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

JOSEE HAMEL, DOMINIQUE DUGOURD, DENIS MARTIN, CHANTAL PROULX, PELE CHONG and BERNARD R. BRODEUR

National Laboratory for Immunology, Laboratory Centre for Disease Control, Ottawa K1A 0L2, Canada

Connaught Centre for Biotechnology Research, Toronto M2R 3T4, Canada

(Received 29 July 1991; revised 27 September 1991; accepted 7 October 1991)

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.*, 1989; 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...
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., 1989). The characterization of three conserved epitopes among the P2 porin proteins from encapsulated strains (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 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

<table>
<thead>
<tr>
<th>Strains*</th>
<th>Reactivity with mAbs†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>division‡</td>
</tr>
<tr>
<td>1</td>
<td>HiNT</td>
</tr>
<tr>
<td>2</td>
<td>HiNT</td>
</tr>
<tr>
<td>3</td>
<td>HiNT</td>
</tr>
<tr>
<td>4</td>
<td>HiNT</td>
</tr>
<tr>
<td>5</td>
<td>HiNT</td>
</tr>
<tr>
<td>6</td>
<td>HiNT</td>
</tr>
<tr>
<td>7</td>
<td>HiNT</td>
</tr>
<tr>
<td>8</td>
<td>HiNT</td>
</tr>
<tr>
<td>9</td>
<td>HiNT</td>
</tr>
<tr>
<td>10</td>
<td>HiNT</td>
</tr>
<tr>
<td>11</td>
<td>HiNT</td>
</tr>
<tr>
<td>12</td>
<td>HiNT</td>
</tr>
<tr>
<td>13</td>
<td>HiNT</td>
</tr>
<tr>
<td>14</td>
<td>HiNT/1</td>
</tr>
<tr>
<td>15</td>
<td>HiNT/2</td>
</tr>
<tr>
<td>16</td>
<td>HiNT/5</td>
</tr>
<tr>
<td>17</td>
<td>HiNT/6</td>
</tr>
<tr>
<td>18</td>
<td>HiNT/4</td>
</tr>
<tr>
<td>19</td>
<td>Hib/I</td>
</tr>
<tr>
<td>20</td>
<td>Hib/I</td>
</tr>
<tr>
<td>21</td>
<td>Hib/I</td>
</tr>
<tr>
<td>22</td>
<td>Hib/I</td>
</tr>
<tr>
<td>23</td>
<td>Hib/I</td>
</tr>
<tr>
<td>24</td>
<td>Hib/I</td>
</tr>
<tr>
<td>25</td>
<td>Hib/I</td>
</tr>
<tr>
<td>26</td>
<td>Hia/II</td>
</tr>
<tr>
<td>27</td>
<td>Hia/II</td>
</tr>
<tr>
<td>28</td>
<td>Hia/I</td>
</tr>
<tr>
<td>29</td>
<td>Hia/I</td>
</tr>
<tr>
<td>30</td>
<td>Hid/I</td>
</tr>
<tr>
<td>31</td>
<td>Hid/I</td>
</tr>
<tr>
<td>32</td>
<td>Hp</td>
</tr>
<tr>
<td>33</td>
<td>Hp</td>
</tr>
</tbody>
</table>

*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).
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 assessed 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), 16388 (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; and Robert S. Musson, Washington University School of Medicine, Saint Louis, MO, USA. Isolates of *H. parainfluenzae*, *Actinobacillus pleuropneumoniae*, *Enterobacter aerogenes* (ATCC 13048), *Edwardsiella tarda* (ATCC 15947), *Neisseria meningitidis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (ATCC 9027), *Shigella sonnei* (ATCC 29200), *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) IsoVitalex (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 immunoadsorbents. 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-
**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...
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 mAbs P2-18 was inhibited by concentrations of less than 0.2 μg ml⁻¹ of peptide P1 (residues 1–14). The binding of mAbs P2-17 and P2-19 was inhibited by 1.55 μg peptide 1 ml⁻¹ (residues 28–55) and 0.4 μg peptide 4 ml⁻¹ (residues 101–129), respectively. No 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 (Klebb 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


H. influenzae P2 porin protein 167


