

Characterization of a Conserved Helper-T-Cell Epitope from Group A Streptococcal M Proteins

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We have previously defined major histocompatibility complex (MHC) class II-restricted T-cell epitopes from the carboxy-terminal region of group A streptococcal type 5 M protein. In this report, T-cell responses to one of these epitopes have been characterized in detail. T-cell clones from recombinant M5-immunized mice and popliteal lymph node cells from peptide-immunized mice were used to show that sM5[300-319] is recognized in the context of I-A alleles of four of nine independent MHC class II haplotypes: I-A^d, I-A^f, I-A^k, and I-A^s. This epitope was also recognized by both helper (Th₂) and inflammatory (Th₁) subsets of murine T cells. The I-A^d-restricted epitope recognized by BALB/c mice was mapped to the 12-amino-acid peptide sM5[308-319] and was shown to provide helper function for an immunoglobulin G anti-peptide antibody response in BALB/c mice. Anti-peptide antibody was shown to be specific for M5[304-315] but failed to recognize intact rM5, suggesting that the conformation of the epitope differed between peptide and protein. However, the results demonstrate that overlapping epitopes can be the focus for both immunoglobulin G antibodies and the T cells which augment their production. Comparison of the available sequences for M proteins indicated that the T-cell epitope within M5[300-319] was highly conserved between M types and hence may elicit helper function for protective antibody responses to a wide range of M types. T-cell epitopes from conserved regions of M proteins which are recognized in the context of multiple MHC haplotypes have potential for the design of multivalent streptococcal vaccines.

M proteins are one of the major virulence factors expressed by *Streptococcus pyogenes*, as well as being the principal protective antigens recognized by the host (9, 15, 29). They form fibrillar α -helical coiled-coil dimers protruding from the surface of group A streptococci which mediate resistance to phagocytosis through their ability to bind mammalian proteins, including fibrinogen (26) and complement factor H (14), preventing effective opsonization by the alternative complement pathway. Sequence variability in the protruding amino-terminal halves of the molecules defines multiple M types, but the carboxy-terminal halves, which are located closer to the cell surface, are highly conserved between M types. Many M types elicit antibodies which cross-react with human fibrillar proteins such as cardiac myosin (7) and glomerular vimentin (18), and it has been suggested that these autoantibodies contribute to the pathogenesis of rheumatic fever (5) and poststreptococcal glomerulonephritis (30).

Effective protection against streptococcal infection is mediated by opsonic antibodies directed against antibody epitopes located in the amino-terminal half of M protein molecules, and attempts to develop M protein vaccines have been complicated because effective protection is predominantly M type specific (2). In addition, the existence of both protective and host cross-reactive epitopes within the same covalent structure requires that M protein vaccines are based on well-characterized, defined epitopes. Extensive efforts have been made in the past 10 years to identify and characterize M protein antibody epitopes to facilitate the design of M protein-based streptococcal vaccines (16). However, until recently the study of antigen-specific T-cell responses to M proteins was limited (3, 8). We have identified

multiple epitopes recognized by T cells of mice immunized with purified, recombinant type 5 M protein (rM5). The responses of rM5-specific, major histocompatibility complex (MHC) class II-restricted T-cell clones to synthetic peptides representing most of the M5 sequence identified at least 13 distinct T-cell recognition sites, distributed throughout both variable amino-terminal and conserved carboxy-terminal regions of M5 (28). Helper-T-cell epitopes located in the carboxy-terminal region of M5 which were conserved between M types could be particularly useful in the design of multivalent, defined-epitope M vaccines. The aim of the present study was to characterize in detail responses to a major carboxy-terminal MHC class II-restricted T-cell epitope from M5.

MATERIALS AND METHODS

Synthetic peptides and rM5. The synthetic peptides representing the M5 sequence 300 to 319 and 15 to 33 were synthesized on an Applied Biosystems (Warrington, United Kingdom) automated peptide synthesizer (model 431A) by using f-moc chemistry and a Beckman 990 peptide synthesizer by using solid-phase t-boc chemistry, respectively. The composition and purity of the peptides were confirmed by amino acid analysis and high-performance liquid chromatography, respectively. Purified peptides were dialyzed against phosphate-buffered saline, filter sterilized, and stored in aliquots at -80°C . Peptides sM5[300-312], -[304-315], and -[308-319] were synthesized by the pin synthesis technique (19) to yield pin-bound as well as cleaved peptides of 12 amino acids in length, overlapping by 8 amino acids. The cloning and expression of the M5 determinant from type 5 group A streptococcal strain Manfredo and the sequence of the cloned gene have been described previously (17, 22). The rM5 was purified to $>95\%$ homogeneity as judged by sodium

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dodecyl sulfate-polyacrylamide gel electrophoresis, from *Escherichia coli* LE392 expressing the cloned M5 molecule, as described previously (28).

Mice and immunization. CBA/Ca (A^kE^k) and C57BL/6 (A^bE^b) mice were purchased from Bantin and Kingman, Hull, United Kingdom; B10.A(4R) ($A^kE^{b/k}$), B10.D2 (A^dE^d), B10.M (A^fE^f), B10.RIII(71NS) (A^fE^f), B10.S (A^sE^s), DBA/1 (A^gE^g), C3H.NB (A^{pEp}), and NZW (A^zE^z) mice were purchased from Olac 1976 Ltd., Cirencester, United Kingdom; and B10.GD ($A^dE^{d/b}$) mice were obtained from the National Institute for Medical Research, London, United Kingdom. B10.A(4R), B10.GD, B10.M, B10.S, C57BL/6, and DBA/1 mice fail to express cell surface I-E molecules (21). BALB/c mice ($H-2^d$) were bred, and all mice were maintained, by the Comparative Biology Centre, University of Newcastle upon Tyne, United Kingdom. For T-cell proliferation assays, groups of mice were footpad immunized once with 25 μ g of rM5 or 100 μ g of synthetic peptide emulsified in complete Freund's adjuvant (FCA; Difco Laboratories, Detroit, Mich.). To measure antibody responses, groups of mice were immunized subcutaneously in the flank with 100 μ g of synthetic peptide in FCA and boosted 12 days later with 100 μ g of peptide in incomplete Freund's adjuvant (FIA; Difco). Mice were bled 12 days later, and serum was stored at -20°C .

rM5-specific T-cell clones. Seven days after footpad immunization, rM5-specific T cells were cloned directly by limiting dilution from popliteal lymph node cells (PLN) as described previously (28). All culture was carried out by using RPMI 1640 (GIBCO Ltd., Paisley, Scotland) supplemented with 3 mM glutamine, 10% fetal bovine serum (Sigma Chemical Co., Poole, Dorset, United Kingdom), and 5×10^{-5} M 2-mercaptoethanol. Clones were maintained by restimulation with rM5 and irradiated spleen cells every 2 to 3 weeks in 24-well plates (Costar; NBL Ltd., Cramlington, Northumberland, United Kingdom). After each restimulation, T-cell blasts were expanded in medium containing 5% of supernatant from concanavalin A-stimulated rat spleen cells for 7 days and maintained in 2% supernatant thereafter.

T-cell proliferation assay. Seven days after immunization, PLN were assayed in round-bottom 96-well plates (Costar) by using 2×10^5 cells per well for 72 h at 37°C in a humidified CO_2 incubator. T-cell clones were assayed in flat-bottom 96-well plates (Costar) by using 2×10^4 cells per well with 7.5×10^5 20-Gy-irradiated syngeneic spleen cells for 48 h. In each case, triplicate wells were pulse-labelled with 15 kBq of tritiated thymidine (TRA 310; specific activity, 74 GBq/mmol; Amersham International plc, Amersham, Bucks, United Kingdom) for the final 4 h of culture and harvested onto glass fiber, and radioactivity was quantitated by scintillation spectroscopy. Responses to the small quantities of peptides made by the pin synthesis technique were performed as above, except that cloned T cells were cultured in inverted 60-well Terasaki plates (Nunc, GIBCO Ltd.) with 5×10^4 irradiated spleen cells and pulsed with 60 kBq of tritiated thymidine. Results are expressed as mean disintegrations per minute \pm standard errors of the mean (SEM) of triplicate wells, taking the peak response from a titration of peptide at doses of 20, 4, 0.8, and 0.16 μ g/ml or rM5 at 10 or 20 μ g/ml. PLN responses were considered positive if disintegrations per minute in the presence of antigen was twofold or more above disintegrations per minute of PLN cells alone.

Cytokine assay. T-cell clones (10^6 cells per well) were stimulated with 25 μ g of rM5 per ml and 7.5×10^5 irradiated spleen cells in 1 ml of medium for 24 h, and supernatants were removed and stored at -20°C . Supernatants were

titrated in twofold dilutions into wells containing 5×10^3 CTLL-2 indicator cells, incubated for 20 h (including a pulse of tritiated thymidine for the final 4 h), and prepared for scintillation spectroscopy as described above. The dilution of supernatant giving the equivalent of 50% maximum CTLL-2 proliferation was used in a subsequent assay of CTLL-2 cells in the presence of a range of dilutions of monoclonal anti-interleukin-2 (IL-2) or anti-IL-4 (saturated ammonium sulfate-precipitated culture supernatants from hybridomas S4B7 and 11B11, respectively).

Antibody assay. Antibodies were measured by enzyme-linked immunosorbent assay (ELISA) with 96-well flexible assay plates (Falcon catalog number 3912; A & J Beveridge, Newcastle upon Tyne, Tyne and Wear, United Kingdom) coated overnight at 4°C with 0.5 μ g of rM5 per ml or 5 μ g of synthetic peptide per ml in buffer A (15 mM Na_2CO_3 , 35 mM NaHCO_3 , 3 mM NaN_3 [pH 9.3]) and washed four times in buffer B (200 mM Tris-HCl, 190 mM NaCl, 0.05% Tween 20 [pH 7.4]). Plates were then treated with 5% bovine serum albumin (BSA) in PBS (BSA-PBS) for 20 min at room temperature and washed four times in buffer B. Sera were diluted twofold in BSA-PBS from 1:200 to 1:6,400, and 100 μ l was added to duplicate wells and incubated for 45 min at room temperature and washed four times in buffer B. Plates were then incubated for 30 min at room temperature with 100 μ l (at 1:3,200) of goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Southern Biotechnology, Lab Impex Ltd., Middlesex, United Kingdom) and washed four times in buffer B, before incubation for up to 15 min in the dark at room temperature with 75 μ l of substrate (0.4 mg of *o*-phenylene diamine- H_2O_2 per ml) in buffer C (24.5 mM citric acid monohydrate, 52 mM Na_2HPO_4 [pH 5.0]). The reaction was stopped by adding 75 μ l of 20% H_2SO_4 , and A_{492} was measured by using a microtiter plate reader (MR5000; Dynatech Ltd., Billingshurst, Sussex, United Kingdom).

RESULTS

MHC restriction phenotype and fine specificity of rM5-specific T-cell clones. We have previously shown that a significant proportion of rM5-specific T-cell clones from BALB/c and CBA.Ca mice immunized with rM5 in FCA recognized the synthetic peptide M5[300-319] *in vitro* (28). The MHC restriction phenotype of BALB/c and CBA M5[300-319]-specific T-cell clones was determined by using spleen cells from intra H-2 recombinant mice as antigen-presenting cells (Fig. 1). The ability of B10.GD spleen cells to present rM5 indicated that all seven BALB/c M5[300-319]-specific clones were A^d restricted, whereas clone X5, which is specific for a different M5 peptide, was E^d restricted (Fig. 1a). Similarly, presentation by B10.A(4R) spleen cells indicated that all seven CBA.Ca M5[300-319]-specific clones were A^k restricted and clone 2, which is specific for a different M5 peptide, was E^k restricted (Fig. 1b). The results were supported by blocking experiments using anti-MHC class II antibodies, but, in general, responses were only partially blocked by the appropriate antibody at antigen doses inducing 50% maximum proliferation (data not shown).

Supernatants from a number of BALB/c T-cell clones activated with rM5 and irradiated spleen cells were typed for their cytokine profile by the ability of anti-IL-2 or anti-IL-4 antibodies to block supernatant induction of proliferation in the CTLL-2 indicator cell line. The results indicated that clones X9, Y2, and Y8 produced IL-2 but little or no IL-4

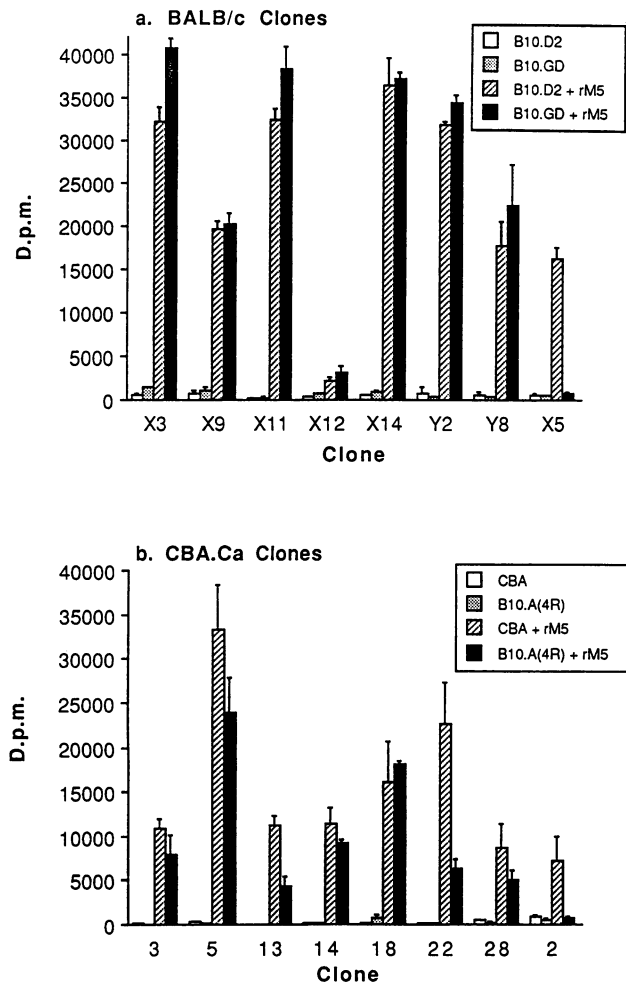


FIG. 1. MHC restriction phenotype of rM5-specific T-cell clones. T-cell clones were stimulated with 10 μ g of rM5 per ml in the presence of irradiated spleen cells from B10.D2 (A^dE^d) or B10.GD ($A^dE^{d/b}$) for BALB/c clones (a) and CBA.Ca (A^kE^k) or B10.A(4R) ($A^dE^{b/k}$) for CBA clones (b) in proliferation assays as described in Materials and Methods. B10.GD and B10.A(4R) mice lack cell surface expression of I-E molecules. Results are shown as mean disintegrations per minute \pm SEM of triplicate cultures for all available M5[300-319]-specific clones, and each figure includes a control clone against a different rM5 peptide. One of the control clones (clone X5) is E^d restricted, and the other (clone 2) is E^k restricted, both being specific for an M5 peptide other than sM5[300-319].

and were presumed to be of the Th_1 phenotype, whereas clone X12 produced IL-4 but little or no IL-2 and was considered to be of the Th_2 type (Fig. 2). From this limited amount of data it can be concluded that M5[300-319] is recognized by both Th_1 and Th_2 subsets of helper-T cells.

The fine specificity of BALB/c M5[300-319]-specific clones was mapped by using a limited range of peptides of 12 amino acids in length (Fig. 3). The three clones tested responded equally well to rM5, sM5[300-319], and only one of the shorter peptides, indicating the presence of a common epitope between residues 308 and 319. A clone specific for a different region of M5 responded to none of the peptides.

PLN responses to synthetic peptides from M5. In previous studies, the peptide sM5[300-319] failed to induce prolifera-

tion in PLN cells from rM5-immunized mice (28) despite being a predominant epitope recognized by rM5-specific T-cell clones in vitro. Therefore, BALB/c mice were immunized with 100 μ g of synthetic peptides to determine their antigenicity in vivo. Groups of BALB/c mice were immunized with sM5[300-319], intact rM5, or another M5 peptide, sM5[15-33], and PLN cells were assayed for proliferation in vitro (Fig. 4). PLN cells from peptide-immunized mice responded specifically to the immunizing peptide and to rM5, indicating that BALB/c mice possess a significant number of M5[300-319]-specific T cells and M5[15-33]-specific T cells which recognize processed rM5 protein. However, the response of PLN cells from rM5-immunized mice to sM5[300-319] was not detectable, as shown previously, whereas sM5[15-33] induced significant responses, as achieved previously with the longer peptide M5[1-35] (28).

Previous experiments established that PLN cells from rM5-immunized mice of C57BL/6 ($H-2^b$), BALB/c ($H-2^d$), B10.M ($H-2^f$), CBA.Ca ($H-2^k$), B10.RIII ($H-2^r$), B10.S ($H-2^s$), C3H.NB ($H-2^p$), DBA/1 ($H-2^q$), and NZW ($H-2^r$) responded in proliferation assays to rM5 in vitro (28; unpublished data). To investigate which of these MHC haplotypes bound and presented the T-cell epitope within M5[300-319], the same strains of mice, representing nine independent MHC haplotypes, were immunized with sM5[300-319] in FCA, before PLN proliferation assays in vitro (Fig. 5). PLN cells from sM5[300-319]-immunized B10.M, B10.S, and BALB/c mice responded well to sM5[300-319] and also recognized rM5. C3H.NB responded to rM5 but not sM5[300-319], whereas the remaining five strains of mice responded poorly or not at all to either sM5[300-319] or rM5. The results indicate that T cells from at least four of nine MHC haplotypes of mice tested recognized a T-cell epitope within M5[300-319]. T-cell responses from B10.M and B10.S, which lack cell surface I-E expression (21), were presumed to be $I-A^f$ and $I-A^s$ restricted, respectively.

Antibody responses to synthetic peptides from M5. Groups of BALB/c mice were immunized with synthetic peptides in FCA and boosted with the same peptide in FIA to determine whether T-cell recognition sites within the peptide could provide help for anti-peptide antibody responses. IgG was measured by ELISA in sera from individual mice immunized with sM5[300-319], sM5[15-33], or rM5 (Fig. 6). Antibody binding to sM5[300-319] (Fig. 6a), sM5[15-33] (Fig. 6b), or rM5 (Fig. 6c) was measured for each serum sample. Serum samples from sM5[300-319]-immunized mice contained IgG specific for M5[300-319], which did not bind rM5 or sM5[15-33], whereas sM5[15-33]-immunized mice made antibody against sM5[15-33], which also reacted with rM5. These results show that each peptide, unlinked to a carrier, induced a specific antibody response, indicating that the T-cell recognition site on the peptide could function as a helper-T-cell epitope for an antibody response directed against an antibody epitope within the same peptide. In addition, sera from rM5-immunized mice contained rM5-specific antibodies that did not react with sM5[15-33] or sM5[300-319], suggesting that antibody epitopes in other regions of the intact antigen are immunodominant.

The antibody epitope within M5[300-319] was mapped by using peptides synthesized on pins. Sera from sM5[300-319]-immunized mice were shown to bind specifically to M5[304-315] (mean absorbance, 0.41) and not sM5[300-319], sM5[308-319], or, as a control, several of 12 amino acid peptides from a different protein (respiratory syncytial virus F protein) (mean absorbance, all <0.1). Also, a mouse serum

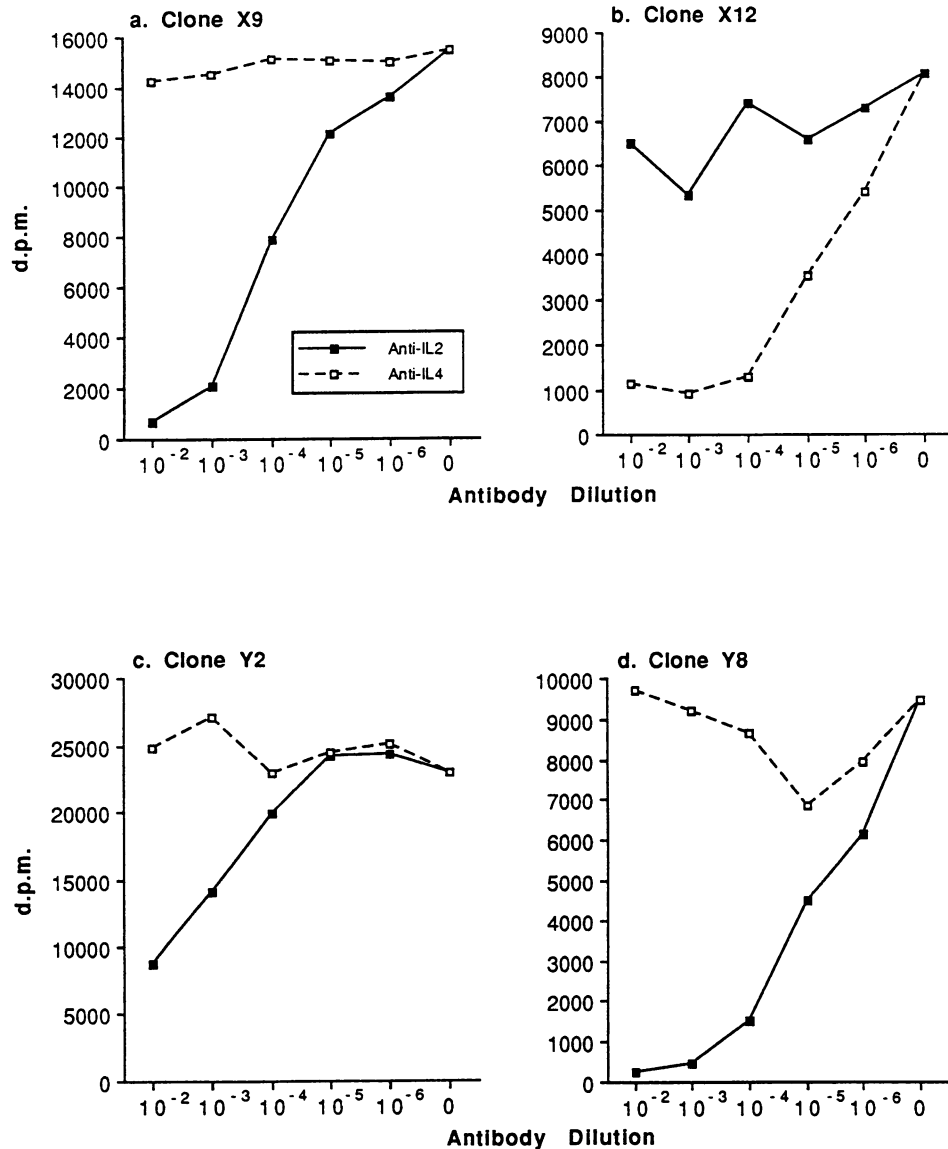


FIG. 2. Cytokine profiles of M5[300-319]-specific T-cell clones. CTLL-2 indicator cells were cultured in the presence of supernatants from BALB/c M5[300-319]-specific T-cell clones X9 (a), X12 (b), Y2 (c), and Y8 (d). Proliferation of 5×10^3 CTLL-2 cells was measured (in disintegrations per minute) in the presence of the range of dilutions of S4B6 anti-IL-2 or 11B11 anti-IL-4 antibodies shown ($10^{-2} = 1:100$). Supernatants were generated by culture of 10^6 cloned T cells in 1 ml of culture medium containing $25 \mu\text{g}$ of rM5 per ml and 7.5×10^5 irradiated spleen cells for 24 h. Blocking proliferation with S4B6 or 11B11 indicated the presence of IL-2 or IL-4, respectively.

raised against a different protein (respiratory syncytial virus F protein) failed to bind to any of the M5 peptides (mean absorbance, all <0.1). These results suggest that the antibody epitope of M5[300-319] maps to within M5[304-315] and overlaps the T-cell epitope located within M5[308-319] on the same peptide.

The M5 sequence 300 to 319 is conserved in many M proteins. Alignment of the amino acid sequence of M5[300-319] with the homologous regions of the remaining five published M protein carboxy-terminal sequences is shown in Table 1. A region of complete identity is found in M6, M12, M24, and M57, and a very similar region is located in M49, indicating that the T-cell epitope within M5[300-319] is conserved between many M types.

DISCUSSION

The expression and purification of rM5 (22) and rM6 (13) proteins of group A streptococci have led to the identification of immune recognition sites throughout the entire length of these molecules. In M5 and M6, antibody epitopes have been mapped to several regions, in both variable and conserved halves of the molecules (2, 10, 29). However protective, opsonic antibodies are M type specific and are directed predominantly towards the amino-terminal half of M proteins (10, 29). In contrast, multiple T-cell epitopes have been located in both variable amino-terminal and conserved carboxy-terminal halves of M5 (28), and it can be predicted that epitopes in the carboxy-terminal regions of M proteins will

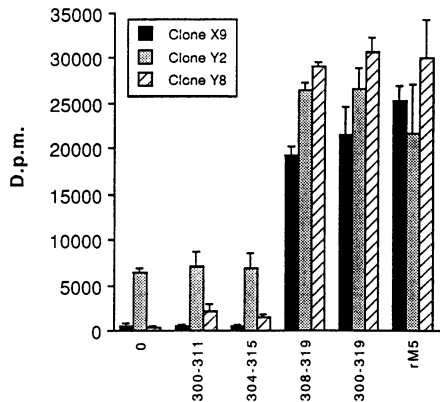


FIG. 3. Fine specificity of rM5-specific BALB/c T-cell clones. Cloned BALB/c T cells were cultured with irradiated BALB/c spleen cells and 10 μ g of rM5 per ml or 20 μ g of peptide per ml in 20- μ l hanging drop cultures in inverted 60-well Terasaki plates as described in Materials and Methods. Results are shown as mean disintegrations per minute \pm SEM of triplicate cultures. A BALB/c M5[15-33]-specific clone was included in this experiment, and dpm of 909 or lower was recorded in wells containing the peptides shown, whereas rM5 and sM5[15-33] induced responses of 39,762 and 37,583 dpm, respectively.

be shared between different M types. However, the conserved carboxy-terminal region of M proteins is located below the cell wall on intact streptococci (25), and it is not known whether T-cell epitopes from this region can be processed and presented to helper T cells and whether these T cells could then augment protective antibody responses directed against the amino-terminal half of the M protein. We have previously localized T-cell recognition in BALB/c mice to at least two distinct synthetic peptides from the carboxy-terminal half of M5, in addition to six synthetic peptides from the amino-terminal half (28). One of the carboxy-terminal peptides, sM5[300-319], was also recognized by T cells of CBA mice. The aim of the present study was to characterize the T-cell epitope(s) within M5[300-319].

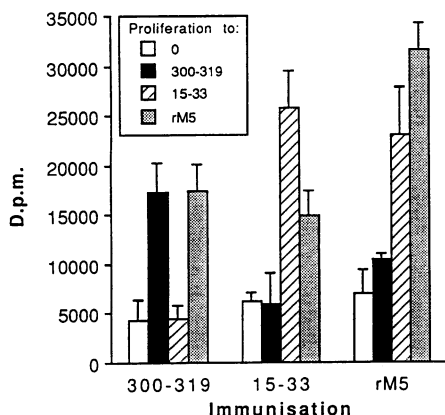


FIG. 4. PLN responses after immunization with synthetic peptides of M5 in vivo. PLN proliferation responses of BALB/c mice immunized with FCA and 25 μ g of rM5 or 100 μ g of synthetic peptide sM5[300-319] or sM5[15-33]. Results from a representative mouse from groups of three are shown as mean disintegrations per minute \pm SEM, taking the peak response following in vitro stimulation with a range of doses of each peptide or 20 μ g of rM5 per ml.

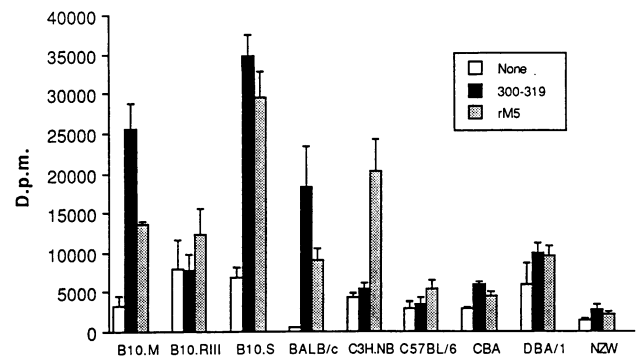


FIG. 5. PLN responses of T cells from sM5[300-319]-immunized mice of different MHC haplotypes. PLN proliferation responses of B10.M (H-2^f), B10.RIII (H-2^r), B10.S (H-2^s), BALB/c (H-2^d), C3H.NB (H-2^p), C57BL/6 (H-2^b), CBA.Ca (H-2^k), DBA/1 (H-2^q), and NZW (H-2^r) mice immunized with 100 μ g of sM5[300-319] in FCA. Results from a representative mouse are shown as mean disintegrations per minute \pm SEM, taking the peak response following in vitro stimulation with a range of doses of sM5[300-319] or 20 μ g of rM5 per ml.

The MHC restriction phenotype of peptide-specific T-cell clones was investigated. Antigen presentation by spleen cells from intra H-2 recombinant mice demonstrated that BALB/c and CBA.Ca T-cell clones were A^d and A^k restricted, respectively. The MHC restriction phenotype was also confirmed by the ability of monoclonal anti-MHC class II antibodies to block proliferation of T-cell clones. However, complete blocking by antibodies was not achieved, suggesting that the affinity of peptide 300 to 319 for A^d and A^k molecules and/or the T-cell receptor was relatively high. Also, PLN proliferation responses elicited from sM5[300-319]-immunized B10.M and B10.S mice were likely to be A^f and A^s restricted, respectively, as these strains fail to express cell surface I-E molecules (21). Thus, sM5[300-319] binds to at least four distinct alleles of I-A. The poor immunogenicity of sM5[300-319] in CBA.Ca mice was unexpected considering the frequency of M5[300-319]-specific T-cell clones derived from CBA.Ca mice (28). The observation that PLN from peptide-immunized C3H.NB mice proliferated in response to rM5, but not to sM5[300-319], suggested that the free peptide was further degraded in vitro by antigen-presenting cells of this strain of mice, since the response to processed rM5 indicated that the immunization against M5[300-319] was successful.

The fine recognition specificity of a limited number of BALB/c M5[300-319]-specific T-cell clones was localized to the 12-amino-acid peptide 308 to 319. This sequence appears once only in M5, despite the large proportion of the molecule represented as regions of repeated sequence (9). This epitope was located adjacent to the C repeats of M5, and a region of identical sequence is observed in M6, M12, M24, and M57, after partial sequence alignment. A closely similar sequence was also found in M49, which has been shown to belong to a structurally distinct class of M protein (4). Therefore, a potential T-cell epitope within M5[300-319] is conserved in all the M proteins which have been sequenced to date. T cells from sM5[300-319]-immunized mice proliferated in vitro to rM5, as well as to the immunizing peptide, indicating that antigen processing of the intact M5 molecule leads to presentation of the M5[300-319] epitope. Similar experiments using other M types would be required to demonstrate that the M5[300-319] epitope was generated

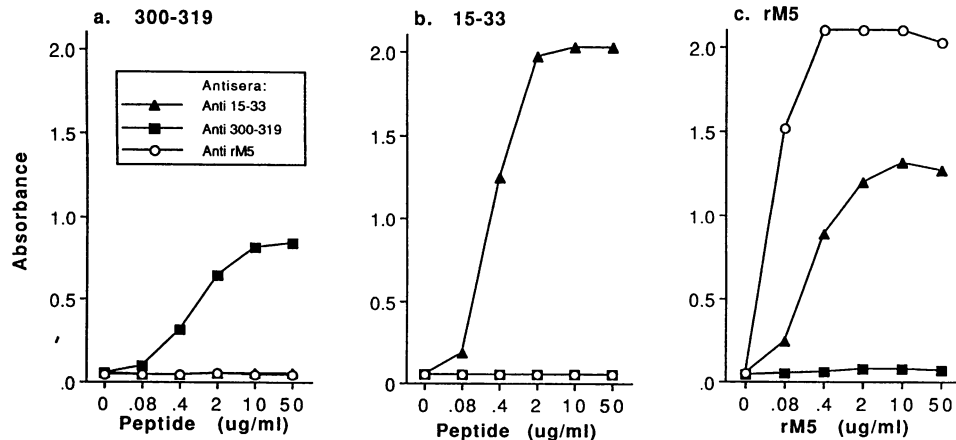


FIG. 6. Synthetic peptides of M5 induce antibody responses in vivo. ELISA of sera from representative BALB/c mice immunized in FCA and boosted in FIA with 100 μ g of peptide sM5[300-319] or sM5[15-33] or with 25 μ g of rM5. IgG binding to plates coated with sM5[300-319] (a), sM5[15-33] (b), or rM5 (c) at the doses shown were measured as described in Materials and Methods. Results are expressed as absorbance for a serum dilution of 1:200 against the range of antigen doses shown.

after antigen processing of M6, M12, M24, or M57. However, this seems likely because the carboxy-terminal regions of M proteins are very similar, and the sequence for a T-cell epitope has been successfully incorporated into a different protein without affecting subsequent processing and presentation to specific T cells (6).

Mice immunized with free peptide sM5[300-319] in adjuvant made antibody responses specific for an epitope between 304 and 315, indicating that the peptide contained a helper-T-cell epitope which augmented the antibody response to an epitope located on the same peptide. Apart from demonstrating the helper function of the epitope within M5[300-319], presumed to be the one localized to 308 to 315, these results show that overlapping epitopes can be the focus for both IgG antibodies and the T cells which augment their production. In addition, the results represent a further example of overlapping T- and B-cell epitopes, as shown for several immunogenic regions of the hemagglutinin of influenza A virus (1). It was also shown that the M5[300-319] epitope was recognized by both Th₁ and Th₂ subsets of MHC class II-restricted T-cell clones. Mosmann and colleagues have shown that antigen-specific mouse CD4⁺ T-cell clones exist in two subsets distinguished by patterns of lymphokine secretion: Th₁ cells secrete IL-2 and gamma interferon but not IL-4 and IL-5, whereas Th₂ cells secrete IL-4 and IL-5 but not IL-2 or gamma interferon (23). Furthermore, Th₂ cells are thought to be the predominant subset which aug-

ment IgG1 antibody responses, whereas the Th₁ subset augment IgG2a antibody and delayed-type hypersensitivity responses in mice (23). In addition, it was shown that anti-sM5[300-319] serum did not react with rM5 in ELISA, suggesting that the conformation of free peptide recognized in vivo differed from that on the intact rM5 molecule. However, anti-M5[15-33] serum bound both sM5[15-33] and rM5, indicating that the conformation of the M5 epitope recognized was preserved in this free peptide.

It has been reported that M proteins have the properties of superantigens (31, 32), with important consequences for the understanding of streptococcal virulence. However, type 5 M proteins cleaved from the surface of streptococci with pepsin (pepM5 fragments) were used in these studies, and it has subsequently been shown that the superantigenicity of pepM1 and pepM5 preparations is due to contamination with streptococcal pyrogenic exotoxins (11).

In conclusion, M5[300-319] includes a helper-T-cell epitope conserved between several M types which was shown to be presented by I-A molecules to different subsets of helper-T cells from multiple MHC haplotypes. Therefore, a helper-T-cell epitope which has potential use in the design of multivalent M protein-based streptococcal vaccines has been defined. We are currently developing such vaccines for evaluation in animal infection models.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

After submission of this paper, we became aware that others have reported conserved epitopes of M protein (S. Pruksakorn, A. Galbraith, R. A. Houghton, and M. F. Good, *J. Immunol.* 149:2729-2735, 1992).

TABLE 1. Partial sequence alignment for M proteins

Protein	Location	Sequence ^a
M5	300-327	DASREAKKQVEKALEEANSKLAALEKLN
M6	333-360	DASREAKKQVEKALEEANSKLAALEKLN
M12	405-431	DASREAKKQVEKALEEANSKLAALEKLN
M24	347-374	DASREAKKQVEKALEEANSKLAALEKLN
M57	335-362	DASREAKKQVEKALEEANSKLAALEKLN
M49	118-145	EASREAKKKVEADLAEANSKLQALEKLN

^a Sequences for M5 (22), M6 (13), M12 (27), M24 (24), M49 (12), and M57 (20) were obtained elsewhere. Disparity in amino acid sequence is marked as l. The underlined sequence of M5 represents the epitope recognized by BALB/c M5[300-319]-specific T-cell clones.

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