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

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**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., 1959). 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)] (Gutormsen *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; Achtmann *et al.*, 1988), IgA1 protease (Brooks *et al.*, 1992; Morelli *et al.*, 1994), Lip antigen (also designated H.8) (Måland & Wedege, 1989; Black *et al.*, 1985), 70 kDa iron-regulated protein (FrpB) (Aoun *et al.*, 1988), transferrin binding protein 2 (Ala’Alden *et al.*, 1994), LPS and other outer-membrane components (Måland & Wedege, 1989).

**Keywords:** *Neisseria meningitidis*, systemic meningococcal disease, PorB protein, B-cell epitope, synthetic peptides
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 (Wolf & 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 transmembrane segments (Delvig et al., 1995), which spanned 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 PIA-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, Guttormsen 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 (Guttormsen 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 cyanogen 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 pepscan 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 (19NHQNGYQVTEVVTT86) 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 were 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), 1–7 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.** 3-1-15P (serotype 15) and 3-1-16P6 (serotype 16) were provided by Dr W. D. Zollinger; AE3 (class 4) was from 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 (Wedge 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 renaturation of the antigens (Wedge 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 represent 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 16FHQNGQVTEVTTAAG12 (D63a1, 16mer) and 16VETRSVFTQHONQVTEVTTAAG14 (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 x 25 cm, Pharmacia) using a 0–40% gradient of acetonitrile/0.1% trifluoroacetic acid. Lyophilized peptides were stored at −20 °C.

Epitope mapping. After incubation with blocking buffer [0.01 M PBS (0.01 M sodium phosphate buffer pH 7.2, containing 0.15 M NaCl), 2% (w/v) BSA, 0.1% Tween 20, 0.05% NaN3] 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 °C, the pins were reacted with horseradish-peroxidase-conjugated rabbit anti-human Ig (γ-chains) (1:500) (Dakopatts) for 90 min, and the reactions were read at 405 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, 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 (γ-chain specific) (Orion Diagnostica). Values showing more than threefold increases in A405 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 °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 Stargraphics 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 vaccinees and 8 non-vaccinees). 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 16FHQNGQVTEVT11 (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 16FHQNGQVTEVT11 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.

Late-convalescent serum (26 months) from another unvaccinated patient (K-54) bound to peptide no. 15 (SFGKLRVGR1NSV), 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 β-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...
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**Fig. 4.** Reactivity of paired sera from an unvaccinated patient (K-54) with systemic meningococcal disease against OMVs from strain 44/76 on immunoblots in the absence of detergent. Tracks: 1, strip developed with murine mAbs specific for PorA, class 4 OMP and Opc protein; 2, acute-phase serum; 3–7, serum taken 26 months later containing 0.004, 0.04, 0.4, 4 nmol or no peptide D63b2, respectively (inhibition experiment); 8, strip with murine serotype-15-specific mAb incubated in the presence of 0.25% Empigen BB.

within the VR1 region consisted of the minimal sequence

\[ ^{19} \text{FHQNQVTE}^{27} \]

plus \(^{17}\text{sv}^{18}\) and/or \(^{18}\text{vt}^{19}\) residues probably being essential for binding. To test this hypothesis, purified synthetic peptides corresponding to different portions of the loop 1 (VR1) region were screened with convalescent-phase sera, and essentially no reactivity was detected with peptide D63a1 that covered only part of the epitope \((^{19} \text{FHQNQVTE}^{27})\) (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 \(^{17}\text{svFHQNQVTE}^{19}\) (Fig. 1). Interestingly, the pin bearing peptide no. 4 with the \(^{19} \text{FHQNQVTE}^{19}\) 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) resulted in a gradual partial reduction in IgG binding to the denatured PorB protein on immunoblots, whereas reactivities 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 Guttmersen *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 (Guttmersen *et al.*, 1993, 1994b; Wetzler *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 (\(^{17}\text{svFHQNQVTE}^{19}\)) 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-terminal parts of the PIA and the PIB porins in *N. gonorrhoeae* have also been reported to be immunogenic and capable of inducing bacterial 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 μg ml\(^{-1}\) and greater) in sera from most SMD patients, as reported in quantitative kinetic studies with the purified PorB protein by Guttmersen *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 (Wedega 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 upregulation of both γ/δ-T-cells and α/β-T-cells with suppressor–inducer phenotype (CD4+CD45R+), and reciprocal down-regulation of helper–inducer-T-cells (CD4+CD29+) (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., 1992; 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.

ACKNOWLEDGEMENTS

We are grateful to Dr M. Achtman for stimulating discussions and for help in synthesizing peptides. We thank Dr J. H. Robinson for support and for critically reading the manuscript. We thank Dr F. Vardal for the HLA-typing of the SMD K patients. We also wish to thank Dr R. Dalsg and Dr F. Oftring for discussions, and K. Bolstad for excellent technical assistance. We are also grateful to Drs W. D. Zollinger, C. T. Sacchi and J. Kolberg for providing murine mAbs. This research project received financial support from the WHO Global Programme for Vaccines (GPV): GPV/V23/181/52.

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Received 16 October 1995; revised 5 March 1996; accepted 24 April 1996.