Immunization with a multiple antigen peptide containing defined B- and T-cell epitopes: production of bactericidal antibodies against group B Neisseria meningitidis

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Previous analysis of the class 1 outer-membrane (OM) protein of Neisseria meningitidis has identified discrete epitopes to be potential targets for immune attack. The conformation of these epitopes is important for inducing antibodies which can react with the native protein and promote complement-mediated lysis of the meningococcus. The multiple antigen peptide (MAP) system, which consists of an oligomeric branching lysine core to which are attached dendritic arms of defined peptide antigens, confers some conformational stability and also allows for the preparation of immunogens containing both B-cell and T helper (Th)-cell epitopes. In this study, MAPs were synthesized to contain (i) the subtype P1.16b meningococcal class 1 protein B-cell epitope (B-MAP), and (ii) the P1.16b epitope in tandem with a defined Th-cell epitope, chosen from tetanus toxin (BT-MAP). The B-MAP was non-immunogenic in animals. In contrast, incorporation of the Th-cell epitope into BT-MAP induced a strong humoral response towards the class 1 protein B-cell epitope. Antisera from immunized mice and rabbits reacted in ELISA with synthetic peptides containing the B-cell epitope, and also cross-reacted with meningococcal OMs from strains of subtype P1.16b and P1.16a. Murine and rabbit antisera showed similar reactivity and epitope specificity, but did not react with denatured class 1 protein in Western blotting, indicating the predominance of antibodies directed towards conformational epitopes. The antisera from rabbits immunized with BT-MAP promoted complement-mediated bactericidal killing not only of the homologous meningococcal subtype P1.16b strain but also of subtype P1.16a.

Keywords: Neisseria meningitidis, multiple antigen peptide, outer membrane protein, T helper-cell epitope, B-cell epitope

INTRODUCTION

The lack of an effective vaccine remains a major obstacle in the prevention of life threatening meningitis and septicaemia caused by Neisseria meningitidis. The vaccines currently in use are based on the meningococcal serogroup-specific capsular polysaccharides, and are of limited efficacy. The capsular polysaccharides from serogroups A and C provide only short-term protection in adults, because of their inability to induce a T helper (Th)-cell response and hence immunological memory (Frasch, 1985). In addition they are non-immunogenic in infants, the age group at greatest risk of infection. Furthermore, the group B capsular polysaccharide is non-immunogenic even in adults, so that current vaccines provide no protection against group B strains, the predominant cause of infection in most temperate countries.

Currently, new experimental vaccines based on outer-membrane (OM) preparations are under investigation. This strategy is based on the observations that the presence of serum bactericidal activity in humans correlates with immunity to infection (Goldschneider et al., 1969) and that antibodies directed against meningococcal

Abbreviations: mAb, monoclonal antibody; MAP, multiple antigen peptide; OM, outer membrane; Th-cell, T helper-cell.
OM proteins activate complement and kill encapsulated strains (Frasch, 1985). Recent field trials with vaccines based on OM preparations have demonstrated some protection against group B infection (Bjune et al., 1991; de Moraes et al., 1992). However the duration of protection appeared to be relatively short-lived (Bjune et al., 1991) and, in addition, at least one study concluded that immunization induced a high proportion of non-functional antibodies, as measured by bactericidal activity (Zollinger et al., 1991). Improved vaccines should therefore be achieved by simultaneously targeting the immune response to specific epitopes on OM proteins known to induce bactericidal antibodies and by prolonging the duration of the effective immune response.

Four major classes of meningococcal OM proteins have been identified (Tsai et al., 1981), of which the class 1 protein is an attractive potential target for immune attack. Unlike several other meningococcal major surface antigens the class 1 protein does not undergo antigenic variation within a strain, and is responsible for sero-subtype specificity (Frasch et al., 1985). Subtype-specific monoclonal antibodies (mAbs) are bactericidal and are particularly effective in preventing infection in the infant rat model of meningococcal infection (Saikkonen et al., 1989). Bactericidal activity in sera from human volunteers immunized with experimental OM protein vaccines appears, in part, to be associated with the production of anti-class 1 antibodies (Wedge & Froholm, 1986; Zollinger et al., 1991). The cloning and sequencing of the porA genes which encode the class 1 protein has permitted detailed structural and antigenic studies which have led to a model of the organization of the protein within the outer membrane (van der Ley et al., 1991). The model predicts that a series of conserved regions form amphipathic transmembrane ß-sheets, so generating eight surface-exposed hydrophilic loops. Structural variations between strains are largely confined to two variable regions VR1 and VR2 which are located in the surface-exposed loops 1 and 4 (McGuinness et al., 1990). Epitope mapping with synthetic peptides has localized the epitopes recognized by the bactericidal, subtype-specific mAbs to the apices of these loops (McGuinness et al., 1990, 1993). Thus a mAb with P1.16a subtype specificity reacted with the peptide TKDTNNNLTL, located at the apex of loop 4. The critical contribution of this region is further demonstrated by the observation that a single amino acid substitution from D to N at position 182 within the epitope results in the generation of a class 1 protein with altered antigenic properties (subtype P1.16b) (McGuinness et al., 1991).

Such detailed antigenic analysis has identified discrete regions of the class 1 protein to be potential targets for immune attack. In a previous study with synthetic linear and cyclic peptides, a cyclic 36mer peptide containing the P1.16b subtype epitope was shown to induce conformationally relevant antibodies which promoted complement-mediated bactericidal killing of the homologous meningococcal strain (Christodoulides et al., 1993). However, the synthesis of conformationally constrained peptides to accurately mimic the three-dimensional structure of the native protein is technically demanding and difficult to monitor. In this report, an alternative approach has been the use of multiple antigen peptides (MAPs), consisting of an oligomeric branching lysine core, to which are attached dendritic arms of defined peptide antigens (Tam, 1988). Consequently, the overall structure of a MAP is of a macromolecule with a high density of surface peptide antigens surrounding a non-immunogenic core matrix of low molecular mass. The high density of peptide antigens seems to enhance the immunogenicity that is often absent when a single copy of the same peptide antigen is used (Francis et al., 1991). Also, because of the close packing of these dendritic antigens in the macromolecular structure, MAPs may have the advantage of providing a tertiary structure that allows some conformational stability to the peptides (DeFoort et al., 1992a). The system also allows the preparation of immunogens consisting of both B-cell and T-cell epitopes in various arrangements.

In this report, peptides containing the meningococcal B-cell epitope TKNTNNNLTQYIKANSFIGITE have been synthesized on MAPs with and without a tandem Th-cell epitope, and investigated as experimental peptide vaccines, with respect to their immunogenicity and the antigenic and biological activities of the antibodies induced.

**METHODS**

**Bacterial strains and growth conditions.** *Neisseria meningitidis* strains MC50 (C:NT:P1.16a), MC58 (B:15:P1.7,16b) and MC51 (C:NT:P1-15) have been described previously (Tinsley & Heckels, 1986). Strain H44/76 (B:15:P1.7,16a) is the subtype P1.7,16 reference strain (Frasch et al., 1985). All strains were grown on protease-peptone agar at 37 °C for 18 h in an atmosphere of 5% (v/v) CO₂ (Tinsley & Heckels, 1986).

**Outer membranes.** Outer membranes were prepared by extraction of whole cells with lithium acetate as described previously (Tinsley & Heckels, 1986).

**Solid-phase peptide synthesis.** Peptides (Table 1) were synthesized using solid-phase Fmoc-[N-α-(9-fluorenlymethoxycarbonyl)] chemistry, on an Applied Biosystems Model 431A Peptide Synthesizer, as described previously (Christodoulides et al., 1993). For MAP synthesis, 4- or 8-branch MAP cores with Fmoc protection were obtained from Applied Biosystems Inc. (ABI) and coupled to 4-(hydroxyethyl)-phenoxyethymethyl (HMP) copolyisoprene-1% divinylbenzene resin (ABI). Linear peptides were also synthesized on HMP resin. Synthesis of both linear and MAP peptides used Fmoc-protected amino acids with tert-buty1 side chain protecting groups, with the exception of asparagine (trityl-) and lysine (Boc). Activation and coupling was done in the presence of hydroxybenzotriazole/
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**Immunization of animals.** Female Balb/C (H-2b) mice of 6–7 weeks of age, and New Zealand half-tail rabbits were used for immunizations. Mice were housed 10 to a cage, from which five were chosen at random and each immunized intraperitoneally on day 1 with 25 μg of peptide emulsified in Freund’s complete adjuvant (Sigma) and on days 14 and 28 with the same peptide emulsified in Freund’s incomplete adjuvant (Sigma). The remaining five mice were immunized controls, which were bled at the same time as immunized animals. Rabbits (two per immunogen) were each immunized subcutaneously on day 1 with 25 μg of peptide emulsified in Freund’s complete adjuvant distributed between two and three sites, and on days 14 and 28 with the same peptide emulsified in Freund’s incomplete adjuvant.

Test bleeds were taken from all animals 10 d after the second booster immunization, to monitor levels of circulating anti-peptide antibodies by ELISA. Up to a further two booster immunizations were then given, after which all animals were terminally bled by cardiac puncture under anaesthesia. All sera were stored at −20 °C.

**Detection of immune response.** (i) ELISA. ELISA determination of antibody was done as described previously (Christodoulides et al., 1993). Flat-bottomed microtitre plates (Sertinl) were coated overnight at 37 °C with antigen (1 μg ml⁻¹) in 0.05 M sodium carbonate buffer, pH 9.6. Antibody binding was detected using the appropriate horseradish peroxidase conjugate (Zymed) with 3,3′,5,5′-tetramethylbenzidine and H₂O₂ as enzyme substrate. Absorbance was measured at 450 nm and the ELISA titre was extrapolated from the linear portion of the serum titration curve and taken as the reciprocal dilution which gave an absorbance of 0.1 h⁻¹.

(ii) Western immunoblotting. Meningococcal outer-membrane protein preparations were separated by preparative SDS-PAGE (250 μg total protein per gel), using a 10–25% (w/v) acrylamide gradient as described previously (Heckels, 1981). Proteins were transferred to nitrocellulose paper (Schleicher and Schuell BA45, 0.45 μm) using a Trans-Blot Semi-Dry transfer cell (Bio-Rad) as described previously (McGuinness et al., 1993). After incubation with sera, immunological reactivity was detected with the appropriate alkaline phosphatase conjugate (Zymed) and substrate solution containing nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad), as described previously (Christodoulides et al., 1993).

**Bacterial killing assays.** The bactericidal activity of antisera was determined essentially as described by Munkley et al. (1991) using guinea-pig serum as a source of exogenous complement. Meningococcal strains MC58 or MC50 were harvested into PBS containing 1% (v/v) acetylene gradient as described previously (Heckels, 1981). Proteins were transferred to nitrocellulose paper (Schleicher and Schuell BA45, 0.45 μm) using a Trans-Blot Semi-Dry transfer cell (Bio-Rad) as described previously (McGuinness et al., 1993). After incubation with sera, immunological reactivity was detected with the appropriate alkaline phosphatase conjugate (Zymed) and substrate solution containing nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad), as described previously (Christodoulides et al., 1993).

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Fig. 1. Structures of multiple antigen peptides (MAPs) used for immunization. (a) MAP dodecamer with meningococcal class 1 protein B-cell epitope (B-MAP). (b) MAP tetramer with class 1 protein B-cell epitope and tetanus toxin Th-cell epitope (BT-MAP). Solid blocks denote the class 1 protein epitope TKNTNNNLTL (P1.16b), and hatched blocks denote the tetanus toxin Th-cell epitope QYIKANNSFIGITE.

dicyclohexylcarbodiimide and at the completion of synthesis all peptides were acetylated at the N-terminus with acetic anhydride.

Crude peptides were washed with ether and purified by reverse-phase HPLC on a semi-preparative Zorbax C8 (DuPont) column using a gradient of 5–80% (v/v) acetonitrile in water containing 0.1% TFA, as described previously (Christodoulides et al., 1993). Purified peptides were obtained as single peaks in >95% yield, and were lyophilized and stored desiccated at −20 °C. The expected amino acid composition of each peptide was verified by amino acid analysis using an automated analyser (ABI).

**Multiple solid-phase synthesis and epitope mapping.** Multiple solid-phase peptides were synthesized by pin technology using a commercially available kit (Chiron Mimotopes) according to the manufacturer’s instructions. The solid phase supports were polyethylene pins, each of which had a Fmoc-protected β-alanine residue coupled via a flexible linker of 1,6-diaminohexane and polyacrylic acid (Geysen et al., 1989). Synthesis used pentafluorophenyl (pfp)-activated esters of Fmoc-L-amino acids with t-butyl side-chain protection and substrate solution containing nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Absorbance was measured at 450 nm and the ELISA titre was extrapolated from the linear portion of the serum titration curve and taken as the reciprocal dilution which gave an absorbance of 0.1 h⁻¹.

(i) Western immunoblotting. Meningococcal outer-membrane protein preparations were separated by preparative SDS-PAGE (250 μg total protein per gel), using a 10–25% (w/v) acrylamide gradient as described previously (Heckels, 1981). Proteins were transferred to nitrocellulose paper (Schleicher and Schuell BA45, 0.45 μm) using a Trans-Blot Semi-Dry transfer cell (Bio-Rad) as described previously (McGuinness et al., 1993). After incubation with sera, immunological reactivity was detected with the appropriate alkaline phosphatase conjugate (Zymed) and substrate solution containing nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad), as described previously (Christodoulides et al., 1993).

**Bacterial killing assays.** The bactericidal activity of antisera was determined essentially as described by Munkley et al. (1991) using guinea-pig serum as a source of exogenous complement. Meningococcal strains MC58 or MC50 were harvested into PBS containing 1% (v/v) heat-inactivated (56 °C, 30 min) foetal calf serum. Bacterial suspension (25 μl, containing approximately 1000 c.f.u.) was added to the wells of a sterile 96-well microtitre plate containing serial dilutions of decomplemented test sera in PBSB (10 μl). Freshly thawed complement or decomplemented serum was then added to a final concentration of 5% (v/v) and the plates were agitated briefly to mix thoroughly. The plates were incubated at 37 °C for 30 min in an atmosphere of 5% (v/v) CO₂, and then 15 μl samples were removed from each well for determination of surviving c.f.u. All sera, with and without exogenous complement, were assayed in triplicate at each serial dilution, and the assays performed in triplicate. As positive controls for bactericidal killing assays, a rabbit polyclonal antiserum raised to P1.16a OM was used with the P1.16a strain (MC50), and similarly a rabbit polyclonal antiserum raised against P1.16b cyclic peptide (Christodoulides et al., 1993) for the P1.16b strain (MC58). For the heterologous strain MC51, a P1-15 specific mAb (MN353C) was used. The
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RESULTS

Immunogenicity of synthetic peptides

Initially, mice and rabbits were immunized with a multiple antigen peptide (MAP) octamer containing the B-cell epitope TKNTNNNL (B-MAP) with triglycyl spacers at the C termini of the peptide chains to provide flexibility away from the lysine core (Fig. 1a). The murine and rabbit antisera raised against this peptide were tested in ELISA. In neither case was any reaction detected with the immunizing B-MAP or with the linear synthetic peptide (B) containing the same epitope (Figs 2a and 3a).

Since the MAP containing the B-cell epitope only, was non-immunogenic, further peptides were synthesized to contain a defined Th-cell epitope (Table 1). A Th-cell epitope comprising amino acids 830-843 from tetanus toxin was chosen, since it has been reported to be a 'universal' human T-cell epitope (Panina-Bordignon et al., 1989) which is also recognized in mice (Valmori et al., 1992). A tetrameric BT-MAP peptide was synthesized.

complement-mediated bactericidal activity of each antiserum, as compared to the corresponding pre-immune serum was analyzed statistically (F- and t-tests) as described previously (Christodoulides et al., 1993).

Fig. 2. ELISA responses of rabbits immunized with the synthetic peptides B-MAP (a), BT (b) and BT-MAP (c), and individual pre-immune and corresponding post-immune sera tested in ELISA against peptides and meningococcal OMs from strains MC58 (P1.16b), MC50 (P1.16a) and H44/76 (P1.16a). The results are shown as log₁₀(titre) for individual rabbits.

Fig. 3. ELISA responses of groups of five mice immunized with the synthetic peptides B-MAP (a), BT (b) and BT-MAP (c), and individual pre-immune and corresponding post-immune sera tested in ELISA against peptides and meningococcal OMs from strains MC58 (P1.16b), MC50 (P1.16a) and H44/76 (P1.16a). The results are shown as log₁₀(titre) for individual mice.
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**Fig. 4.** Epitope mapping of antisera raised against synthetic peptides. Sera were reacted in ELISA with overlapping decapeptides corresponding to the amino acid sequence of loop 4 of the class I protein in which adjacent peptides differed by a single residue, and where X is either amino acid N (P1.16b) or D (P1.16a) at position 182. Pairs of rabbits were immunized with peptides BT or BT-MAP. Each rabbit post-immune serum was then tested and the absorbance of the corresponding pre-immune serum subtracted as a background control. Representative data from individual rabbits are shown, since reactivity within each pair was similar. Sera from groups of five mice immunized with the same antigen were pooled and tested, with the absorbance of the corresponding sera from groups of five unimmunized mice subtracted as the negative background control. Antisera were raised against peptide BT in rabbits (a) and mice (c) and against peptide BT-MAP in rabbits (b) and mice (d), and were reacted with peptides corresponding to the P1.16b (filled bars) or P1.16a (open bars) epitopes.

(Fig. 1b) and used to immunize both mice and rabbits. In addition, the linear monomeric BT peptide, together with control linear peptides B (B-cell epitope) and T (Th-cell epitope), were synthesized (Table 1) and used for immunization. All anti-peptide antisera were subsequently tested in ELISA against each peptide synthesized and also against meningococcal OMs.

Antisera raised in mice and rabbits to the control peptides B or T did not react with homologous peptides or meningococcal OMs in ELISA (data not shown). Animals were also immunized with a non-covalent mixture of peptides B and T and the antisera raised were similarly non-reactive. In contrast, antisera from mice and rabbits immunized with the linear BT peptide both showed reactions with peptides in ELISA. The antisera reacted weakly with homologous peptide BT, and more strongly with BT-MAP (Figs 2b and 3b), in accord with previous observations that MAPs may provide increased sensitivity over linear peptides in solid-phase ELISA (Tam & Zavala, 1989). Both rabbit and mouse antisera to peptide BT also reacted with meningococcal OMs, showing approximately four- to eightfold increases in titre against all three OMs (Figs 2b and 3b).

The BT-MAP proved to be the best peptide immunogen in both rabbits and mice (Figs 2c and 3c). ELISA reactivity was greatest with BT-MAP as the coating antigen, with both murine and rabbit antisera showing approximately $10^2$-$10^4$-fold increases in titre (Figs 2c and 3c). Antisera from both species also showed significant increases in titre with meningococcal OMs. The rabbit antisera showed a tenfold rise in titre with OM from the subtype P1.16b strain MC58, and also cross-reacted with OMs from
subtype P1.16a strains MC50 and H44/76 (fivefold rises) (Fig. 2c). The murine antisera showed approximately tenfold rises in mean titre against OM from both subtype P1.16b and P1.16a meningococci (Fig. 3c). No increase in titre was observed when either rabbit or murine antisera to BT and BT-MAP were reacted with OM from a meningococcal strain of unrelated serosubtype, MC51 (C:NT:P1-15).

**Epitope mapping of rabbit and murine anti-peptide antisera**

To determine the epitope specificity of the antisera raised, all sera were reacted with overlapping decapeptides corresponding to the amino acid sequence of loop 4 of the class 1 OM protein which contained the B-cell epitope. Peptides were synthesized on polyethylene pins with adjacent peptides differing by a single residue and containing either the N182 or D182 residue corresponding to the P1.16b and P1.16a epitopes, respectively. As expected from the ELISA results, immunization of rabbits and mice with the non-immunogenic peptides B, T or B-MAP induced antisera which failed to react with any of the decapeptides constituting loop 4 of the class 1 OM protein (data not shown).

In contrast, the immunogenic peptides BT and BT-MAP induced antisera which reacted with a variety of the decapeptides. Rabbit (Fig. 4a) and murine (Fig. 4c) antisera raised against peptide BT showed essentially similar patterns of reactivity, both reacting with the decapeptide sequence lsoTKXTNNNLTL1sg, where X is either N (the subtype P1.16b amino acid sequence) or D (the subtype P1.16a amino acid substitution). Equally, little difference was seen between rabbit and murine antisera raised against peptide BT-MAP. In this case, rabbit antisera reacted with peptides incorporating amino acid residues 174–199 from the class 1 protein, and was centred around the decapeptide sequence lsoTKXTNNNLTL1sg (Fig. 4b). Murine antisera reacted similarly, with peptides incorporating amino acid residues 175–189 (Fig. 4d). In addition, both rabbit and murine antisera raised against BT-MAP also reacted with additional peptides from the N-terminus of the sequence (Figs 4b and 4d).

**Reactivity of rabbit and murine antisera on Western blots**

The specificity of reactivity of all anti-peptide antisera was also determined by Western blotting against OM from meningococcal strains MC58 (P1.16b) and MC50 (P1.16a). Neither rabbit nor murine antisera raised against the immunogenic peptides BT and BT-MAP reacted with the class 1 protein from the strains MC58 or MC50.

**Bactericidal activity of rabbit and murine antisera**

Rabbit and murine antisera raised against synthetic peptides were tested for their ability to promote complement-mediated bactericidal killing of meningococcal strains MC58 (P1.16b) and MC50 (P1.16a) using guinea-pig serum as a complement source. The rabbit antisera raised against peptides BT-MAP and BT both demonstrated bactericidal killing of meningococci. Antiserum raised against peptide BT was bactericidal for the homologous strain MC58 (P1.16b) with a 50% endpoint at a dilution of approximately 1:80 (Fig. 5a), and also showed weaker, but significant (P < 0.05) killing of the subtype P1.16a strain MC50 at dilutions of 1:10 (25%) and 1:40 (16%) (Fig. 5b).

Bactericidal activity was greatest in rabbit antisera raised against the BT-MAP peptide. This antiserum was highly bactericidal for the homologous subtype P1.16b strain MC58 with a 50% endpoint of approximately 1:320 (Fig. 5c), and also showed significant (P < 0.05) killing of the subtype P1.16a strain MC50 at dilutions of 1:10 (44%) and 1:40 (32%) (Fig. 5d). The subtype specificity of the killing was demonstrated by the fact that sera raised against BT and BT-MAP did not promote complement-mediated bactericidal killing of an unrelated strain, MC51 (C:NT:P1-15) (data not shown).
In contrast to the rabbit antisera, murine antisera raised to peptides BT and BT-MAP did not show any bactericidal activity (data not shown).

**DISCUSSION**

A model for the organization of the class 1 protein within the OM of *N. meningitidis* predicts that a series of conserved regions form amphipathic β-sheets, thus generating eight surface-exposed hydrophilic loops (van der Ley et al., 1991). The protective, subtype-specific epitopes have been mapped to the apices of loops 1 and 4, which are the most surface-exposed and accessible to immune surveillance. In a previous report (Christodoulides et al., 1993), linear synthetic peptides corresponding to the apex of loop 4 containing the protective B-cell epitope, induced antisera which reacted poorly with native protein in OMs, and were non-bactericidal. In contrast, antisera raised by immunization with a large 36mer cyclic peptide incorporating the B-cell epitope, showed bactericidal activity against meningococci. In solution, synthetic peptides are likely to adopt a wide range of conformations, only a fraction of which may be relevant to the stable conformations of the native protein (Arnon, 1991). The important difference between linear peptides and the cyclic peptide was that the latter appeared to induce antisera which recognized conformational determinants. Consequently, reconstruction of the protective epitopes which induce antibodies cross-reactive with the native protein and bactericidal for the meningococcus, must involve some stabilization of peptide conformation. The synthesis of cyclic peptides which mimic precisely the structure of the native antigenic determinant is time-consuming and experimentally difficult to achieve, so that the possible use of cyclic peptides for human vaccines poses significant problems. An alternative method for preparing chemically-defined immunogens with conformational stability is the multiple antigen peptide (MAP) system (Tam & Lu, 1991). The MAP system also provides a method for introducing defined Th-cell epitopes into the peptide immunogen. This obviates the need for covalent coupling of peptide B-cell epitopes to high molecular mass protein carriers such as keyhole limpet haemocyanin (KLH) to provide Th-cell activity. Furthermore, there are other major limitations associated with the conjugation of peptides to carrier proteins which the MAP system was designed to overcome. The peptide antigen represents only a minor fraction of the total molecular mass of the peptide–carrier protein conjugate, in contrast to the multiple copies of peptide assembled on the low molecular mass lysine core of a MAP. Also, the coupling procedures used in the production of peptide–carrier protein conjugates may alter the structural and immunogenic properties of the peptide. In addition, immunization with peptide–carrier protein conjugates may induce undesirable immune responses to carrier molecules and/or the cross-linking reagent. The MAP system, however, produces carrierless, chemically-defined peptide immunogens, and can be considered more likely candidates for human use. In fact, the MAP system has been used to prepare experimental peptide vaccines, many of which include Th-cell epitopes, against protozoa (Darcy et al., 1992; Calvo-Calle et al., 1993), schistosomes (Reynolds et al., 1994), chlamydia (Zhong et al., 1993), hepatitis B (Tam & Lu, 1989) and HIV (DeFoort et al., 1992b).

Initially, an octameric MAP containing the meningococcal P1.16b B-cell epitope TKNTNNNLTTL was found to be non-immunogenic in animals, highlighting the need for an effective Th-cell epitope to induce a humoral immune response. For the synthesis of a potential meningococcal MAP vaccine containing the B-cell epitope, use was made of a well-characterized 'universal' human Th-cell epitope comprising amino acids 830–843 from tetanus toxin (Panina-Bordignon et al., 1989). This Th-cell epitope has also been used in a MAP to enhance the immunogenicity of malarial B-cell epitopes in mice (Valmori et al., 1992). The meningococcal MAP was synthesized with the B-cell epitope at the N terminus of the assembled chain and the Th-cell epitope proximal to the tetrameric lysine core, i.e. as BT-MAP (Fig. 1b). This configuration of peptide presentation has been shown to be the most immunogenic for a B-cell epitope component (Tam et al., 1990). Incorporation of the Th-cell epitope into peptides significantly enhanced the humoral response of immunized animals towards the meningococcal B-cell epitope. The tetrameric BT-MAP was highly immunogenic, eliciting murine and rabbit antisera which reacted strongly in ELISA with peptides, and not only with OM from the homologous subtype P1.16b strain, but also with OM from the subtype P1.16a strain. The immunogenicity of BT-MAP may be due to a combination of factors. First, amino acid residues located at the N terminus and distal to the core matrix are usually the most antigenic, due to greater flexibility and accessibility (Lu et al., 1991). Also, since the density of the Th-cell epitope determines the concentration of the MHC-epitope on the surface of the antigen-processing cell, a large number of MHC proteins displaying their epitope simultaneously may amplify the subsequent T and B lymphocyte activation, proliferation and differentiation processes (Esposito et al., 1993). Structural studies have also shown that the synthesis of a 10mer T-cell epitope on a tetrameric MAP stabilizes peptide conformation, leading to an enhancement in the T-cell proliferation response (Esposito et al., 1993).

The specificity of antisera raised in mice and rabbits to peptide BT-MAP was also confirmed by epitope mapping. Antisera reacted specifically with the meningococcal P1.16b subtype B-cell epitope (180TKNTNNNLTTL), and also cross-reacted equally well with the P1.16a subtype. Therefore, immunization with BT-MAP induced polyclonal antisera which, in contrast to the P1.16 mAbs (McGuinness et al., 1993), reacted with peptides containing a minor variation in the subtype epitope. A surprising finding in this study was that both murine and rabbit antisera raised against BT-MAP did not react with the class 1 protein in Western blots. Antisera to synthetic peptides contain antibodies directed towards conformational and non-conformational structures adopted by the peptide. Since MAP peptides may be constrained in some manner, this would favour the predominance of con-
formational antibodies which may not react with denatured proteins in Western blots. However, these antisera also reacted strongly with linear peptides in M. natured proteins in Western blots. However, these non-reactivity with the denatured proteins may be that solid-phase assays with peptides detect a low proportion of non-formational antibodies which cannot be detected in Western blots, possibly because of a lower molar amount of available epitope in the immunoblotted proteins and the reduced sensitivity of the assay.

It is generally accepted that immunity to meningococcal infection correlates with bactericidal activity in vitro (Goldschneider et al., 1969), so that any candidate group-B meningococcal vaccine must have the ability to induce an effective complement-mediated bactericidal response (Frasch, 1985). Thus, the most important property of the antisera raised during the current study is that antisera from rabbits immunized with peptide BT-MAP were highly bactericidal for the homologous subtype P1.16b strain, MC58. Furthermore, these antisera also recognized and promoted bactericidal killing of the heterologous P1.16a strain, MC50. Thus, in contrast with the P1.16-specific mAbs (McGuinness et al., 1993), the polyclonal response to the BT-MAP is such that the protective effect is extended to a strain which shows an important variation within the subtype epitope. Since minor variations within subtype epitopes appear to be relatively common (Feavers et al., 1992; McGuinness et al., 1993), such a cross-protective effect would be an important property for a potential vaccine antigen. Despite the specificity of reactivity of antisera with OMs in ELISA, no unequivocal correlation could be drawn between the bactericidal activity of rabbit antisera for meningococci and the observed increases in OM titres. This finding is in accord with the conclusion of the previous study with cyclic peptides, that ELISA OM assays are not reliable indicators of protection, and reinforces further the importance of relevant biological assays such as bactericidal killing (Christodoulides et al., 1993).

Although rabbit and murine antisera raised to peptides BT and BT-MAP showed similar reactivities in ELISA and epitope mapping experiments, in contrast to rabbit antisera, murine antisera did not induce complement-mediated bactericidal killing of meningococci in vitro. Bactericidal activity is dependent not only on the amount, epitope specificity and high avidity of antibodies induced, but also on the class and ratio of complement-activating to non-complement-activating antibodies (Verheul et al., 1993). Murine antibodies of subclasses IgG2a, IgG2b, IgG3 and IgM are effective activators of complement, whereas IgA and IgG1 display little or no activity, with the latter also capable of inhibiting complement-activating antibodies (Ey et al., 1980; Neuberger & Rajewsky, 1981). The fact that murine antisera did not kill meningococci in the presence of complement may be in accord with the observation that IgG1 antibodies are also the predominant isotype induced in Balb/C mice by immunization with Freund's adjuvant (Hadjipetrou-Kourounakis & Moller, 1984).

In humans, immunization with a synthetic peptide immunogen must induce antibodies that are cross-reactive with the epitope in its native conformation. Also, the use of alternative non-toxic adjuvants with these peptide immunogens is essential, since Freund's adjuvant is unacceptable for human immunization. It is desirable that these non-toxic adjuvants preferentially elicit high-affinity antibodies of the correct isotypes which are effective in activating complement and so conferring protection. In addition, the humoral response is dependent on the presence of Th-cell epitopes which can be recognized promiscuously by human MHC class II molecules. Although a human tetanus toxin Th-cell epitope would be expected to induce an immune response in human vaccinees, it would not trigger specific meningococcal T-cell memory in immunized individuals who subsequently encounter meningococcal infection. Recently, Th-cell epitopes have been identified within the class I protein of N. meningitidis which are recognized by immune humans (Wiertz et al., 1992). Thus, information is now available which would permit the design of vaccines to include a protective meningococcal B-cell epitope together with Th-cell epitopes, recognized promiscuously by human MHC class II molecules. Certainly, the observation that a MAP containing defined B- and Th-cell epitopes induces a bactericidal response against group B meningococci, demonstrates the potential of such synthetic antigens as components of new meningococcal vaccines.

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REFERENCES


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