Location of a Blocking Epitope on Outer-membrane Protein III of Neisseria gonorrhoeae by Synthetic Peptide Analysis

By MUMTAZ VIRJI*† AND JOHN E. HECKELS
Department of Microbiology, Southampton University Medical School, Southampton General Hospital, Tremona Road, Southampton SO9 4XY, UK

(Received 25 January 1989; revised 20 March 1989; accepted 6 April 1989)

A series of overlapping peptides spanning the deduced amino acid sequence of outer-membrane protein PIII of Neisseria gonorrhoeae have been synthesized on solid-phase supports. The peptides were used in an attempt to locate the epitopes recognized by anti-PIII monoclonal antibodies with defined biological properties. Four bactericidal and two nonbactericidal antibodies were reacted with the synthetic peptides. None of the bactericidal antibodies reacted with the linear peptides. However, the two nonbactericidal antibodies were found to react within the disulphide loop thought to be exposed on the bacterial surface. Monoclonal antibody SM51 recognized a decapeptide corresponding to amino acid residues 24–33, while monoclonal antibody SM50 recognized an octapeptide contained within the decapeptide. The difference in the ability of the two antibodies to block the bactericidal effect of antibodies directed against other surface antigens therefore appears to be related to a difference in their ability to activate complement rather than to the location of the epitope recognized.

INTRODUCTION

The outer-membrane protein III (PIII) of Neisseria gonorrhoeae is highly conserved (Judd, 1982; Lytton & Blake, 1986), is expressed by all strains of gonococci (Gotschlich et al., 1987) and serological studies have shown the presence of surface-exposed immunogenic regions on the protein (Swanson et al., 1982). Since two important requirements of a suitable vaccine candidate are its expression on diverse strains and its immunogenicity, PIII could be regarded as a potential candidate for vaccination against gonorrhoea. However, antibodies directed against PIII, which are present in some convalescent sera, have the ability to block the bactericidal effect of antibodies directed against other surface antigens (Rice et al., 1986). The ability of PIII to elicit such blocking antibodies is a major obstacle to its consideration as a candidate vaccine antigen.

In order to investigate the ability of PIII to elicit protective antibodies, we have previously used monoclonal antibodies directed against surface-exposed regions of the protein. These studies have shown the presence of at least two regions on PIII which elicit bactericidal antibodies. In addition, one further immunogenic region was identified which proved to be an ineffective target for antibody–complement-mediated killing by two different mAbs (SM50 and SM51) which bind to the same or very closely positioned epitopes within this region (Virji et al., 1987). Further studies with these monoclonal antibodies revealed that complement-mediated bactericidal activity of several highly effective mAbs against many diverse gonococcal antigens could be blocked by the presence of the mAb SM50 (Virji & Heckels, 1988).

† Present address: University of Oxford, Department of Paediatrics, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK.

Abbreviations: PIII, outer-membrane protein III; mAb, monoclonal antibody; FMOC, Nα-(9-fluorenylemethoxycarbonyl); DMF, N,N'-dimethylformamide.
In view of the ability of PIII to elicit blocking antibodies, its use in a vaccine would require strategies designed to avoid the induction of an immune response against the blocking epitopes. This would require identification of the structure and location of the protective and blocking epitopes on PIII. In the present study we have used the amino acid sequence of PIII, derived from the DNA sequence as reported by Gotschlich et al. (1987), in order to synthesize a series of overlapping peptides spanning the PIII molecule. By the use of these peptides, we have identified the blocking epitope on PIII recognized by mAb SM50.

METHODS

Monoclonal antibodies. Hybridomas producing mAbs against several distinct epitopes on PII1 were produced by fusion of the NS-1 myeloma cell line with spleen cells from a Balb/c mouse which had been immunized with outer-membrane preparations from two strains of gonococci. The details of immunization schedule and fusion procedure have been described previously (Virji et al., 1987). Antibody reactivity was determined by ELISA and Western blotting, and the specificity of mAbs was confirmed by radioimmunoprecipitation assay (RIPA), reactivity with reduction-modified PII1 and with the cloned PII1 gene product (Virji et al., 1987). The current studies have used the two nonbactericidal mAbs SM50 and SM51 to identify their binding sites on the PII1 molecule. The bactericidal mAbs SM52–55 were also tested for their reactivities against the synthesized peptides.

Synthesis of peptides. The solid-phase peptide synthesis was carried out using a commercially available kit (Cambridge Research Biochemicals) based on the methods of Geyser et al. (1984, 1987). Peptides were synthesized on to polystyrene rods (diameter 4 mm, length 40 mm) assembled into holders designed to hold 96 rods in the format of a microtitre plate. Synthesis was carried out with pentafluorophenyl (pfp) active esters of Nα-(9-fluorenlymethoxyxycarbonyl) (FMOC)-L-amino acids with t-butyl side chain protecting groups (Millipore), except in the case of methionine which had a trimethylsulphonyl side chain protecting group, and serine and threonine in which the oxybenzotriazine active ester was used. All solvents used were of analytical grade and were supplied by Romil (Leicester, UK) and were used without further purification with the exception of piperidine (BDH) which was redistilled before use and N,N'-dimethylformamide (DMF) which was stored over molecular sieve (Union carbide, grade 4A) to remove amine impurities.

The rods were supplied with FMOC-protected β-alanine coupled to the rods via acryllic acid and hexamethylene diamine. The peptide synthesis was initiated by deprotection of the rod-linked β-alanine by removal of the base-labile N'-FMOC group in 20% (v/v) piperidine in DMF; the rods were washed in DMF and methanol prior to coupling. The appropriate N'-FMOC amino acid esters were activated by dissolving in 30 mM-1-hydroxybenzo-triazole in DMF and were dispensed into 96-well polystyrene plates. The deprotected rods were placed in the wells and coupling was allowed to proceed for 18 h at room temperature. The deprotection, washing and coupling procedures were repeated until all amino acids were coupled. Finally, the terminal amino group was acetylated by reaction with acetic anhydride/diisopropylethylamine/DMF (5:1:50, by vol.) and the side chains were then deprotected in a mixture of trifluoroacetic acid/phenol/ethanedithiol (95:2.5:2.5, by vol.) followed by washing in dichloromethane/diisopropylethylamine (95:5, v/v). The pins were washed in methanol then dried in vacuo before use in immunological screening. Each synthesis was done in duplicate and control peptides reacting with a test mAb supplied by the manufacturer were included to check the coupling during each synthesis.

Detection of immunological reactivity. Reactivities of the synthesized peptides on the rods was performed essentially as described by Geyser et al. (1987). The rods were blocked against nonspecific binding by preincubation with 1% (w/v) bovine serum albumin (Sigma, fraction V) and 1% (w/v) ovalbumin (Sigma) in PBS containing 0.1% Tween-20 (blocking buffer). Ascites containing mAbs were diluted 1:1000 in blocking buffer and dispersed at 100 µl per well in 96-well microtitre plates. The blocked rods were incubated with the antibody at 4 °C overnight. Unbound antibody was removed by washing four times in PBS containing 0.05% Tween-20 (PBST). Bound antibody was reacted with goat anti-mouse IgG conjugated to horseradish peroxidase (Zymed) for 1 h at room temperature, and the excess conjugate was removed by four PBST washes. Substrate solution was prepared by dissolving 50 mg of 2,2'-azinodip(3-ethylbenzthiazodinsulphonate) (ABTS) in 100 ml of 0.1 M-phosphate, 0.08 M-citrate buffer, pH 4. Immediately prior to use, 30 µl of 120 vol. hydrogen peroxide was added. The rods were placed in microtitre plates containing 100 µl of substrate solution until sufficient colour had developed. Absorbance was measured at 405 nm in a Titretek ELISA plate reader.

The solid-phase peptides were reused after bound antibody was dissociated by sonication of the rods in 1% (w/v) SDS, 0.1% 2-mercaptoethanol in 0.1 M-phosphate buffer, pH 7.2, at 60 °C for 30 min. The blocks were then thoroughly washed in hot water (55 °C) and then immersed in boiling methanol for 2 min. Immunological reactivity was always observed in duplicate peptides and in assays repeated on at least two occasions.

Database searches. The occurrence in other proteins of sequences similar to immunologically reactive peptides was sought using the FASTP program (Wilbur & Lipman, 1983) to search the NIH Protein Identification Resource and the EMBL SwissPro databases.
**Fig. 1.** Reaction of monoclonal antibodies SM50 and SM51 with solid-phase synthetic peptides spanning the PIII molecule. A series of decapeptides were synthesized with a five amino acid overlap (i.e. the last five residues of one peptide were shared with the first five residues of the next) and reacted in ELISA with monoclonal antibodies SM50 (■) and SM51 (□). Residue number denotes the position within the PIII molecule of the first amino acid in each peptide.

**Fig. 2.** Reaction of monoclonal antibodies with solid phase synthetic peptides spanning amino acid residues 21–40 of the PIII molecule. All possible decapeptides (a) and hexapeptides (b) were synthesized and reacted in ELISA with monoclonal antibodies SM50 (■) and SM51 (□).

**RESULTS AND DISCUSSION**

In order to detect continuous epitopes, in the initial screening of mAb reactivities a peptide length of 10 residues was selected. Using the predicted amino acid sequence of PIII (Gotschlich et al., 1987), series of decapeptides spanning the entire molecule were synthesized with adjacent peptides overlapping by five residues. This identified a region of reactivity with the two nonbactericidal mAbs SM50 and SM51 (Fig. 1) although not with any of the four bactericidal mAbs which had previously been shown to recognize at least two distinct epitopes (Virji et al., 1987). Antibody SM50 reacted strongly with a single peptide corresponding to amino acid residues 26–35, while mAb SM51 reacted with the overlapping peptide 21–30. However, the reaction of SM51 with peptide 21–30 was weak, suggesting that the SM51 epitope may be only partly represented in the synthesized peptides.

To further define both epitopes, deca- and hexapeptides were synthesized between residues...
21 and 40, such that the adjacent peptides shared all but the first residue. ELISA analysis of the peptides with the mAbs is shown in Fig. 2. Neither SM50 nor SM51 reacted with the hexapeptides. SM50 reacted almost equally well with three adjacent decapeptides which have in common the sequence WKNAYFDK, corresponding to amino acid residues 26–33, but not with either of the adjacent peptides, suggesting that this octapeptide sequence constitutes the minimum SM50-reactive epitope. In contrast mAb SM51 reacted strongly only with the decapeptide ECWKNAYFDK corresponding to residues 24–33. Its reaction with the adjacent peptides was considerably lower, demonstrating that the optimum SM51 epitope spans all 10 amino acid residues.

Antibodies to PIII have until recently been implicated only in blocking the bactericidal effect of antibodies in normal human serum and immune serum (Rice et al., 1986). However, immunization of mice with native protein yielded some mAbs which were bactericidal and others which were nonbactericidal (Virji et al., 1987). Thus, immunogenic protective epitopes could be demonstrated on this highly conserved protein. In addition, one of the nonbactericidal antibodies (SM50) was shown to block complement-mediated killing in the presence of many mAbs of a wide range of specificities (Virji & Heckels, 1988). The mechanism of blocking by this antibody seemed to be related to its ability to fix complement since mAb SM51 did not fix complement and did not block, although competitive radioimmunoassay experiments suggested that the two antibodies bound to identical or closely positioned epitopes.

The epitope mapping studies described above confirm the close proximity of the epitopes recognized by mAbs SM50 and SM51, suggested by the previous observations of mutual inhibition in competitive radioimmunoassay. Indeed the octapeptide SM50 epitope is entirely contained within the decapeptide required by SM51. Thus it appears that the difference in biological effects of the two antibodies is indeed related to the difference in their ability to activate complement as previously suggested (Virji & Heckels, 1988). The precise mechanism of blocking is unclear, but one possibility is that binding of mAb SM50 to its nonbactericidal epitope diverts complement components away from bactericidal antibodies (Joiner et al., 1985) which bind at remote sites without any direct steric interference. The lack of blocking by mAb SM51, which binds to the same region, is therefore simply a consequence of its inability to fix complement (Virji & Heckels, 1988).

Analysis of the amino acid sequence of the PIII molecule has revealed significant homology with a segment of the OmpA outer-membrane protein of Escherichia coli. By comparison of the PIII sequence with the predicted map of the surface organization of OmpA, Gotschlich et al. (1987) have suggested a model for the surface organization of PIII. In this model only a relatively small segment of the protein, approximately between residues 1 and 44, is available to be exposed on the surface. This region includes a disulphide loop between residues 25 and 41. The location of the blocking epitope(s) between residues 24 and 33 is therefore in that region of the molecule which would be predicted to be most exposed and hence most accessible to the immune system. Such a location would be in accord with the propensity of PIII to induce blocking antibodies. It is also interesting to note that the blocking epitope lies within that part of the PIII molecule which is distinct from the OmpA protein and that, in addition, it shows no significant homology with any amino acid sequences currently within the protein databases.

The lack of reactivity of the bactericidal anti-PIII mAbs precluded the direct identification of these bactericidal epitopes in the current study. This lack of reactivity may be due to the fact that the peptides synthesized do not contain the complete epitope(s), or that there is some requirement for secondary structure for binding of the bactericidal mAbs, which is absent in the relatively short peptides which were synthesized. Nevertheless the identification of the blocking epitopes now permits consideration of immunochemical or genetic methods to produce a modified PIII vaccine designed to induce a protective, cross-reacting immune response, while avoiding production of blocking antibodies.

This work was supported by a Medical Research Council project grant. We are grateful to Dr W. Conlon for advice on peptide synthesis.
REFERENCES


