Immune responses to linear epitopes on the PorB protein of Neisseria meningitidis in patients with systemic meningococcal disease

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Neisserial porins, the major protein constituents of the outer membrane capable of inducing antibody responses in humans, are considered to be meningococcal vaccine candidates, so it is important to map the relevant B-cell epitopes. For B-cell epitope analyses of the serotype 15 PorB protein in Neisseria meningitidis, paired sera from selected patients with systemic meningococcal disease (SMD) were screened with synthetic 12mer peptides spanning the PorB protein sequence, and/or its variable region 1 (VR1). A 'SMD-related' linear B-cell epitope was found within the VR1 region consisting of 14 residues ("svFHQNGQVTEvt8"). A 23mer soluble peptide (D63b2) that covered the VR1 region, including the complete "svFHQNGQVTEvt8" sequence, was recognized, whereas no detectable binding was observed to a 16mer peptide (D63a1) containing most of the essential sequence ("FHQNGQVTEvt8"). A low frequency of IgG responses specific for the PorB linear epitopes was found in convalescent-phase sera from 132 SMD patients studied, as judged from both immunoblotting studies (24/132; 18.2%) and reactivity with peptide D63b2 (18/132; 13.6%). Peptide D63b2 significantly inhibited IgG binding to the denatured PorB protein on immunoblots, suggesting that this B-cell epitope was one of the main linear epitopes on the PorB protein recognized by sera from some SMD patients.

Keywords: Neisseria meningitidis, systemic meningococcal disease, PorB protein, B-cell epitope, synthetic peptides

INTRODUCTION

Protective immunity against systemic meningococcal disease has been ascribed to antibody-dependent bactericidal killing, and to opsonophagocytic killing by neutrophils (Figueroa & Densen, 1991; Ross et al., 1987; Griffiss, 1982) and by tissue macrophages mainly involved in the clearance and destruction of bacteria (Benacerraf et al., 1989). At present, no fully efficient vaccine exists against serogroup B meningococci, at least partly because of the lack of immunogenicity of the group B capsular polysaccharide in humans. Convalescents from meningococcal disease have been reported to mount immune responses against other non-capsular surface antigens (Mandrell & Zollinger, 1989), which are therefore considered to be meningococcal vaccine candidates (Romero & Outshoorn, 1994). For example, patients with systemic meningococcal disease (SMD) have been shown to respond to epitopes on the meningococcal PorA protein [class I outer-membrane protein (OMP)] (Guttormsen et al., 1994b; Mandrell & Zollinger, 1989), suggesting that the PorA protein could be an important component of meningococcal vaccines (Orren et al., 1992; Mandrell & Zollinger, 1989). In addition, SMD induced significant antibody responses to class 5 OMPS (Poolman et al., 1983; Mandrell & Zollinger, 1989), Opc protein (Rosenqvist et al., 1993; Achtman et al., 1988), IgA1 protease (Brooks et al., 1992; Morelli et al., 1994), Lip antigen (also designated H.a) (Mølgaard & Wedege, 1989; Black et al., 1985), 70 kDa iron-regulated protein (FrpB) (Aoun et al., 1988), transferrin binding protein 2 (Ala'Aldeen et al., 1994), LPS and other outer-membrane components (Mølgaard & Wedege, 1989).
Neisserial porins have attracted significant attention as major protein constituents of the outer membrane (Blake & Gotschlich, 1986). Studies on the classes 2 and 3 OMPs (also designated PorB proteins) from Neisseria meningitidis identified two alleles of the porB gene (Hitchcock, 1989) sharing sequence homology with other neisserial (Wolff & Stern, 1991; Ward et al., 1992) and Escherichia coli (Gotschlich et al., 1987) porins. Molecular modelling of the topology of neisserial porins (van der Ley et al., 1991) predicted that the PorB molecule consisted of eight surface-exposed hydrophilic loops, with loops 1, 5, 6 and 7 bearing four variable regions designated VR1–VR4, respectively (Bash et al., 1995; Feavers et al., 1992; Zapata et al., 1992). Murine PorB-specific mAbs have been reported to recognize mainly surface-exposed conformational epitopes (Delvig et al., 1995; Zapata et al., 1992), as did PLA-specific mAbs (Mee et al., 1993), and some of them were bactericidal and protective in the infant rat infection model (Saukkonen et al., 1987; Moran et al., 1994). Taken together, the animal studies provided evidence that the PorB protein might also be involved in protection against SMD. Indeed, convalescent-phase sera obtained from patients with SMD showed a high rate of seroconversion to the major OMPs (PorB protein, PorA protein and class 5 OMP) (Poolman et al., 1983; Mandrell & Zollinger, 1989). Using the purified meningococcal porin proteins, Guttmersen et al. (1994b, 1993) found that SMD stimulated IgG antibodies specific for both the PorA and PorB proteins in most SMD patients, with the latter antibodies directed to surface-exposed epitopes. Furthermore, affinity-purified antibodies from patients with SMD were found to be bactericidal and opsonic (Guttmersen et al., 1994a), suggesting that the PorB protein might contain potentially protective B-cell epitopes.

Several approaches have been developed to identify epitopes on bacterial and viral antigens, including fragmentation of an antigen by cyanojen bromide treatment (Rothbard et al., 1984), screening large collections of small peptides displayed on filamentous bacteriophage (Meola et al., 1995) and solid-phase synthesis of peptides on polyethylene pins (pepscan method) (Geyser et al., 1984). Using the pepsan method, we have previously observed that the majority of human post-vaccination sera obtained after three doses of the Norwegian meningococcal group B outer-membrane vesicle (OMV) vaccine reacted with a relatively small linear B-cell epitope (199HQNGQVTEVTT586) located on loop 1 (VR1) of the PorB protein from strain 44/76 (Delvig et al., 1995). The aim of the present study was to map B-cell epitopes targeted by immune responses in surviving patients during the course of meningococcal disease.

**METHODS**

**Patients and serum samples.** The first group of SMD patients (group K) included 99 individuals (aged 12–21 years; 52 females and 47 males) infected in Norway during 1988–1991. Of these, 41 patients were participants in the Norwegian group B vaccination trials (Bjune et al., 1991) (14 vaccinees plus 27 placebo controls), and 58 patients were non-participants. Forty-eight group K patients were infected with serotype 15 meningococci, whilst strains of other serotypes were isolated from the remaining subjects (Holby et al., 1991). The 14 vaccine-failure patients were immunized at weeks 0 and 6 with 25 μg of meningococcal group B OMV vaccine. Late-convalescent sera collected at least 6 months after the onset of disease, as described by Garred et al. (1993). In addition, paired sera from 30 patients, obtained at admission (acute-phase sera), 1–6 weeks later (convalescent-phase sera) and, for some patients, 1–2 months later, were available.

The second group of patients (group U) consisted of 33 individuals (aged 5–50 years; 20 males and 13 females), who were consecutively admitted to Ullevål University Hospital, Oslo, between 1985 and 1987 (Brandtzæg et al., 1989). Eighteen patients were infected with serotype 15 strains, and the strains isolated from the remaining subjects belonged to other serotypes. Sera were obtained at admission (acute-phase sera), 3–7 weeks later (convalescent-phase sera) and, for some patients, also about 1 year after the SMD episode.

Approval for this study was obtained from the Regional Ethical Committee for Medical Research in Norway. Informed consent was obtained from all the participants or from their parents.

**Murine mAbs.** 3-1-P15 (serotype 15) and 3-1-P16 (serotype 16) were provided by Dr W. D. Zollinger, AE3 (class 4) was donated by Dr C. T. Sacchi and 279-5c (Opc) was produced at the National Institute of Public Health, Norway. All mAbs were available as ascites fluids.

**Immunoblotting.** All sera from patients were tested on immunoblots against whole-cell preparations (group U) or OMVs (group K) from strain 44/76 as described previously (Wedeg et al., 1988, 1991). After SDS-PAGE and electrotransfer, blots were incubated with human sera (diluted 1:200) in the absence of Empigen BB (Albright & Wilson), or murine mAbs in the presence/absence of the detergent to enhance reautorization of the antigens (Wedeg et al., 1988). The intensity of IgG binding to the PorB protein was visually scored from 0 to 4, where scores of 0–1, 2–2.5 and 3–4 represented weak, medium and strong binding, respectively.

**Solid-phase peptide synthesis.** Multiple N-terminally acetylated peptides were synthesized on pins to span the sequence of the serotype 15 PorB protein from strain 44/76 by 12mers overlapping by 6 amino acids (Fig. 1) using a commercially
available Epitope Scanning kit V2.0 (Cambridge Research Biochemicals). Other pin-bound peptides covering the VR1 region of the serotype 15 PorB protein by 12mers with an 11 amino acid overlap were synthesized using Multipin peptide synthesis (non-cleavable block, Chiron Mimotopes Peptide Systems). Peptide synthesis was performed in duplicate.

The synthetic peptides \(^{19}\text{FHQNGQVTEVTATTG}^{24}\) (D63a1, 16mer) and \(^{19}\text{VE}^{22}\text{RSR}^{24}\) \(^{27}\text{FHQNGQVTEVTATTG}^{34}\) (D63b2, 23mer) covering different portions of loop 1 of the serotype 15 PorB protein (Fig. 1) were synthesized as described previously (Delvig et al., 1995). Briefly, synthesis was performed at 0.25 mmol scale using FastMoc technology with an automated peptide synthesizer (model 431A, Applied Biosystems) on the Rink resin (TentaGel S RAM, Rapp Polymere) providing a C-terminal amide cap. After synthesis, peptides were purified by reversed-phase HPLC (Pep-S, C2/C18, 100 Å pore size, 22.5 mm × 25 cm, Pharmacia) using a 0–40% gradient of acetonitrile/0.1% trifluoroacetic acid. Lyophilized peptides were stored at \(-20^\circ\text{C}\).

**Epitope mapping.** After incubation with blocking buffer [0.01 M PBS (0.01 M sodium phosphate buffer \(\text{pH 7.2},\) containing 0.15 M NaCl), 2% (w/v) BSA, 0.1% Tween 20, 0.05% \(\text{NaN}_3\)] for 90 min at room temperature, the pin-bound synthetic peptides were screened in ELISA with sera (diluted 1:500 in blocking buffer) obtained from SMD patients. After incubation for 18 h at \(4^\circ\text{C}\), the pins were reacted with horseradish-peroxidase-conjugated rabbit anti-human Ig (\(\gamma\)-chains) (1:500) (Dakopatts) for 90 min, and the reactions were read at \(450\) nm. Each serum sample was tested on two occasions against two separate pin sets.

**Synthetic peptide ELISA.** Peptide D63b2 (5 μg ml\(^{-1}\)) was coated on MaxiSorp Nunc-Immuno plate F96 (Nunc) in 0.05 M carbonate buffer, \(\text{pH 9.6}\) for 16 h at 35 °C. The plates were then blocked with PBS containing 2% BSA and 0.05% Tween 20 for 90 min at room temperature. Tests with human sera (diluted 1:50) were performed in triplicate. The reaction was developed with alkaline phosphatase conjugate (diluted 1:500) of swine anti-human IgG (\(\gamma\)-chain specific) (Orion Diagnostica). Values showing more than threefold increases in \(A_{405}\) above the mean background level were considered significant.

**Inhibition experiments.** Increasing, 10-fold amounts of peptide D63b2 (from 0.004 to 4 nmol) were added to sera from selected patients and incubated for 16 h at \(4^\circ\text{C}\). Inhibition by the soluble peptide was observed on immunoblots as a decrease in the intensity of the immunoreactive PorB protein band.

**Statistics.** Statistical analyses were performed using CSS: Statistics for Windows (Statsoft) and/or Statgraphics 3.01 (STSC) and an IBM-compatible PC.

**RESULTS**

**PorB- and peptide D63b2-specific IgG responses in SMD patients**

Convalescent-phase sera from 99 group K patients and from 33 group U patients were screened for reactivity against linear epitopes on the denatured PorB protein on immunoblots and to peptide D63b2 in ELISA. Late-convalescent sera from 21/99 (21.2%) of group K patients bound to the PorB protein on immunoblots with medium/high intensity, of which 15/21 (71.4%) patients developed significant IgG responses to peptide D63b2 (7 vaccines and 8 non-vaccines). Of the 15 responders, 10 were infected with the serotype 15 strains, one with serotype 4, and the remaining four patients were culture-negative cases with clinically confirmed diagnosis of SMD (Høiby et al., 1991). Except in three cases, convalescent-phase sera from the unvaccinated group U patients reacted with neither the PorB protein nor peptide D63b2. Sera from the three exceptional subjects (serotype 15 strains; two patients with septicaemia, and one with meningococcaemia), obtained 3–6 weeks or 1 year after the disease, reacted weakly both with the denatured serotype 15 PorB protein on immunoblots and with peptide D63b2 in peptide ELISA (data not shown).

Altogether, a low frequency of IgG specific for the linear epitopes was found in sera from all 132 SMD patients studied: 24/132 patients responded to the denatured PorB protein on immunoblots (mean 18.2%; 95% confidence interval 12.0–25.8%), and 18/132 patients (mean 13.6%; 95% confidence interval 8.3–20.7%) responded to peptide D63b2 in ELISA.

**Mapping of linear B-cell epitopes on the serotype 15 PorB protein**

Epitope mapping experiments were performed with paired sera from four SMD patients (two patients with meningitis – K-131 and K-199, and two patients with meningococcal septicaemia – K-15 and U-17) infected with virulent ET-5 complex strains (B:15:P1.7,16), plus one patient (K-54) infected with B:15:P1.12,13a strain. The current selection was based on the pronounced reactivity of the sera with the denatured PorB protein on immunoblots (data not shown), suggesting the presence of detectable levels of antibodies reactive with linear epitopes. In addition, the protocol used allowed the study of epitopes recognized under different clinical forms of SMD.

Convalescent sera, unlike the acute-phase sera, obtained from both patients with meningitis (K-131 from the vaccinees group, and K-199 from the placebo control group) mainly reacted with a single peptide \(^{19}\text{FHQNGQVTEVT}^{28}\) (peptide no. 4) (Fig. 2b, c), whilst no increased reactivity to other peptides was found. Infection induced a strong response against this 12mer peptide in the vaccinated patient K-131 14 d after admission but no such reactivity was detected in the paired late-convalescent serum obtained 19 months later (data not shown). In the unvaccinated patient K-199, peptide 4 reactivity was detected in the late-convalescent serum obtained 9 months after the SMD episode, but not in the acute-phase serum taken 9 d after admission (data not shown).

Early convalescent-phase sera (15–21 d) from two patients with meningococcal septicaemia (K-15 and U-17) showed two- to fivefold increases in reactivity against the majority of peptides (Fig. 2d, e). A significant response against peptide \(^{19}\text{FHQNGQVTEVT}^{28}\) was detected only in the serum from the previously vaccinated patient K-15. This pattern of polyclonal reactivity is likely to reflect the immunomodulating activity of meningococcal LPS.
Fig. 2. ELISA reactivity of paired sera from five patients with systemic meningococcal disease with synthetic 12mer peptides on pins spanning the PorB molecule. Data are presented as difference in reactivities between immune serum versus pre-immune serum obtained at admission. (a) Late-convalescent serum from unvaccinated patient K-54, 26 months after disease; (b) acute-phase serum from vaccinated patient K-131 with meningitis, 14 d after admission; (c) late-convalescent serum from the unvaccinated patient K-199 with meningitis, 9 months after disease; (d) acute-phase serum from vaccinated patient K-15 with septicaemia, 15 d after admission; (e) acute-phase serum from unvaccinated patient U-17 with septicaemia, 21 d after admission. Results are the means of two separate experiments obtained on two different sets of pins. The filled bars represent the means, and the error bars show SEM.

(Melancon Kaplan & Murgita, 1987) and/or elevated antigenic load in septic patients (Brandtzæg et al., 1989).

Late-convalescent serum (26 months) from another unvaccinated patient (K-54) bound to peptide no. 15 (\textit{\textsuperscript{85}FGKLVRGLNSV\textsuperscript{98}}), in addition to peptide no. 4, implying the presence of other linear B-cell epitopes on the PorB protein (Fig. 2a). According to the topology model for neisserial porins (van der Ley et al., 1991), this additional B-cell epitope was located on the transmembrane amphipathic $\beta$-strand between loops 2 and 3 plus a short loop on the periplasmic face, and probably was not exposed on the cell surface. To study other putative B-cell epitopes in more detail, late-convalescent sera from an additional six group K patients, which reacted with medium intensity with the denatured PorB protein on immunoblots, were subjected to epitope mapping, but no significant responses with single peptides were detected (data not shown).

**Further definition of the B-cell epitope within the VR1 region**

To define the linear structure of this B-cell epitope in more detail, 12mer peptides, having 11 amino acids in common, were synthesized on pins to span the VR1 region. Paired sera from only three of the above-mentioned patients (K-199, K-131 and K-54) were tested. All three showed a similar pattern of reactivity (Fig. 3a–c, respectively). These data suggested that the B-cell epitope
within the VR1 region consisted of the minimal sequence
\[
^{19}\text{FHQNGQVTE}^{27} + ^{17}\text{sv}^{18}\text{t} + ^{8}\text{vt}^{30}
\]
residues probably being essential for binding. To test this hy-
pothesis, purified synthetic peptides corresponding to di-
ferent portions of the loop 1 (VR1) region were screen-
ed with convalescent-phase sera, and essentially no re-
activity was detected with peptide D63a1 that covered only part of the epitope \(^{19}\text{FHQNGQVTE}^{30}\) (data not shown). In contrast, peptide D63b2 containing the complete B-cell epitope was efficiently recognized by these sera; thus the complete epitope comprises the 14 amino acids \(^{17}\text{svFHQNGQVTE}^{30}\) (Fig. 1). Interest-
ningly, the pin bearing peptide no. 4 with the \(^{19}\text{FHQNGQVTE}^{30}\) sequence was strongly reactive with human sera. This apparent discrepancy between pin-
bound and free peptides might reflect some limitations of
the pepscan method since high peptide concentrations on
the solid-phase can facilitate bivalent reactions and cross-
reactivity (Van Regenmortel, 1992).

Inhibition efficiency of peptide D63b2

To test if the ‘SMD-related’ B-cell-epitope-specific IgG
response was immunodominant, peptide D63b2 was
added to sera from patients K-199, K-131 and K-54, and
data on patient K-54 is shown in Fig. 4. The presence of
increasing amounts of peptide D63b2 (0.004–4 nmol) re-
sulted in a gradual partial reduction in IgG binding to the
denatured PorB protein on immunoblots, whereas reac-
tivities against other OMPs remained intact (Fig. 4, tracks
3–6). Absorption of the sera with epidemic strain
44/76 resulted in complete inhibition of binding to the
PorB protein (data not shown). These data are in
agreement with observations by Gutormsen et al. (1993)
that the PorB-specific antibodies in SMD patients are
mainly directed to surface-exposed epitopes.

**DISCUSSION**

Recent studies have shed light on the target antigens
recognized by the immune system during infection by
pathogenic *Neisseria* species. In this respect, neisserial
porins are receiving increasing attention as abundant
components of the outer membrane (Blake & Gotschlich,
1986) capable of activating T-lymphocytes through the
HLA class II-restricted pathway (Wiertz et al., 1991,
1992), as well as stimulating B-lymphocyte proliferation
and differentiation into antibody-secreting cells (Gut-
Gutormsen et al., 1993, 1994b; Wertzler et al., 1988).
In addition, neisserial porins appear to be potent immuno-
modulators, and are therefore used as protein carriers in
different experimental vaccines (Lowell et al., 1988;
Livingston et al., 1993; Siber, 1994).

Here, we demonstrate that some SMD patients develop
IgG antibody responses to peptide D63b2 bearing a linear
B-cell epitope \(^{14}\text{svFHQNGQVTE}^{30}\) mapped to the
N-terminus (VR1) of the serotype 15 PorB protein. This
antigenic PorB variant has been shown to be expressed
by ET-5 complex strains which have caused epidemics of
meningococcal disease in Norway and other countries
since the mid-1970s (Caugant et al., 1989). The N-termi-
lar parts of the FIA and the PIB porins in *N. meningitidis*
have also been reported to be immunogenic and capable of
inducing bactericidal antibodies (Elkins et al., 1992). As
mentioned above, only a small proportion of 132 SMD
patients studied here responded to the denatured PorB
protein on immunoblots (24/132; 18.2\%) or with peptide
D63b2 (18/132; 13.6\%), and these responders were
infected mainly with serotype 15 meningococcal strains.
By contrast, 74\% of post-vaccination sera obtained from
vaccinees after three doses of the Norwegian group B
OMV vaccine bound to the denatured PorB protein and
peptide D63b2 (Delvig et al., 1995), suggesting that,
unlike SMD, vaccination gave rise to a higher frequency
of antibodies directed to the linear B-cell epitopes. The
apparent differences between human responses to the
linear B-cell epitope observed after vaccination and SMD
could have resulted from the immunization protocol
involving several administrations of the OMV vaccine
during the Norwegian vaccination trial (Bjune et al.,
1991), or could have reflected more efficient mechanisms
of antigen processing and presentation for the OMV
vaccine compared to that for bacteria. On the other hand,
SMD has been shown to give rise to high levels of PorB-
specific antibodies (up to 100 \(\mu\)g ml\(^{-1}\) and greater) in sera
from most SMD patients, as reported in quantitative
kinetic studies with the purified PorB protein by
Gutormsen et al. (1993); these antibodies were probably
targeted to conformational epitopes and were not detected
in the present study.

Under the experimental conditions used, SMD was found
to induce anamnestic IgG responses against peptide
D63b2 in early convalescent-phase sera from 7/14 group
K patients, previously immunized with the Norwegian
OMV vaccine, whereas only late-convalescent sera from
unvaccinated group K patients contained detectable levels
of peptide-specific IgG antibodies. The delayed appearance of the D63b2-specific antibodies in the latter sera described here is in agreement with earlier observations that the PorB-specific IgG can only be detected in late-convalescent phase sera from patients on immunoblots (Wedige et al., 1991). This phenomenon may to some extent be attributed to the carriage of serotype 15 strains, which is of relatively low rate in the Norwegian population (Caugant et al., 1994). Alternatively, the delayed D63b2-specific responses could have originated from the impaired T-helper-cell function for B-lymphocytes described in patients with bacterial meningitis, namely up-regulation of both γδ-T-cells and αβ-T-cells with suppressor–inducer phenotype (CD4+CD45R+), and reciprocal down-regulation of helper–inducer-T-cells (CD4+CD25+) (Raziuddin et al., 1991, 1994). Interestingly, the loop 1 region in the serotype 15 PorB protein covered by peptide D63b2 shares 16 out of 23 amino acids (69.6% homology) with its serotype 4 counterpart (Zapata et al., 1992; Ward et al., 1992). This homology may explain the observed cross-reactivity for peptide D63b2 of a convalescent-phase serum from the patient infected with a serotype 4 strain (data not shown), although we cannot rule out that this patient became a carrier of a serotype 15 strain.

To be effective, a peptide-based vaccine has to contain different appropriate epitopes in order to overcome the variation in immune responses resulting from histocompatibility leucocyte antigen (HLA) heterogeneity, and to induce T- and B-cell activation and differentiation (Ada, 1992). The HLA heterogeneity could therefore account for the relatively low frequency of the peptide-D63b2-specific responses in SMD patients infected with serotype 15 strains that was observed here. Although an HLA-DR1 binding motif has been shown to overlap with the B-cell epitope (Delvig et al., 1995), there was no significant association between expression of a particular HLA-DR haplotype in K patients and antibody responses to peptide D63b2 (unpublished data).

So far, information about human B- and T-cell epitopes on meningococcal antigens is limited to capsular polysaccharide (Kabat et al., 1988), and some non-capsular antigens (Wiertz et al., 1992; Delvig et al., 1994; de Cossio et al., 1994; Morelli et al., 1994). In this context, the B-cell epitope described in this report will add to the limited collection of epitopes recognized by human immune sera.

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REFERENCES


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