Localization of conserved B-cell epitopes among encapsulated and non-encapsulated *Haemophilus influenzae* P2 porin proteins using synthetic peptides

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The P2 outer membrane protein of *Haemophilus influenzae* belongs to a class of apparently ubiquitous proteins in Gram-negative bacteria that function as porins. Murine hybridomas raised to the P2 protein and synthetic peptides were used to investigate the structural and antigenic relationships among P2 proteins of encapsulated and non-encapsulated *H. influenzae*. Three monoclonal antibodies (mAbs), P2-17, P2-18 and P2-19, recognizing epitopes on the P2 protein, as shown by Western immunoblotting of outer membrane preparations, and purified and recombinant P2 proteins are described. The epitopes reactive with the mAbs were widely distributed among *H. influenzae* strains since 70–100% of strains of encapsulated and non-encapsulated isolates collected worldwide were recognized by individual mAbs. None of the mAbs reacted with *H. parainfluenzae* or other bacterial species. The peptide composition of P2 epitopes was determined by analysis of mAb reactivity with a series of overlapping synthetic peptides that covered the amino acid sequences of *H. influenzae* type b. The domains recognized by these mAbs were completely distinct. mAb P2-18, reactive with an epitope conserved among all *H. influenzae* P2 porin molecules which were screened, recognized a peptide corresponding to the N-terminal segment (residues 1–14). The P2-17- and P2-19-specific epitopes were located between residues 28 and 55, and 101 and 129, respectively. None of the epitopes were exposed on the cell surface since no mAbs bound to intact live bacteria. The identification of epitopes that are shared among P2 proteins from different strains of encapsulated and non-encapsulated *H. influenzae* strains suggests that the P2 porin protein from all *H. influenzae* strains has been highly conserved and that these epitopes may represent amino acid residues critical to the porin's structure.

Introduction

Haemophilus influenzae is responsible for a variety of diseases in humans, ranging from chronic respiratory infection to meningitis. Virtually all cases of meningitis and other forms of invasive infection are caused by organisms that possess a serotype b capsule (Turk, 1984). Although strains expressing other capsule types or lacking a capsule are usually associated with superficial infections or asymptomatic carriage, these strains have now been recognized as important invasive pathogens in some human populations (Greene, 1978; Gratten *et al.*, 1985; Munson *et al.*, 1989b; Weinberg *et al.*, 1989). This has been particularly well documented in Pakistan where

bacteremic cases of lower respiratory tract infection were shown to be caused by non-encapsulated strains (Weinberg *et al.*, 1989). For many years it has been known that encapsulated strains frequently lose the ability to synthesize a capsule (Pittman, 1931; Hoiseth *et al.*, 1985). Recently, however, Musser *et al.* (1986) demonstrated conclusively that most non-encapsulated clinical isolates are genetically unrelated to the type b strains and are not phenotypic variants of encapsulated isolates that have recently lost the polysaccharide capsule. These findings emphasize the need for a new vaccine capable of protecting against a wide range of *H. influenzae* strains, both encapsulated and non-encapsulated, and suggest that a vaccine may require the inclusion of non-capsular antigens.

Several outer membrane proteins are currently being considered as potential vaccine candidates since they

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Abbreviations: mAb, monoclonal antibody.

have induced antibodies protective in an animal model of infection (Munson *et al.*, 1983; Kimura *et al.*, 1985; Munson & Granoff, 1985; Granoff & Munson, 1986; Green *et al.*, 1987; Loeb, 1987; Deich *et al.*, 1990; Thomas *et al.*, 1990). One of these proteins, P2, has porin activity (Vachon *et al.*, 1985). Porins belong to a class of apparently ubiquitous proteins which form water-filled channels across the outer membrane and have remarkably conserved physical properties (Nikaido & Vaara, 1985). In addition to its porin activity, P2 protein may play an important role in the pathogenesis of *H. influenzae* infection, since the absence of its expression drastically reduced the ability of *H. influenzae* type b to grow *in vitro*, and to produce bacteremia in an animal model (Cope *et al.*, 1990). A major step in the understanding of *H. influenzae* P2 protein was achieved by the recent cloning of the structural gene (Hansen *et al.*, 1988; Munson & Tolan, 1989). Comparative analysis of the nucleotide and amino acid sequences of four different type b strains indicated that the P2 genes were highly conserved (Munson *et al.*, 1989a). This observation was substantiated by the isolation of P2-specific monoclonal antibodies (mAbs) reactive with a large panel of *H. influenzae* type b isolates (Hamel *et al.*, 1987a, b; Hamel & Brodeur, 1990; Hansen *et al.*, 1989; Martin *et al.*, 1990). Moreover, recent immunological studies on the epitope mapping of P2 protein from *H. influenzae* type b have revealed the presence of at least four highly conserved regions (Martin *et al.*, 1991).

In contrast to the type b strains, the P2 protein in non-typable *H. influenzae* seems to exhibit considerable variation. The molecular mass of P2 protein is more variable among non-typable strains than among type b strains (Loeb & Smith, 1980; Barenkamp *et al.*, 1981, 1982; Murphy *et al.*, 1983). Several studies have suggested that many of the antigenic differences among non-typable strains reside in the P2 molecule (Murphy & Bartos, 1988a, b; Groeneveld *et al.*, 1989; van Alphen *et al.*, 1991; Haase *et al.*, 1991). Further antigenic analysis of P2 protein may provide information relevant to future vaccine development, and to further definition of structure-function relationships. In this paper, we studied the antigenic relationship between P2 proteins by use of mAbs and synthetic peptides. We report the identification and localization of three conserved epitopes among the P2 porin proteins from encapsulated and non-encapsulated *H. influenzae* strains.

Methods

Bacterial strains. The 31 isolates of *H. influenzae* used in this study are presented in Table 1. The samples include 18 non-typable isolates, 7

Table 1. Characteristics of *H. influenzae* strains and mAbs tested in this study

Strains*				Reactivity with mAbs†		
No.	Serotype/ division‡	Designation	Source	P2-18	P2-17	P2-19
1	HiNT	Q108	Eye	+	+	+
2	HiNT	Q101	Nasopharynx	+	+	+
3	HiNT	Q103	Ear	+	—	—
4	HiNT	Q316	Sputum	+	+	—
5	HiNT	Q373	Sputum	+	+	—
6	HiNT	MTL3	Non-INV	+	+	+
7	HiNT	MTL5	Non-INV	+	+	+
8	HiNT	MTL6	Non-INV	+	—	+
9	HiNT	Just209	INV	+	+	+
10	HiNT	4069	INV	+	+	+
11	HiNT	4144	INV	+	—	—
12	HiNT	4147	INV	+	+	+
13	HiNT	4389	INV	+	+	+
14	HiNT/1	10095	INV	+	+	+
15	HiNT/2	10108	INV	+	—	—
16	HiNT/5	12037	INV	+	+	+
17	HiNT/6	12049	INV	+	+	+
18	HiNT/4	12085	INV	+	+	+
19	Hib/I	3068	Non-INV	+	+	+
20	Hib/I	MinnA	INV	+	+	+
21	Hib/II	KIL7	INV	+	+	—
22	Hib/II	WAR28	INV	+	+	—
23	Hib/I	WHAEM294	INV	+	+	+
24	Hib/I	Durst	Unknown	+	+	+
25	Hib/I	1613	INV	+	+	+
26	Hia/II	531	Sputum	+	+	—
27	Hia/II	541	Nasopharynx	+	+	—
28	Hia/I	506	INV	+	+	+
29	Hia/I	728	INV	+	+	+
30	Hid/I	571	Unknown	+	+	+
31	Hid/I	580	Unknown	+	+	+
32	Hp	M523/85	Non-INV	—	—	—
33	Hp	M481/85	Non-INV	—	—	—

* Abbreviations: HiNT, non-typable *H. influenzae*; Hia, Hib and Hid; *H. influenzae* serotypes a, b and d, respectively; Hp, *H. parainfluenzae*; INV, strain recovered from patients with invasive infection; non-INV, strain recovered from asymptomatic carriers or from patients with superficial infection.

† A dot enzyme immunoassay described in Methods was used for the screening of mAbs against bacterial strains.

‡ Multilocus enzyme electrophoretic analysis was used to determine the genetic relatedness among bacterial isolates. The characterization of encapsulated strains of *H. influenzae* was performed by J. M. Musser as described elsewhere (Musser *et al.*, 1990). Clonally restricted non-typable isolates recovered from Islamabad, Pakistan, were characterized by Weinberg *et al.* (1989).

isolates of serotype b, 4 isolates of serotype a and 2 isolates of serotype d. The non-typable isolates were cultured from asymptomatic carriers, patients with superficial infections or patients with invasive disease. Of the non-typable invasive strains, one was recovered in Canada, one in the United States, one in Papua New Guinea and two in Sweden; the

last five were representatives of the most common non-typable clonal groups from the Pakistan study (Weinberg *et al.*, 1989). Encapsulated *H. influenzae* are clonally diverse, as analysed by their outer membrane protein profile (Barenkamp *et al.*, 1981) and by multilocus enzyme electrophoresis (Musser *et al.*, 1988, 1990). Representatives of the most prevalent clones were studied. *H. influenzae* serotype b isolates included prototype strains MinnA (OMP 1H), Durst (OMP 2L), 1613 (OMP 3L), WHAEM294 (OMP 6U), and strains KIL7 and WAR28, both from divergent clones assigned to primary phylogenetic division II (Musser *et al.*, 1990). The *H. influenzae* isolates were obtained from James M. Musser, Pennsylvania State University, Philadelphia, PA, USA; Michel G. Bergeron, Université Laval, Sainte-Foy, Quebec, Canada; Serge Montplaisir, Sainte-Justine Hospital, Montreal, Quebec, Canada; and Robert S. Munson, Washington University School of Medicine, Saint Louis, MO, USA. Isolates of *H. parainfluenzae*, *Actinobacillus pleuropneumoniae*, *Enterobacter aerogenes* (ATCC 13048), *Edwardsiella tarda* (ATCC 15947), *Neisseria perflava* (ATCC 14799), *Neisseria meningitidis*, *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 9027), *Shigella sonnei* (ATCC 9290), *Escherichia coli* and *Proteus vulgaris* (ATCC 13315) were obtained from the American Type Culture Collection (Rockville, MD, USA) or a collection held by the National Laboratory for Immunology (Ottawa, Canada).

H. influenzae isolates were grown overnight at 37 °C in an atmosphere with 5% CO₂ on chocolate agar plates supplemented with 1% (v/v) IsoVitalX (BBL Microbiology Systems). Cultures were stored at -70 °C in brain heart infusion broth containing 20% (v/v) glycerol.

Antigen preparations. Various *H. influenzae* antigens were prepared for immunization and immunoassays. The extraction of outer membranes from all *H. influenzae* isolates was performed by the lithium chloride method as described previously (Hamel *et al.*, 1987a). Sarcosyl-insoluble preparations enriched in the major outer membrane proteins from non-typable *H. influenzae* MTL6 were prepared by the method described by Barenkamp *et al.* (1981). The P2 protein from non-typable *H. influenzae* 12085 was isolated according Munson *et al.* (1983). P2 protein purified from *H. influenzae* type b MinnA and the sarcosyl-insoluble fraction from recombinant *E. coli* JM101(pRSM478), which expressed the P2 from MinnA, were generously provided by R. S. Munson (Munson *et al.*, 1983; Munson & Tolan, 1989).

Production of mAbs. Balb/c mice were immunized intraperitoneally with 20 µg sarcosyl-insoluble proteins of non-typable *H. influenzae* MTL6 suspended in Freund's complete adjuvant. Two weeks later, the mice were given another injection of Freund's incomplete adjuvant. Three days before the fusion, the mice were boosted with 10 µg P2 protein from non-typable *H. influenzae* 12085. Hybridomas were produced by fusion of splenic lymphocytes from immunized mice with non-secreting Sp2/0 myeloma cells as previously described (Hamel *et al.*, 1987a). P2-protein-specific hybridomas were cloned by sequential limiting dilutions, expanded and frozen in liquid nitrogen. The class, subclass and light-chain type of each mAb was determined by ELISA as described previously (Martin *et al.*, 1990). Preliminary characterization of mAb P2-17 has been reported previously (Martin *et al.*, 1990).

Peptide synthesis. The complete nucleotide and derived amino acid sequences of the P2 protein of *H. influenzae* type b MinnA have been published (Munson & Tolan, 1989). A series of 17 overlapping peptides (12–30 amino acid residues each) covering the entire P2 protein sequence were synthesized as previously described (Martin *et al.*, 1991). The sequence and the position of all peptides synthesized are shown in Fig. 1.

SDS-PAGE and Western immunoblotting. Outer membrane proteins were resolved by electrophoresis on homogeneous 12.5% polyacryl-

Fig. 1. Peptides of the P2 protein.

amide gels using the PhastSystem (Pharmacia). After separation, the proteins were stained with Coomassie blue or transferred from the gel to a nitrocellulose membrane (Bio-Rad) using the PhastTransfer Semi-dry Transfer Kit (Pharmacia). Luminescent detection of antigens reactive with the mAbs was performed by an indirect antibody immunoassay using peroxidase-labelled anti-mouse immunoglobulins and the luciferin-enhanced substrate (Proulx *et al.*, 1991). The blot was exposed for 5 min at room temperature to Kodak XAR-5 film in a Kodak X-omatic cassette fitted with intensifying screens. The following proteins (Sigma) were used as molecular mass standards: bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and α -lactalbumin (14.2 kDa).

Enzyme immunoassay procedures. The ELISA procedure, using outer membrane preparations from *H. influenzae* as coating antigens, was performed as detailed previously (Hamel *et al.*, 1987a). The peptide ELISA (Martin *et al.*, 1991) was carried out by coating synthetic peptides onto microtitration plates (Maxisorp immunoplate; Nunc) at a concentration of 5 µg ml⁻¹ in 50 mM-sodium carbonate buffer, pH 9.6. A peptide inhibition assay was done using plates coated with outer membrane preparations (Martin *et al.*, 1991).

A dot enzyme immunoassay (Martin *et al.*, 1991) was used for the rapid screening of mAbs against a large number of bacterial strains. Briefly, bacteria were suspended in PBS (0.9% NaCl; 10 mM-potassium phosphate buffer, pH 7.4) with or without 2% (v/v) SDS, boiled for 5 min, and then filtered through a nitrocellulose membrane by gravity using the Bio-Dot microfiltration apparatus (Bio-Rad). The membrane was blocked by incubation with PBS containing 3% (w/v) skimmed milk. This was followed by incubation with culture supernatants containing the mAbs. Identification of the strains reactive with the mAbs was then achieved using peroxidase-labelled anti-mouse immunoglobulins as secondary antibody.

Antibody accessibility radioimmunoassay. Absorption of mAbs at the surface of live bacteria was performed (Martin *et al.*, 1988; Proulx *et al.*, 1991) in order to determine whether mAbs were directed against surface exposed epitopes. Briefly, hybridoma culture supernatant was mixed with 10⁹ live intact *H. influenzae* type b 3068 or non-typable strain 12085 and incubated for 2 h at 4 °C. After three washes with PBS, the bacterial cells were suspended in a solution containing ¹²⁵I-labelled anti-mouse immunoglobulins (Amersham) and incubated for 1 h at room temperature. The bacteria were washed twice with PBS and the amount of cell-bound ¹²⁵I was determined.

Results

Specificity of mAbs for P2 protein

Culture supernatants of hybridomas obtained from fusion of primed spleen cells and SP2/0 myeloma cells were screened initially by ELISA using outer membrane preparations from *H. influenzae* type b 3068, and non-typable *H. influenzae* JUST209 and 12085. In order to recover the hybridomas secreting P2-protein-specific mAbs, supernatants that were reactive with these preparations were retested with purified P2 protein from non-typable strain 12085. Several antibodies, all IgG₁κ, were identified and were divided, based on their pattern of reactivity, into three groups which are represented by mAbs P2-17, P2-18 and P2-19.

The specificities of the mAbs were determined by immunoblotting using outer membrane preparations, purified P2 proteins and extracts from *E. coli* expressing or not expressing *H. influenzae* P2 protein as antigens. Molecular mass heterogeneity among the P2 proteins of encapsulated and non-encapsulated strains was observed by SDS-PAGE analysis of outer membrane preparations (Fig. 2a). When tested by luminescent immunoblotting, all mAbs recognized epitopes present on a protein band with an approximate molecular mass of 40 kDa corresponding to the P2 protein (Fig. 2b, c and d). P2 proteins from all studied non-typable, type a, b and d strains were reactive with the mAbs with the exception of mAbs P2-17 and P2-19 which did not react with non-typable strain MTL6 and serotype a strain 541, respectively. mAbs also recognized their epitopes on purified and recombinant P2 proteins (Fig. 3). None of the mAbs reacted with extracts of non-transformed *E. coli* JM101 which were used as controls.

Reactivity of mAbs against a panel of *Haemophilus* strains

ELISA and dot enzyme immunoassays were performed to determine the reactivity of mAbs against type a, b and d, and non-typable *H. influenzae*. Results obtained from this study are summarized in Table 1. Each mAb had its own pattern of recognition. mAb P2-18 reacted with all strains while mAbs P2-17 and P2-19 missed 4 strains and 9 strains, respectively. Previous experiments with mAb P2-17 have suggested that isolates other than type b, including type a and d, and non-typable isolates, were not reactive with mAb P2-17 (Martin *et al.*, 1990). However, in the present study, immunoassays using outer membrane preparations and SDS-treated organisms instead of intact bacteria indicate that mAb P2-17 reacted with all capsulated and most non-typable *H. influenzae* strains. mAb P2-19 reacted with type a, b and

Fig. 2. SDS-PAGE and Western blot analysis of outer membrane preparations from different *H. influenzae* strains. (a) Coomassie blue staining, and (b, c, d) luminescent immunoblot using mAbs P2-18, P2-17 and P2-19, respectively. Lanes: 1, non-typable strain 12085; 2, type b strain 3068; 3, non-typable strain 4147; 4, non-typable strain Q108; 5, type a strain 541; 6, type d strain 571; 7, non-typable strain MTL6; 8, molecular mass standards.

Fig. 3. SDS-PAGE and Western blot analysis. (a) Coomassie blue staining, and (b, c, d) luminescent immunoblot using mAbs P2-18, P2-17 and P2-19, respectively. Lanes: 1, molecular mass standards; 2 and 3, sarcosyl-insoluble fractions from *E. coli* JM101(pRSM478) which expressed the P2 protein, and JM101(pT7-7) which only contained the cloning vector; 4 and 5, purified P2 protein and outer membrane preparations from *H. influenzae* type b strain MinnA; 6 and 7, purified P2 protein and outer membrane preparations from non-typable *H. influenzae* strain 12085.

d strains allied in division I whereas encapsulated strains allied in division II, as well as some non-typable strains, were not recognized. None of the mAbs reacted with *H. parainfluenzae*.

Specificity of mAbs to *H. influenzae*

Various Gram-negative species were tested with the mAbs, including *Actinobacillus pleuropneumoniae*, *Enterobacter aerogenes*, *Edwardsiella tarda*, *Neisseria perflava*, *Neisseria meningitidis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Escherichia coli* and *Proteus vulgaris*. Dot enzyme immunoassays using bacterial suspensions boiled in the presence or absence of SDS were negative with all strains, suggesting that only *H. influenzae* contained a protein reactive with the mAbs.

Cell surface accessibility of epitopes

To determine if our mAbs were directed against cell-surface-exposed epitopes, hybridoma culture supernatants were incubated with live bacteria. mAbs did not bind to live intact type b or non-typable *H. influenzae* as measured by radioimmunoassay indicating that the epitopes recognized by mAbs were not accessible on the cell surface. Furthermore, mAbs reacted very poorly in dot enzyme immunoassay using freshly prepared or multiple frozen and thawed bacterial suspensions. However, a positive response was always obtained using outer membrane preparations or bacteria boiled in the presence of SDS as antigens.

Localization of mAb-specific epitopes

Overlapping peptides covering the entire P2 protein sequence from *H. influenzae* MinnA were used in ELISA to map the epitopes recognized by the mAbs precisely. Fig. 4 shows the reactivities of mAbs with the synthetic peptides. mAbs P2-18, P2-17 and P2-19 reacted with peptide P1 (residues 1–14), peptide 1 (residues 28–55) and peptide 4 (residues 101–129), respectively. An inhibition assay was performed to confirm the results obtained by direct ELISA. The amount of peptide required to inhibit 50% of the binding of the mAb to outer-membrane-coated plates was determined. The binding of mAbs to outer membrane preparations was inhibited specifically with the reactive peptide. The binding of mAb P2-18 was inhibited by concentrations of less than $0.2 \mu\text{g ml}^{-1}$ of peptide P1 (residues 1–14). The binding of mAbs P2-17 and P2-19 was inhibited by $1.55 \mu\text{g ml}^{-1}$ peptide 1 (residues 28–55) and $0.4 \mu\text{g ml}^{-1}$ peptide 4 (residues 101–129), respectively. No

Fig. 4. Reactivity of mAbs with synthetic peptides in ELISA. Microtitration plates were coated with peptides and the binding of mAbs P2-18 (a), P2-17 (b) and P2-19 (c) was determined using alkaline-phosphatase-labelled anti-mouse immunoglobulins. Results are expressed as OD₄₁₀ read 1 h after the addition of substrate.

inhibition was observed with control peptides. The absence of reactivity of the mAbs with peptide 10, which gave non-specific reaction with the conjugated anti-mouse immunoglobulins, was confirmed by peptide inhibition assay.

Discussion

Outer membranes of Gram-negative bacteria must allow the influx of nutrients and efflux of waste products (Nikaido & Vaara, 1985). A class of outer membrane proteins called porins is known to be involved in the non-specific transmembrane diffusion of solutes. So far, protein P2 is the only species of outer membrane proteins

of *H. influenzae* that has known porin activity (Vachon *et al.*, 1985). In this study, mAbs have been produced to investigate the antigenic and structural relationships among encapsulated and non-encapsulated *H. influenzae* P2 porin proteins. The reactivity spectrum of the mAbs was examined with a collection of carefully selected encapsulated and serologically non-typable isolates. These represented the major genetic clone families expressing serotype a, b and d capsules, and naturally occurring non-encapsulated organisms obtained from a variety of clinical conditions, in many geographic areas, including Islamabad in Pakistan where invasive disease-producing isolates were assigned to major clonal groups. Our study demonstrates that the mAbs react with B-cell epitopes that are widely distributed among both encapsulated and non-encapsulated *H. influenzae*. The data obtained with mAb P2-18 clearly indicate that there are epitopes common to all strains of this pathogen. However, P2 proteins exhibit some antigenic heterogeneity among non-encapsulated isolates since the epitope defined by mAb P2-17 was present on all encapsulated strains but only on 78% of non-typable isolates.

Recently, Musser *et al.* (1990) has demonstrated that the genetic structure of encapsulated *H. influenzae* is basically clonal, with divergent lineages divided in two deep phylogenetic divisions. In contrast, very extensive genetic diversity exists among non-typable isolates and, with few exceptions, none of the non-typable *H. influenzae* isolates shared the same clonal group (Musser *et al.*, 1986; Porras *et al.*, 1986). In this context, the identification of epitopes shared by highly divergent encapsulated and non-encapsulated isolates was unpredictable. However, it was not surprising to notice that mAb P2-19 failed to react specifically with serotype a and b isolates assigned to division II. This result was expected since division II is composed of clones rarely associated with invasive disease and highly divergent in overall chromosomal character from those clones in primary division I (Musser *et al.*, 1988). In addition, previous studies revealed that none of the 17 P2-specific mAbs reacted with genetically divergent serotype a or b clones assigned to primary division II (Hamel *et al.*, 1987b; Martin *et al.*, 1990). Although significant associations of clones with particular clinical syndromes or particular geographic areas have previously been reported (Musser *et al.*, 1990), the lack of reactivity of mAbs P2-17 and P2-19 with some non-typable isolates could not be correlated to any known factors.

The native state of P2 protein in outer membranes and in purified form is most probably a trimer (Vachon *et al.*, 1988). These trimers are dissociated into monomers by treatment with ionic detergents such as SDS. Our mAbs reacted with both native and SDS-denatured P2 protein, suggesting that the mAbs were directed against

sequence-continuous epitopes. Thus, it is possible that synthetic peptides constituting these well-conserved epitopes might be reactive with our mAbs as long as the synthetic peptides cover all the critical residues. This study demonstrates that the highly conserved P2-18-specific epitope is carried by the N-terminal segment (residues 1–14) whereas the epitopes reactive with P2-17 and P2-19 were located between residues 28 and 55, and 101 and 129, respectively. The lack of reactivity of mAb P2-17 and P2-19 with some isolates may be a consequence of nucleotide sequence variation occurring in the P2 protein structural gene.

All together, six distinct epitopes have been localized on the primary structure of *H. influenzae* P2 proteins. In previous studies, we reported the identification of one conformational and two linear surface-exposed epitopes of P2 protein that were highly conserved among *H. influenzae* type b strains (Hamel *et al.*, 1987a, 1990; Martin *et al.*, 1990, 1991). The linear epitopes were carried by the C-terminal end of the protein (residues 314–341) and residues 158–174, respectively. A segment of P2 protein (residues 148–174) in very close proximity to the surface of the bacteria carry a distinct internal epitope. In contrast to the epitopes described in the current study, these epitopes were only present on capsulated *H. influenzae* strains.

The location of the epitopes with respect to the membrane was studied in binding experiments performed with intact bacteria and outer membrane preparations. These studies clearly indicated that mAbs did not react with surface exposed epitopes. The positive reaction observed with membrane preparations might suggest that the epitopes recognized by mAbs constitute loops in the periplasm, since the membrane vesicles obtained from the extraction with lithium chloride are likely to be accessible from both sides. However, we cannot exclude the possibility that P2 proteins could have been dissociated during the outer membrane extraction procedure, allowing antibody to bind to epitopes normally embedded in the membrane.

Examining the amino acid sequences of porins from different *H. influenzae* type b strains reveals that the amino acid residues are fairly conserved (Munson *et al.*, 1989a). The low homology found between the sequences of P2 and other available porin proteins (Munson *et al.*, 1989a) is in good agreement with the specificity of the mAbs to *H. influenzae*. Although the structural genes for the porins of non-serotype b isolates have not been determined, our data strongly suggest a very well-conserved structure of porin proteins. These observations are in accord with the highly conserved function of porin.

In summary, we have demonstrated that epitopes of native *H. influenzae* P2 proteins are conserved among

different encapsulated and non-encapsulated isolates, as reported for porins of enterobacteriaceae (Klebba *et al.*, 1990). The outer membrane protein OmpF, a porin of *E. coli*, contains a conserved and internal region composed of residues which are involved in the determination of channel permeability properties. Immunochemical analyses of internal OmpF deletions which alter porin permeability indicated that these mutations cause internal structural changes that do not affect porin's surface epitopes. Whether the N-terminal portion of the P2 protein is critical to the structure of *H. influenzae* porins still remains to be determined. Our future studies will focus on identifying conserved epitopes on P2 protein that are accessible to antibodies in whole cells and that may be useful as vaccine components.

References

- VAN ALPHEN, L., EIJK, P., GEELLEN-VAN DEN BROEK, L. & DANKERT, J. (1991). Immunochemical characterization of variable epitopes of outer membrane protein P2 of nontypeable *Haemophilus influenzae*. *Infection and Immunity* **59**, 247–252.
- BARENKAMP, S. J., MUNSON, R. S., JR & GRANOFF, D. M. (1981). Subtyping isolates of *Haemophilus influenzae* type b by outer membrane protein profiles. *Journal of Infectious Diseases* **143**, 668–676.
- BARENKAMP, S. J., MUNSON, R. S. & GRANOFF, D. M. (1982). Outer membrane protein and biotype analysis of pathogenic nontypeable *Haemophilus influenzae*. *Infection and Immunity* **36**, 535–540.
- COPE, L. D., PELZEL, S. E., LATIMER, J. L. & HANSEN, E. J. (1990). Characterization of a mutant of *Haemophilus influenzae* type b lacking the P2 major outer membrane protein. *Infection and Immunity* **58**, 3312–3318.
- DEICH, R. A., ANILIONIS, A., FULGINITI, J., METCALF, B. J., QUATAERT, S., QUINN-DEY, T., ZLOTNICK, G. W. & GREEN, B. A. (1990). Antigenic conservation of the 15000-dalton outer membrane lipoprotein PCP of *Haemophilus influenzae* and biologic activity of anti-PCP antisera. *Infection and Immunity* **58**, 3388–3393.
- GRANOFF, D. M. & MUNSON, R. S., JR (1986). Prospects for prevention of *Haemophilus influenzae* type b disease by immunization. *Journal of Infectious Diseases* **153**, 448–461.
- GRATTEN, M., BARKER, J., SHANN, F., GEREDA, G., MONTGOMERY, J., KAJOI, M. & LUPUWA, T. (1985). Non-type b *Haemophilus influenzae* meningitis. *Lancet* **i**, 1343–1344.
- GREEN, B. A., QUINN-DEY, T. & ZLOTNICK, G. W. (1987). Biological activities of antibody to a peptidoglycan-associated lipoprotein of *Haemophilus influenzae* against multiple clinical isolates of *H. influenzae* type b. *Infection and Immunity* **55**, 2878–2883.
- GREENE, G. R. (1978). Meningitis due to *Haemophilus influenzae* other than type b: case report and review. *Pediatrics* **62**, 1021–1025.
- GROENEVELD, K., VAN ALPHEN, L., VOORTER, C., EIJK, P. P., JANSEN, H. M. & ZANEN, H. C. (1989). Antigenic drift of *Haemophilus influenzae* in patients with chronic obstructive pulmonary disease. *Infection and Immunity* **57**, 3038–3044.
- HAASE, E. M., CAMPAGNARI, A. A., SARWAR, J., SHERO, M., WIRTH, M., CUMMING, C. U. & MURPHY, T. F. (1991). Strain-specific and immunodominant surface epitopes of the P2 porin protein of nontypeable *Haemophilus influenzae*. *Infection and Immunity* **59**, 1278–1284.
- HAMEL, J. & BRODEUR, B. R. (1990). Induction of an immune response to the porin of *Haemophilus influenzae* type b by monoclonal anti-idiotypic antibodies. *Microbial Pathogenesis* **9**, 81–93.
- HAMEL, J., BRODEUR, B. R., LAROSE, Y., TSANG, P. S., BELMAAZA, A. & MONTPLAISIR, S. (1987a). A monoclonal antibody directed against a serotype-specific, outer membrane protein of *Haemophilus influenzae* type b. *Journal of Medical Microbiology* **23**, 163–170.
- HAMEL, J., BRODEUR, B. R., BELMAAZA, A., MONTPLAISIR, S., MUSSER, J. M. & SELANDER, R. K. (1987b). Identification of *Haemophilus influenzae* type b by a monoclonal antibody coagglutination assay. *Journal of Clinical Microbiology* **25**, 2434–2436.
- HANSEN, E. J., GONZALES, F. R., CHAMBERLAIN, N. R., NORGARD, M. V., MILLER, E. E., COPE, L. D., PELZEL, S. E., GADDY, B. & CLAUSELL, A. (1988). Cloning of the gene encoding the major outer membrane protein of *Haemophilus influenzae* type b. *Infection and Immunity* **56**, 2709–2716.
- HANSEN, E. J., PELZEL, S. E., ORTH, K., MOOMAW, C. R., RADOLF, J. D. & SLAUGHTER, C. A. (1989). Structural and antigenic conservation of the P2 porin protein among strains of *Haemophilus influenzae* type b. *Infection and Immunity* **57**, 3270–3275.
- HOISETH, S. K., CONNELLY, C. J. & MOXON, E. R. (1985). Genetics of spontaneous, high-frequency loss of b capsule expression in *Haemophilus influenzae*. *Infection and Immunity* **49**, 389–395.
- KIMURA, A., GULIG, P. A., MCCracken, G. H., JR, LOFTUS, T. A. & HANSEN, E. J. (1985). A minor high-molecular-weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. *Infection and Immunity* **47**, 253–259.
- KLEBBA, P. E., BENSON, S. A., BALA, S., ABDULLAH, T., REID, J. SINGH, S. P. & NIKAI, D. H. (1990). Determinants of OmpF porin antigenicity and structure. *Journal of Biological Chemistry* **265**, 6800–6810.
- LOEB, M. R. (1987). Protection of infant rats from *Haemophilus influenzae* type b infection by antiserum to purified outer membrane protein a. *Infection and Immunity* **55**, 2612–2618.
- LOEB, M. R. & SMITH, D. H. (1980). Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. *Infection and Immunity* **30**, 709–717.
- MARTIN, D., LAROSE, Y., HAMEL, J., LAGACÉ, J. & BRODEUR, B. R. (1988). Heterohybridomas secreting human monoclonal antibodies against *Haemophilus influenzae* type b. *European Journal of Immunology* **18**, 601–606.
- MARTIN, D., HAMEL, J., BRODEUR, B. R. & MUSSER, J. M. (1990). Antigenic relationships among the porin proteins of encapsulated *Haemophilus influenzae* clones. *Journal of Clinical Microbiology* **28**, 1720–1724.
- MARTIN, D., MUNSON, R., JR, GRASS, S., CHONG, P., HAMEL, J., ZOBRIK, G., KLEIN, M. & BRODEUR, B. R. (1991). Mapping of B-cell epitopes on the outer membrane P2 porin protein of *Haemophilus influenzae* by using recombinant proteins and synthetic peptides. *Infection and Immunity* **59**, 1457–1464.
- MUNSON, R. S., JR & GRANOFF, D. M. (1985). Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. *Infection and Immunity* **49**, 544–549.
- MUNSON, R., JR & TOLAN, R. W., JR (1989). Molecular cloning, expression, and primary sequence of outer membrane protein P2 of *Haemophilus influenzae* type b. *Infection and Immunity* **57**, 88–94.
- MUNSON, R. S., JR, SHENEP, J. L., BARENKAMP, S. J. & GRANOFF, D. M. (1983). Purification and comparison of outer membrane protein P2 from *Haemophilus influenzae* type b isolates. *Journal of Clinical Investigation* **72**, 677–684.
- MUNSON, R., JR, BAILEY, C. & GRASS, S. (1989a). Diversity of the outer membrane protein P2 gene from major clones of *Haemophilus influenzae* type b. *Molecular Microbiology* **3**, 1797–1803.
- MUNSON, R. S., JR, KABERR, M. H., LENOIR, A. A. & GRANOFF, D. M. (1989b). Epidemiology and prospects for prevention of disease due to *Haemophilus influenzae* in developing countries. *Reviews in Infectious Diseases* **11**, S588–S597.
- MURPHY, T. F. & BARTOS, L. C. (1988a). Purification and analysis with monoclonal antibodies of P2, the major outer membrane protein of nontypeable *Haemophilus influenzae*. *Infection and Immunity* **56**, 1084–1089.
- MURPHY, T. F. & BARTOS, L. C. (1988b). Human bactericidal antibody response to outer membrane protein P2 of nontypeable *Haemophilus influenzae*. *Infection and Immunity* **56**, 2673–2679.
- MURPHY, T. F., DUDAS, K. C., MYLOTTE, J. M. & APICELLA, M. A. (1983). A subtyping system for nontypeable *Haemophilus influenzae* based on outer-membrane proteins. *Journal of Infectious Diseases* **147**, 838–846.

- MUSSEY, J. M., BARENKAMP, S. J., GRANOFF, D. M. & SELANDER, R. K. (1986). Genetic relationships of serologically nontypable and serotype b strains of *Haemophilus influenzae*. *Infection and Immunity* **52**, 183–191.
- MUSSEY, J. M., KROLL, J. S., MOXON, E. R. & SELANDER, R. K. (1988). Clonal population structure of encapsulated *Haemophilus influenzae*. *Infection and Immunity* **56**, 1837–1845.
- MUSSEY, J. M., KROLL, J. S., GRANOFF, D. M., MOXON, E. R., BRODEUR, B. R., CAMPOS, J., DABERNAT, H., FREDERIKSEN, W., HAMEL, J., HAMMOND, G., ARNE HØIBY, E., JONSDOTTIR, K. E., KABEER, M., KALLINGS, I., KHAN, W. N., KILIAN, M., KNOWLES, K., KOORNOHOF, H. J., LAW, B., LI, K. I., MONTGOMERY, J., PATTISON, P. E., PIFFARETTUM, J.-C., TAKALA, A. K., THONG, M. L., WALL, R. A., WARD, J. I. & SELANDER, R. K. (1990). Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. *Reviews of Infectious Diseases* **12**, 75–111.
- NIKAIDO, H. & VAARA, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews* **49**, 1–32.
- PITTMAN, M. (1931). Variation and type specificity in the bacterial species *Haemophilus influenzae*. *Journal of Experimental Medicine* **53**, 471–492.
- PORRAS, O., CAUGANT, D. A., GRAY, B., LAGERGARD, T., LEVIN, B. R. & SVANBORG-EDÉN, C. (1986). Difference in structure between type b and nontypable *Haemophilus influenzae* populations. *Infection and Immunity* **53**, 79–89.
- PROULX, C., MUNSON, R. S., JR, GRASS, S., HAMEL, J., MARTIN, D. & BRODEUR, B. R. (1991). Identification of a surface-exposed immunodominant epitope on outer membrane protein P1 of *Haemophilus influenzae* type b. *Infection and Immunity* **59**, 963–970.
- THOMAS, W. R., CALLOW, M. G., DILWORTH, R. J. & AUDESCH, A. A. (1990). Expression in *Escherichia coli* of a high-molecular-weight protective surface antigen found in nontypeable and type b *Haemophilus influenzae*. *Infection and Immunity* **58**, 1909–1913.
- TURK, D. C. (1984). The pathogenicity of *Haemophilus influenzae*. *Journal of Medical Microbiology* **18**, 1–16.
- VACHON, V., LYEW, D. J. & COULTON, J. W. (1985). Transmembrane permeability channels across the outer membrane of *Haemophilus influenzae* type b. *Journal of Bacteriology* **162**, 918–924.
- VACHON, V., KRISTJANSON, D. N. & COULTON, J. W. (1988). Outer membrane porin protein of *Haemophilus influenzae* type b: pore size and subunit structure. *Canadian Journal of Microbiology* **34**, 134–140.
- WEINBERG, G. A., GHAFOR, A., ISHAQ, Z., NOMANI, N. K., KABEER, M., ANWAR, F., BURNEY, M. I., QURESHI, A. W., MUSSEY, J. M., SELANDER, R. K. & GRANOFF, D. M. (1989). Clonal analysis of *Haemophilus influenzae* isolated from children from Pakistan with lower respiratory tract infections. *Journal of Infectious Diseases* **160**, 634–643.