protection only against the homologous strain (1, 18). Groeneveld et al. demonstrated that rabbits immunized with intact nontypeable *H. influenzae* isolates from patients with COPD containing a minor variation in the amino acid sequence of P2 elicited strain-specific bactericidal antibody (12). Otitis media in children is known to stimulate the production of strain-specific bactericidal antibodies (2, 9, 40); recurrent otitis media episodes are usually due to different nontypeable *H. influenzae* strains, indicating a lack of cross-protection by the bactericidal antibodies developed during previous episodes (9, 33). We hypothesize that the strain-specific bactericidal antibodies which develop following infection in humans are directed to the epitopes defined by the bactericidal MAbs characterized here.

Variations within the surface-exposed hypervariable regions are one means this organism has to evade the immune system. This permits reinfection of the host despite a prior immune response to other strains of the same organism (6). If cross-reacting antibody is generated, it may not be bactericidal or protective (49). Strain-specific bactericidal antibody response in conjunction with other immune mechanisms such as opsonophagocytosis is likely to clear the bacteria and resolve the infection (36, 38, 42). If a subsequent episode of otitis media or an exacerbation of COPD occurs, it is usually caused by a new strain or a variant of the previous strain (9, 11, 33).

Further study of the P2 protein with MAbs to other surface-exposed regions will help to delineate how the loops are arranged topologically in the outer membrane and which residues are potentially immunogenic and immunoprotective.

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