# Identification of T Cell Epitopes Occurring in a Meningococcal Class 1 Outer Membrane Protein Using Overlapping Peptides Assembled with Simultaneous Multiple Peptide Synthesis

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## Summary

The meningococcal class 1 outer membrane protein (OMP) plays an important role in the development of protective immunity against meningococcal infection, and is therefore considered to be a promising candidate antigen (Ag) for a meningococcal vaccine. The induction of an effective antibody response entirely depends upon T helper cells. To identify T cell epitopes of the OMP, we prepared 45 overlapping synthetic peptides representing the entire sequence of the class 1 protein of reference strain H44/76. Fully automated simultaneous multiple peptide synthesis (SMPS) was used to assemble the 45 twenty mer which overlapped by 12 amino acid residues on a 12 μmol scale. The peptides were tested for recognition by peripheral blood mononuclear cells (PBMC) obtained from 34 volunteers. Surprisingly, all synthetic peptides induced proliferative responses of PBMC isolated from one or more human histocompatibility leukocyte antigen (HLA)-typed immune adults. With PBMC from seven nonimmune donors, no proliferative response was observed. Immunodominant regions were found, recognized by PBMC from many volunteers, irrespective of their HLA type. Most of the immunodominant T cell epitopes are located outside the variable regions and, thus, will be conserved among different meningococcal (and gonococcal) strains. Furthermore, the overlapping peptides could be used to identify the epitopes recognized by OMP-specific T cell clones with known HLA restriction. It is interesting that the epitopes defined with the clones occur in highly conserved areas, shared by all neisserial porin proteins. In summary, this analysis of the T cell response to the meningococcal class 1 OMP constitutes a complete study of reactivity to a foreign protein, and illustrates some important features of Ag recognition by T cells. Our data demonstrate unexpected diversity in the T cell recognition of the OMP, and imply that the T cell repertoire against foreign Ag may be greater than previously assumed. This observation is supported by recent data on the interaction of peptide and major histocompatibility complex (MHC) class II, the latter being much less selective than MHC class I. Finally, a comparative analysis pointed out the limitations of algorithms predicting T cell determinants, and the importance of the empirical methodology provided by SMPS.

A vaccine against meningococci will aim at the induction of bactericidal and opsonic antibodies directed against capsular polysaccharides (CPS)[1], lipopolysaccharides (LPS) and outer membrane proteins (OMP) (1-3). In addition, LPS-neutralizing antibodies will contribute to the prevention of

septic shock. The effectiveness of these antibody responses depends on simultaneous activation of B and T cells by the vaccine (4, 5). Ig class switch, affinity maturation, magnitude of response, and induction of memory are regulated by T cells, although the molecular mechanisms involved in this regulation are largely unknown. The disadvantage of a lack of T cell activation can clearly be observed with vaccines containing purified groups A and C CPS. For reasons that are yet unknown, saccharide antigens fail to induce functional T cell help. As a consequence of this, the immunogenicity

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[1] *Abbreviations used in this paper:* CPS, capsular polysaccharides; FAB, fast atom bombardment; OMP, outer membrane proteins; SMPS, simultaneous multiple peptide synthesis; SI, stimulation index.

of these vaccines in infants is poor, and the immunity is of short duration (6, 7). This problem can be circumvented by conjugation of the saccharide epitopes to protein carrier molecules that provide the T cell epitopes (8–10).

In contrast to groups A and C CPS, the group B CPS is not immunogenic in humans (11). Alternative vaccine candidates are some OMP and LPS. Class 1 OMP especially induce antibodies that are very potent in a bactericidal assay and in an infant rat challenge model (3). The amino acid sequences of the protective B cell epitopes in the class 1 protein have been identified (12, 13). This sequence information is now being used to prepare synthetic peptides containing these epitopes. Furthermore, isolated or chemically synthesized core oligosaccharides of LPS can be used as protective B cell epitopes (14, 15).

Like the meningococcal groups A and C CPS, synthetic OMP B cell epitopes and protective saccharide epitopes of LPS have to be presented to the immune system in association with T cell epitopes to achieve an optimal response. Apart from the established concept of conjugating B cell epitopes with carrier proteins like tetanus or diphtheria toxoid, the use of homologous, i.e., in this case meningococcal, T cell epitopes has many advantages. Therefore, we have investigated recognition sites for human T cells in the meningococcal class 1 OMP.

In a previous paper (16) we described T cell recognition of class 1 OMP-derived peptides containing possible T epitopes, as predicted by the methods of Margalit et al. (17), and Rothbard and Taylor (18). In this study, we screened the entire sequence of the OMP for occurrence of T cell epitopes. For this purpose, a series of 45 peptides (20 mer with 12 overlapping amino acid residues; see Fig. 1) was assembled on micromolar scale using fully automated simultaneous multiple peptide synthesis (SMPS). The peptides were tested for recognition by PBMC obtained from HLA-typed volunteers. To investigate OMP T cell epitopes in detail and to define MHC restriction elements involved in T cell recognition, OMP-specific T cell clones were isolated from immune adults. The epitopes recognized by the clones could be identified using the overlapping peptides.

## Materials and Methods

*Volunteers.* A group of 30 healthy adults with negative anamnesis for meningococcal disease participated in this study. PBMC obtained from 27 individuals demonstrated a positive proliferative response to the isolated class 1 OMP (i.e., stimulation index (SI) >3, see below). Sera from these volunteers contained high antibody titers to the class 1 OMP (data not shown). PBMC from three adults and from the umbilical cord blood of four neonates, demonstrated no T cell response to the class I OMP. Sera from these nonresponders contained no anti-class 1 OMP antibodies. HLA typing of the volunteers was performed by standard National Institutes of Health lymphocytotoxicity test for HLA-ABC and by the propidium iodide method for HLA-DR and -DQ (19), using well defined sets of alloantisera and mAbs.

*Outer Membrane Protein.* The class 1 OMP was isolated from meningococcal reference strain H44/76 (B:15:P1.7,16, originally

obtained from Dr. E. Holten, Akershus Central Hospital, Nordbyhagen, Norway), as described earlier (20). Because it is very difficult to separate the class 1 protein from the class 2/3 protein, a mutant strain was used which lacks the class 2/3 OMP (strain HIII-5) (21).

*Synthetic Peptides.* Fig. 1 shows the amino acid sequence of the overlapping peptides 1–45. Peptides with uncharged termini, i.e., N-acetylpeptide amides, containing 20 amino acid residues (with the exception of peptide 45 which is a 21 mer) were prepared. The native Met-144 residue was replaced by isosteric Nle (X) in peptides 17 and 18. All sequences were assembled simultaneously using an automated multiple peptide synthesizer, equipped with a 48-column reaction block (AMS 422; ABIMED Analysen-Technik GmbH, Langenfeld, Germany), and $N^\alpha$-fluorenylmethoxycarbonyl (Fmoc)-protected (22, 23) amino acids (Novabiochem, Läufelfingen, Switzerland). The following side-chain protecting groups were used: tBu, *tert*-butyl ether (Ser, Thr, Tyr) or ester (Asp, Glu); Boc, *tert*-butyloxycarbonyl (Lys); Trt, triphenylmethyl (Asn, Gln, His), and Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Arg). The syntheses were performed on a 12 μmol scale. Thus, each column was charged with 33 mg of 4-(2′,4′-dimethoxyphenyl-Fmoc-amino-methyl)-phenoxy resin (polystyrene, cross-linked with 1% divinylbenzene, 100–200 mesh, functionalized to the extent of 0.37 mmol/g resin; Novabiochem). Cleavage of Fmoc-groups was effected with piperidine/N,N-dimethylacetamide (DMA), 2:8 (vol/vol), 2 × 5 min (basic reaction time in cycles 1–5, thereafter gradually increased to 12.5 min, an automated option of the AMS 422). Stock-solutions (0.67 M) of Fmoc-amino acids were prepared in high quality DMA, with three exceptions. Fmoc-Phe-OH and Fmoc-His(Trt)-OH were dissolved in N-methylpyrrolidone (NMP) and Fmoc-Arg(Pmc)-OH in DMA/NMP, 1:1 (vol/vol). A 0.77 M stock solution of the activating reagent 1-benzotriazolyloxy-tris-[N-pyrrolidino]-phosphonium hexafluorophosphate (PyBop) was prepared in DMA/NMP, 4:1 (vol/vol), and a 4.0 M stock solution of N-methylmorpholine (NMM) in DMA. In coupling steps, solutions of Fmoc-amino acid (75 μl, 50 μmol), PyBOP (65 μl, 50 μmol), and NMM (25 μl, 100 μmol), and an extra quantity (5 μl) of DMA were added to each column. The coupling time was 30 min in cycles 1–6, and was gradually increased to 60 min in further steps. Double couplings were performed in each cycle of the syntheses. Each Fmoc cleavage or double coupling was separated by six washings with DMA. The final Fmoc cleavage and DMA washings were followed by manual addition of 0.5 ml acetic anhydride/triethylamine/DMA, 5:1:44 (vol/vol/vol) to the columns. After 10 min, the resin-bound protected peptides were washed with DMA (3 × 0.5 ml), 2-methyl-2-butanol (2 × 0.5 ml), 2-methyl-2-butanol/acetic acid, 9:1 (vol/vol) (2 × 0.5 ml), 2-methyl-2-butanol (3 × 0.5 ml), and diethyl ether (3 × 3 ml), successively, and dried in vacuo. Side-chain deprotection of the peptides and cleavage from the resin was performed as proposed in reference 23. Peptides containing Arg (but not Trp) were deprotected with trifluoroacetic acid (TFA)/phenol, 95:5 (vol/vol); peptides containing Trp (but not Arg) with TFA/anisole/1,2-ethanedithiol (EDT), 95:2.5:2.5 (vol/vol/vol); peptides containing Arg and Trp with TFA/phenol/anisole/EDT, 94:2:2:2 (vol/vol/vol), and the remaining peptides with TFA/water, 95:5 (vol/vol). The appropriate TFA/scavenger mixture was added to the columns, containing the resin-bound protected peptides, at $t = 0$, 15, 30, 45, and 60 min (200 μl each time). The combined filtrates (from individual columns) were left at room temperature for an additional hour. Next, diethyl ether (10 ml) was added, and the suspensions obtained were cooled to −20°C. The peptide precipitates were collected by centrifugation (−20°C, 10 min, 6240 g),

washed with diethyl ether (3 × 10 ml), dissolved (or suspended) in 1 ml of acetic acid/water, 9:1 (vol/vol), and lyophilized.

The purity of the 45 peptides, as determined by analytical reverse phase HPLC (C$_8$-column; water/acetonitrile/trifluoroacetic acid, gradient from 99:1:0.1 to 30:70:0.1), varied between 50 and 90%. Small aliquots of peptides 9, 18, 27, 36, and 45 were purified by semi-preparative HPLC and subjected to fast atom bombardment (FAB) mass spectrometry. FAB mass spectra were recorded in the positive ion mode on a JEOL HX 110/HX 110 mass spectrometer, equipped with a standard FAB source (JEOL, Tokyo, Japan) operated at 3 kV. Spectra were obtained using a magnet scan rate of 35 s from m/z 1800 to 2600. The peptides were deposited on acid-etched stainless-steel probe tips with glycerol as matrix. In all cases, the protonated molecular ion (MH$^+$) was detected at the expected value of m/z (monoisotopic values found/calculated: 9, 2141.5/2141.1; 18, 2448.2/2448.2; 27, 2183.0/2183.1; 36, 2159.0/2159.1; 45, 2302.1/2302.2).

The crude peptides were used in immunological experiments. To obtain stock solutions, the peptides were dissolved in 1 ml of 6 M urea/tris(hydroxymethyl) aminomethane, pH 7.0, and diluted with culture media (see below) to a concentration of 0.44 mM. In the immunological experiments, a final peptide concentration of 5 $\mu$M was used.

*Proliferation Assays.* To investigate the presence of peptide-specific T cells in the peripheral blood, freshly isolated PBMC (10$^5$/well) were cultured in the absence or presence of antigen (6 $\mu$M) in 96-well roundbottomed microculture plates (150 $\mu$l/well). After 5 d, the cultures were pulsed with 0.5 $\mu$Ci of [$^3$H]thymidine. 16–18 h later, all 96 wells were harvested simultaneously with a 96-well sample harvester (Pharmacia/LKB, Turku, Finland). The complete filters containing 96 spots were placed in plastic bags. Scintillation liquid (Pharmacia/LKB) was added, and the bags were sealed. Radioactivity incorporated into DNA was determined with a Pharmacia/LKB 1205 Betaplate counter. The results obtained with six replicate cultures are expressed as the stimulation index (SI) which was calculated as the ratio of cpm obtained in the presence of Ag to the cpm obtained in the absence of Ag (media alone).

*Establishment of T Cell Clones.* PBMC were isolated by Ficoll-Isopaque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) gradient centrifugation. Cells were cultured in RPMI 1640 (Gibco Laboratories, Paisley, Scotland) supplemented with L-glutamine (2 mM), $\beta$-ME (10$^{-5}$ M), benzylpenicillin (100 IU/ml), streptomycin (100 $\mu$g/ml) and 10% heat-inactivated, pooled human AB serum. PBMC (10$^5$/well) were restimulated in vitro with antigen (10 $\mu$M) in 96-well roundbottomed microculture plates (150 $\mu$l/well). After 7 d, T cell blasts were cloned by limiting dilution (0.5 cells/well) in the presence of irradiated (25 Gy) autologous feeder cells (5 × 10$^4$ PBMC/well) that had been pulsed with antigen (10 $\mu$M) for 2 h. The cells were cultured in 96-well roundbottomed microculture plates in the presence of recombinant IL-2 (Boehringer Mannheim, Mannheim, Germany) (12 U/ml) in a volume of 150 $\mu$l. Growing cultures were expanded by restimulation with Ag-pulsed irradiated feeder cells (5 × 10$^4$ PBMC/well) every 2 wk and addition of human rIL-2 (2 U/well) weekly. To investigate the proliferative response of the clones, APC (5 × 10$^4$ PBMC/well) were preincubated with antigen (6 $\mu$M) or media (controls) in triplicate in 96-well roundbottomed microculture plates for 2 h. After irradiation (25 Gy) of the APC, 1–2 × 10$^4$ washed T cells were added to the wells. The cells were cultured in a volume of 150 $\mu$l/well for 3 d. 16–18 h before harvest, cultures were pulsed with [$^3$H]thymidine. Radioactivity incorporated into DNA was determined as indicated previously.

## Results

*Screening of the P1.7,16 Protein for Occurrence of T Cell Epitopes Using Series of Overlapping Peptides Spanning the Entire Sequence.* To identify T cell epitopes occurring in the menin-
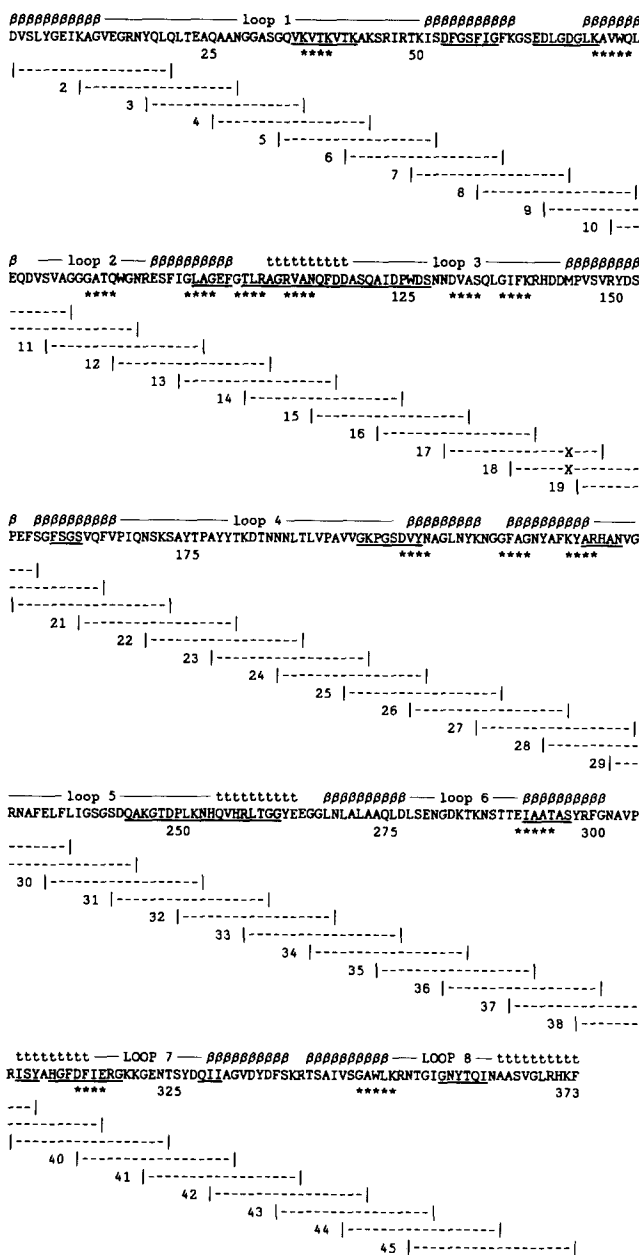


**Figure 1.** Amino acid sequence of the P1.7,16 class 1 OMP of *Neisseria meningitidis*, strain H44/76. Synthetic peptides and their code numbers are indicated. N-acetylpeptide amides, 20 mer with 12 overlapping amino acid residues, were prepared. Peptide 45 is a 21 mer. The native Met-144 residue was replaced by isosteric Nle (X) in peptides 17 and 18. Based on the putative model of the OMP (13), eight surface loops are represented. Protective antibodies have been mapped to loops 1, 4, and 5. Most of the *trans*-membrane regions (*tttt*, above the P1.7,16 sequence) occur as amphipathic $\beta$ sheets ($\beta\beta\beta\beta\beta$). T cell sites predicted by the AMPHI algorithm (17) are underlined. (*) T cell motifs described by Rothbard and Taylor (18).
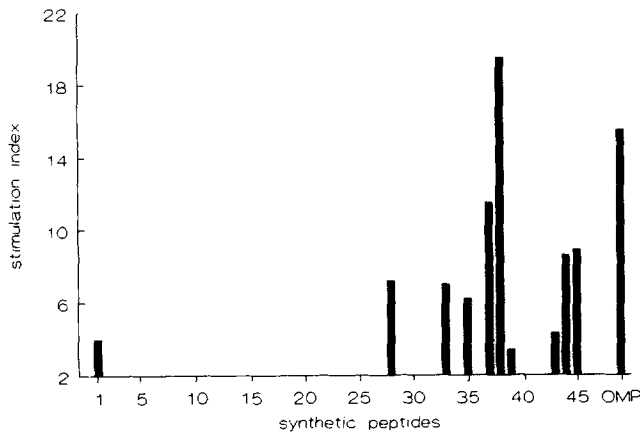
**Figure 2.** Proliferative response of PBMC isolated from an immune individual (donor 15) to 45 overlapping peptides spanning the entire sequence of the class 1 OMP. The peptides which consisted of 20 amino acid residues and overlapped by 12 residues are numbered according to Fig. 1. Proliferation was measured after 5 d of culture as indicated in Materials and Methods. Results of six replicate cultures are represented as the stimulation index (SI) which was calculated as (experimental cpm + Ag)/(control cpm − Ag). The background was used as the cutoff level twice (background proliferation: 379 ± 14 cpm). The response to the purified P1.7,16 protein is indicated as OMP.

gococcal class 1 OMP, a series of 45 peptides consisting of 20 mer with an overlap of 12 amino acid residues (Fig. 1) was tested for T cell recognition. Results of a representative experiment performed with PBMC from an immune individual are shown in Fig. 2. The positive responses to pep-

tides 1, 28, 33, 35, 37–39 and 43–45 indicate recognition of at least six different T cell epitopes by this donor. Results obtained with PBMC from 27 immune, HLA-typed adults are represented in Fig. 3. A proliferative response to the purified class 1 OMP was observed with PBMC from these volunteers (Fig. 3, far right). Sera from these donors displayed antibody titers to meningococcal antigens. A positive response was recorded to one or more peptides with PBMC from each immune donor. All peptides were recognized. Because of the large number of positive responses it is impossible to discover association of T cell recognition of a distinct peptide with the occurrence of a particular HLA haplotype. In three volunteers, no T or B cell response was seen to the class 1 OMP. With PBMC obtained from these nonimmune donors, no response was observed to any of the peptides. Because it is very difficult to find adults who lack immunity to meningococci, results obtained with lymphocytes isolated from umbilical cord blood from four neonates were included in this study as additional negative controls. With the neonatal PBMC, no proliferative response was observed to the class 1 OMP or any of the peptides (results not shown).

A stacked bar diagram of the positive responses that were observed to each of the peptides in the individual donors is shown in Fig. 4 A. Results obtained with a threshold of SI = 2 and SI = 3 were qualitatively the same. It is interesting that many positive responses were recorded for peptides corresponding to the COOH-terminal part of the OMP.

*Correlation of Predicted Structural Properties with T Cell Recognition of Synthetic Peptides.* The class 1 OMP is a cation-selective porin (21) which occurs in the outer membrane as
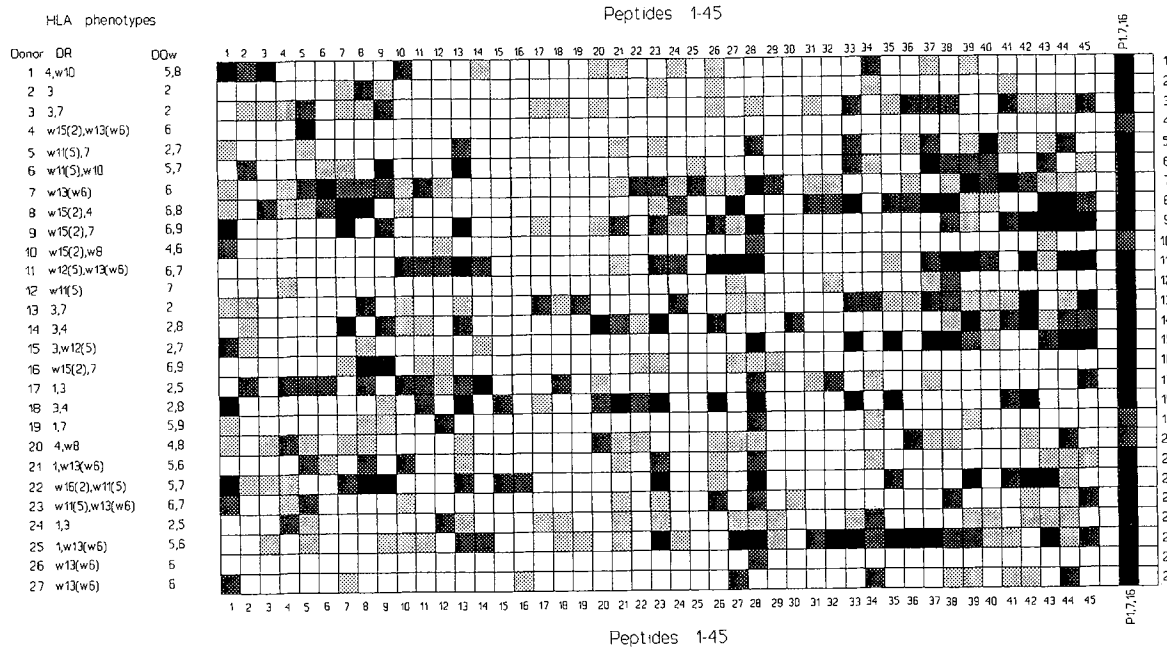


**Figure 3.** Schematic diagram of proliferative responses obtained with PBMC from HLA-typed immune adults to 45 overlapping peptides spanning the meningococcal class 1 OMP. The sequence of the 20 mer which overlapped by 12 residues is represented in Fig. 1. The HLA-DR and -DQ phenotypes of the donors are shown. Proliferative responses were measured after 6 d of culture as indicated in Materials and Methods. SI were calculated as (experimental cpm + Ag)/(control cpm − Ag). (*Open boxes*) SI <2; (*light stippled boxes*) SI = 2–3; (*dark stippled boxes*) SI = 3–5; (*solid boxes*) SI >5.

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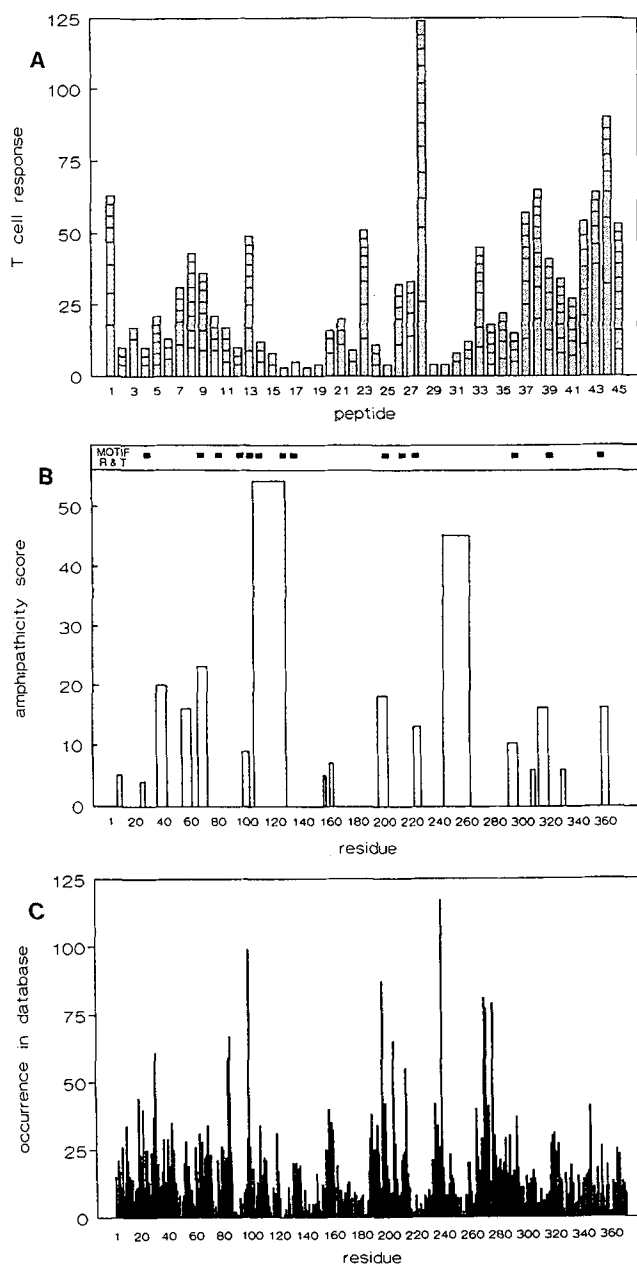
Figure 4. (A) Histogram representing the T cell responses to each of the 45 overlapping peptides spanning the meningococcal class 1 OMP. For each peptide, the stacked bar is a cumulation of SI (only SI ≥3) as obtained with PBMC from 27 immune volunteers. Individual responses are shown in Fig. 3. (B) Prediction of amphipathic α helices from the primary sequence of the H44/76 class 1 OMP using the computer program AMPHI (block length = 11; threshold = 4) (17). The amphipathicity score of each segment is shown. (Black boxes, top) Presence of T cell epitope motifs according to Rothbard and Taylor (18). (C) Tetrapeptide frequency profile of the H44/76 class 1 OMP. For each position along the sequence, the frequency of the local tetrapeptide is noted. The frequency was computed from the number of occurrences of this amino acid pattern within all known human somatic protein sequences (NBRF-PIR database). Regions corresponding to quadruples which do not occur in the human somatic reference set indicate putative T cell epitopes.

a trimer. *Trans*-membrane regions presumably occur as amphipathic β sheets. The protective B cell epitopes were found to be located on top of surface loops 1, 4, and 5 (12, 13). Amphipathic β sheets and surface loops are indicated in the primary sequence of the OMP (Fig. 1) to facilitate association with the overlapping peptides. Segments with high propensity to form amphipathic α helices, proposed to be related to T cell epitope formation, were predicted with the computer program AMPHI (17). Results of this analysis are shown in Fig. 4 B. Sequences containing predicted amphipathic α helices are underlined in the primary sequence of the P1.7, 16 protein (Fig. 1). The T cell epitope motif described by Rothbard and Taylor (18) (charged residue or glycine, followed by two or three hydrophobic residues and then polar residues or glycine) occurred 14 times in the OMP. These sequences are indicated with asterisks in Fig. 1. Amphipathic α helices do not always coincide with the Rothbard motif.

Finally, the degree of the OMP homologies with human somatic self sequences was evaluated. Previous investigations have suggested a coincidence between the occurrence of T cell epitopes and "rare" sequence patterns not found in the host somatic self proteins (25). The OMP sequence was analyzed as a series of single residue overlapping tetrapeptides and compared to a reference set of human somatic self protein sequences available in the NBRF-PIR database (release 29.0, June 1991). The catalog of tetrapeptide frequencies was calculated over this set of reference. The OMP sequence was represented by corresponding tetrapeptide frequencies (noted at positions 1, 2 etc. of the sequence). The graphical representation of the results (Fig. 4 C) allows the immediate location of putative T cell epitopes (i.e., regions of quadruples absent from the human somatic self reference set).

To evaluate the predictive value of the algorithms described by Margalit et al. (17), and by Rothbard and Taylor (18), the results were cross-classified into four groups: (a) T cell epitope was predicted; peptide was recognized; (b) T cell epitope was predicted; peptide was not recognized; (c) T cell epitope was not predicted; peptide was recognized; and (d) T cell epitope was not predicted; peptide was not recognized. The results were analyzed using a normal distribution that was found to be a fair approximation of the binomial distribution. Neither algorithm demonstrated a positive correlation with T cell recognition (tested at a 99% level of significance, $T_0$ ≤0.1). It is interesting that some association was found to exist between T cell recognition of a given peptide and occurrence of surface loops in the corresponding region of the OMP (tested at a 99% level of significance, $T_0$ = 4.1). *Trans*-membrane regions or β sheets did not occur more frequently in peptides that were found to contain T cell epitopes ($T_0$ ≤1.9). The prediction of "nonself" regions by comparing the class 1 OMP sequence with a catalog of all known human somatic self proteins was found to be indicative for some epitopes, but failed to predict others.

*Recognition of Overlapping Synthetic Peptides by OMP-specific T Cell Clones.* T cell clones were established from healthy adults with high antibody titers to the class 1 OMP. PBMC from these volunteers demonstrated a strong proliferative re-

sponse to the P1.7,16 protein. All class 1 OMP-specific T cell clones isolated were found to be of the helper phenotype. Analysis of surface molecules by flow cytometry revealed expression of CD3, CD4 and the TCR-$\alpha/\beta$ (results not shown). Clones AZ5.4 and AZ14.0 which had been selected with the peptide OMP(49–61) were found to recognize the overlapping peptides 6 and 7 (Table 1). Blocking experiments with mAbs against MHC class I/II molecules, as well as the use of a panel of HLA-typed APC, revealed HLA-DRw15(2) restriction of the AZ clones (16, and Wiertz et al., manuscript in preparation). The T cell clone JP5.3 was isolated from the peripheral blood of donor 6 using the P1.7,16 class 1 OMP. Out of the 45 overlapping peptides tested for recognition by this clone, peptide 13 induced a strong proliferative response. In addition, some proliferation was observed to peptide 12. Ag recognition by clone JP5.3 was HLA-DR10 restricted. The JV1 T cells that were isolated from donor 15 responded to peptides 38 and 39. In this case, recognition was found to be associated with HLA-DRw11(5). Clone JS17.4 responded to peptide 38 as well. However, this clone was HLA-DR1 restricted (16). Clone JS21.6 demonstrated a positive response to only one single peptide, i.e., peptide 45. In this case, antigen recognition occurred in the context of HLA-DR1 exclusively.

## Discussion

T cell recognition of a peptide antigen requires stabilization of the oligopeptide in the Ag binding groove of a MHC molecule. The TCR interacts (presumably via its CDR 3 region) with certain TCR-exposed side chains of the Ag. At the same time, the TCR binds to the $\alpha$ helices of the MHC molecule (presumably via the CDR 1 and 2 regions) (26). A peptide that contains the appropriate side chains to interact with the Ag-binding pocket(s) of a particular MHC molecule, will adopt its final conformation in the trimolecular complex with MHC and TCR. As a consequence of this, chemically assembled peptides lacking a prerequisite conformation can be used to study the trimolecular interaction of TCR, Ag, and MHC. This is in contrast with Ag recognition by Ig Fab regions which usually requires a defined secondary and tertiary structure of the epitope. In case of MHC class II–restricted T cell recognition, processing of the protein Ag by proteolytic enzymes usually precedes or coincides with the association of antigenic determinants and MHC. T cell epitopes may therefore occur throughout a protein and are not limited to surface-exposed regions. Consequently, fragmentation of a protein Ag is required to enable systematic identification of T cell epitopes. In vitro cleavage with proteolytic enzymes, as well as the construction of overlapping fragments by recombinant DNA methods, have led to successful identification of T cell epitopes in many proteins. However, a fruitful alternative is provided by SMPS which enables assembly of large numbers of overlapping peptides spanning the entire protein.

SMPS using polyethylene rods as the solid support was introduced by Geysen et al. (27). In its original form the

method has been used to detect the interaction of antibodies with synthetic peptides, which remain attached to the support ("Pepscan"). However, in T cell proliferation assays, free peptides are required. Recently, two procedures for cleaving the peptides from the rods were proposed (28–30). In both cases, small quantities (nanomolar range) of peptides with unnatural COOH-terminal modifications, i.e., an additional aspartyl residue (28), or an artificial diketopiperazine (29, 30), have been obtained. Diketopiperazine-extended peptides have been used for T cell epitope mapping of hen egg-white lysozyme (31). A thorough investigation of an antigen like the meningococcal class 1 OMP, using PBMC from a large panel of blood donors and numerous T cell clones, requires the availability on micromolar scale of overlapping peptides spanning the entire sequence. A method of SMPS which yields larger, i.e., micromolar, quantities of free peptides, has been developed by Houghton (32). In this approach, conventional solid supports are compartmentalized in permeable polypropylene bags (the "tea bag" approach). Finally, several multiple column systems for peptide synthesis on micromolar scale have been introduced. These systems can be operated manually (33) or automatically (34–37). Fully automated multiple-column synthesis is very suitable to support immunological research, and was used in the present study. A series of overlapping peptides spanning the entire sequence of the meningococcal OMP was prepared on 12 $\mu$mol scale. To minimize the risk of missing determinants, 20 mer were chosen which overlapped by 12 amino acid residues (Fig. 1).

Results of a representative proliferation experiment applying the synthesized overlapping 20 mer are shown in Fig. 2. Four individual peptides and two sets of adjacent peptides were capable of recalling proliferation of PBMC obtained from an immune donor. Thus, at least six T cell determinant regions could be defined. The results of proliferation experiments with PBMC from 27 immune donors (Figs. 3 and 4 A) revealed strong diversity of the T cell repertoire involved in recognition of the OMP. To a large extent, this will reflect the genetic polymorphism in the MHC of the population. In addition, the in vitro T cell response may be influenced by the relative frequency of OMP-specific precursors and the state of activation in the host before the experiment.

It is surprising that all 45 peptides were recognized by one or more volunteers. Thus, numerous T cell determinants must be present in one single protein. This finding can, at least in part, be explained by recent data on the interaction of peptide with MHC class II. In contrast to MHC class I, class II seems to bind peptides with low affinity in the processing compartment (fast-on/fast-off) (38, 39). This may allow the capture of a large variety of peptides. Indeed, characterization of naturally processed peptides eluted from MHC class II revealed the presence of different determinants of a single protein. In addition, several overlapping peptides corresponding to the same core determinant were identified (40). Even within a single T cell epitope, different ways of MHC binding may occur. For a peptide corresponding to amino acid residues 49–61 of the meningococcal class 1 OMP, putative MHC (and TCR) contact residues were elucidated using

**Table 1.** *Proliferative Response of Class 1 OMP-specific T Cell Clones to Overlapping Synthetic Peptides*

| Antigen | Resides | Peptide sequence | Recognition by T cell clones (SI) | |
|---|---|---|---|---|
| | | | AZ5.4 | AZ14.0 |
| Purified OMP | | – | 169 | 55 |
| Peptide 4 | 25–44 | QAANGGASGQVKVTKVTKAK | <2 | <2 |
| Peptide 5 | 33–52 | GQVKVTKVTKAKSRIRTKIS | <2 | <2 |
| Peptide 6 | 41–60 | TKAKSRIRTKISDFGSFIGF | 6 | 64 |
| Peptide 7 | 49–68 | TKISDFGSFIGFKGSEDLGD | 57 | 65 |
| Peptide 8 | 57–76 | FIGFKGSEDLGDGLKAVWQL | <2 | <2 |
| | | | | JP5.3 |
| Purified OMP | | – | | 103 |
| Peptide 11 | 81–100 | SVAGGGATQWGNRESFIGLA | | <2 |
| Peptide 12 | 89–108 | QWGNRESFIGLAGEFGTLRA | | 4 |
| Peptide 13 | 97–116 | IGLAGEFGTLRAGRVANQFD | | 63 |
| Peptide 14 | 105–124 | TLRAGRVANQFDDASQAIDP | | <2 |
| Peptide 15 | 113–132 | NQFDDASQAIDPWDSNNDVA | | <2 |
| | | | | JV1 |
| Purified OMP | | – | | 19 |
| Peptide 36 | 281–300 | GDKTKNSTTEIAATASYRFG | | <2 |
| Peptide 37 | 289–308 | TEIAATASYRFGNAVPRISY | | <2 |
| Peptide 38 | 297–316 | YRFGNAVPRISYAHGFDFIE | | 39 |
| Peptide 39 | 305–324 | RISYAHGFDFIERGKKGENT | | 31 |
| Peptide 40 | 313–332 | DFIERGKKGENTSYDQIIAG | | <2 |
| | | | | JS17.4 |
| Purified OMP | | – | | 7 |
| Peptide 36 | 281–300 | GDKTKNSTTEIAATASYRFG | | <2 |
| Peptide 37 | 289–308 | TEIAATASYRFGNAVPRISY | | <2 |
| Peptide 38 | 297–316 | YRFGNAVPRISYAHGFDFIE | | 13 |
| Peptide 39 | 305–324 | RISYAHGFDFIERGKKGENT | | 5 |
| Peptide 40 | 313–332 | DFIERGKKGENTSYDQIIAG | | <2 |
| | | | | JS21.6 |
| Purified OMP | | – | | 94 |
| Peptide 42 | 329–348 | IIAGVDYDFSKRTSAIVSGA | | <2 |
| Peptide 43 | 337–356 | FSKRTSAIVSGAWLKRNTGI | | <2 |
| Peptide 44 | 345–364 | VSGAWLKRNTGIGNYTQINA | | 2 |
| Peptide 45 | 353–372 | NTGIGNYTQINAASVGLRHK | | 68 |

APC were pulsed with Ag (6 $\mu$M peptide or 0.08 $\mu$M OMP) in 96-well microculture plates for 2 h. After irradiation, T cells were added to the wells. 3 d later, proliferation was measured as indicated in Materials and Methods. Results of triplicate cultures are expressed as SI calculated as (experimental cpm + Ag)/(control cpm − Ag).

a number of HLA-DR2-restricted OMP$_{(49-61)}$-specific T cell clones. Different modes of interaction with DR2 (and the clonotypic T cell receptors) were observed (Wiertz et al., manuscript in preparation). This also suggests that peptide-MHC class II binding is less stringent.

In the meningococcal class 1 protein, immunodominant regions were found, recognized by PBMC of most volunteers, irrespective of their HLA type. This observation can be explained in several ways. The 20 mer could accommodate several overlapping T cell epitopes, recognized in the

context of different restriction elements (41). Alternatively, one or more of the peptides may contain a single immunodominant T cell epitope that can interact with several or all MHC class II haplotypes (42–44). To define the MHC molecule(s) involved in presentation of a particular region of the meningococcal class 1 OMP, T cell clones were isolated from immune donors. The epitopes recognized by the T cell clones could be identified with the overlapping 20 mer (Table 1). The HLA restriction elements involved in presentation to the clones were elucidated using a panel of APC with known HLA specificity (i.e, PBMC, homozygous EBV-BC, L cell transfectants) (16, and Wiertz et al., manuscript in preparation).

Most of the immunodominant T cell determinants are located outside the variable regions which are limited to the protective surface loops 1, 3, and 5 (13). Consequently, these T cell epitopes will be conserved among different meningococcal (and gonococcal) strains (13, 45), indicating that they are common T helper antigenic sites. All epitopes defined with the (limited number of) T cell clones correspond to putative *trans*-membrane regions of the OMP. These regions, in particular, are highly conserved in all neisserial porins, including the meningococcal class 1 and 2 OMP and the gonococcal PIA and PIB proteins (13, 45). The T cell epitopes identified, in combination with different B cell neutralizing determinants, may be useful in the development of a synthetic or recombinant multivalent meningococcal (and gonococcal) vaccine.

A number of algorithms designed to predict T cell determinants were applied to the meningococcal class 1 OMP. The results were compared with the data obtained with the overlapping peptides in proliferation assays. Segments with high propensity to form amphipathic $\alpha$ helices, amphipathic $\beta$ sheets, putative nonself regions and T cell epitope motifs according to Rothbard and Taylor (18) defined some determinant regions, but missed others. There is some correlation between T cell recognition and the presence of surface loops in the corresponding region of the OMP. This observation may be explained in several ways. In vivo binding of antibodies to the OMP is limited to surface loops. At the site of infection,

antigen-specific B cells, that have been found to be very efficient antigen-presenting cells (46, 47), may selectively capture OMP surface loops from debris that contains (partially) degraded meningococci. In addition, surface loops may be more easily accessible to proteolytic enzymes during processing and may associate with MHC molecules more frequently.

Cleavage sites for proteolytic enzymes involved in antigen processing may be used as a predictive tool for the identification of potential MHC-binding sequences, and, thus, potential T cell epitopes (24). In the primary sequence of the meningococcal class 1 OMP, preferential cleavage sites for cathepsin D were found to precede several immunodominant T cell epitopes. Especially at the COOH terminus, a high frequency of putative cleavage sites for cathepsin D coincided with the presence of immunodominant regions.

In conclusion, prediction of MHC class II–restricted T cell epitopes is still intricate. Recently, some data have become available on primary sequence motifs present in MHC class I and II–bound peptides (40, 48–50). Analysis of the contents of MHC class I grooves revealed a number of anchoring residues at certain positions of the peptide antigens (48–50). Crystallographical data on peptide-loaded HLA-B27 molecules indicated the presence of nonamers bound in an extended conformation in the antigen-binding groove (51). In contrast, peptides eluted from MHC class II molecules showed no positions with conserved residues, whereas the length of the peptides eluted was found to vary from 13 to 17 amino acid residues (40). Overlapping peptides corresponding to the same core determinant were identified, having identical $NH_2$ termini, but varying in length. Similar observations were made when the MHC and TCR contact residues were identified for a peptide corresponding to amino acid residues 49–61 of the meningococcal class 1 OMP (Wiertz et al., manuscript in preparation). Until now, no consistent MHC class II anchoring residues, particular conformation, size, or sequence motifs have been identified. In summary, the most reliable approach to identify MHC class II–restricted T cell determinants within a protein will be a systematical screening using chemically synthesized overlapping peptides spanning the entire sequence.

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