Mitogenicity of M5 Protein Extracted from \textit{Streptococcus pyogenes} Cells Is due to Streptococcal Pyrogenic Exotoxin C and Mitogenic Factor MF

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M proteins of \textit{Streptococcus pyogenes} are virulence factors which impede phagocytosis, bind to many plasma proteins, and induce formation of cross-reactive autoimmune antibodies. Recently, it has been reported that some M proteins, extracted with pepsin from streptococci (pep M), are superantigens. One of these, pep M5, was investigated in detail and was shown to stimulate human T cells bearing \( V_B^2 \), \( V_B^4 \), and \( V_B^8 \). In the present study, we extracted and purified M5 protein by different biochemical methods from two M type 5 group A streptococcal strains. The crude extracts were fractionated by affinity chromatography and ion-exchange chromatography. All fractions were tested in parallel for M protein by immunoblotting and for T-cell-stimulating activity. Although several crude preparations of M5 protein were associated with mitogenicity for \( V_B^2 \) and \( V_B^8 \) T cells, the M5 proteins, irrespective of the extraction method, could be purified to the extent that they were no longer mitogenic. The mitogenic activity was not destroyed during the purification procedures but was found in fractions separated from M protein. In these fractions, streptococcal pyrogenic exotoxin C and mitogenic factor MF could be detected by protein blotting and enzyme-linked immunosorbent assay. Moreover, anti-M protein sera did not inhibit the mitogenic activity of crude extracts, but antisera which contained anti-streptococcal pyrogenic exotoxin C antibodies showed inhibition. The inability of M5 protein to stimulate T cells was confirmed with recombinant pep M5 produced in \textit{Escherichia coli}. Our data strongly suggest that the mitogenic activity in M protein preparations is caused by traces of streptococcal superantigens different from M protein.

M proteins of \textit{Streptococcus pyogenes} are important pathogenicity factors of pyogenic streptococci (4, 18, 32). They are receptor proteins which can show multiple binding to different plasma proteins, like fibrinogen, albumin, and immunoglobulin G (11, 16, 30, 31); complement factor H (9); and fibronectin (30). These interactions are involved in the ability of M protein to protect streptococci against phagocytic attack (29, 36, 39). M proteins form a family of closely related proteins protruding from the cell surface. The N-terminal end of M protein is a highly variable region that carries the type-specific epitopes of the molecule (4). M protein fragments can be produced by limited proteolytic digestion of whole streptococci (pep M; reference 1). They are released from the cells and have a molecular mass of approximately 28 kDa. It has been proposed that these soluble fragments are superantigens (13, 15, 33, 34, 38). The superantigenic activity of pepsin-extracted M protein from type 5 strain Manfredo (pep M5) was characterized in more detail. It was shown to stimulate human but not murine T cells (13, 14, 38). By use of peptides blocking the response of T cells to intact pep M5, a region on pep M5 was defined that apparently plays a role in T-cell stimulation (37). A recombinant pep M5 preparation was reported to be mitogenic for T cells (38).

It was reported that pep M5 selectively stimulates T cells bearing \( V_B^2 \), \( V_B^4 \), and \( V_B^8 \) (33, 35). More recently, it was reported that other M protein serotypes also have superantigenic properties. M proteins purified by limited pepsin digestion of type 2, 5, 6, 18, 19, and 24 streptococci were also stimulators of human T cells and stimulated T cells with different individual \( V_B \)s (38). The investigators concluded that in their N termini, variable M proteins represent a family of streptococcal superantigens analogous to the structurally related family of staphylococcal enterotoxins.

In contrast, in studies with M1, M3, M5, and M12 proteins extracted by different methods, it has been shown that the T-cell-stimulating activity could be separated from M1 and M12 proteins by affinity chromatography on immobilized anti-M antibodies (12) and from M1, M3, and M5 proteins by affinity chromatography on fibrinogen-Sepharose and albumin-Sepharose (5). Moreover, other researchers failed to detect nonspecific mitogenic activity with a recombinant M5 protein preparation (26). Data were reported suggesting that a pepsin digest of group A type 12 streptococci is superantigenic for murine T cells, but the mitogenic component in that extract was not due to M12 protein (3).

An argument against the claim that the superantigenic properties of M protein could be due to contamination by streptococcal pyrogenic exotoxins (SPE) is the difference in \( V_B \) specificity between pep M5 and SPEA, SPEB, and SPEC (35). pep M5 preferentially stimulates human T cells bearing \( V_B^2 \), \( V_B^4 \), and \( V_B^8 \) (34, 35); SPEA stimulates T cells bearing \( V_B^2 \), \( V_B^{12} \), \( V_B^{14} \), and \( V_B^{15} \); SPEB stimulates T cells bearing \( V_B^8 \); and SPEC stimulates T cells bearing \( V_B^1 \), \( V_B^2 \), \( V_B^{5.1} \), and \( V_B^{10} \) (35). However, we have found, by using recombinant SPE, that
SPEA stimulates Vβ12 but not Vβ2 (2) and that the Vβ8-stimulating activity attributed to SPEB (the streptococcal protein expressed in reference 7) is due to a novel superantigen different from all known SPE and tentatively designated SPEX (2, 6, 8). Moreover, novel SPE, such as SSA (21), have recently been discovered, and it is likely that SPE form a large, polymorphic family. It is intriguing that the newly described mitogenic factor MF (10), also tentatively designated SPEF, was found to have the same TCR Vβ stimulation pattern as pep M5 (22).

These contradictory reports show the question of whether M proteins are superantigens or not has not been answered satisfactorily. Because of the importance of superantigens in acute streptococcal diseases and their sequelae, the question of whether M proteins are superantigens or not has become very important. In the present study, we extracted M protein from two M5-type strains by pepsin extraction and by several physicochemical extraction methods. The extracted M protein was purified, and M protein and T-cell mitogenicity was assayed in different preparations. Moreover, the pep M5 fragment was cloned and expressed in *Escherichia coli* and included in our investigations.

**MATERIALS AND METHODS**

**Bacteria and plasmids.** The *S. pyogenes* M type 5 strain (TSB/P/S) used in this study came from the Public Health Laboratory Service, Streptococces Reference Laboratory, London, England. Strain M5 Manfredo was provided by Malak Kotb, Memphis, Tenn. Chromosomal DNAs of both strains were tested with probes specific for the genes for SPEA, SPEB, and SPEC by Southern blot hybridization (25). Both M5 strains carry the genes for SPEB and SPEC but not for that for SPEA. For isolation of M proteins, streptococci were grown in 5 liters of Todd-Hewitt broth in a 1-liter fermentor. The cultures were inoculated with 500 ml of an overnight stand culture, and fermentation was performed for 18 h at 38°C and pH 7.2 with regulation of the glucose concentration at 0.2%. Bacteria were then sedimented by centrifugation and washed twice with saline. The washing solution was added to the culture filtrate. The washed sediments were treated as described below. For cloning and expression of recombinant pep M5, expression vector pQE50 and *E. coli* M15 carrying regulating plasmid plpREPs were used from QIAGEN were used.

**Extraction of M protein.** M5 protein was found in the culture filtrates (CF) of both type 5 strains. To purify cell-associated M proteins, two different extraction procedures were performed. In the first procedure, streptococci were washed in phos- phate-buffered saline before T-cell stimulation assays and further purification of the cells by hydrolysis with hot HCl (resulting in M5-HCl) at 95°C for 10 min. The second preparation of M5 proteins was obtained without proteolytic cleavage (see Fig. 1). N-terminal amino acid sequencing.

Sequence was performed with polyacrylamide gel electrophoresis (SDS-PAGE) and protein blotting.

**Expression of recombinant pep M5 (r-pep M5) in *E. coli*.** The region encoding pep M5 was amplified by PCR from genomic DNA of streptococci strain M5 Manfredo prepared by standard methods. The sequences of PCR primers were synthesized by using the M5 nucleotide sequence reported by Miller et al. (20). As a control, the 68-mer oligonucleotide (5'-AACGCTATATAGGCTGACCATGCTGCT-3') including a *Bam* H1 restriction site was used. This primer omitted the signal sequence and started at position 112 of the *emm*-5 gene. The reverse primer pm25 (5'-GGCCTCACTTTTTTTACCCGATAACAGGGC-3') was constructed with a stop codon before the *Pvu*I restriction site. The amplified PCR fragment was ligated to the PQE50 expression vector and transformed in *E. coli* M15(pREP4). The construction resulted in a fusion protein with the N-terminal heteroligogenic amino acids M-R-G-S, resulting from in-frame ligation in PQE50 (see below). The clones were screened for expression of r-pep M5 by induction of liquid cultures with isopro- pyl-β-D-thiogalactopyranoside (IPTG) and Western blotting as described in the QIAGEN procedure.

**Purification of M proteins.** The binding of M protein to fibrinogen and albumin (30, 31) was used to isolate M5 protein by successive affinity chromatography on albumin-Sepharose and fibrinogen-Sepharose as also described recently for other M proteins (5, 16, 30, 31). The columns were washed with 0.05 M sodium chloride, pH 7.4. M protein was released from the columns with 0.05 M sodium acetate, pH 5.0, containing 2 M KSCN. M5 protein purified by this method was isolated from CF and from the various extracts of streptococcal cells. These M proteins contain molecules larger than 52 kDa.

Pep-extended M protein (pep M5) was purified on fibrinogen-Sepharose by using a different buffer system. Because the affinity to fibrinogen is decreased in the pepsin-degraded peptide, the columns were eluted with 0.05 M Tris buffer containing 0.3 M NaCl. A second fractionation of pep M5 on albumin-Sepharose was not possible, because after pepsin cleavage the C-terminal part of M5 protein (including the C repeats and the anchor region) was removed from the N-terminal part (19, 27), resulting in loss of albumin binding (Fig. 2; reference 5). Alternatively, crude pep M5 was fractionated by fast protein liquid chromatography (FPLC) on a Mono-Q HR 5/5 column (Pharmacia, Freiburg, Federal Republic of Germany). The chromatography was started with 0.01 M sodium phosphate buffer, pH 8.0 (19), and after washing with starting buffer, the proteins were eluted with an NaCl gradient from 0 to 1 M. r-pep M5 was purified by using the same procedures as used for streptococcal-derived pep M5.

**SDS-PAGE and protein blotting.** SDS-PAGE was performed with a total acrylamide concentration of 10% and 2.6% cross-linker. Proteins were blotted onto nitrocellulose membranes (Bio-Rad, Munich, Federal Republic of Germany) by electrophoretic transfer from SDS-PAGE gels. The nitrocellulose was blocked with 2% defatted skim milk in phosphate-buffered saline containing 0.01% Tween 20. Binding of M protein was assayed by incubation of the strips with horseradish peroxidase-labeled plasma proteins (31) or with immune rabbit serum (1:500) for 3 h and staining with peroxidase-labeled anti-rabbit immunoglobulin (G).

**N-terminal amino acid sequencing.** Sequencing was performed with lyophilized samples of M5 protein purified by affinity chromatography, as well as with electrophoretically separated peptides of different M5 protein preparations after treatment with Immobilon membranes (Millipore). Sequence was determined by Edman degradation on Applied Biosystems gas-liquid phase sequences 470A and 473A. Phenyliodhydroxyl acid amino acids were identified by on-line, reversed-phase high-pressure liquid chromatography. M5 peptides from both strains isolated from the CF, from extracts with obtained with dissociating agents, and from the pep M5 extract revealed the same N-terminal sequence, AV-T-R-G-F-T-N-D-P, which has been previously reported for M5 protein (19). This corresponds to the translated DNA sequence beginning with amino acid 43 of recombinant M5 protein (20) and represents the N terminus of the M5 protein expressed by streptococci. This was not the theoretically calculated cleavage site of the signal sequence which was calculated, with the sequence program package HUSAR (DKFZ, Heidelberg, Federal Republic of Germany), to start at position 38 with the sequence N-E-V-S-A-A-V-T. Mostly in lyophilized samples, a second minor sequence was detected (AV-T-R-G-F-D-P). In smaller M5 protein species, starting at amino acid 58, that is present in most preparations obtained without proteolytic cleavage (see Fig. 1). N-terminal sequencing of r-pep M5 revealed the sequence M-R-O-S-N-E-S-V-A-A, showing absence of the expected sequence. Construction of the constructed fusion protein (M-R-G-S-N-E-V-S-A-A), showing absence of the expected sequence.

**Cells.** Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors. The human T-cell leukemia line Jurkat, which expresses Vβ8*, and the murine T-cell hybridoma FRN2.7, which expresses a transfected human Vβ2* T-cell receptor, were used as interleukin-2-producing responder cells (2, 28). The interleukin-2 produced by these cells was measured by using CTL2 cells. Burkitt's lymphoma Raji cells were used as accessory cells (2).

**T-cell assays.** Cellular assays were performed as described recently (2, 5). Briefly, proliferative responses were measured in 200-μl triplicates containing 10^6 PBMC as responders. The assay was pulsed with [3H]thymidine after 48 h for 16 h. Interleukin-2 was produced by incubating 5 x 10^5 Jurkat or FRN2.7 cells with 10^5 Raji cells per well with various concentrations for 24 h. Secreted interleukin-2 was measured in a bioassay using the proliferative response of CTL2 cells. Results are expressed as mean counts per minute incorporated by triplicates or as a mean stimulation index (stimulation index = test counts per minute/background counts per minute). Inhibition of PBMC stimulation with rabbit antiserum was performed by addition of 10 μl of the corresponding serum dilution to the reaction mixture.

**Antibodies and antibodies.** Anti-M1, -M3, and -M5 sera were raised in rabbits by subcutaneous immunization with entire M proteins (purified by affinity chromatography from the culture filtrates of strains M1 40/58, M3 4/55, and M5 TSB/P3 on fibrinogen-Sepharose and albumin-Sepharose) with complete Freund's adjuvant (23). Anti-SPEB (7) and anti-SPEC (23) were previously produced as previously described.

A complex antiserum against a concentrated culture filtrate of strain NY5, originally M type 12, was also produced by subcutaneous injection of rabbits. SPEC was identified by enzyme-linked immunosorbent assay (ELISA) with a polyclonal antibody against SPEC (40). Anti-M5 was raised against the purified protein fragment AX as described in reference (6).
RESULTS

Presence of M5 protein and mitogenic activity in different preparations. Culture supernatants and the different cell extracts were analyzed by SDS-PAGE, and M protein was detected by protein blotting. As shown in Fig. 1, some M protein was spontaneously released in the CF of both strains and the major fraction of cell-bound M protein could be washed off of the cells by sequential treatment with dissociating buffers. The different extraction procedures released M protein peptides with different molecular weights. High-molecular-mass peptides of M5 protein (>52 kDa) were found in the CF (M5-CF) and in the HCl extract (M5-HCl) of both strains. In M5-HCl, low-molecular-weight peptides were also found (Fig. 1A and B, lanes 1 and 6). Extraction with KSCN and urea released a high-molecular-weight species of M protein from T5/B/PS cells but not from M5 Manfredo cells (Fig. 1A and B, lanes 4 and 5). The peptides identified in CF and in the KSCN and urea extracts represent the complete functional M5 molecule because they carry the binding regions for albumin and fibrinogen. The M5 protein from strain M5 Manfredo was found to be 2 kDa larger than that from strain T5/B/PS, but both revealed the same N-terminal amino acid sequence.

Enzymatic extraction with pepsin also yielded a heterogeneous mixture of smaller M5 peptides (Fig. 2, pep M5). These peptides had lost the ability to bind albumin but still bound fibrinogen. The identity of the various extracted peptides with M5 protein was confirmed not only by probing with labeled fibrinogen (Fig. 1) and anti-M5 serum (Fig. 2) but also by N-terminal amino acid sequencing. Independently of the extraction procedure, most of the M5 peptides started at the N terminus with the sequence A-V-T-R-G-T-I-N-D-P. Washing of streptococcal cells with 0.3 or 1 M NaCl did not result in significant extraction of M protein from both strains (Fig. 1A, lane 3; Fig. 1B, lanes 2 and 3).

These different extracts were tested for T-cell stimulation to determine if the stimulating activity could be separated from M5 protein. For this purpose, M protein samples from all extraction and purification steps were tested. We used a Vβ21 and a Vβ81 T-cell line to detect Vβ21 and Vβ81 cell-specific superantigens, respectively. PBMC were used to detect mitogens for the full T-cell spectrum. Vβ21 cells react with SPEC but not with SPEA or SPEB (2); the Vβ81 Jurkat T-cell line has previously been used to detect M5 protein (14) and MF (6) but does not react with SPEA, SPEB, or SPEC (2).

T-cell stimulation was found in nearly all crude extracts independently of the presence of M protein (Fig. 3). Remarkably, the HCl extract of strain T5/B/PS, although containing M5 protein, did not stimulate T cells, indicating that the cell-bound mitogenic components had already been removed by the preceding washing steps. The highest mitogenic activity was detected in each case in the CF of both strains, which also contained, among other exoproducts, spontaneously excreted M5 protein (Fig. 1, lane 1). Surprisingly, significant differences were found in the abilities of various extracts to stimulate the Vβ21 and Vβ81 cell lines. Whereas most extracts stimulated PBMC as well as Vβ21 cells, stimulation of Vβ81 cells was found only in crude pep M5 (Table 1), in the CF, and in the first saline washing solution (Fig. 3), although the latter solution did not contain M protein. Washing of the cells with higher concentrations of NaCl released neither M protein nor Vβ81 cell-stimulating activity (Fig. 1 and 3). The Vβ81 cell-stimulating activity was also strongly reduced or was not present in any other extract.

![FIG. 1. SDS-PAGE of crude M5 protein released successively by different buffers from strains T5/B/PS (A) and M5 Manfredo (B). Lanes: 1, crude M5-CF; 2, extract released with 0.05 M Tris-0.3 M NaCl, pH 7.4 (shown in panel B only); 3, extract released with 0.05 M Tris-1 M NaCl, pH 7.4-4. M5-KSCN, extracted with 2 M KSCN; 5, M5-urea, extracted with 6 M urea; 6, M5-HCl, extracted with hot HCl. Fifteen microliters was applied to each lane. Left panels are gels stained with Coomassie blue, and right panels are protein blots. M5 protein was stained with peroxidase-labeled fibrinogen.](http://iai.asm.org/pic/1995/63/14/4571/F1.jpg)

![FIG. 2. SDS-PAGE and protein blot of crude preparations of r-pep M5 and pep M5. Gels were stained with Coomassie blue (left panel) or blotted onto filters and the filters were developed with peroxidase-labeled albumin (HSA), fibrinogen (FBG), immunoglobulin G, and rabbit antiserum against M5 protein, SPEC (ETC), or MF. The crude r-pep M5 (28-kDa band) stained with fibrinogen and with anti-M5 serum but not with antisera to SPEC and MF, whereas SPEC and MF (bands in the 28-kDa region) were present in crude pep M5.](http://iai.asm.org/pic/1995/63/14/4571/F2.jpg)
The protein blot is indicated by a plus or minus sign, respectively. Hand 810 b VI 8; PBMC, 560 the results of three different experiments comparable. Medium control with adjusted to a protein concentration of 10\(^{-6}\) g/ml. The stimulation index (counts per minute with sample/counts per minute with medium only) was used to make the results of three different experiments comparable. Medium control with PBMC, 560 ± 240 cpm; medium controls for the cell lines, 1,100 ± 700 (\(V_{b}^{2}\)) and 810 ± 600 (\(V_{b}8\)) cpm. The presence or absence of M protein as revealed by the protein blot is indicated by a plus or minus sign, respectively. □, \(V_{b}2\); △, \(V_{b}8\); ■, PBMC.

Present in the M5-KSCN, M5-urea, and M5-HCl extracts containing high concentrations of M5 protein (Fig. 3, A and B). Thus, simple differential washing of the cells led to separate release of \(V_{b}2\) and \(V_{b}8\) cell-stimulating activities, indicating that they belong to different components.

**Identification of contaminants and specific inhibition of mitogenicity with anti-SPEC.** To identify the mitogenic component(s) in our M5 preparations, we tested crude pep M5 (and r-pep M5 as a control) by Western blotting with different antisera directed against crude culture filtrate of strain NY5, against SPEA, against SPEC, and against the recently described mitogenic factor MF (Fig. 2). Furthermore, in crude pep M5, but not in crude r-pep M5, a specific reactivity with antisera directed against SPEC and MF was found by Western blotting. The stained bands were located in the same 28-kDa region as pep M5. The lack of reactivity of anti-SPEC and anti-MF sera with the r-pep M5 preparation demonstrates that there is no cross-reaction with M5 protein. In addition, there was no reactivity of anti-SPEC with MF protein and anti-MF with SPEC (data not shown).

We next used our antisera to neutralize the T-cell stimulation obtained with crude pep M5. pep M5 was described as a moderate superantigen, and at a concentration of 1 \(\mu\)g/ml, which we normally use for T-cell stimulation, pep M5 should have been mitogenic if the activity were located in the molecule. At this concentration, however, crude pep M5 showed clear stimulation of PBMC (Fig. 4A and Table 1) but purified pep M5 did not (Table 1; see below). Because our purified pep M5 did not activate T cells, we used crude pep M5 for the neutralization experiments.

Addition of polyclonal anti-M5 rabbit serum and sera raised against other purified M proteins caused no inhibition of T-cell stimulation (Fig. 4B). Antisera raised against SPEA (not shown) and SPEB (serum K40) also did not decrease mitogenicity. However, with anti-SPEC rabbit serum (K121) and with a serum raised against the complete culture supernatant of S. pyogenes type 12 strain NY5 (K-CF), concentration-dependent inhibition of T-cell-stimulating activity of crude pep M5 was found. K-CF serum contains antibodies against SPEA, SPEC, MF, and probably other mitogens but not against M5. Antisera against purified MF (6) did not inhibit T-cell stimulation (data not shown). The sera alone had no stimulating effect (Fig. 4).

**Separation of M protein and mitogenic activity.** Crude extracts which contained the entire M protein molecule (Fig. 1) and showed T-cell stimulation (Fig. 3) were further purified by sequential affinity chromatography on albumin-Sepharose and fibrinogen-Sepharose. All fractions were tested for T-cell stimulation (Table 1). The results show that the purified, intact M protein in the eluates, regardless of the extraction procedure, did not stimulate any of the different T-cell preparations used. The stimulating activity was, in all cases, found in the flowthrough fractions.

Because mitogenicity of M protein preparations has been demonstrated mostly with pep M5 from strain M5 Manfredo (15, 33, 34, 37), we performed more detailed investigations with this protein. As shown by Western blotting, crude pep M5 contained SPEC and MF as contaminants. Therefore, crude pep M5 was fractionated on fibrinogen-Sepharose or, alternatively, by ion-exchange FPLC (Fig. 5) to separate pep M5 from these proteins. Adsorption to fibrinogen-Sepharose did not always result in a pep M5 preparation free of high-molecular-weight peptides because these high-molecular-weight peptides likewise show affinity for fibrinogen (Fig. 2, pep M5 blot; Fig. 5, blot). Crude pep M5 stimulated PBMC and \(V_{b}^{2}\) and \(V_{b}8\) cells (Table 1). After affinity chromatography on fibrinogen-Sepharose, the entire stimulating activity was found in the flowthrough fraction. The pep M5 fraction retained fibrinogen-Sepharose and eluted with 0.3 M NaCl showed no mitogenic activity (Table 1).

We next used a completely different pep M5 purification protocol, ion-exchange chromatography on an FPLC Mono-Q HR column under nondenaturing conditions (Fig. 5). SDS-PAGE and protein blotting revealed that with the Mono-Q HR column, separation of pep M5 (Fig. 5, fraction 9, 28-kDa band) from the minor amount of high-molecular-weight peptides present in fractions 10 to 12 could be achieved. Furthermore, we found the PBMC-stimulating activity in flowthrough fractions 3, 4, and 5, which were eluted at the initial sodium phosphate concentration. The pep M5 characterized by fibrinogen binding was clearly separated from the mitogenic frac-
tions and appeared in fractions 8 to 10 (Fig. 4, protein blot). These fractions showed no stimulation of PBMC. Since the protein concentration in mitogenic fractions 3, 4, and 5 was very low, no bands could be detected either by staining with Coomassie blue or by Western blotting with anti-M5 serum. However, by ELISA with a monoclonal anti-SPEC antibody, SPEC could be identified in fractions 3, 4, and 5 whereas no SPEC was detectable in pepM5-containing fractions 8, 9, and 10 (Fig. 5). This indicates that SPEC, and not pepM5, is one of the T-cell-stimulating components in fractions 3, 4, and 5.

![Graphs](http://iai.asm.org/)  
**FIG. 4.** Effects of different antisera on T-cell stimulation by crude pep M5. (A) Titration of crude pep M5 on PBMC. (B) Inhibition of PBMC response to 2 μg of crude pep M5 by different rabbit antisera. The antisera used were K40 (anti-SPECB), K121 (anti-SPEC), K-CF (anti-CF of strain NY5), and three type-specific anti-M protein sera; they were added at the final dilutions indicated. Con A, concanavalin A.
Possible technical problems. (i) The indicator cell system. We used PBMC to detect mitogenicity independently of Vβ2-specific and Vβ8-specific stimulation. Vβ2+ FRN2.7 cells have been previously shown to react specifically with SPEC (2) and toxic shock syndrome toxin 1 (38). Vβ8+ Jurkat cells have even been shown, by Koth et al. (14), to detect the mitogenicity of the M5 protein. Therefore, detection of the M protein-associated superantigen(s) by the T cells used does not pose a problem.

(ii) Purification. It is unlikely that M protein mitogenic activity was destroyed during purification, since we used the nondenaturing FPLC protocol for purification of intact M5 protein, and we were able to elute pep M5 from immobilized fibrinogen with only 0.3 M NaCl. Furthermore, we could recover the entire superantigen activity in other fractions not containing M protein. In such fractions and in the crude M5 preparation, pep M5 SPEC and MF, known mitogens, were identified.

(iii) Additional N-terminal amino acids in r-pep M5. Our r-pep M5 has four additional anionic amino acids at its N terminus. However, this should not influence its putative mitogenicity because Wang et al. have reported that the region important for the superantigenicity of this molecule is localized at residues 157 to 197, far away from the N terminus (37).

(iv) Strain differences. We used two different M type 5 strains of *S. pyogenes*; one of them was strain M5 Manfredo, which has been used as a source of pep M5 protein in all previous studies.

M protein is a receptor molecule able to bind a variety of different serum proteins (4, 9, 11, 30, 31). It is therefore not surprising that streptococcal proteins also associate and copurify with M proteins. We identified two of the associated proteins as SPEC and MF with specific antisera. We have previously reported that during purification of SPEC, M protein is copurified and is difficult to remove (24). The neutralization tests suggest that SPEC is the component responsible for the Vβ2-specific stimulation in the M5 protein preparations (2). The Vβ8-specific stimulator could be MF, which has been reported to stimulate Vβ2+, Vβ4+, and Vβ8+ T cells (22). However, the separation of Vβ2+ and Vβ8+ cell-specific stimulation in the present study argues against this notion and is also compatible with the idea that a Vβ8+ cell-specific mitogen, different from MF and possibly novel, is present. In this case, the mitogenicity would not be due to MF. This issue is still unresolved.

Although there are numerous reports about the superantigen activity of M protein (13–15, 34, 35, 37, 38), all data about the superantigenicity of M protein are derived from the preparations of a single laboratory and have not been confirmed by an independent study. The evidence described here strongly suggests that the superantigenicity of M protein is not an intrinsic property of this protein but is due to contamination with extremely potent streptococcal superantigens; one of them is SPEC. The reported reactivity of pep M with human PBMC but not mouse spleen cells (13, 14, 37) can be explained. At low concentrations, most streptococcal exotoxins and SPE, similar to pep M, show a preference for human as opposed to mouse cells, because most streptococcal pyrogenic exotoxins and staphylococcal enterotoxins bind much better to human class II molecules. Our study is another demonstration of the extreme potency of SPE and is a caveat for work with proteins purified from *S. pyogenes* and *Staphylococcus aureus*.

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