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Crystallization and preliminary X-ray diffraction analysis of antigen-binding fragments which are specific for antigenic conformations of sialic acid homopolymers

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Abstract

Meningococcal meningitis is a severe childhood disease which often results in significant disability or death. Two major etiological agents of meningitis are the group B meningococci and capsular type K1 *E. coli*. The virulence of these organisms is attributable to structural mimicry between their common $\alpha(2\text{–}8)$-polysialic acid capsular polysaccharide and human tissue antigens, which allows the bacteria to evade immune surveillance. There is currently no effective vaccine to protect against this infection. It has been demonstrated that the capsular polysaccharide of the bacteria can adopt a unique 'antigenic conformation'. This antigenic conformation has formed the basis for the development of an *N*-propionylated polysialic acid vaccine. Immunization trials in mice with this vaccine show the production of two groups of antibodies, of which only *N*-propionylated polysialic acid-specific were protective. Knowledge of the structure of the antigen-binding site which recognizes the protective epitope is essential to determining the antigenic conformation of the polysaccharides, and is a critical aspect in understanding and improving the action of potential vaccines. The antigen-binding fragments (Fab) of one protective (13D9) and one non-protective (6B9) monoclonal antibody specific for the capsular polysaccharides of group B meningococci have been crystallized and have undergone preliminary X-ray diffraction analysis. Both crystals are observed to scatter X-rays to approximately 1.7 Å resolution at the A1 station at the Cornell High-Energy Synchrotron Source. 13D9 has an orthorhombic unit cell with $a = 41.8$, $b = 102.3$, $c = 134.7$ Å, with space group $P2_12_12_1$. Fab 6B9 has an orthorhombic unit cell with $a = 89.6$, $b = 132.0$ and $c = 36.9$ Å, with space group $P2_12_12$.

1. Introduction

Meningococcal meningitis is a severe childhood disease caused by bacterial infection of the meninges of the brain and spinal chord, and often results in significant disability or death. Group B *Neisseria meningitidis* and capsular type K1 *Escherichia coli* are responsible for a large number of cases of meningococcal meningitis in developed countries (Peltola *et al.*, 1977), and are considered to be major human pathogens. The virulence of this disease is attributable to structural mimicry between their capsular polysaccharides and human tissue antigens, which allows these bacteria to evade immune surveillance (Pon *et al.*, 1997). The group B meningococcal polysaccharide (GBMP) and type K1 *E. coli* capsular polysaccharides consist of a homopolymer of $\alpha(2\rightarrow8)$-linked sialic acid (Jennings *et al.*, 1984). Structurally similar antigens in human tissue are found in gangliosides and neural cell adhesion glycoproteins (Finne *et al.*, 1983; Troy, 1992).

GBMP is the only surface antigenic structure that is conserved across group B meningococci and research has focussed on developing a GBMP vaccine (Jennings, 1997); however, this has been hampered by the poor immunogenicity of the polysaccharide in all humans (Wyle *et al.*, 1972). A common strategy to overcome poor immunogenicity is to covalently link polysaccharide antigens to protein carriers. Such glycoconjugate vaccines have displayed initial promise for meningococcal groups A and C; however, this approach has been shown to be ineffective for group B meningococcal polysaccharides (Jennings & Lugowski, 1981). At the present time, no effective vaccine against group B meningococci is commercially available (Diaz-Romero & Outschoorn, 1994; Jennings, 1997).

NMR and other experiments have shown that the key to immune recognition of the GBMP antigen lies in the propensity of these long-chain homopolymers to form helical structures (Brisson *et al.*, 1992). These helical structures are thought to display a specific conformation of sialic acid which is unattainable for the smaller oligomers found in human tissue antigens. This 'antigenic conformation' forms the basis for potential immune recognition of GBMP, and the subsequent neutralization of pathogens.

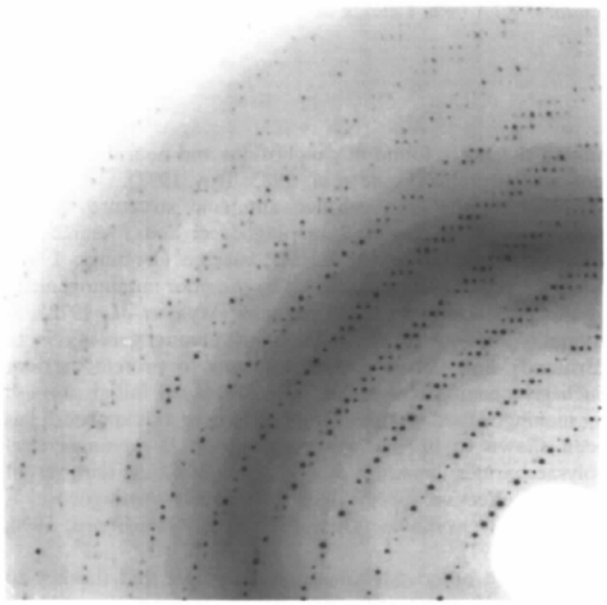
The concept of antigenic conformations has been reported previously, and a significant advance came with the isolation of the murine mAb735 specific for GBMP by direct immunization of NZB mice with viable group B meningococci (Frosch *et al.*, 1985), where it was shown that the binding surface of the Fab was highly complementary to the helical structures observed by NMR experiments of GBMP (Evans *et al.*, 1995). The binding site of the Fab from mAb735 (like any Fab) can at the most accommodate approximately eight $\alpha(2\rightarrow8)$-linked sialic acid residues; however, the antigenic form of GBMP is only favoured by much longer oligomers (Jennings *et al.*, 1984, 1985; Finne & Mäkelä, 1985; Kabat *et al.*, 1988; Häyrinen *et al.*, 1989). Indeed, mAb735 will not effectively bind oligomers of less than about ten residues (Michon *et al.*, 1987).

A potential breakthrough in vaccination technology for meningitis came with the use of chemically modified GBMP in an attempt to create a molecule that would display the required antigenic conformation but with lower immune tolerance. One of the compounds investigated was *N*-propionylated (NPr)GBMP. When coupled to tetanus toxoid as a protein carrier, this small change in chemical structure induced a high titer of antibodies against all group B meningococci (Jennings *et al.*, 1986; Ashton *et al.*, 1989). Surprisingly, the major population of NPrGBMP-specific antibodies showed no significant cross-reactivity with $\alpha(2\rightarrow8)$-polysialic acid, but

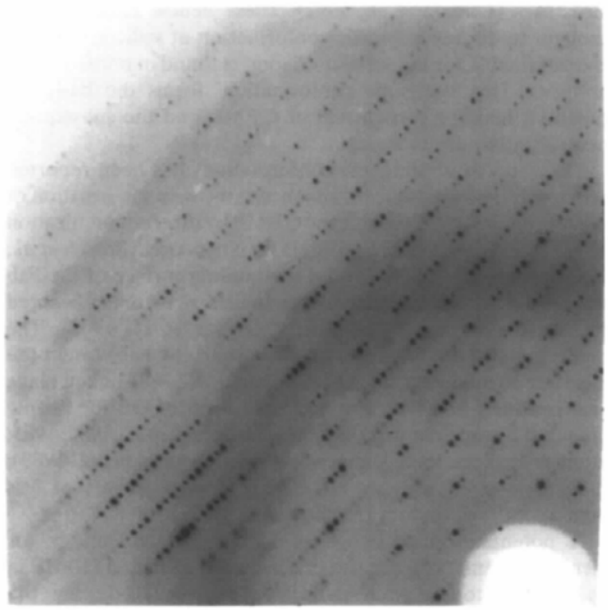
contained all bactericidal activity. Those antibodies which did cross react with $\alpha(2\rightarrow8)$-polysialic acid showed no bactericidal activity, and were not protective (Pon *et al.*, 1997). Further, only antibodies specific for extended helical epitopes of the NPrGBMP were protective; antibodies specific for short lengths of NPrGBMP displayed no protective activity. Thus, the NPrGBMP mimics a unique capsular epitope on the surface of GBM which could be formed by the interaction of the extended helical epitope of GBMP with another molecule (perhaps GBMP itself), and it is this structure that is mimicked by NPrGBMP (Jennings, 1997; Pon *et al*, 1997).

Given that these antibodies bind a maximum of eight sugar residues, and that the antigenic conformation of GBMP is not formed until the polymer is at least ten residues in length, it is not surprising that there have been no reports of crystals of Fab grown in complex with GBMP. Nevertheless, determining the exact nature of the interaction of GBMP with protective and non-protective antibodies is critical to exploiting this phenomenon in the development of vaccines.

We have developed a methodology to combine structural information on the antigen-binding fragment gained from high-resolution crystallographic analysis with the antigenic conformations of the polysaccharide antigens as determined by NMR to produce a model of antigen binding through computer-aided docking experiments (Evans *et al.*, 1995). In order to understand more fully the difference between protective and non-protective antibodies, one representative of each class was selected for crystallographic study, where mAb 13D9 recognizes a protective epitope and mAb 6B9 recognizes a non-protective epitope of GBMP. Here we report the generation, purification, crystallization and preliminary X-ray diffraction analysis of the Fabs from antibodies 13D9 and 6B9.



(*a*)



(*b*)

Fig. 1. Diffraction images for (*a*) Fab 13D9 and (*b*) Fab 6B9 taken on the 1k CCD detector at MacCHESS with an oscillation of 1° and exposures of 10 and 5 s, respectively. The beam stop can be seen in the lower right corner. The resolution limits are approximately 2.1 Å at adjacent corners and 1.65 Å at the opposite corner. Crystals of both antigen-binding fragments scatter to at least 1.7 Å resolution.

2. Experimental

The 6B9 and 13D9 monoclonal antibodies were generated, screened and purified as previously described (Pon *et al.*, 1997). Fabs were prepared from the purified mAb preparations using an 'ImmunoPure Fab Preparation Kit' (Pierce).

A Shodex CM-825 (Phenomenex) ion-exchange high-pressure liquid chromatography (HPLC) column was equilibrated with 20 m*M* HEPES pH 7.5. The dialyzed 13D9 and 6B9 digests were applied in turn to the column in several 1 ml injections. The 13D9 Fab was eluted as the major peak using a 0–1.2 *M* NaCl gradient, collected and concentrated in a Centricon-10 (Amicon) to a final concentration of 3.6 mg ml^{-1}. The 6B9 Fab was eluted as the major peak using a 0–4 *M* NaCl gradient, collected and concentrated in a Centricon-10 (Amicon) to a final concentration of 4.1 mg ml^{-1}.

Crystals of both 13D9 and 6B9 Fabs were obtained by the hanging-drop vapour-diffusion method (McPherson, 1982) using Linbro tissue-culture plates (Flow Labs). Crystals of 6B9 were obtained by suspending a 10 µl drop containing 1.3 mg ml^{-1} 6B9 Fab, 0.1 *M* calcium acetate, 0.05 *M* sodium cacodylate pH 6.5 and 6.8%(*w*/*v*) PEG 8000 over 1 ml of reservoir solution containing 0.2 *M* calcium acetate, 0.1 *M* sodium cacodylate pH 6.5 and 13.6%(*w*/*v*) PEG 8000. Well formed crystals of ~0.5 × 0.2 × 0.3 mm were obtained after 6 to 8 weeks. Crystals of 13D9 were obtained by suspending a 10 µl drop containing 1.55 mg ml^{-1} 13D9 Fab, 0.04 *M* HEPES pH 7.0 and 10.4%(*w*/*v*) PEG 10 000 over 1 ml of reservoir solution containing 0.08 *M* HEPES pH 7.0 and 20.8%(*w*/*v*) PEG 10 000. Crystals of up to 1.5 × 0.2 × 0.2 mm were obtained within a few days.

Diffraction data at 160 K was obtained by mounting crystals measuring no more than 0.2 mm on each side onto the 1K CCD at the A1 station of the Cornell High Energy Synchro-

tron Source (CHESS) at Cornell University. Rayon loops (Hampton Research) were used to remove the crystals from the hanging drops. The crystals were immersed in cryoprotectant solution for a few seconds, then snared again with the rayon loop and placed directly in the nitrogen cooling stream (140 K). The rayon loops were selected to minimize the volume of mother liquor that would be frozen with the drop. The most suitable cryoprotectant was found to be a solution of mother liquor with 20% of the water replaced by glycerol. The frozen drops held in the rayon loops were completely transparent, with no evidence of cracks or stress in the crystal. Sample diffraction images for each crystal are shown in Fig. 1. Fab 6B9 was found to have an orthorhombic unit cell with a = 89.6, b = 132.0, and c = 36.9 Å, with systematic absences consistent with space group $P2_12_12$. 13D9 was found to have an orthorhombic unit cell with a = 41.8, b = 102.3, c = 134.7 Å, with systematic absences consistent with space group $P2_12_12_1$. The calculated values of V_m (Matthews, 1968) are 2.34 Å^3 Da^{-1} for 6B9 and 3.10 Å^3 Da^{-1} for 13D9, leading to an estimated solvent content of 47 and 60%, respectively.

For 6B9 41 312 of 48 680 possible reflections were collected to 1.7 Å resolution (85–92% complete to 1.79 Å resolution), while for 13D9 45 234 of 55 929 possible reflections were collected to 1.78 Å resolution (81–93% complete to 1.92 Å resolution). Preliminary molecular replacement trials showed a clear rotation–translation solution for 13D9 using the constant domain of Fab YsT9.1 as a model (Evans *et al.*, 1994). No clear solution was immediately apparent for 6B9; however, a preliminary solution was finally obtained for the same model using the brute-force PC-refinement method outlined in Evans *et al.* (1994).

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