

## Antigenic Relationships among the Porin Proteins of Encapsulated *Haemophilus influenzae* Clones

DENIS MARTIN,<sup>1,2</sup> JOSEE HAMEL,<sup>2</sup> BERNARD R. BRODEUR,<sup>2\*</sup> AND JAMES M. MUSSER<sup>3</sup>

Connaught Centre for Biotechnology Research, Toronto,<sup>1</sup> and National Laboratory for Immunology, Laboratory Centre for Disease Control, Ottawa K1A 0L2,<sup>2</sup> Ontario, Canada, and Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania 19104<sup>3</sup>

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**Monoclonal antibodies (MAbs) specific for *Haemophilus influenzae* were generated to identify antigenic determinants shared among encapsulated *H. influenzae* clones. Sixteen MAbs reacted by Western immunoblot with a protein of an approximate molecular size of 40 kilodaltons corresponding to the P2 major outer membrane protein (porin). These MAbs also reacted with purified and recombinant *H. influenzae* porin. Fourteen of the MAbs recognized cell surface-exposed epitopes, and two of the MAbs, P2-16 and P2-17, identified epitopes that are not present or are not accessible on the cell surface. The reactivity spectrum of the MAb panel was studied by dot immunoassay against 32 serologically nontypeable and 119 encapsulated *H. influenzae* strains recovered worldwide, representing the major serotype a, b, and d clone families. MAbs P2-4 and P2-6 recognized only serotype b clones assigned to primary phylogenetic division I. These clones account for more than 99% of all invasive episodes worldwide. MAbs P2-3, P2-8, and P2-11 reacted with division I serotype b isolates and also identified all genetically allied strains expressing serotype a and d polysaccharide capsules. In contrast, none of the 16 MAbs reacted with genetically divergent serotype a or b clones assigned to primary phylogenetic division II. These results demonstrate that, in general, the patterns of P2 protein surface epitope exposure are cognate with genetic lineages of encapsulated *H. influenzae* strains and support the hypothesis that the population structure of encapsulated *H. influenzae* is predominantly clonal.**

Encapsulated strains of *Haemophilus influenzae* are a major cause of meningitis and other serious invasive diseases (33, 34). Several techniques have been developed to characterize variation among serotype b isolates and to study their in vivo transmission. These methods include electrophoretic profiling of major outer membrane proteins (OMP) (2, 3, 7, 18, 37), metabolic enzyme analysis (28-31), and chromosomal restriction fragment length polymorphism pattern analysis (1, 15, 22, 24, 29-32). The data obtained from studies of large samples of encapsulated *H. influenzae* strains recovered worldwide have demonstrated that, in general, the population structure is clonal. In addition, despite the occurrence of 182 distinct chromosomal genotypes expressing the serotype b polysaccharide capsule, the great majority of cases of invasive *H. influenzae* disease in North America, Europe, and other regions are caused by type b strains assigned to only three major clone families. It has been hypothesized that extant encapsulated *H. influenzae* strains have evolved from relatively few progenitor strains; for type b isolates this number may be three (1, 29).

For immunoprophylactic and immunodiagnostic development, much research effort has been directed toward the characterization of noncapsular surface antigens of *H. influenzae*. It has been demonstrated that noncapsular epitopes present on OMP induce the synthesis of antibodies that cross-react with a variety of *H. influenzae* strains. Moreover, some of these antigens are protective in an infant rat model of invasive infection (8, 9, 16, 17, 19, 26, 27). One of the surface molecules, the P2 protein (b/c protein [18]), is the most abundant protein present in the OM of *H. influenzae* strains (2, 18, 27). The protein has an apparent molecular mass of between 37 and 40 kilodaltons (2, 5, 6, 18, 36, 37) and is a porin (5, 35, 36). The P2 protein from several *H.*

*influenzae* type b strains was also cloned and sequenced (12, 13, 23, 25). Munson et al. (27) have demonstrated with polyclonal antibodies directed against OMP P2 that antigenic heterogeneity exists among the P2 proteins expressed by different type b strains. Recently, Hamel et al. (10, 11) and Hansen et al. (14) have generated porin-specific monoclonal antibodies (MAbs) that recognize 99 and 100%, respectively, of type b strains tested. Taken together, these data suggest the existence of an array of antigenic epitopes associated with the P2 protein, some of which may be more highly conserved than others.

In this report, we describe the reactivity pattern of a panel of P2-specific MAbs against a sample of 119 genetically characterized encapsulated *H. influenzae* strains expressing type a, b, or d polysaccharide. The data demonstrate that in general the patterns of P2 protein surface epitope exposure are cognate with genetic lineages of encapsulated *H. influenzae* strains and support the hypothesis (29) that the population structure of this group of organisms is predominantly clonal.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** We examined a collection of 151 isolates of *H. influenzae* recovered from patients worldwide. The sample included 10 isolates of serotype a, 90 isolates of serotype b, 19 isolates of serotype d, and 32 isolates that do not react with antisera directed against any of the six known capsule types. The chromosomal genotypes of the encapsulated isolates were previously characterized, and the sample included isolates representing the major genetically distinct clone families expressing serotype a, b, and d capsule polysaccharides (29). The chromosomal genotypes of the 32 nontypeable isolates have not been determined.

The 32 serologically nontypeable isolates and type b strain

\* Corresponding author.

3068 were recovered from patients at Sainte-Justine Hospital, Montreal, Quebec, Canada, and Centre Hospitalier Universitaire de Laval, Quebec, Canada. Isolates of 19 gram-negative species were obtained from the American Type Culture Collection or a collection held by the Laboratory Centre for Disease Control.

*H. influenzae* strains were grown overnight on chocolate agar plates at 37°C in an atmosphere with 5% CO<sub>2</sub>. Cultures were stored at -70°C in brain heart infusion broth with 20% (vol/vol) glycerol.

**Antigen preparation.** Lithium chloride extraction of OM preparations from *H. influenzae* serotype b strain 3068 was performed as described by Brodeur et al. (4). P2 protein purified from serotype b strain Minn A and recombinant *H. influenzae* serotype b porin were generously provided by R. S. Munson, Washington University School of Medicine, St. Louis, Mo.

**Production of MAbs.** Immunization and cell fusion procedures were performed as described by Hamel et al. (11). Supernatants of hybridoma cell cultures were screened for antibodies directed against *H. influenzae* OM preparations or purified or recombinant porin by an enzyme-linked immunosorbent assay (ELISA) (11). Class and subclass specificities of the MAbs were determined by an ELISA with commercially available reagents (Fisher Biotech, Ottawa, Canada).

**Proteinase K and sodium periodate treatment of the OM preparations.** Proteinase K (Boehringer GmbH, Mannheim, Federal Republic of Germany) treatment of OM preparations has been described previously (21). Sodium periodate oxidation was used to determine if a MAb reacted specifically with carbohydrate antigenic determinants present on the lipopolysaccharide. The method described by Woodward et al. (39) was used with the following modifications. Microtiter plates (Linbro E.I.A.; Flow Laboratories, Inc., McLean, Va.) were coated with OM preparations adjusted to 7.5 µg of protein per ml and were incubated with phosphate-buffered saline (PBS) containing 0.05% (vol/vol) Tween 20 (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C. The plates were washed five times with PBS-Tween 20 and rinsed with 50 mM sodium acetate buffer, pH 4.5, prior to treatment with sodium periodate (Sigma). Sets of wells were then exposed to various concentrations of sodium periodate (0 to 100 mM) in sodium acetate buffer for 1 h at room temperature in the dark. Following a brief rinse with sodium acetate buffer, 0.2 ml of 50 mM sodium borohydride (Sigma) in PBS was added to each well and the plates were incubated for 30 min at room temperature. Following five washes with PBS-Tween 20, 0.1 ml of culture supernatants containing the MAbs was added to the appropriate wells. After 1 h of incubation at 37°C, the plate was washed three times and 0.1 ml of alkaline phosphatase-conjugated goat anti-mouse or anti-human immunoglobulins (Sigma; Cappel, Organon Teknika Corp., West Chester, Pa.), diluted 1 in 1,000 in PBS containing 3% (wt/vol) bovine serum albumin was added. After incubation for 1 h at 37°C, the plates were washed and 0.1 ml of diethanolamine (10% [vol/vol], pH 9.8) containing *p*-nitrophenylphosphatase (Sigma) at 1 mg/ml was added. After 60 min, the absorbance was determined spectrophotometrically at 410 nm with a microplate reader (MR 600; Dynatech Industries, Inc., McLean, Va.).

**Western immunoblotting procedure.** OM preparations from *H. influenzae* serotype b 3068 were resolved by electrophoresis with the Mini Protean system (Bio-Rad Laboratories, Richmond, Calif.) and transferred electrophoretically

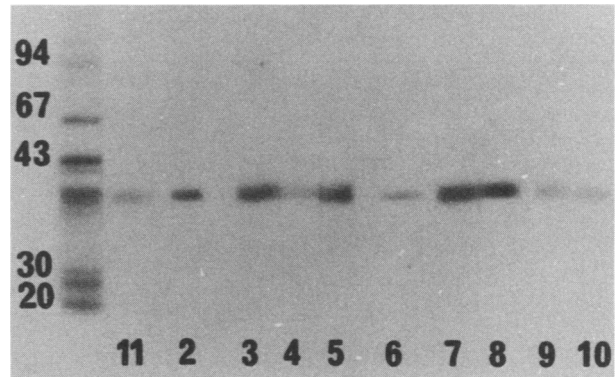


FIG. 1. Immunoblots of *H. influenzae* serotype b 3068 strain outer membrane preparations after probing with a mouse hyperimmune serum (leftmost lane) and 10 P2-specific MAbs: P2-11 (lane 11), P2-2 (lane 2), P2-3 (lane 3), P2-4 (lane 4), P2-5 (lane 5), P2-6 (lane 6), P2-7 (lane 7), P2-8 (lane 8), P2-9 (lane 9), and P2-10 (lane 10). Standard proteins are identified with their kilodalton values on the left.

to nitrocellulose transfer membrane paper (Bio-Rad) (20, 21).

**Reaction of MAbs to intact bacterial cells.** One milliliter of hybridoma culture supernatant was mixed with 10<sup>9</sup> live intact *H. influenzae* type b cells and was incubated with constant shaking for 2 h at 4°C. After three washes in PBS, the bacterial cells were suspended in a 1% (wt/vol) bovine serum albumin-PBS solution containing 0.25 µCi of <sup>125</sup>I-labeled sheep anti-mouse immunoglobulins (Amersham Corp., Arlington Heights, Ill.) per ml and incubated with constant agitation for 1 h at room temperature. The bacteria were washed twice with PBS, and the <sup>125</sup>I radioactivity was measured by gamma scintillation (1282 Compugamma; LKB Instruments, Inc., Rockville, Md). Bacterial counts performed before and after reaction with MAbs revealed no loss of viability.

**Dot enzyme immunoassay.** A dot enzyme immunoassay (20) was used for rapid screening of the MAbs against a large number of bacterial strains.

## RESULTS

**Properties of the MAbs.** Detection of antibodies present in hybridoma culture supernatants was performed by ELISA, with *H. influenzae* type b strain 3068 OM preparations used as coating antigens. In order to recover only the hybridomas secreting porin-specific MAbs, supernatants derived from positive wells were retested with porin purified from strain Minn A. In this fashion, 16 hybridomas secreting antibodies that reacted strongly with purified P2 were subcloned twice by limiting dilution; this panel of MAbs also reacted strongly in an ELISA with recombinant type b P2 protein expressed in *Escherichia coli*. Western immunoblotting demonstrated that all the MAbs recognized a protein band with an apparent molecular mass of 40 kilodaltons (Fig. 1), a value that is similar to that reported for the porin protein. ELISA analysis revealed that the hybridomas secreted immunoglobulin G immunoglobulins with a variety of subclass specificities; all MAbs were κ light chain restricted.

Sodium periodate and proteinase K treatments were used to confirm the protein character of the epitopes. Proteinase K treatment altered the OMP structures such that the

TABLE 1. Reactivity of the P2-specific MABs with *H. influenzae* strains

MAB or MAB group	No. of <i>H. influenzae</i> isolates recognized by MAB(s)					
	Serotype b cluster				Serotype a cluster B2 (5) <sup>a</sup>	Serotype d cluster B1 (19)
	A1 (25)	A2 (57)	B1 (7)	Total (%) <sup>b</sup>		
P2-2	25	56	0	81 (90)	0	0
A <sup>c</sup>	25	57	0	82 (91)	5	19
B	25	57	7	89 (99)	0	0
C	17	52	0	69 (77)	0	2
D <sup>d</sup>	25	57	6	88 (98)	0	0
P2-14	25	57	0	82 (91)	0	2
P2-15 <sup>d</sup>	25	57	0	82 (91)	0	0
P2-16 <sup>e</sup>	25	57	7	89 (99)	0	18
P2-17 <sup>e</sup>	15	51	7	73 (81)	0	0
Hb-2	25	57	7	89 (99)	0	19

<sup>a</sup> Total number of strains tested for each group. All five serotype a isolates assigned to division II (clusters H1 and I1) clones failed to react with all MABs.

<sup>b</sup> Total number of serotype b strains tested including clusters A1, A2, B1 and J1.

<sup>c</sup> Each MAB in a group had the same reactivity pattern: group A, P2-3, P2-8, and P2-11; group B, P2-4 and P2-6; group C, P2-5, P2-7, P2-9, and P2-12; group D, P2-10 and P2-13.

<sup>d</sup> Weak reaction with serotype b, B1 family of strains.

<sup>e</sup> Strains were boiled for 5 min before the lysates were applied to the nitrocellulose paper.

ELISA reactivity was reduced by more than 90%. Treatment with sodium periodate did not produce significant alteration in the reactivity of the MABs for OM preparations. In contrast, the reactivity of HiH-2, a human MAB specific for *H. influenzae* lipopolysaccharide (21), was greatly diminished by sodium periodate but not proteinase K treatment.

Hybridoma culture supernatants were incubated with intact *H. influenzae* cells to determine the ability of the MABs to bind to surface-exposed porin epitopes. MABs P2-14, P2-16, and P2-17 weakly bound to the surface of intact bacterial cells, as measured by radioimmunoassay. In contrast, the 14 other MABs strongly reacted with the surface of intact cells, presumably with their corresponding porin epitopes. MAB gp 41-1, which was raised against an unrelated viral antigen, was used as a negative control and did not bind to the surface of type b *H. influenzae* cells.

**Phylogenetic distribution of the epitopes recognized by porin-specific MABs.** A dot enzyme immunoassay was used to index the distribution of the epitopes recognized by the P2-specific MABs in a sample of strains representing the major clonal lineages of encapsulated *H. influenzae* (Table 1). In addition, the reactivity of MAB Hb-2, which was previously described (11), was also studied. All the MABs reacted to varying degrees with populations of encapsulated type a, b, and d clones assigned to primary phylogenetic division I (Fig. 2). However, in contrast, none of the porin-specific MABs reacted with those highly divergent serotype a and b clones in primary division II (Table 1 and data not shown).

MABs P2-4, P2-6, P2-10, P2-13, and P2-15 recognized only serotype b clones in primary division I. All serotype b clones present in the three major clone families of division I (A1, A2, and B1) reacted with MABs P2-4 and P2-6; MABs P2-10 and P2-13 identified 88 of 89 type b isolates assigned to these three clone families and, hence, were closely similar in reactivity pattern. MAB P2-15 specifically recognized an epitope present only on serotype b isolates in the two closely allied major clone families A1 and A2 (Table 1).

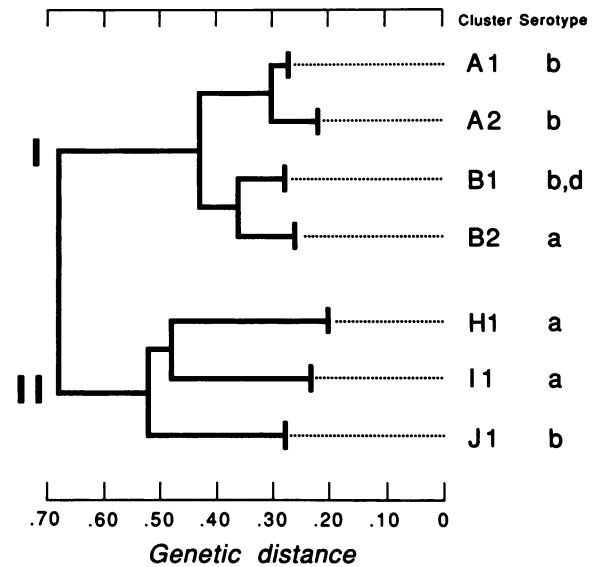


FIG. 2. Genetic relationships among multilocus enzyme genotypes (ETs) of three serotypes of *H. influenzae*. The dendrogram is a simplified version of that described by Musser et al. (29) and is based on analysis of genetic variation of 2,209 encapsulated isolates at 17 enzyme-encoding loci. There are two primary phylogenetic divisions (I and II) that diverge from one another at a genetic distance of 0.66. Clusters represented by multiple ETs diverging from one another at genetic distances <0.20 were truncated; the level of truncation indicates the deepest level of divergence of clusters of ETs within a lineage. Virtually all invasive serotype a and serotype b episodes worldwide are caused by isolates assigned to ETs in primary phylogenetic division I (29).

The other MABs recognized epitopes more widely distributed among division I clones and were not serotype b specific. For example, MAB Hb-2 reacted with an epitope shared by all division I serotype b and d clones. MABs in group C were unusual in that they reacted with only 77% of type b clones in families A1 and A2, failed to react with type b clones in family B1, and recognized 2 of 19 serotype d isolates tested (Table 1).

Radioimmunoassays revealed that MABs P2-16 and P2-17 bound very weakly to epitopes expressed on the surface of intact bacterial cells. In order to increase reactivity, bacteria were boiled before application to nitrocellulose paper; with this treatment, MAB P2-16 reacted with all serotype b and 94% of serotype d clones in division I but did not react with serotype a clones. MAB P2-17 failed to recognize several type b clones assigned to families A1 and A2 or any serotype a or d clones. Boiling of bacteria did not affect the reactivity pattern of the other MABs (data not shown).

None of the 32 isolates which failed to react with antisera directed against the six known *H. influenzae* capsule types and none of the isolates representing 19 other gram-negative species was recognized by the P2-specific MABs (data not shown).

## DISCUSSION

The need for new, more efficient vaccines against pathogenic *H. influenzae* clones prompted our search for highly conserved immunoreactive surface epitopes. Since recent studies have demonstrated that on a global scale the great majority of serious disease episodes are caused by only a few highly pathogenic *H. influenzae* clones (29) and that the

molecular structure of the P2 OMP is well conserved among clones of a known level of chromosomal divergence (23), we reasoned that MAbs could be generated to identify P2 epitopes shared by *H. influenzae* clones commonly recovered from severe infections.

The failure of the P2-specific MAbs to react with *H. influenzae* clones assigned to primary division II was not unexpected. Multilocus enzyme electrophoresis has revealed that division II clones are highly divergent in overall chromosomal character from those clones in primary division I (Fig. 2). The lack of reactivity of the panel of MAbs generated in this study with division II serotype a and b clones is consistent with the hypothesis advanced previously that horizontal transfer of genes involved in capsule synthesis accounts for the expression of the same polysaccharide antigen in evolutionarily divergent lineages of *H. influenzae*. Under this hypothesis, division II cell lines fail to react with the MAbs because mutations resulting in antigenic drift have sufficiently altered P2 epitopes shared by a common ancestral strain. Sequence comparison of the P2 genes carried by serotype a and b clones assigned to division II with those recently described (23) from four strains representing three serotype b clone families in primary division I will yield additional insight regarding the evolution of this locus and the structure-function relationships of the P2 protein.

Group A MAbs reacting with serotype a and d clones in division I and also recognizing type b strains assigned to clone families A1 and A2 do not identify strains of electrophoretic type (ET) 25.6 which are in the B1 clone family (29). Strains of ET 25.6 are rarely recovered from invasive disease episodes in North America or Europe, but they are common causes of severe pneumonia, sepsis, and meningitis in Papua New Guinea and Pakistan (29, 38). Isolates of this phylogenetic line are usually OMP subtype 6U. Munson et al. (23) have demonstrated that the P2 structural genes from a representative ET 25.6 OMP 6U isolate and an ET 1.9 OMP 1H isolate (A1 clone family) diverge at 13 nucleotides; six of these differences cause four amino acid changes located between amino acids 181 and 195 of the P2 protein. The data suggest that some of the heterogeneity we identified in MAb reactivity pattern may be a consequence of nucleotide sequence variation occurring in this region of the P2 structural gene. Consistent with this hypothesis is the observation that the reactivity of these MAbs for the porin protein is inhibited by a synthetic peptide composed of the amino acid sequence of this region of protein P2 (D. Martin et al., unpublished data).

Group C MAbs and P2-14 also failed to react with ET 25.6 clones and did not recognize serotype a clones and the great majority of type d clones. Interestingly, these antibodies did react with two serotype d isolates recovered in invasive episodes in Malaysia. This pattern of reactivity can be explained by either of two hypotheses: these two serotype d strains may have retained an epitope that all other serotype d strains examined have lost; alternatively, the putative epitope may have been lost and then regained, probably through mutational convergence.

Two of the MAbs, P2-16 and P2-17, identified epitopes on the P2 protein that are not exposed or not accessible on the surface of intact cells. MAb P2-16 recognized all serotype b clones in division I and 18 of 19 genetically allied serotype d clones. Hansen et al. (14) have also described a MAb (9F5) that is directed against a P2 epitope not exposed on the surface of intact cells; this antibody recognized all serotype b strains tested.

In summary, our study identified a panel of 16 MAbs

directed against a family of epitopes located on the P2 porin protein of *H. influenzae*. In general, the reactivity patterns of this group of MAbs were cognate with current understanding regarding the evolution of encapsulated *H. influenzae* clones (23, 29). Some of these MAbs may be useful for immunoidentification of bacterial antigens present in clinical specimens and may also be useful for immunoprophylactic intervention. Indeed, group B MAbs are particularly noteworthy since they specifically identify all serotype b clones in primary division I. Epitope mapping and animal protection studies are under way to identify immunodominant antigenic sites recognized by the MAbs.

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