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Identification of a Surface-Exposed Immunodominant Epitope on Outer Membrane Protein P1 of *Haemophilus influenzae* Type b

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Eight murine monoclonal antibodies (MAbs) directed against outer membrane protein P1 of *Haemophilus influenzae* type b were generated and characterized. Seven of the eight MAbs reacted with recombinant P1 and purified P1 protein from *H. influenzae* type b strains MinnA and 1613; MAb P1.8 was specific for the latter strain. A panel of 32 nontypeable and 140 encapsulated *Haemophilus* strains recovered worldwide representing the major clonal families of serotypes a, b, and d was used to evaluate the distribution among *Haemophilus* strains of the epitopes identified by the P1-specific MAbs. The epitope reactive with the seven MAbs which recognized P1 from strains MinnA and 1613 was shared by 92% of the encapsulated *Haemophilus* isolates tested. The epitope is present in the *H. influenzae* type b strains from clonal families commonly recovered from cases of invasive disease in North America and Europe. A series of nested 5′ and 3′ deletions of the P1 gene were constructed and analyzed to localize the determinants on P1 recognized by the MAbs. MAbs P1.2, P1.4, P1.5, P1.6, and P1.7 recognized an epitope localized to the carboxy-terminal portion of P1. Murine MAbs P1.1 and P1.3 and two human MAbs, HiH-7 and HiH-10, recognized a complex epitope which was partially localized to the carboxy-terminal portion of the P1 protein. These data indicate that an immunodominant surface-exposed epitope is present on the carboxy-terminal portion of the P1 protein of type b *Haemophilus* isolates responsible for the majority of invasive disease in North America.

Haemophilus influenzae type b is a frequent cause of invasive diseases in children. Alloenzyme electrophoretic typing analysis indicated that the encapsulated *H. influenzae* isolates are clonal in nature. Two major phylogenetic divisions (I and II) have been proposed (19). Division I contains the major clonal groups (A1 and A2) of type b organisms responsible for the majority of invasive disease in North America and Europe, as well as organisms of a third electrophoretic group, designated B1. *H. influenzae* type b strains from this latter group are a common cause of invasive disease in children in the developing world but are rarely seen in the developed world. Division I also contains all electrophoretic types of serotype c and d strains. Division II includes a genetically heterogeneous second group of electrophoretic types of serotype b isolates referred to as group J. Isolates of serotype a occur in both major divisions.

Previous studies have demonstrated the importance of anticapsular antibody in host defenses against *H. influenzae* type b (5, 7). In addition, antibody directed against noncapsular surface antigens of *H. influenzae* type b may also play a protective role. Antibody directed against the outer membrane proteins (OMPs) P1, P2, P6, and a 98-kDa protein as well as antibody directed against lipooligosaccharide (LOS) have protective activity in the infant rat model of bacteremia (5). Among the best studied of these antigens is the P1 protein. It has been demonstrated that both monoclonal and polyclonal antibodies directed against OMP P1 have protective activity (4, 10). The cloning and sequence of the P1 genes from prototype isolates representative of the clonal groups A1, A2, and B1 have been reported (15, 16). These P1 proteins vary in deduced molecular weight (M_r): strains designated H (heavy) in the OMP subtyping scheme have a P1 protein with an M_r of 47,752 (based on the derived amino acid sequence), L (light) strains have a P1 protein with an M_r of 46,937, and strains which have a P1 protein with an M_r of 46,111 are called U for unclassified. Significant variability in the primary sequence of these three proteins is localized to three small variable regions (16); one or more of these variable regions is likely to be responsible for the reported antigenic heterogeneity of the P1 proteins.

We report here the generation of eight monoclonal antibodies (MAbs) specific for the OMP P1 of *H. influenzae* type b obtained from two different mouse cellular fusions. Seven MAbs recognized a surface-exposed epitope on the P1 protein present in 92% of *H. influenzae* type b strains analyzed from the worldwide collection. Two human MAbs, HiH-7 and HiH-10, described elsewhere (14) also recognized a surface-accessible determinant on the P1 protein of the same *H. influenzae* type b strains reactive with the seven murine MAbs. The purpose of this study was to localize conserved and immunogenic regions on the P1 protein among strains of *H. influenzae* type b. The identification of a surface-exposed epitope present on the P1 protein from type b *Haemophilus* strains in the A1 and A2 clonal groups may prove useful for the development of a future vaccine based on synthetic peptides.

MATERIALS AND METHODS

Bacteria and plasmids. *H. influenzae* type b strain MinnA was isolated from the cerebrospinal fluid of a child with meningitis in Minneapolis (1). It has the OMP subtype designated 1H and is representative of the clonal group A1.

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H. influenzae type b strain 1613 was isolated from the blood of a patient in St. Louis with periorbital cellulitis (17). Strain 1613 has the OMP subtype 3L and is representative of the clonal group A2. Strain 8358, a gift from Janet Montgomery, was isolated from a patient in Papua New Guinea. It has the OMP subtype 6U and is representative of the clonal group B1. Other encapsulated strains were kindly provided by J. M. Musser, Hospital of the University of Pennsylvania, Philadelphia, Pa. (19), and D. M. Granoff, Washington University, St. Louis, Mo. Included were 111 *Haemophilus* type b strains, 44 of clonal family A1, 58 of clonal family A2, two of clonal family B1, and 2 of clonal family J. Ten type a *Haemophilus* strains were tested, 5 of division I and 5 of division II. Nineteen type d *Haemophilus* strains were tested. Thirty-two nontypeable *Haemophilus* strains were also tested. These strains were obtained from several regions in Canada (6). *H. influenzae* strains were grown overnight at 37°C in 5% CO_2 on chocolate agar plates supplemented with IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) (1%, vol/vol).

The plasmid pRSM188 contains the P1 gene from strain MinnA (15). The bacteriophage T7 expression vector pT7-7 and the M13 phage mGP1-2 containing the T7 RNA polymerase gene were gifts of Stan Tabor (20). The plasmid vector pEX2 and *Escherichia coli* pop 2136 were gifts of Jelle Thole (21). Strain pop 2136 contains a chromosomal copy of the λ*c*I857 gene encoding the temperature-sensitive lambda repressor. At 30°C, genes expressed from the λ p_R promoter are not expressed, whereas at 42°C genes under control of this promoter are highly expressed. Thus, the Cro-LacZ-P1 fusion proteins in pEX2 were expressed in *E. coli* pop 2136 after a temperature shift to 42°C. *E. coli* JM101 was obtained from New England BioLabs, Inc. (Beverly, Mass.). *E. coli* strains were grown in L broth supplemented with 50 μg of ampicillin per ml as appropriate.

Outer membrane preparation. Lithium chloride extraction of outer membranes from the bacteria was performed as described elsewhere (6). Alternatively, total membrane or sarcosyl-insoluble preparations enriched in OMPs were prepared as described previously (2). Protein concentration was determined by the method of Lowry et al. (11) adapted to membrane fractions or by the bicinchoninic acid method (BCA Protein Assay Kit; Pierce Chemical Co., Rockford, Ill.).

Immunization of mice and fusion procedure. BALB/c mice were immunized by injections given intraperitoneally. One set of mice received 10 μg of purified P1 protein from strain 1613 (15, 16) suspended in Freund complete adjuvant (GIBCO Laboratories, Grand Island, N.Y.) and were boosted twice at 15-day intervals with the same amount of protein in Freund incomplete adjuvant (fusion 1). A second group of mice received 50 μg of OMPs from strain 1613 suspended in Freund complete adjuvant and were boosted once 15 days later with the same amount of antigen in Freund incomplete adjuvant (fusion 2). Both groups of mice were injected intravenously with the respective antigens 4 days before hybridoma production.

Hybridomas were produced by fusion of spleen cells recovered from immunized animals with nonsecreting SP2/0 myeloma cells as described elsewhere (6). Specific hybrids were cloned by sequential limiting dilutions, expanded, and frozen in liquid nitrogen. The class, subclass, and light-chain type of each MAb were determined as reported elsewhere (13) and are presented in Table 2. Human hybridomas were described previously by Martin et al. (14). Both murine and human MAbs used in assays were taken from 3-day-old culture supernatants at a concentration estimated at approximately 5 μg/ml.

Construction of 3′ deletions of P1 gene. To create deletions from the 3′ end of the strain MinnA P1 gene, we cloned the P1 gene from pRSM188 as a first codon fusion into the plasmid vector pT7-7. For convenience, the *Pst*I site in the multiple cloning site of pT7-7 was removed. The signal peptide coding sequence 5′ to the *Pst*I site of the P1 gene was reconstructed with complementary oligonucleotides. The first codon of the T7 capsid gene in pT7-7 is part of an *Nde*I site; therefore, the oligonucleotides were constructed to generate a 5′ *Nde*I site and a 3′ *Pst*I site. The complementary-strand oligonucleotide was phosphorylated with T4 polynucleotide kinase, annealed to the second oligonucleotide, and ligated to the *Pst*I-*Eco*RI fragment containing the P1 gene. The ligated fragments were digested with *Eco*RI and cloned into pT7-7 which had been digested with *Nde*I and *Eco*RI. A plasmid with the correct restriction map was designated pRSM459 (Fig. 1). In this plasmid, P1 synthesis is under the control of the bacteriophage T7 promoter and P1 is synthesized after infection of JM101/pRSM459 with mGP1-2, an M13 phage containing the structural gene for the bacteriophage T7 RNA polymerase. Induction of P1 and confirmation by Western immunoblot analysis with rabbit anti-P1 antibody were performed as described previously (15, 18).

A series of truncated P1 genes were then constructed from pRSM459 as shown in Fig. 1. Only the relevant sites in the multiple cloning site of pT7-7 are shown in the pRSM459 construct. Sequences 3′ to the *Rsa*I site were deleted to construct pRSM526. The construct was generated by cloning the *Pst*I-to-*Rsa*I fragment of pRSM459 into pRSM459 which had been digested with *Pst*I and *Sma*I. Sequences 3′ to the *Pvu*I site were deleted by digestion of pRSM459 with *Pvu*I, blunt ending, and digestion with *Pst*I. The *Pst*I-to-blunt-end fragment was ligated into pRSM459 which had been digested with *Pst*I and *Sma*I to create pRSM520. The sequences 3′ to the *Hin*dIII site were deleted by digestion of pRSM459 with *Hin*dIII and religation. This construct was designated pRSM474. Sequences 3′ to the *Bgl*II site were deleted by cloning the *Pst*I-to-*Bgl*II fragment into pRSM459 which had been digested with *Pst*I and *Bam*HI. This construct was designated pRSM500. Plasmids were characterized by restriction analysis, and the truncated proteins were characterized by Western immunoblot with rabbit anti-P1 antisera.

Construction of 5′ nested deletions of P1 gene. Nested deletions from the 5′ end of the MinnA P1 gene were constructed in the plasmid vector pEX2. The vector pEX2 codes for a Cro-LacZ fusion protein under the control of the bacteriophage λ promoter p_R. A multiple cloning site is present near the 3′ end of the *lacZ* gene. pEX2 was digested with *Hin*dIII, blunt ended, and digested with *Pst*I. pRSM188 was linearized with *Eco*RI, blunt ended, and digested with *Pst*I. The *Pst*I–blunt-end fragment containing the P1 gene was ligated into pEX2 and transformed into *E. coli* pop 2136 at 30°C. Clones were analyzed by restriction analysis and by Western immunoblot analysis for synthesis of a Cro-LacZ-P1 fusion protein at 42°C. The plasmid designated pRSM724 was saved for further study. pRSM724 contains two *Xba*I sites, one of which is in the P1 gene. pRSM724 was partially digested with *Xba*I, blunt ended, religated, and transformed back into *E. coli* pop 2136. Plasmids were again characterized by Western immunoblot and restriction analysis. A plasmid which retained the *Xba*I site in the P1 gene no longer contained the second *Xba*I site and was designated pRSM734 (Fig. 1). pRSM734 was digested with *Pst*I and

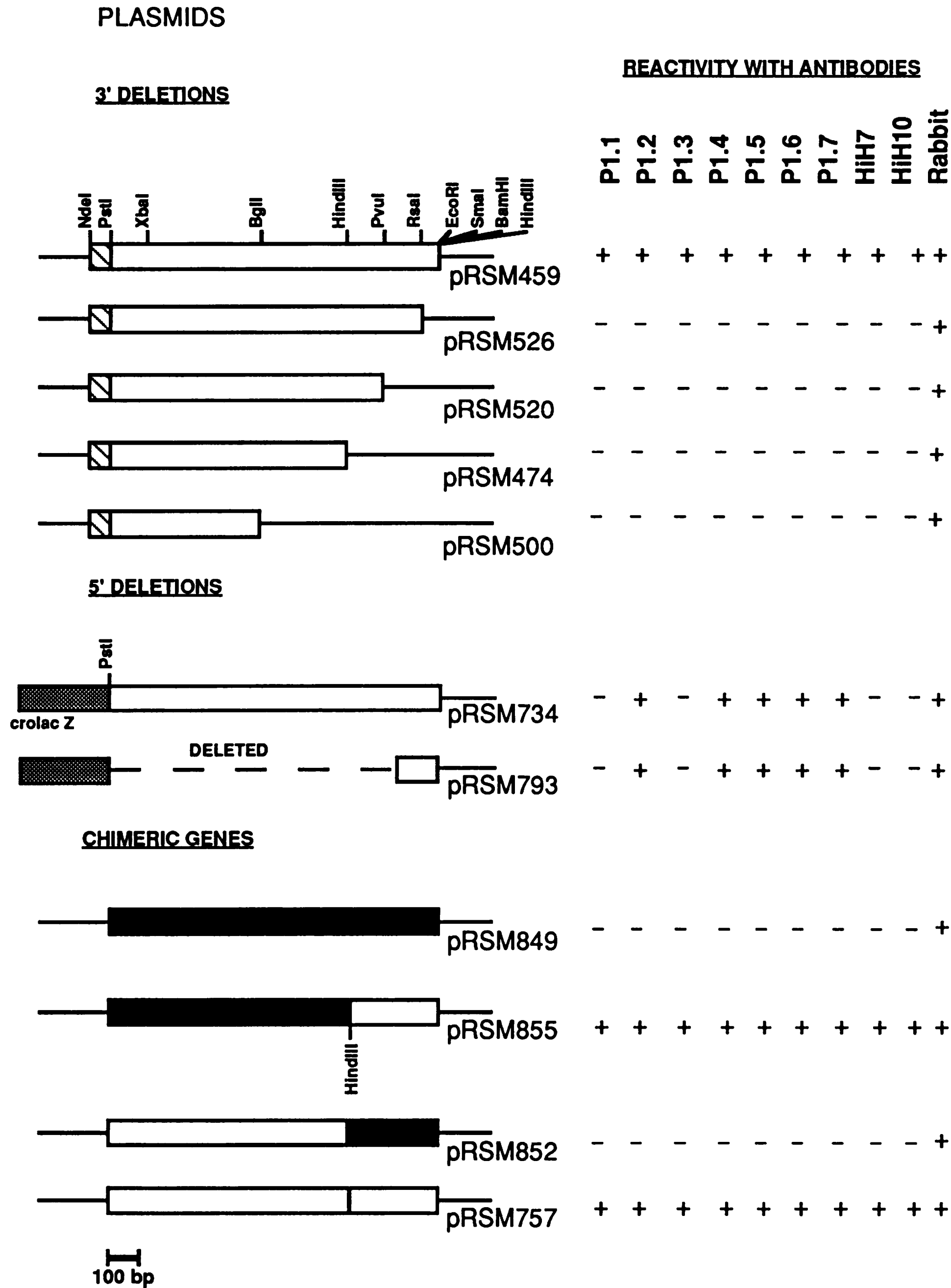

FIG. 1. Reactivity of human and murine MAbs with 5′ and 3′ deletion products of the MinnA P1 gene (OMP subtype 1H) and with chimeric gene products. Summary of data shown in Fig. 3 and 4. Symbols: ■, OMP subtype 6U; □, OMP subtype 1H; ▧, leader peptide.

*Xba*I, and unidirectional deletions into the P1 gene were constructed by incubation with exonuclease III employing an Erase-a-Base Kit system (Promega). Aliquots were blunt ended and religated, and plasmids were transformed into *E. coli* pop 2136 at 30°C. Ampicillin-resistant clones were grown at 30°C to the mid-log phase and shifted to 42°C for 2 h to induce synthesis of the fusion proteins, and clones were screened for production of fusion proteins larger than the Cro-LacZ fusion protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Junctions between the *lacZ* and *ompP1* genes were determined by DNA sequence analysis as described previously (15, 16).

Construction of chimeric P1 genes. Surprisingly, we were unable to clone the OMP subtype 6U P1 gene into pT7-7. We were also not successful in constructing a chimeric gene containing the OMP subtype 1H P1 sequences 5′ to the *Hin*dIII site and OMP subtype 6U P1 sequences 3′ to the *Hin*dIII site. To reduce the apparent toxicity of the cloned gene products, we constructed OMP subtype 1H and 6U P1 genes which had no leader peptide sequences. We used the polymerase chain reaction to clone the modified 6U P1 gene such that an ATG translational start was positioned immediately 5′ to the coding sequences for the mature 6U P1 gene. The oligonucleotide primer 5′ to the P1 gene was 5′GGT

GGTGCACATATGGCAGCGTTTCAATTGGCGGA. This oligonucleotide contains the ATG as part of an *Nde*I site. The oligonucleotide primer 3′ to the gene contained an *Eco*RI site and has been described (17). The P1 gene was amplified from genomic DNA of strain 8358 as described previously (16), digested with *Nde*I and *Eco*RI, and cloned into pT7-7. A plasmid with the correct restriction map was designated pRSM849 (Fig. 1). Similarly, the 1H gene was cloned without the leader peptide from PCR-amplified DNA from strain MinnA; the plasmid was designated pRSM757. Full-size P1 was detected by Western immunoblot of extracts of JM101/pRSM849 and JM101/pRSM757 after induction. A chimeric gene containing the 6U P1 sequences 5′ to the *Hin*dIII site and the 1H P1 sequences 3′ to the *Hin*dIII site was constructed by cloning the *Hin*dIII-to-*Eco*RI fragment of pRSM188 into pRSM849; this construct was designated pRSM855. Similarly, pRSM852 contains the *Nde*I-to-*Hin*dIII fragment of pRSM757 cloned into pRSM849.

Enzyme immunoassay procedures. The enzyme-linked immunosorbent assay (ELISA) procedure was performed as described by Brodeur et al. (3). Coating antigens were OMP preparations from *H. influenzae* type b strain 1613 or MinnA or a mutant of strain 1613 deficient in P1 protein (17) (7.5 μg of antigen per ml of 0.05 M carbonate buffer, pH 9.6). Alternatively, purified P1 protein from *H. influenzae* type b strain 1613 or strain MinnA or recombinant MinnA P1 protein (15) was used at a concentration of 5 μg of antigen per ml.

Dot-blot immunoassay was used to screen the MAbs against the *Haemophilus* strain collection as previously described (12). Extracts of *E. coli* producing recombinant P1 derivatives were blotted onto nitrocellulose, blocked with gelatin, and then incubated sequentially with MAbs and secondary antibody. Blots were then developed as described previously (15).

Western immunoblots were performed after separation of OMPs by SDS-PAGE in 1.5-mm-thick slab gels as described by Laemmli (8). The resolved proteins were transferred electrophoretically onto nitrocellulose paper (Bio-Rad) by the method of Towbin et al. (22). The nitrocellulose paper was soaked in 0.5% milk (Carnation) in phosphate-buffered saline (PBS) (pH 7.2) for 1 h at 37°C. The blot was incubated for 1 h at 37°C with supernatants from 3-day-old culture clones or mouse hyperimmune sera (mouse immunized with OMP from *H. influenzae* type b strain 1613) and then washed three times for 10 min each in PBS–0.02% Tween (Sigma Chemical Co., St. Louis, Mo.). The blot was then soaked for 60 min at 37°C in 3% milk–PBS containing peroxidase-conjugated affinity-purified goat anti-mouse immunoglobulins (Cappel) diluted 1/800. After several washes, the paper was incubated in the luciferin-enhanced substrate for 2 min at room temperature as described by Laing (9). For luminescent detection of antibody, the blot was placed between polyethylene sheets and exposed for 5 min at room temperature to Kodak XAR-5 film in a Kodak X-Omatic cassette fitted with intensifying screens.

Antibody accessibility radioimmunoassay. Three-hour-old culture plate-grown cells (10^9 live cells per tube) were incubated with undiluted supernatants of 3-day-old culture clones (0.5 ml) for 2 h at 4°C with agitation. The suspensions were then pelleted and washed three times with PBS. Anti-mouse antibody labeled with ^{125}I (0.25 μCi/ml; Amersham Corp., Arlington Heights, Ill.) was added for 30 min at room temperature. After three washes in PBS, the radioactivity in each sample was counted to determine bound antibody.

TABLE 1. Reactivity of MAbs with OMP preparations and purified and recombinant P1 proteins from *H. influenzae* type b strains

MAb	OMP from *H. influenzae*[a]		Purified P1 protein[a]		Recombinant P1 protein (MinnA)[a]	OMP from P1-deficient *H. influenzae* (1613)[a]
	1613	MinnA	1613	MinnA		
P1.1	>2.0	0.89	0.95	0.85	0.58	0.00
P1.2	>2.0	0.95	0.99	1.01	0.72	0.00
P1.3	>2.0	0.84	1.20	0.91	0.48	0.00
P1.4	0.87	0.58	0.38	0.27	0.12	0.00
P1.5	0.95	0.54	0.35	0.35	0.10	0.00
P1.6	0.93	1.09	0.55	0.61	0.10	0.00
P1.7	>2.0	1.03	1.78	1.65	0.23	0.00
P1.8	1.10	0.00	0.51	0.00	0.00	0.00
LO-1[b]	1.10	0.65	0.00	0.00	0.00	0.91
P2-7[c]	>2.0	1.80	0.00	0.00	0.00	1.89

[a] ELISA value (A_{410}) taken 1 h after the addition of substrate; the value for supernatants from a control mouse myeloma culture has been subtracted from each value.

[b] LO-1 is an MAb specific for an LOS determinant.

[c] P2-7 is an MAb specific for the P2 OMP of *H. influenzae* type b.

RESULTS

Specificity of MAbs for OMP P1 of *H. influenzae* type b. Supernatants of hybrid clones obtained from fusion of primed spleen cells and nonsecreting myeloma cells were first tested by ELISA against OMP preparations of *H. influenzae* type b strains 1613 and MinnA as coating antigens. Further screenings were performed with purified P1 protein from strains 1613 and MinnA, recombinant P1 protein, and OMP preparations of a mutant of *H. influenzae* type b strain 1613 deficient in P1 protein. From two cellular fusions, a total of eight hybridomas producing anti-P1 MAbs were obtained: P1.1 to P1.3 from fusion 1 and P1.4 to P1.8 from fusion 2 (Table 1). All but one recognized the purified P1 protein of both strains as well as the recombinant MinnA P1 protein. P1.8 recognized an epitope on the P1 protein of strain 1613 which is not present on P1 of strain MinnA. The MAbs were not reactive with OMP preparations of a P1-deficient *Haemophilus* strain. Control MAbs which were specific for P2 (P2-7) or an LOS epitope (LO-1) did not react with purified or recombinant P1 proteins but did react with extracts of the mutant *Haemophilus* strain lacking the P1 protein (Table 1).

Preparations of OMPs from *H. influenzae* type b strain 1613 were probed with MAbs by the Western immunoblotting technique. The luminescent immunoblot demonstrated that P1.1 to P1.8 were specific for an epitope present on P1 (Fig. 2, lanes a to h). The reactivity of anti-P1 MAbs in Western immunoblot after SDS-PAGE treatment at 100°C suggests that these MAbs recognized a continuous amino acid sequence. OMP P2 specific- and LOS-specific MAbs (Fig. 2, lanes i and k, respectively) served as controls since no reaction was observed with the P1 protein. Mouse hyperimmune serum against OMPs (Fig. 2, lane j) was included as a positive control. All MAbs were tested with representatives of OMP subtype 1H strains (MinnA, 1H) and L strains (*H. influenzae* type b Eagan, 1L, and MHAEM 1476, 2L) both in Western immunoblot and accessibility assays; results were the same as the ones obtained with the immunizing strain (1613, 3L) except for P1.8, which does not react with 1H strains.

Reactivity of MAbs against a panel of *Haemophilus* strains. The eight MAbs were tested in an immunodot assay for their

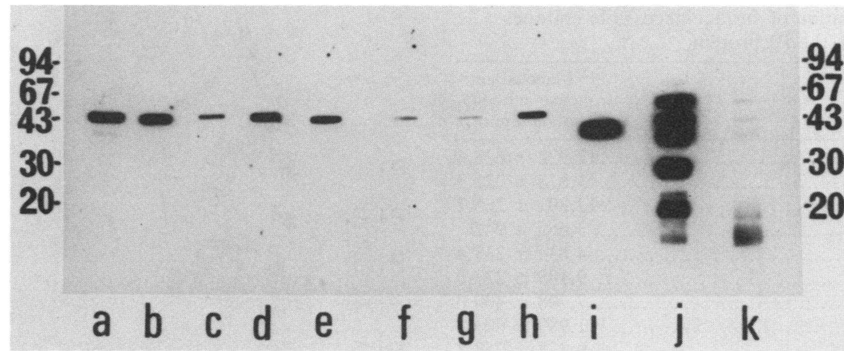

FIG. 2. Luminescent immunoblot. OMPs from *H. influenzae* type b strain 1613 were probed with MAbs by the Western immunoblot technique. Lanes a to h, Murine MAbs P1.1 to P1.8; lane i, P2-7, murine anti-P2 MAb specific for the P2 OMP of *H. influenzae* type b; lane j, polyvalent sera from mice immunized with OMP preparations from strain 1613 (pooled sera were diluted 1/200 in PBS–0.5% milk); lane k, LO-1, murine anti-LOS MAb, specific for an LOS epitope. Numbers on sides show size in kilodaltons.

reactivity against 111 type b *Haemophilus* strains, 10 type a *Haemophilus* strains, 19 type d *Haemophilus* strains, and 32 nontypeable *Haemophilus* strains. All, except for P1.8, recognized an epitope on the P1 protein of all *H. influenzae* type b strains grouped in the two closely related major clusters, A1 and A2 (Table 2). Serotype b strains in cluster B1 were not recognized by the MAbs. In contrast, serotype a strains in cluster B1 were recognized by all the MAbs except P1.8. Serotype d strains in cluster B1 were recognized by the human MAbs but not by the murine MAbs. Serotype b strains in cluster J of division II and serotype a strains of division II are distantly related to the division I clusters; strains representative of these groups did not react with any of the MAbs. Five of 32 nontypeable strains were reactive with a subset of the MAbs. Strain M15 was reactive with two of the MAbs; the other strains were reactive with only one of the MAbs. As the pattern of recognition of the MAbs was different from that seen with the type b organisms, it is unlikely that these nontypeable strains are unencapsulated mutants of the commonly observed strains representative of the clonal group A1 or A2.

Binding properties of MAbs. To determine whether the epitopes recognized by the anti-P1 MAbs are surface exposed in intact bacteria, we sequentially incubated cells of *H. influenzae* type b strain 1613 with MAb supernatants and ^{125}I-labeled anti-mouse antibody. P1.1 to P1.7 recognized a surface-exposed epitope on the *Haemophilus* cells (Table 3). ^{125}I binding to cells incubated with P1.8 was comparable to that observed when cells were incubated with PBS or with a supernatant containing an anticytomegalovirus MAb, indicating that the epitope recognized by P1.8 was not exposed on the surface of intact *Haemophilus* cells (Table 3).

Identification of immunodominant epitope on P1 protein. The mouse MAbs P1.1 to P1.7 and the two human MAbs were reactive with the recombinant P1 expressed in JM101/pRSM459 (Fig. 1 and 3). Extracts from JM101/pRSM508 containing vector only were not reactive with the MAbs (Fig. 3). A series of 3′-truncated genes were constructed by removal of sequences 3′ to the *Rsa*I, *Pvu*I, *Hin*dIII, and *Bgl*II sites. None of these truncated proteins were reactive with the MAbs, although all the proteins were reactive with rabbit anti-P1. Data are shown only for the protein truncated at the *Rsa*I site, JM101/pRSM526 (Fig. 1 and 3).

To construct a series of 5′ nested deletions, we fused the P1 gene in frame with β-galactosidase in the vector pEX2. This plasmid was designated pRSM734 (Fig. 1). This fusion protein was strongly reactive with P1.2, P1.4, P1.5, and the rabbit antibody. The fusion protein was also consistently

TABLE 2. MAb reactivity with different strains of *H. influenzae* taken from a panel of strains recovered worldwide[a]

MAb	Class, subclass[b]	Type a strains		Type b strains				Nontypeable (32)[d]
		Division I (5)[c]	Division II (5)	Divison I			Division II, J (2)	
				A1 (44)	A2 (58)	B1 (7)		
P1.1	IgG1, κ	5	0	44	58	0	0	0
P1.2	IgG2b, κ	5	0	44	58	0	0	1 (M15)
P1.3	IgG1, κ	5	0	44	58	0	0	1 (M15)
P1.4	IgG3, κ	5	0	44	58	0	0	1 (Q191)
P1.5	IgG3, κ	5	0	44	58	0	0	0
P1.6	IgG2b, κ	5	0	44	58	0	0	0
P1.7	IgG1, κ	5	0	44	58	0	0	0
P1.8	IgG3, κ	0	0	37	53	0	0	3 (Q156, Q169, Q188)
HiH-7	IgG, λ	5	0	44	58	0	0	0
HiH-10	IgG3, λ	5	0	44	58	0	0	0

[a] Reactivity was tested by immunodot assay; strains used are described in Materials and Methods. Nineteen type d *H. influenzae* strains were tested against the MAbs; none were recognized by murine MAbs, but all reacted with human MAbs.
[b] IgG, Immunoglobulin G.
[c] Numbers in parentheses are total number of strains tested in the group.
[d] Five different nontypeable strains were recognized by MAbs.

TABLE 3. Determination of surface-accessible epitopes on the P1 protein

MAb	^{125}I-labeled anti-mouse antibody bound (cpm)[a]
P1.1	12,922 ± 628.6
P1.2	11,818 ± 221.3
P1.3	12,801 ± 265.2
P1.4	3,665 ± 94.1
P1.5	4,634 ± 233.4
P1.6	9,183 ± 227.0
P1.7	7,708 ± 400.0
P1.8	1,989 ± 80.0
LO-1	9,475 ± 258.3

[a] PBS and unrelated MAb (anticytomegalovirus antibody) gave background levels of 930 and 1,020 cpm, respectively. Numbers are averages of triplicates ± standard error; the experiment was done at least twice.

reactive with P1.6 and P1.7. The fusion protein was not reactive with the human MAbs and was poorly and inconsistently reactive with P1.1 and P1.3 (Fig. 1 and 3). The MAbs did not react with extracts of *E. coli* pop 2136/pRSM618, the strain containing the cloning vector. The series of nested deletions were tested with the panel of MAbs. All the data were identical to that shown in Fig. 3 for *E. coli* pop 2136/pRSM793, the strain expressing a fusion protein containing only the carboxy-terminal portion of P1. That is, the MAbs reacted with fusion proteins containing the amino-terminal deletions with the same pattern of specificity as they did with the whole fusion protein.

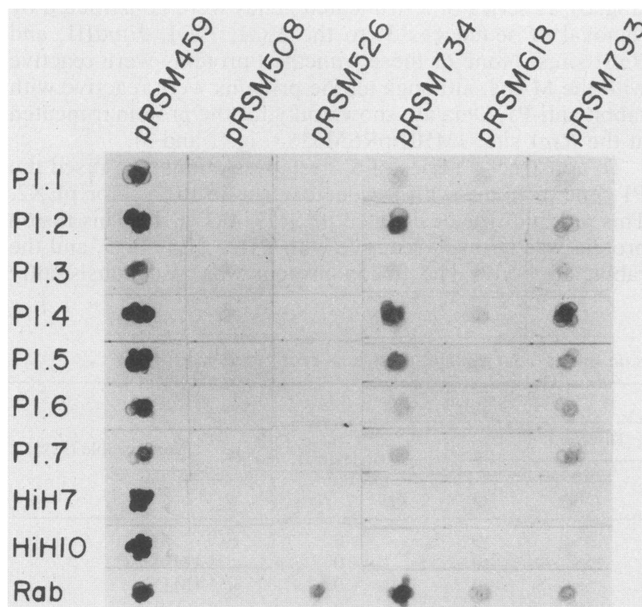

FIG. 3. Dot-blot immunoassay demonstrating the reactivity of truncated P1 proteins with the panel of P1 MAbs. Sonicates of *E. coli* strains containing the indicated plasmids (3 μg) were applied to nitrocellulose and then incubated sequentially with undiluted MAbs and alkaline phosphatase-conjugated second antibody and developed. The whole P1 gene from strain MinnA is present in pRSM459. The P1 gene in pRSM526 is truncated at the *Rsa*I site. The P1 gene in pRSM734 is fused in frame to β-galactosidase. Plasmid pRSM793 contains only the 3′ portion of the P1 gene fused to β-galactosidase. These constructs are shown schematically in Fig. 1. Plasmids pRSM508 and pRSM618 contain vector sequences only.

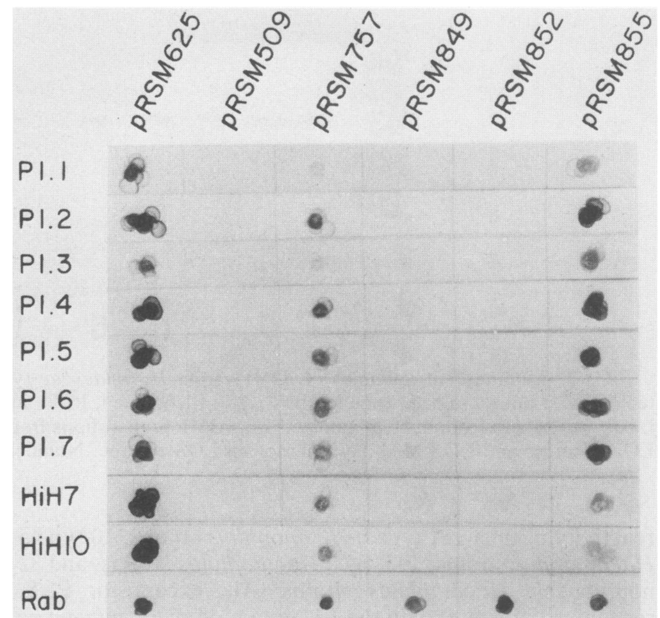

FIG. 4. Dot-blot immunoassay demonstrating the reactivity of chimeric P1 proteins with the panel of MAbs. The experiment was performed as described in the legend to Fig. 3. Plasmid pRSM625 contains the complete strain MinnA P1 gene. The leader peptide coding sequences of the remaining constructs have been replaced with a methionine codon. Plasmid pRSM757 contains the gene from strain MinnA. Plasmid pRSM849 contains the gene from strain 8358. Plasmid pRSM852 contains the 5′ portion of the MinnA gene and the 3′ portion of the 8358 gene. Plasmid pRSM855 contains the 5′ portion of the 8358 gene and the 3′ portion of the MinnA gene. These constructs are shown schematically in Fig. 1. Plasmid pRSM509 contains vector sequences only.

The reactivity of P1.4 with *E. coli* pop 2136/pRSM793 extracts was strong; however, the reactivity of P1.2 and P1.5 appeared weaker than that observed with the whole fusion protein. Further, a subset of the MAbs either reacted poorly and inconsistently or did not react with the extract. Thus, we constructed a series of chimeric genes to further confirm the carboxy-terminal localization of the epitope(s). JM101/pRSM625 extracts (construct contains the complete OMP subtype 1H gene) were strongly reactive with all the MAbs (Fig. 4). To stabilize some of the constructs, the leader peptide coding sequences were deleted and mature P1 proteins were expressed with an amino-terminal methionine. As expected, JM101/pRSM849 extracts (expressing the OMP subtype 6U protein) and JM101/pRSM509 (vector only) were not reactive with the MAbs. JM101/pRSM757 extracts containing the OMP subtype 1H methionine-P1 were poorly reactive with P1.1 and P1.3 but were moderately reactive with the other MAbs. The chimeric gene product present in extracts of JM101/pRSM852 was not reactive with the MAbs, while the extract of JM101/pRSM855 was reactive with the MAbs. Extracts of JM101/pRSM855 contain the carboxy-terminal portion of the OMP subtype 1H protein, confirming that a portion of the epitope recognized by the MAbs is localized to the carboxy-terminal portion of the P1 protein.

DISCUSSION

OMP P1 of *H. influenzae* exhibits antigenic and size heterogeneity. Size differences in the P1 protein from dif-

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                         PvuI
1H   AGTGCTGCAATTCCAGATACCGATCGCACTTGGTATAGTTTAGGTGCAACCTATAAATTC   1260
     SerAlaAlaIleProAspThrAspArgThrTrpTyrSerLeuGlyAlaThrTyrLysPhe    380
6U   .....................A......................G...............
     .....................Asn....................................

                                       -> pRSM793
1H   ACGCCGAATTTATCTGTTGATCTTGGCTATGCTTACTTAAAAGGCAAAAAAGTTCACTTT   1320
     ThrProAsnLeuSerValAspLeuGlyTyrAlaTyrLeuLysGlyLysLysValHisPhe    400
6U   ............................................................
     ............................................................

                                 RsaI
1H   AAAGAAGTAAAAACAATAGGTGACAAACGTACATTGACATTGAATACAACTGCAAATTAT   1380
     LysGluValLysThrIleGlyAspLysArgThrLeuThrLeuAsnThrThrAlaAsnTyr    420
6U   .......C.C..CA.GCT.CA.GTGGCTTC         A.A.CA.....C........C
     ......AlaGlnGlnAlaAlaGlyGlyPhe         IleThr...............

1H   ACTTCTCAAGCACACGCAAATCTTTACGGTTTGAATTTAAATTATAGTTTCTAATCCGTT   1440
     ThrSerGlnAlaHisAlaAsnLeuTyrGlyLeuAsnLeuAsnTyrSerPhe             437
6U   .............................C..A..C...............
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FIG. 5. Nucleotide and derived amino acid sequences of the 3′ portion of the P1 genes from strains MinnA and 8358 as described elsewhere (15, 16). The positions of the *Pvu*I and *Rsa*I sites and the fusion junction of pRSM793 are shown.

ferent serotype b strains were used by Barenkamp et al. (2) as part of an OMP-subtyping scheme. The subtyping scheme, in general, agrees well with a more extensive subclassification of *Haemophilus* strains based on electrophoretic differences in 17 enzyme loci (19). In this analysis, two major phylogenetic divisions, designated I and II, containing type b strains were identified. Division I strains are further subdivided into A1, A2, B1, and B2 clusters. The MAbs characterized in this study were prepared against a strain with the OMP subtype 3L of the A2 group in division I. Seven of the eight MAbs characterized in this study, as well as two previously characterized human anti-P1 MAbs, were reactive with all group A1 and A2 strains tested. The mouse MAb designated P1.8 recognized a subset of strains in both the A1 and A2 clonal groups. The strain specificity of the P1.8 MAb was examined in more detail. Strains representative of OMP subtypes 2L and 3L from both A1 and A2 clonal groups were reactive with MAb P1.8. Strains with the OMP subtypes 1H and 2H are in clonal group A1; these strains were not reactive with P1.8.

Division I strains in the B lineage contain serotype b strains as well as serotype a and d strains. None of the lineage B strains tested were recognized by P1.8. The seven remaining murine MAbs and the two human MAbs reacted with serotype a strains in the B2 lineage. The serotype d strains in the B1 lineage were reactive with the human MAbs but not with the murine MAbs. None of the MAbs were reactive with the serotype b strains in lineage B1. These serotype b strains are commonly seen in the developing world but have rarely been isolated from patients in the developed world. Serotype b strains of the J lineage and serotype a strains of the H lineage of division II were not reactive with the anti-P1 MAbs. These data indicate that the epitope(s) recognized by these P1 MAbs is not present in the distantly related serotype a and b strains of division II. No additional data are available on the OMPs of these division II strains.

The mouse MAbs P1.1 through P1.7 recognized one or more epitopes present at the surface of intact *Haemophilus* cells. Therefore, it was of interest to define the portion(s) of the P1 protein which reacted with these MAbs. We first constructed a series of P1 proteins which were truncated from the C-terminal end. Surprisingly, none of the MAbs reacted with any of these truncated proteins (Fig. 1). Western immunoblots with rabbit antisera indicated that the truncated P1 proteins were detectable and were of approximately the predicted size. Therefore, the lack of reactivity of the MAbs could not be explained by degradation of the truncated proteins in *E. coli*. These data suggested that the epitope(s) recognized by these MAbs was localized to the C-terminal portion of P1. Indeed, P1.2, P1.4, P1.5, P1.6, and P1.7 were reactive with a β-galactosidase fusion protein containing residues 392 to 437 of the subtype 1H P1 protein, thus localizing the epitope recognized by these MAbs to the C-terminal portion of P1. The P1 proteins of prototype *H. influenzae* type b strains representative of A1, A2, and B1 strains have been sequenced. The derived amino acid sequences of the P1 proteins from the A1 and A2 strains were identical in this portion of P1, consistent with the observed reactivity of the MAbs with the group A1 and A2 strains. The P1 protein of the OMP subtype 6U isolates from group B1 was not reactive with these MAbs. The sequence of the gene from a prototype OMP 6U isolate differs in derived amino acid sequence between residues 403 and 415 (Fig. 5), further confirming the localization of the epitope(s) to this portion of P1.

P1.1, P1.3, and the two human MAbs reacted poorly with the β-galactosidase fusion protein and also reacted poorly with a fusion protein containing the complete P1 gene. However, the lack of reactivity with the C-terminal-truncated protein and the lack of reactivity of these MAbs with the OMP subtype 6U protein suggested that a portion of the epitope was localized to the same portion of P1 recognized by the P1.2-like MAbs. To confirm this hypothesis, we constructed chimeric genes. Chimeric proteins containing the carboxy-terminal portion of the OMP subtype 1H protein were reactive with the MAbs, while chimeric proteins containing the carboxy-terminal portion of the OMP subtype 6U protein were not reactive. Therefore, these MAbs recognize a more complex epitope which is partially localized to the same carboxy-terminal portion of the P1 protein as that recognized by the P1.2-like MAbs. The remaining portion of this epitope has not been identified, although it might be speculated that it is near the amino terminus of the P1

protein because the β-galactosidase fusion protein containing the whole P1 gene is not reactive with the P1.1 and P1.3 MAbs.

No information is available on the conformation of P1. It is noteworthy that MAbs recovered from two mouse fusions and one human fusion defined a surface-exposed epitope which is, in part, localized to the carboxy-terminal portion of P1. This portion of the P1 protein is very hydrophilic; 5 of the 13 residues between residues 403 and 415 and 6 of 11 residues between residues 392 and 402 are charged or polar. The more complex epitope recognized by P1.1 and P1.3 contains this portion of the P1 protein as well as another undefined portion of the protein. Although complex, this epitope is still recognizable on Western immunoblots.

The surface-exposed epitope defined in this study appears to be important; however, it is not the only surface-exposed epitope present in P1. Hansen and coworkers (7) isolated anti-P1 MAbs which recognized a surface-exposed epitope(s) on approximately 50% of the isolates they tested. It is likely that these MAbs react, at least in part, with one of the two variable portions of P1 which differentiate the OMP subtype 1H and 3L proteins. Further studies will be required to identify all the surface-exposed epitopes of P1.

H. influenzae type b polysaccharide conjugate vaccines appear to have good efficacy against invasive type b disease. In these vaccines, carrier proteins such as diphtheria and tetanus toxoids are employed. In future vaccines, *Haemophilus* OMPs or peptides representative of portions of *Haemophilus* OMPs might prove to be more useful carriers. As well as acting as a carrier for the polysaccharide portion of the vaccine, these proteins would also induce a protective antiprotein response. Antibody directed against OMP P1 has protective activity in the infant rat model of bacteremia, and therefore P1 or peptides derived from the sequence of P1 may be suitable for inclusion in future vaccines.

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