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

KARL-HERMANN SCHMIDT,[1]* DIETER GERLACH,[1] LEO WOLLWEBER,[2] WERNER REICHARDT,[1] KARLHEINZ MANN,[3] JÖRG-HERMANN OZEGOWSKI,[1] AND BERNHARD FLEISCHER[4]

Institute of Experimental Microbiology[1] and Institute of Molecular Biotechnology,[2] Friedrich Schiller University, D-07745 Jena,[1] Department of Protein Chemistry, Max Planck Institute of Biochemistry, D-82152 Martinsried,[3] and Bernhard Nocht Institute of Tropical Medicine, D-20359 Hamburg,[4] Federal Republic of Germany

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M proteins of *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 Vβ2, Vβ4, and Vβ8. 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 Vβ2 and Vβ8 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 *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 *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 Vβ2, Vβ4, and Vβ8 (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 Vβs (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 enterotoxin superantigens.

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 Vβ specificity between pep M5 and SPEA, SPEB, and SPEC (35). pep M5 preferentially stimulates human T cells bearing Vβ2, Vβ4, and Vβ8 (34, 35); SPEA stimulates T cells bearing Vβ2, Vβ12, Vβ14, and Vβ15; SPEB stimulates T cells bearing Vβ8; and SPEC stimulates T cells bearing Vβ1, Vβ2, Vβ5.1, and Vβ10 (35). However, we have found, by using recombinant SPE, that

* Corresponding author. Mailing address: Institute of Experimental Microbiology, Friedrich Schiller University, Jena, Winzerlaer Strasse 10, D-07745 Jena, Federal Republic of Germany. Phone: (049) (0)3641-65-7117. Fax: (049) (0)3641-65-7133. Electronic mail address: kschmidt@imb-jena.de.

SPEA stimulates Vβ12 but not Vβ2 (2) and that the Vβ8-stimulating activity attributed to SPEB (the streptococcal proteinase precursor; 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 that 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 (T5/B/PS) used in this study came from the Public Health Laboratory Service, Streptococcus 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 that for SPEA. For isolation of M proteins, streptococci were grown in 5 liters of Todd-Hewitt broth in a 7-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 pREP4 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, streptococcal cells washed in saline were successively treated with buffers that differed in dissociating potency. The cells (20 g [wet weight]) were suspended at room temperature in 50 ml of the respective buffer and centrifuged after 1 h of incubation. The supernatant was collected, and the sedimented cells were suspended in the next extraction buffer. Extractions were performed sequentially with (i) saline; (ii) 0.3 M NaCl in 0.05 M Tris buffer, pH 7.5; (iii) 1 M NaCl in 0.05 M Tris buffer, pH 7.5; (iv) 2 M KSCN in 0.05 M sodium acetate, pH 5.0 (resulting in the preparation M5-KSCN); and (v) 6 M urea (resulting in M5-urea) in 0.1 M glycine, pH 2.5. Finally, the cell-bound M protein remaining after these procedures was extracted from the cells by hydrolysis with hot HCl (resulting in M5-HCl) at 95°C (17). Pepsin extraction (pep M5) was performed with cells washed twice with 0.067 M phosphate buffer, pH 5.8, as previously described (1).

The presence of M protein in each extraction step was determined in a dot blot assay with peroxidase-labeled fibrinogen and albumin (30) or with rabbit anti-M5 serum by protein blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5, 30). The crude extracts were dialyzed against phosphate-buffered saline before T-cell stimulation assays and further purification of M protein as described below.

Expression of recombinant pep M5 (r-pep M5) in *E. coli*. The region encoding pep M5 was amplified by PCR from genomic DNA of streptococcus 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 forward primer, the oligonucleotide pm5-f, including a *Bam*HI restriction site in the 5 end (5′-aaccggatccAATGAAGTTAGTGCAGCCGTG-3′), was used. This primer omitted the signal sequence and started at position 112 of the *emm-5* gene. The reverse primer, pm5-r (5′-ggccctgcagttaATCTTTTTTA GCTAATTCTTGTTTAAGGGC-3′), was constructed with a stop codon before the *Pst*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 heterologous 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 isopropyl-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 Tris–0.2 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 M5 preparations consisted of molecules larger than 52 kDa.

Pepsin-extracted 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 streptococcus-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 transfer onto Immobilon membranes (Millipore). Sequences were determined by Edman degradation on Applied Biosystems gas-liquid phase sequences 470A and 473A. Phenylthiohydantoin amino acids were identified by on-line, reversed-phase high-pressure liquid chromatography. M5 peptides from both strains isolated from the CF, from extracts obtained with dissociating agents, and from the pepsin extract revealed the same N-terminal sequence, A-V-T-R-G-T-I-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 (A-L-D-K-Y-E-L) was detected. This is possibly derived from a 2-kDa smaller M 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-G-S-N-E-V-S-A-A, showing exactly the expected sequence of the constructed fusion peptide with the heterologous amino acids M-R-G-S and the start of M5 at N-E-V-S-A-A.

Cells. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors. The human T-cell leukemia line Jurkat, which expresses $V\beta8^+$, and the murine T-cell hybridoma FRN2.7, which expresses a transfected human $V\beta2^+$ T-cell receptor, were used as interleukin-2-producing responder cells (2, 28). The interleukin-2 produced by these cells was measured by using CTLL-2 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 10^5 PBMC as responders. The assay was pulsed with [^{3}H]thymidine after 48 h for 16 h. Interleukin-2 was produced by incubating 5×10^4 Jurkat or FRN2.7 cells with 3×10^4 Raji cells per well with various additions for 24 h. Secreted interleukin-2 was measured in a bioassay using the proliferative response of CTLL-2 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 antisera was performed by addition of 10 μl of the corresponding serum dilution to the reaction mixture.

Antisera 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 T5/B/PS on fibrinogen-Sepharose and albumin-Sepharose) with complete Freund's adjuvant (30). Anti-SPEB (7) and anti-SPEC (23) were 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 monoclonal antibody against SPEC (40). Anti-MF serum was raised against the purified protein from peak AX as described recently (6).

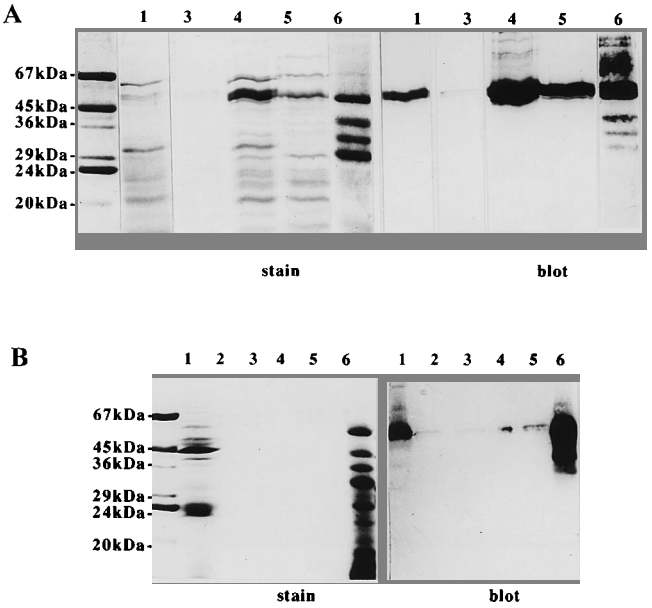

FIG. 1. SDS-PAGE of crude M5 protein released successively by different buffers from strains T5/B/PS (A) and M5 Manfredo (B). Lanes: 1, crude M5-CF; 2, extract released with 0.05 M Tris–0.3 M NaCl, pH 7.4 (shown in panel B only); 3, extract released with 0.05 M Tris–1 M NaCl, pH 7.4; 4, M5-KSCN, extracted 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.

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 $V\beta2^+$ and a $V\beta8^+$ T-cell line to detect $V\beta2^+$ and $V\beta8^+$ cell-specific superantigens, respectively. PBMC were used to detect mitogens for the full T-cell spectrum. $V\beta2^+$ cells react with SPEC but not with SPEA or SPEB (2); the $V\beta8^+$ 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 $V\beta2^+$ and $V\beta8^+$ cell lines. Whereas most extracts stimulated PBMC as well as $V\beta2^+$ cells, stimulation of $V\beta8^+$ 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 $V\beta8^+$ cell-stimulating activity (Fig. 1 and 3). The Vβ8 cell-stimulating activity was also strongly reduced or was not

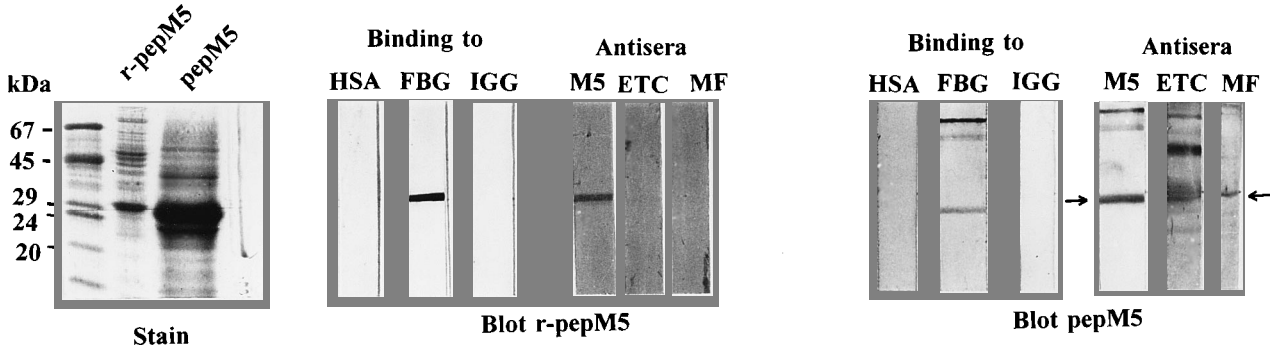

FIG. 2. SDS-PAGE and protein blot of crude preparations of r-pep M5 and pep M5. Gels were stained with Coomassie blue (left panel) or blotted onto filters and the filters were developed with peroxidase-labeled albumin (HSA), fibrinogen (FBG), immunoglobulin G, and rabbit antisera against M5 protein, 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.

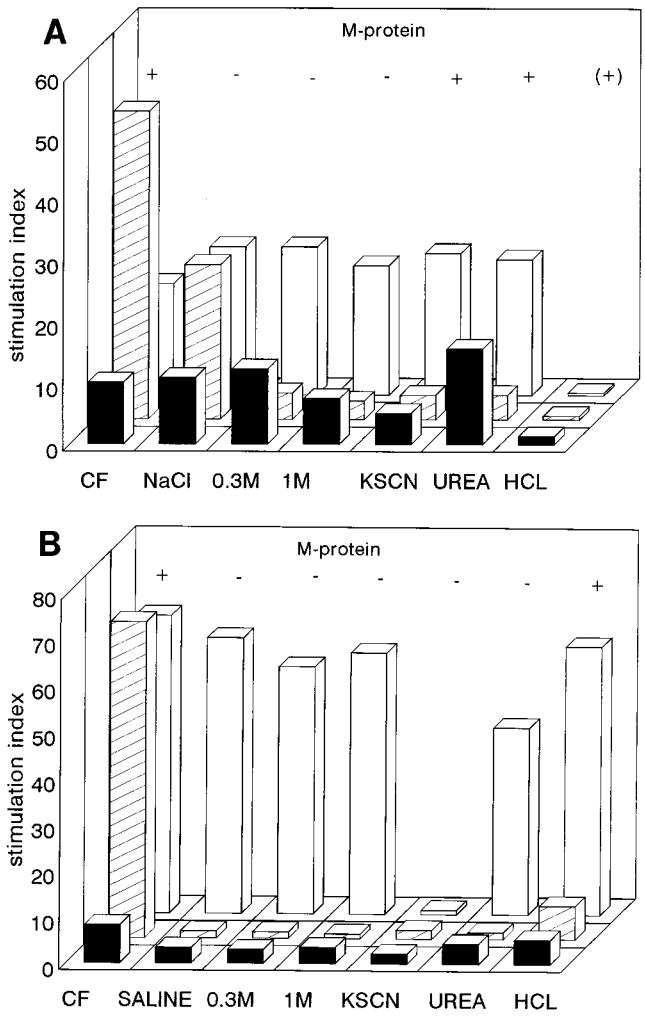

FIG. 3. Stimulation of Vβ2$^+$ and Vβ8$^+$ T-cell lines and PBMC by extracts released from strains T5/B/PS (A) and M5 Manfredo (B) with different buffers. CF, culture filtrate; NaCl, saline wash; 0.3 M, extract obtained with 0.05 M Tris–0.3 M NaCl, pH 7.4, buffer; 1M, extract obtained with 1 M NaCl in the same buffer; KSCN, extract obtained with 2 M KSCN (M5-KSCN); UREA; extract obtained with 6 M urea (M5-urea); HCL, HCl extract (M5-HCl). Cells were stimulated in each case with a 1:100 dilution of the appropriate dialyzed extract adjusted to a protein concentration of 10 μg/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 (Vβ2) and 810 ± 600 (Vβ8) cpm. The presence or absence of M protein as revealed by the protein blot is indicated by a plus or minus sign, respectively. □, Vβ2; ▨, Vβ8; ■, 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 Vβ2 and Vβ8 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 μg/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 Vβ2$^+$ and Vβ8$^+$ cells (Table 1). After affinity chromatography on fibrinogen-Sepharose, the entire stimulating activity was found in the flowthrough fraction. The pep M5 fraction retained by 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-

TABLE 1. Detection of T-cell-stimulating activity in different M5 protein preparations during purification by affinity chromatography[a]

Purification step	PBMC (cpm [10^2])		Vβ2 (cpm [10^2])		Vβ8 (cpm [10^2])	
	M5 Manfredo	T5/B/PS	M5 Manfredo	T5/B/PS	M5 Manfredo	T5/B/PS
Crude pep M5	42 ± 9	35 ± 18	612 ± 190	NT[b]	217 ± 50	NT
Flowthrough	31 ± 11	NT	601 ± 220	NT	181 ± 65	NT
Purified pep M5	8 ± 4	NT	19 ± 10	NT	8 ± 4	NT
Medium	7 ± 3	7 ± 3	11 ± 5	NT	9 ± 4	NT
Crude r-pep M5	6 ± 4	NT	4 ± 3	NT	10 ± 6	NT
Flowthrough	7 ± 4	NT	NT	NT	NT	NT
Purified r-pep M5	4 ± 3	NT	3 ± 2	NT	13 ± 9	NT
Medium	6 ± 3	NT	3 ± 2	NT	7 ± 5	NT
Crude M5-CF	57 ± 12	51 ± 9	524 ± 190	167 ± 6	322 ± 90	181 ± 41
Flowthrough	49 ± 13	53 ± 10	511 ± 210	142 ± 31	277 ± 75	175 ± 65
Purified M5-CF	5 ± 4	9 ± 4	21 ± 8	6 ± 4	7 ± 4	9 ± 5
Medium	5 ± 2	8 ± 3	11 ± 5	3 ± 2	5 ± 2	3 ± 2
Crude M5-KSCN	12 ± 4	30 ± 4	18 ± 9	92 ± 17	8 ± 3	35 ± 18
Flowthrough	NT	15 ± 5	NT	59 ± 12	NT	22 ± 9
Purified M5-KSCN	NT	5 ± 3	NT	4 ± 3	NT	6 ± 2
Medium	5 ± 2	6 ± 3	11 ± 5	4 ± 2	5 ± 2	5 ± 3
Crude M5-urea	14 ± 6	48 ± 12	34 ± 5	69 ± 25	7 ± 4	12 ± 4
Flowthrough	NT	21 ± 7	NT	42 ± 22	NT	22 ± 7
Purified M5-urea	NT	6 ± 2	NT	5 ± 4	NT	7 ± 3
Medium	5 ± 2	6 ± 3	11 ± 5	4 ± 2	5 ± 2	5 ± 3
SPEC (0.5 μg/ml)	208 ± 81		210 ± 75		9 ± 5	
MF (0.5 μg/ml)	91 ± 22		80 ± 15		127 ± 31	

[a] pep M5 and r-pep M5 were purified on fibrinogen-Sepharose and eluted with 0.3 M NaCl; M5 proteins from CF or extracted by KSCN, urea, or HCl were purified by successive affinity chromatography steps on albumin-Sepharose and fibrinogen-Sepharose and eluted from the affinity columns with 2 M KSCN. Crude extracts and flowthrough fractions were tested at a protein concentration of 10 μg/ml. The positive controls SPEC and MF were purified as described recently (6, 23) and applied at a concentration of 0.5 μg/ml.

[b] NT, not tested.

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 pep M5-containing fractions 8, 9, and 10 (Fig. 5). This indicates that SPEC, and not pep M5, is one of the T-cell-stimulating components in fractions 3, 4, and 5.

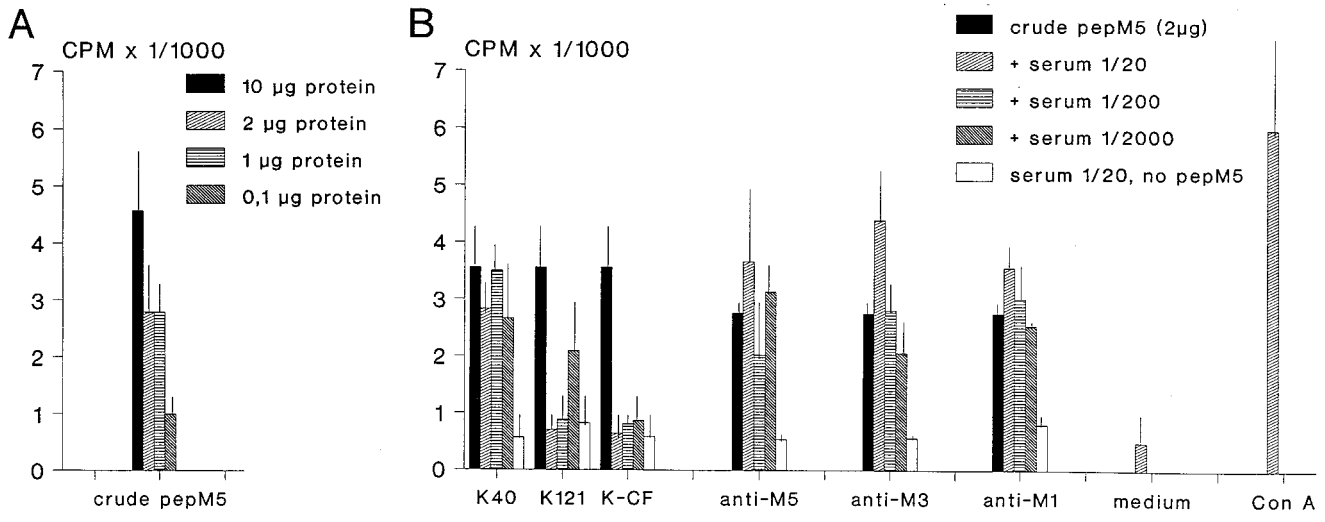

FIG. 4. Effects of different antisera on T-cell stimulation by crude pep M5. (A) Titration of crude pep M5 on PBMC. (B) Inhibition of PBMC response to 2 μg of crude pep M5 by different rabbit antisera. The antisera used were K40 (anti-SPEB), K121 (anti-SPEC), K-CF (anti-CF of strain NY5), and three type-specific anti-M protein sera; they were added at the final dilutions indicated. Con A, concanavalin A.

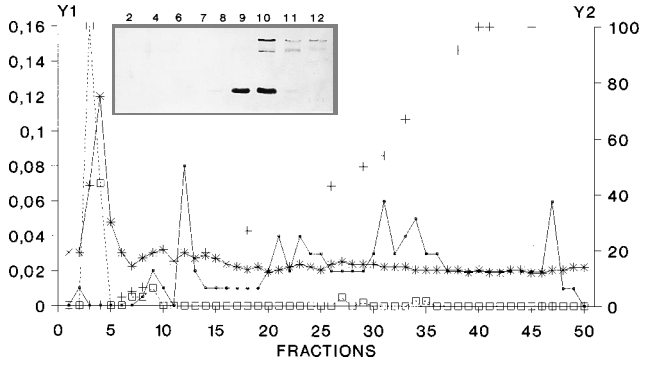

FIG. 5. Fractionation of crude pep M5 on Mono-Q HR (Pharmacia). A 0.01 M sodium phosphate buffer, pH 8.0, and a sodium chloride gradient from 0 to 1 M (axis Y2, percent gradient) were used. One-milliliter fractions were collected and monitored for protein content (axis Y1 values are optical densities at 280 nm), for mitogenicity of PBMC (axis Y2 values are counts per minute × 1/30), for the presence of SPEC by ELISA with a monoclonal anti-SPEC antibody (axis Y1 values are optical densities at 492 nm × 1/12), and for the presence of M5 protein fragment pep M5 by protein blotting. (Inset) The indicated fractions were separated by SDS-PAGE, and the gel was blotted onto filters; M protein was detected with peroxidase-labeled fibrinogen. PBMC were stimulated with 1:200 dilutions of the fractions, corresponding to 500 ng of pep M5 in fractions 9 and 10. Mitogenicity was detected in fractions 3, 4, and 5, which were eluted with the starting buffer. In these fractions, no M5 protein was found (insert) but SPEC was detected by ELISA. Symbols: ●, optical density at 280 nm (Y1); +, percent NaCl gradient (Y2); □, ETC (optical density at 492 nm; 1/12; Y1); ∗, MIT (counts per minute, 1/30; Y2).

Thus, careful purification of M5 protein by both affinity chromatography and ion-exchange chromatography on Mono-Q HR resulted in separation of T-cell-stimulating activity from M5 protein. Moreover, we generated and produced recombinant pep M5 in *E. coli*. We found no T-cell-stimulating activity with either PBMC or either of the T-cell lines used (Table 1).

DISCUSSION

Crude M5 protein preparations extracted from streptococci with dissociating buffers or by pepsin digestion (pep M5) contain superantigenic activity. However, here we have demonstrated that purification of M5 proteins from different crude extracts by affinity chromatography on albumin-Sepharose and/or fibrinogen-Sepharose, as well as by ion-exchange chromatography on Mono-Q HR, resulted in clear separation of M5 protein from the T-cell-stimulating activity. Such a detailed documentation of the purification steps of M5 protein in parallel with determination of the T-cell-stimulating activity of the fractions has not been described in previous reports. None of the purified M5 protein preparations, regardless of whether they consisted of the complete molecule or of smaller fragments down to the 28-kDa pep M5 peptide, stimulated any of the T cells used. Taken together, the following observations argue against the notion that M protein is a superantigen. (i) Mitogenic components can be separated from the M protein fraction by different methods, and purified M protein is not mitogenic. (ii) Mitogenicity in crude pep M5 protein preparations can be neutralized with anti-SPEC sera but not with anti-M sera. (iii) Our recombinant pep M5 did not stimulate T cells, and the same is true of other recombinant M proteins, like recombinant M3 and recombinant M1 (28a). In previous work with recombinant M5, mitogenicity was not reported (26). Because the putative mitogenicity of M protein has been known for many years and has attracted much attention, we wondered whether our failure to find mitogenicity associated with M5 protein could be due to technical problems.

Possible technical problems. (i) The indicator cell system. We used PBMC to detect mitogenicity independently of Vβ2-specific and Vβ8-specific stimulation. $V\beta2^+$ FRN2.7 cells have been previously shown to react specifically with SPEC (2) and toxic shock syndrome toxin 1 (38). $V\beta8^+$ Jurkat cells have even been shown, by Kotb 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 $V\beta2^+$, $V\beta4^+$, and $V\beta8^+$ T cells (22). However, the separation of $V\beta2^+$ and $V\beta8^+$ cell-specific stimulation in the present study argues against this notion and is also compatible with the idea that a $V\beta8^+$ 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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