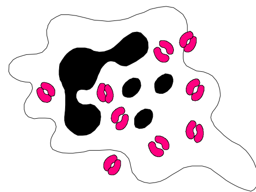
Structure  of Neisserial Proteins

Characterization of the relative conformational stability of *Neisseria meningitidis* porins

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Porins are water filled channels which play important roles as molecular sieves in the outer membrane of gram-negative bacteria. The major porins from *Neisseria meningitidis* are the (PorB) class 2 and (PorB) class 3 proteins, which are mutually exclusive among meningococcal strains (1). Both proteins have been overexpressed, isolated, and refolded from *E. coli* inclusion bodies (2), and characterized by a combination of functional and physicochemical techniques. Our investigation has focused primarily on the trimeric assembly and conformational stability of these neisserial porins in the native state. The resultant data have been interpreted on the basis of x-ray crystallographic structures determined for other bacterial porins (3,4) in conjunction with topology models proposed for neisserial porins (5,6).

Our studies indicate that recombinant (PorB) class 2 and (PorB) class 3 proteins refold and assemble as trimers, thereby retaining the overall structural features of their native counterparts. Preliminary observations regarding the SDS-resistant properties of these trimeric structures prompted us to investigate the relative conformational stabilities of both proteins and correlate specific differences with sequence and topology variations described in the literature (5,6). Comparative studies on the chemical and thermal denaturation of neisserial porins monitored by UV, circular dichroism, and fluorescence spectroscopy facilitate elucidation of specific domain interactions which govern the stability of these proteins. Experiments have been performed on porins isolated from strain M986 [(PorB) class 2], strain M981 [(PorB) class 3], a mutant strain 44/76 D1/D4 [(PorB) class 3], and their respective recombinant counterparts. The conformational properties of a native (PorA) class 1 protein obtained from mutant strain 44/76 D3/D4 have also been investigated.

Spectrophotometric studies conducted in the presence of SDS or Gdn•HCl have revealed increases in both tyrosine and tryptophan exposure accompanying the detergent- and chemical-induced unfolding processes, respectively. Significantly, (PorB) class 3 protein exhibits a transition over the range of 0.25 - 0.5 % SDS, whereas only minor changes are observed for (PorB) class 2 protein at comparable SDS concentrations. Unfolding transitions involving the concomitant disruption of secondary and tertiary structure occur at midpoints of 2.5 M and 4.75 M Gdn•HCl for (PorB) class 3 and (PorB) class 2, respectively. These results clearly indicate a significant correlation between the SDS-resistant properties of (PorB) class 2 protein and its enhanced stability with respect to chemical denaturation (7). Reversible thermal unfolding transitions are observed for (PorB) class 2 and (PorB) class 3 proteins, although the relative stabilities and pathways are quite distinct. Specifically, (PorB) class 3 exhibits a biphasic melting profile with transitions at 62.5 °C and 90.0 °C, while (PorB) class 2 unfolds via a single cooperative transition at a midpoint of 85.5 °C. The observed differences in thermal stability may be correlated with the relative susceptibility of both porins to chemical denaturants.

Envisioning the possibility that heterotrimers of class 1/2 or class 1/3 may exist *in vivo,* we recently extended our studies on the conformational stability of meningococcal porins to include (PorA) class 1 protein. Analysis of wild type strains expressing both PorA and PorB genes indicate that those expressing class 1/2 (strain M986) contain predominantly SDS-resistant species, whereas those expressing class 1/3 (strain 44/76) are comprised exclusively of SDS-sensitive species. Consequently, it is conceivable that the presence of either class 2 or class 3 within heterotrimers in wild type strains is the limiting factor that renders these oligomeric structures inherently more (class 1/2) or less (class 1/3) stable.

In summary, functional and physicochemical studies of (PorA) class1, (PorB) class 2, and (PorB) class 3 proteins provide significant insight into *Neisseria meningitidis* porins, particularly structure-stability relationships within the porin superfamily.

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Studies on the PorA protein of *Neisseria meningitidis* by X-ray crystallography and NMR.

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The antigenic variability of the PorA protein of the meningococcus has attracted study as a result of the use of this protein as a target for serosubtyping and in vaccines (1,2,3,4). Most of the antigenic variability of this protein resides in two loops (loop I or VR1 and loop IV or VR2) protruding from its proposed β-barrel porin structure (5,6). The variability of these loops is not continuous and there are a number of distinct families of peptide sequence in both VR1 and VR2. In addition, minor variation also occurs in these families (7). In order to investigate the structural consequences of the antigenic variability of PorA, we have instigated a systematic analysis of the three-dimensional structures of a range of linear peptide antigens in complex with antibodies against different serosubtype variants. Such an investigation will reveal whether different serosubtype variants are related in structure, despite the absence of significant sequence homology between many variants.

We now have crystals of two Fab fragments in complex with linear peptide antigens derived from VR1 and VR2 sequence variants. A complete data set of reflections has been collected from crystals of a P1.7 serosubtype variant in complex with Fab fragment to a resolution of 2.9 Angstroms (R_{merge}= 3.1% for all reflections from 30 to 2.9 Angstroms resolution; 91% complete). Data has also been collected from crystals of a second Fab fragment in complex with a P1.10 linear peptide antigen, although diffraction is not yet to a sufficiently high resolution to permit a determination of the structure. We are currently investigating solutions of both these structures by molecular replacement methods. Both crystal complexes contain a single domain from Streptococcal protein G, which binds exclusively to the C_H1 domain within Fab: inclusion of protein G was required to obtain satisfactory crystals, and may assist in the solution of the structure of the complex by molecular replacement (8).

Nuclear magnetic resonance offers an alternative approach to the structural analysis of antibody-antigen complexes. We have synthesised a linear peptide antigen corresponding to the P1.7 serosubtype, incorporating ^{15}N into all alanine and glycine residues and studied the complex of this peptide with Fab fragment from a mouse monoclonal against serosubtype P1.7. Heteronuclear single and multiple quantum coherence methods were employed to distinguish proton resonances originating from protons bound to ^{15}N nuclei, thus removing interfering proton signals from the Fab fragment. Small but significant changes in amide proton chemical shift were found on binding of the peptide to the Fab fragment. A two-dimensional ^{15}N-filtered NOESY conducted on the Fab-peptide complex demonstrated that several NOEs could be detected from the peptide amide protons to adjacent Cα protons. In addition, three NOEs were also visible to methyl protons, presumed to be from the three alanine residues in the peptide. Experiments are currently in progress to assign each NOE to a specific residue, and thus determine the conformation of the P1.7 peptide in its bound state.

By collating the results derived from NMR and protein crystallography experiments we hope to build up a detailed description of the molecular basis for immune recognition of meningococcal antigens. It should be readily apparent from the results why particular monoclonal typing antibodies cross-react with some serosubtype variants, but not others. This approach will also provide more general information about the three-dimensional structural relationships between different serosubtype variants.

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High resolution model of the *Neisseria* pilus fiber

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The atomic resolution structure of *Neisseria gonorrhoeae* pilin (1) was used along with published biochemical, genetic and biophysical data to model the pilus fiber. Five pilin molecules form each 4.1 nm repeat of the helical fiber. The long conserved N-terminal alpha helices pack against each other on the interior of the fiber, wrapped by continuous beta sheet. The hypervariable C-terminal region is exposed on the surface.

The fiber model facilitates rational design of further experiments to probe the function and structure of the fiber. For example. new anti- peptide antibodies can be raised against small structural motifs such as turns, and used to verify the exposed or buried nature of these regions in the fiber. Furthermore, peptides could be chosen which are conserved but partially accessible in the assembled fiber, with the goal of eliciting an antibody response that would play a role in immunity. Mutations have been designed to investigate the structural importance of the hypervariable region, and to add reactive sites for metal labeling to use in electron microscopy.

This model also allows us to interpret previous experimental results. in the context of the pilus. For example, epitopes encompassing residues 94-108 and 37-56 are known from immunoelectron microscopy experiments to be exposed on the ends of fibers (2) and are indeed located at the ends of the proposed fiber model, buried by longitudinal contacts within the fiber. The covalently linked saccharide (1,3) is exposed on the fiber surface, where it could affect antigenic variation, adhesivity or target cell specificity.

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Opsonophagocytosis responses to meningococcal antigens adsorbed to beads

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In order to identify serogroup B meningococcal structures that mediate antimeningococcal opsonophagocytosis, *in vitro* flow cytometry (FCM) and chemiluminescence (CL) assays evaluating functional antigen-specific opsonophagocytic responses were developed. Serogroup B meningococcal outer membrane vesicles (OMV) (1) were adsorbed to fluorescent latex beads (OMV-beads) of similar size to that of meningococci (1µm in diameter) and opsonized with acute phase and convalescence sera from patients with serogroup B meningococcal disease (MCD). Phagocytosis of these "meningococci-mimicking" beads with selected bacterial components and subsequently OMV antigen-specific opsonins exposed on the surfaces, depended on both the amount of adsorbed antigen and on the concentration of opsonizing sera. FCM cytograms and histograms gated for phagocytosing cells confirmed and visualized the antigen-specific nature of the opsonophagocytosis responses.

OMV-beads opsonized with 5% serum from a patient recovering from MCD, caused 97% of the donor monocyte- and polymorphonuclear leukocyte population to phagocytose an average of 15.8 beads per cell with a CL response of 46,550 mVs, whereas opsonized control beads coated with bovine serum albumin were phagocytosed by 19% of the cells with 1.1 beads per cell and a CL response of 53 mVs (initial bead:phagocyte ratio of 20:1). Opsonization with MCD convalescence serum induced higher phagocytosis than acute phase serum from the same patient, indicating that increased amounts of anti-OMV opsonins are induced during infection. When a pneumococcal antigen preparation was adsorbed to beads and opsonized with the same acute and convalescence MCD patient sera, the difference in opsonophagocytic effects between the sera was abolished. Heat-inactivation of sera and replacement of MCD patient sera with sera from non-infected patients with hypogammaglobulinemia reduced the phagocytosis.

Opsonized OMV-beads elicited phagocyte responses of similar magnitude to those of opsonized, fluorescein isothiocyanate-labelled whole meningococci. Serial confocal laser scanning microscopy sections (z-series) were generated through the incubated leukocytes and merged into one image to visualize intraphagocyte location of fluorescent particles.

We conclude that epitopes on the meningococcal outer membrane seem to be recognized by patient anti-meningococcal opsonins in these functional phagocytosis assays, which provide a basis for evaluation of various purified meningococcal components as mediators of human opsonophagocytic responses and hence future vaccine constituents.

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Noncapsular Vaccines, Poster

Serum bactericidal activity elicited by two outer membrane protein serogroup B meningococcal vaccines among infants, pre-school children, and adults in Santiago, Chile

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Background: Two serogroup B vaccines have been developed by Finlay Institute (FI) in Cuba and the National Institute of Public Health (NIPH) in Norway (1,2). Each vaccine is based on specific isolates from epidemics in Cuba (CU385, B:4:P1.15) and Norway (44/76-SL, B:15:P1.7,16). Vaccine efficacy (VE) has been demonstrated among children and young adults 11 to 16 years of age in randomized, double-blind controlled trials (RDCT) using 2-dose regimens of the FI-produced vaccine in Cuba, and the NIPH-produced vaccine in Norway (estimated VE, 83% and 57%, respectively). However, VE has not been demonstrated in children <5 years of age (1-3). In 1993, Santiago had a clonal, serogroup B epidemic; 60% of cases occurred among children <5 years of age (4). Because the Chilean epidemic strain (#539, B:15:P1.3) was different than the strains used to produce the FI and NIPH vaccines, an immunogenicity study was conducted as a potential correlate for VE.

Methods: Standardized SBA elicited by the FI-produced and NIPH-produced vaccines, and a non-meningococcal control vaccine was determined in a RDCT among infants <1 year of age (N=187), pre-school children 2-4 years of age (N=183), and adults 17-30 years of age (N=173). Participants received 3 doses of vaccine, 2 months apart; blood samples were obtained prior to dose 1 and dose 3, and 4-6 weeks following dose 3. Response was defined as a $\geq$4-fold rise in titer compared with prevaccination. All sera were tested against the Chilean outbreak strain. Based on these results, additional assays were performed; adult sera were tested against the Cuban vaccine type strain and infant sera were tested against the Norwegian vaccine type strain.

Results: Local and systemic reactogenicity profiles for both the FI-produced and the NIPH-produced vaccines were satisfactory and consistent with previous studies (1,2).
Among infants, there was no significant difference in the proportion of responders against the heterologous Chilean epidemic strain between those vaccinated with the control vaccine (bleed 2, 1.8%; bleed 3, 5.6%) and either the FI-produced (bleed 2, 1.9%; bleed 3, 9.6%) or the NIPH-produced vaccine (bleed 2, 5.8%; bleed 3, 11.5%). Among pre-school aged children, recipients of the FI-produced vaccine (bleed 2, 14.3%; bleed 3, 30.6%), and recipients of the NIPH-produced vaccine (bleed 2, 22.2%; bleed 3, 34.5%) were more likely than control vaccine recipients (bleed 2, 1.8%; bleed 3, 5.3%) to be responders ($p<0.05$ vs. control). Similarly, adult recipients of the FI-produced vaccine (bleed 2, 26.9%; bleed 3, 36.5%), and adult recipients of the NIPH-produced vaccine (bleed 2, 49.9%; bleed 3, 60.0%) were more likely than adult control vaccine recipients (bleed 2, 1.9%; bleed 3, 3.8%) to be responders ($p<0.05$ vs. control). Among adults, the proportion of responders was significantly higher among recipients of the NIPH-produced vaccine when compared to the FI-produced vaccine at bleed 2 ($p<0.04$) and bleed 3

(p<0.03). SBA geometric mean titers revealed similar response patterns across all three age groups.

Among adult sera tested against the Cuban type strain, there was no significant difference in the proportion of responders between those vaccinated with the FI-produced vaccine (bleed 2, 30.4%; bleed 3, 65.2%) and the NIPH-produced vaccine (bleed 2, 24.0%; bleed 3, 48.0%). Against the Norwegian type strain, infant recipients of the NIPH-produced vaccine (bleed 2, 100%; bleed 3, 100%) were significantly more likely than control vaccine recipients (bleed 2, 2%; bleed 3, 2%) to be responders (p<0.001).

Conclusions: SBA results for infants and pre-school aged children suggest that the FI-produced and NIPH-produced vaccines may not confer protection against the Chilean outbreak strain. Among pre-school aged children and adults, a third dose of either the FI-produced or NIPH-produced vaccine was associated with a higher proportion of responders than two doses. However, the proportion of responders among pre-school aged children after two or three doses of either vaccine was relatively low. Among adults, SBA results against the Chilean strain were consistent with VE data for the NIPH-produced vaccine. Among adult recipients of the FI-produced vaccine, the proportion of SBA responders against the Cuban type strain was less than expected based on VE data for the FI-produced vaccine. Although SBA against the Chilean outbreak strain among infants vaccinated with the NIPH-produced vaccine was disappointingly low, the 100% SBA response rate against the Norwegian type strain among NIPH-vaccinated infants was surprising, suggesting that the NIPH-vaccine may confer protection against the homologous strain in this age group. Although SBA may not be an ideal serologic correlate for VE for serogroup B meningococcus, a high proportion of SBA responders to the circulating strain is likely to predict VE in the effected population.

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