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Superantigenicity of helper T-cell mitogen (SPM-2) isolated from culture supernatants of *Streptococcus pyogenes*

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SUMMARY

A superantigen (*Streptococcus pyogenes* mitogen-2; SPM-2) that stimulates human helper T cells bearing unique types of variable domains of T-cell receptor β-chain (TCR Vβ) was isolated from the culture supernatant of *S. pyogenes* strain T12. The active molecule isolated by diethylaminoethyl (DEAE)–cellulose chromatography and isoelectric focusing was a protein with a molecular weight (MW) of 29 000 and isoelectric point (pI) of 6·0. This new superantigen was found to activate preferentially Vβ4$^+$, 7$^+$, and 8$^+$ T cells, whereas recombinant streptococcal pyrogenic exotoxin A and C activated Vβ12$^+$ and Vβ2$^+$ T cells, respectively, as determined by flow cytometry and reverse transcriptase-polymerase chain reaction (RT–PCR) methods. This proliferative response was significantly inhibited by anti-HLA-DR monoclonal antibody, and required paraformaldehyde-fixed antigen-presenting cells (APC), indicating that this action is dependent on major histocompatibility complex (MHC) class II molecules without processing. Analysis of the amino-terminal amino acid sequence of the molecule failed to find any identical or significantly homologous proteins. We have previously reported that cytoplasmic membrane-associated protein (CAP), a streptococcal superantigen isolated from the cell membranes of *S. pyogenes* T12 strain, stimulated mainly Vβ8$^+$ T cells. Both SPM-2 and CAP preferentially stimulated helper T cells, and rabbit antiserum against SPM-2 completely neutralized the T-cell-stimulating activities of CAP, suggesting that SPM-2 and CAP belong to a family of streptococcal mitogenic proteins. The SPM-2 activity with stimulation of Vβ8$^+$ T cells was detected extensively in the culture fluids of group A streptococci, but not in those of other streptococcal species, including groups B and D streptococci, and most of the activities detected were completely inhibited by anti-SPM-2 serum. These results indicate that SPM-2 may be a newly discovered superantigen molecule, which can be commonly synthesized by group A streptococci.

INTRODUCTION

The genus *Streptococcus* is one of the most harmful Gram-positive cocci to humans (as well as the genus *Staphylococcus*), and infection by bacteria included in this genus causes various diseases. In particular *Streptococcus pyogenes* is the most severely pathogenic bacteria within the genus *Streptococcus*. It is known that *S. pyogenes* is principally distributed over the tunica mucosa pharynges, and occasionally brings on adenopharyngitis, peritonsillar abscess, tympanitis, erysipelas, impetigo, scarlet fever and toxic shock-like syndrome.[1] Toxic shock-like syndrome has recently been given a particular attention by us as the most important streptococcal infectious disease leading to septic shock and rapid systemic necrosis.[2] *Streptococcus pyogenes* is also involved in the pathogenesis of autoimmune arthritis such as reactive arthritis, the arthritis associated with rheumatic fever, and rheumatoid arthritis.[3,4]

Streptococcal pyrogenic exotoxins (SPE) produced by *S. pyogenes* were indicated as causative factors for these pyogenic streptococcal infectious diseases, but the precise mechanisms for pathogenesis of these SPE remain unclear.[5–7] SPE consist of three types (SPE-A, -B and -C), one of which at least is produced by most of the clinical isolates of *S. pyogenes*.[8,9] It has been established recently that these toxins, called superantigens, are apparently distinct from conventional toxins such as diphtheria, cholera, tetanus or botulinus toxin. Superantigenic SPE bind to both the proper sites of the major histocompatibility complex (MHC) class II

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Abbreviations: APC, antigen-presenting cell; CAP, cytoplasmic membrane-associated protein; MHC, major histocompatibility complex; PBL, peripheral blood lymphocyte; SEB, staphylococcal enterotoxin B; SPE, streptococcal pyrogenic exotoxin; SPM-2, *Streptococcus pyogenes* mitogen-2; TCR Vβ, variable domain of T-cell receptor β-chain.

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molecule on antigen-presenting cells (APC) and the variable domain of the T-cell receptor β-chain (TCR Vβ) of T lymphocytes, and then stimulate the proliferation of T lymphocytes with this specific Vβ.[10–12] Recently, two novel products of *S. pyogenes*, SSA and SPE-F (previously referred to as mitogenic factor), have been identified and reported to exhibit superantigenic activities.[13,14] And although the problem of contaminants present in pep M preparations has been raised, superantigenic properties of M proteins have been reported.[15] Taken together, these data indicate that most of the streptococcal infectious diseases or post-infectious sequelae might be attributable to the active response of T cells stimulated by streptococcal superantigens, although other superantigens yet to be discovered might also be involved.[5,7]

We first isolated a human T-cell mitogen from the cytoplasmic membrane of *S. pyogenes* strain T12, and named this mitogen as a cytoplasmic membrane-associated protein (CAP).[16] There was evidence that CAP was a novel streptococcal superantigen distinct from the known SPE, which selectively stimulated $V\beta8^+$ T lymphocytes.[17] We could detect helper T-cell-stimulating activity in the culture supernatants of *S. pyogenes* strains T12 and T19 and it was designated as *Streptococcus pyogenes* mitogen-2 (SPM-2), unlike the SPM recently reported.[18] In this paper, we report the isolation and superantigenic characterization of this molecule from the T12 strain, with similar immunological characteristics to those of CAP. The activity of SPM-2 was also detected in the culture supernatants of most of *S. pyogenes* strains tested, irrespective of laboratory or clinical strains, but not in those of other groups of streptococci. Based on these observations, it is concluded that SPM-2 might be a newly isolated superantigenic molecule that could be synthesized commonly by group A streptococci, and we discuss the possible roles of SPM-2 in the pathogenicity of autoimmune diseases caused by *S. pyogenes* infection in humans.

MATERIALS AND METHODS

Bacteria

Streptococcus pyogenes T12 strain (ATCC 12353), *S. agalactiae* (types Ia, Ib and II), *S. mitis*, *S. faecalis*, *S. sanguis* (ATCC 10556) and *S. mutans* (Ingbritt and GS-5), stored in our laboratory (Department of Microbiology, Tohoku University School of Dentistry), were used. *Streptococcus pyogenes* strains T18, T19, C203, B220, SF130, OSLO85 and NY5 were kindly provided by Dr H. Ohkuni (Nippon Medical School, Kawasaki, Japan). Clinical isolates of *S. pyogenes* and other streptococci were provided by Dr S. Murayama (Yamagata Prefectural Institute of Public Health, Yamagata, Japan). Each bacterial strain was grown at 37° in Todd–Hewitt broth (THB; Oxoid, Basingstoke, Hampshire, UK).

Isolation and purification of SPM-2

Streptococcus pyogenes strain T12 was mainly grown at 37° in 20 l of THB. After 24 h incubation, the batch culture was continuously centrifuged at 13000 ***g*** to obtain the culture supernatants. These supernatants were precipitated at 60% ammonium sulphate saturation in an ice-bath. The precipitate was dissolved in distilled water, and insoluble substances were removed by centrifugation. The concentrate was desalted by dialysis against distilled water at 4°, and acetate buffer (pH 5·2) was added to give a final concentration at 0·01 M. The desalted sample was applied to a diethylaminoethyl (DEAE)–cellulose (DE-52; Whatman, Maidstone, UK) column (2·6 × 28·5 cm) equilibrated with 0·01 M acetate buffer (pH 5·2). Finally, the active fractions in passing through the column, were purified by preparative isoelectric focusing (IEF) in a Rotofor cell (Bio-Rad Laboratories, Richmond, CA) at a pH gradient of 3·5–10·0 at 40 V constant voltage for 4 h. The purified materials were dialysed against 1 M NaCl and then distilled water to remove ampholytes. The isolated SPM-2 was tested for purity by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (12%; SDS–PAGE) under reducing conditions, followed by Coomassie Brilliant Blue staining. The electroeluates from successive slices of unstained gels of SDS–PAGE were also tested for mitogenic activity of human peripheral blood lymphocytes (PBL).[16] Then, CAP was also prepared from cytoplasmic membranes of the T12 strain, as described previously.[16]

Determination of the amino-terminal sequence

After SDS–PAGE of purified SPM-2, the gel was electrotransferred onto a polyvinilidene fluoride membrane, and stained with Coomassie Brilliant Blue. The amino-terminal amino acid sequence of the excised band was determined by an amino acid sequencer (model 473A; Applied Biosystems Inc., Foster City, CA).

Cell culture and proliferative assay

PBL were isolated from heparinized blood of healthy donors by Ficoll–Isopaque density gradient centrifugation.[16,17] The isolated PBL were washed three times with phosphate-buffered saline (PBS) and suspended in RPMI-1640 medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 10% heat-inactivated autologous sera and 2 mM L-glutamine. Highly purified T cells were obtained by passage through a nylon-wool column, as described previously.[19] PBL were suspended at a concentration of 5×10^5 cells/ml in the medium described above. Two hundred microlitres of this cell suspension was added to each well of a 96-well round-bottomed microculture plate (Falcon Labware, Oxnard, CA) and incubated with various mitogens at 37° in a humidified atmosphere of 5% CO_2 in air for 4 days. To assess APC function, the cells were treated with 1% paraformaldehyde solution in PBS for 15 min at room temperature, and used as fixed APC.[20] Anti-HLA-DR (Nichirei Co., Tokyo, Japan) or -DP monoclonal antibody (mAb) and control mouse IgG (Becton Dickinson, Mountain View, CA) were also used at a concentration of 5 μg/ml for the assay. The cells from each well were pulsed with 0·5 μCi [^{3}H]thymidine ([^{3}H]TdR) during the final 18 h of culture, and harvested onto glass fibre filters, after which radioactive incorporation was determined in a liquid scintillation counter.[16,17] The results represent the mean c.p.m. ± SD of triplicate cultures.

Flow cytometry

Analysis of Vβ expression on T cells stimulated with superantigens was performed as follows. PBL were incubated in RPMI-1640 medium for 4 days in the presence of various mitogens, followed by the addition of 100 IU/ml of recombinant human interleukin-2 (rIL-2; Shionogi Pharmaceutical Co.

Ltd, Osaka, Japan) for 1 day unless otherwise indicated. More than 96% of responding cells expressed CD3 marker in this culture. Then cells were stained with phycoerythrin (PE)-conjugated anti-CD3 or anti-CD4 mAb (Becton Dickinson), or fluorescein isothiocyanate (FITC)-conjugated anti-CD8 mAb (Becton Dickinson). Cells were also stained with biotin-conjugated anti-Vβ2 mAb (Immunotech, Marseille, France) followed by PE-conjugated streptavidin (Becton Dickinson), or stained with anti-Vβ5·3, 8, or 12 mAb (T Cell Science, Cambridge, MA) followed by FITC-conjugated goat anti-mouse IgG (Caltag, San Francisco, CA). The fluorescence intensities were determined by using a FACScan (Becton Dickinson).

RT-PCR-based analysis of TCR gene expression

Total RNA was extracted from SPM-2-stimulated PBL using guanidinium thiocyanate. The first-strand cDNA was synthesized in a 30-μl final volume at 42° for 1 h, using approximately 2 μg of total RNA, 1 μg of random hexanucleotides, and 5 U of reverse transcriptase (Promega, Madison, WI). The samples were heated for 5 min at 95° to terminate the reaction. The cDNA was aliquoted into 25 tubes, each containing a Vβ-specific primer with a Cβ primer and two Cα primers (Clontech Lab. Inc., Palo Alto, CA). A 35-cycle PCR reaction was performed in a 30-μl volume containing 0·3 μM of each primer and 2·5 U of *Taq* polymerase (TaKaRa, Kyoto, Japan). The cycle conditions for PCR were 1 min at 95°, 55° and 72°, respectively. These products were resolved on 2% agarose gels by electrophoresis and visualized by ethidium bromide staining and exposure to UV light.

Neutralization by anti-SPM-2 antiserum

Antibody against SPM-2 was prepared in New Zealand white rabbits (6 weeks old) by repeated injection of purified SPM-2 with Freund's complete adjuvant. For the neutralization of stimulating activity of superantigens for T cells, PBL (5×10^5 cells/ml) were stimulated with various superantigens in the medium containing anti-SPM-2 or normal rabbit serum at a final dilution of 1:80. After incubation for 4 days, the effects on DNA synthesis of whole PBL and specific induction of Vβ8-expressed T cells were measured.

Reagents

Concanavalin A (Con A), phytohaemagglutinin (PHA) and staphylococcal enterotoxin B (SEB) were purchased from Sigma Chemical Co. (St Louis, MO). Recombinant (r)SPE-A and -C were kindly provided by Shionogi Pharmaceutical Co. Ltd and by Dr Y. Nemoto (Iwate Medical University, Morioka, Japan), respectively.

RESULTS

Isolation of SPM-2 from culture supernatants of *S. pyogenes*

SPM-2 was precipitated at 60% ammonium sulphate saturation from culture supernatant of *S. pyogenes* strain T12, followed by separation on a DEAE–cellulose column with step-wise NaCl gradient, and the resulting fractions were tested for mitogenic activity to PBL and TCR Vβ expression of reactive T cells by flow cytometry. Although major proteins were eluted in passing through column (fraction I), 0·1 M NaCl (fraction II) and 0·5 M NaCl (fraction III), as shown in Fig. 1a, significant mitogenic activities were detected in fractions I and II but not in fraction III. Analysis of Vβ expression by flow cytometry showed that the eluate in fraction I activated Vβ8$^+$ T cells, and in fraction II activated Vβ2$^+$ and 8$^+$ T cells. In SDS–PAGE analysis, the protein at 29 000 MW was identified in fraction I, while several proteins were revealed in fraction II (Fig. 1b). When fraction I was further subjected to separation by preparative IEF in a Rotofor cell, a protein with mitogenic activity was eluted at an isoelectric point (pI) of 6·0 (Fig. 1c). The biological assay for the eluates from successive slices of unstained gels of SDS–PAGE showed a peak of mitogenic activity to PBL in the region corresponding to the band of 29 000 MW (Fig. 2). The purified SPM-2 stimulated PBL at concentrations greater than 1 ng/ml with DNA synthesis, yielding a maximal response at 1 μg/ml.

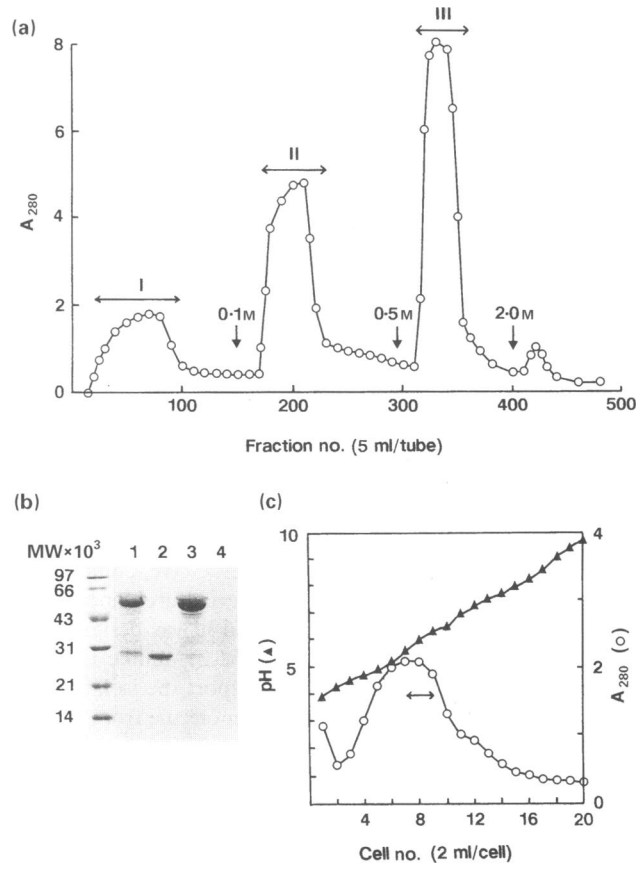

Figure 1. Purification profile of SPM-2. (a) Culture supernatant of strain T12 after ammonium sulphate precipitation was applied to a DEAE column equilibrated with 0·01 M acetate buffer (pH 5·2) and eluted with 0·1 M, 0·5 M and 2·0 M NaCl in acetate buffer as indicated with arrows. Passing through fractions and eluates with 0·1 M and 0·5 M NaCl were designated as fraction I, II and III, respectively. (b) Each of the fractions separated in (a) was analysed at a concentration of 10 μg of protein by 12% SDS–PAGE. Left lane, molecular weight marker proteins; lane 1, ammonium sulphate precipitate; lane 2, fraction I; lane 3, fraction II; lane 4, fraction III. (c) Fraction I in (a) was subjected to preparative IEF in a Rotofor cell at a pH gradient of 3·5–10·0 (▲). Active fractions, indicated by a bar with arrowheads, were collected.

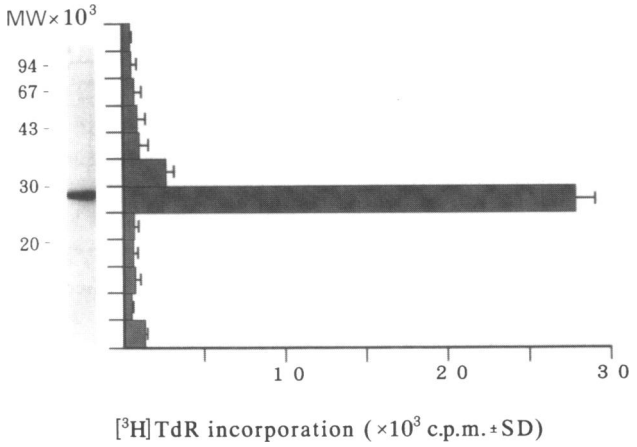

Figure 2. Recovery of mitogenic activity from SDS–PAGE. Samples were loaded at a concentration of 10 μg of protein per lane. The gel section shown was stained with Coomassie brilliant blue. The SPM-2 was extracted from unstained gels and detected by [³H]TdR incorporation into PBL. The positions of standard marker proteins are indicated on the left of the gel.

HLA-DR-dependent stimulation of T cells with SPM-2

Because superantigens possess the property of T-cell mitogenicity in an MHC class II-dependent but MHC-unrestricted manner, it was determined whether SPM-2-induced T-cell proliferation was MHC class II dependent using anti-HLA-DR, -DP and control mouse IgG. Staphylococcal enterotoxin B (SEB) was tested as a superantigen control. The results in Fig. 3 demonstrate that both SPM-2- and SEB-induced proliferative responses of PBL were significantly inhibited by the addition of anti-HLA-DR mAb, but not anti-HLA-DP and control IgG. However, it had little effect on the response to mitogens such as PHA. Furthermore, purified T cells reacted to SPM-2 in the presence of paraformaldehyde-fixed APC (25% of reduction in [³H]TdR incorporation), but not in the absence of APC, indicating that stimulation of T cells with SPM-2 depended on MHC class II molecules with APC without processing.

TCR Vβ expression of T cells reactive with SPM-2

Since it is well-known that almost all strains of *S. pyogenes* often produce one or more kinds of SPE as extracellular factors, we ascertained the possible appearance of T cells reactive with SPE-A or -C that might contaminate SPM-2 preparations by flow cytometry using Vβ-specific mAb. Table 1 shows that SPM-2 induced a striking increase in Vβ8$^+$ T cells that was similar to that of T cells stimulated with CAP,[17] but values outside the normal range were not observed for Vβ2$^+$, 5$^+$ and 12$^+$ subsets by SPM-2 stimulation. In contrast, rSPE-A stimulated Vβ12$^+$ T cells and rSPE-C stimulated Vβ2$^+$ T cells. No selective increase of any Vβ$^+$ T cells in Con A-stimulated T-cell blasts was observed. To confirm TCR Vβ elements responsive to SPM-2 precisely, RNA was isolated from SPM-2-induced T-cell blasts and examined for TCR Vβ expression by the reverse transcription-polymerase chain reaction (RT-PCR) method. RNA from Con A-induced T-cell blasts was used as a control. The results in Fig. 4 demonstrate that SPM-2 stimulated preferential expansion of Vβ4$^+$ and 7$^+$ T cells in addition to Vβ8$^+$ T cells, whereas no significant Vβ signals were observed in Con A-induced T-cell blasts.

Amino-terminal amino acid sequence of SPM-2

The sequence of amino-terminal amino acids of the SPM-2 protein was determined by an amino acid sequencer (Fig. 5).

Table 1. Vβ expression of human T cells stimulated with SPM-2

	% positive cells				
Stimulus	CD3	Vβ2	Vβ5·3	Vβ8	Vβ12
SPM-2	98·7	3·5	0·8	34·3	4·1
CAP	96·8	0·8	0·8	42·7	2·7
rSPE-A	99·1	0·7	0·9	2·3	13·7
rSPE-C	97·8	73·1	1·2	0·4	0·1
Con A	98·1	6·3	6·5	4·6	3·9

PBL (5×10^5/ml) were stimulated with Con A (1 μg/ml) for 2 days, or with streptococcal superantigens (1 μg/ml) for 4 days, and further expanded with rIL-2 (100 IU/ml) for 1 day. Analysis of Vβ expression was carried out by flow cytometry.

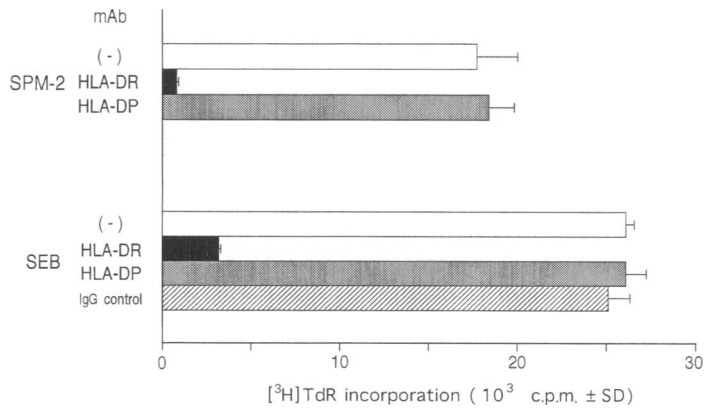

Figure 3. Requirement of MHC class II antigens for SPM-2-induced proliferation. PBL were cultured with SPM-2 or SEB (100 ng/ml) for 4 days in the presence of anti-HLA-DR or -DP mAb and control mouse IgG (5 μg/ml).

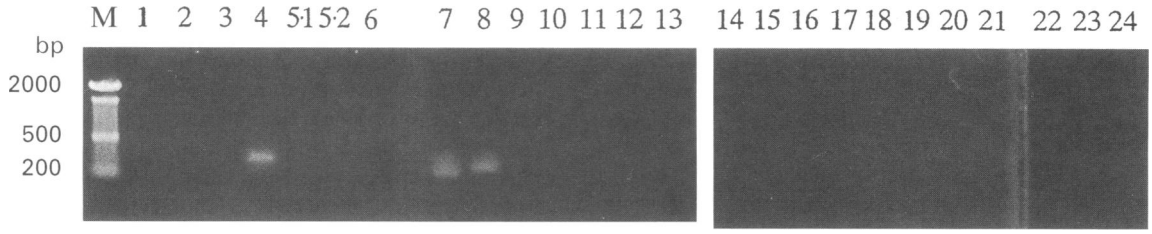

Figure 4. PCR analysis of the TCR Vβ usage of SPM-2-stimulated T cells. PBL were stimulated with 50 ng/ml of SPM-2 for 3 days, followed by expansion with 100 IU/ml of rIL-2 for 2 days. RNA was extracted from the cells, and its cDNA was prepared and analysed by the PCR method. M (bp) shows marker DNAs.

	1								10
SPM-2	: H	R	V	P	T	R	P	N	P
SPE-A(W)	: Q	Q	D	P	D	P	S	Q	L H
SPE-A(J)	: S	T	R	P	K	P	S	Q	L Q
SPE-B	: Q	P	V	V	K	S	L	L	D S
SPE-C	: D	S	K	K	D	I	S	N	V K
SSA	: S	S	Q	P	D	P	T	P	E Q
MF	: Q	T	Q	V	S	N	D	V	V L

Figure 5. Comparison of amino-terminal amino acid sequence with streptococcal superantigens. The SPE-A, -B, -C, SSA and MF sequences were derived from the cloned gene sequences.[13,21–25] SPE-A (W) was reported by Weeks & Ferretti[24] and SPE-A (J) was reported by Johnson & Schlievert.[23]

This sequence of the first nine amino acids was different from the sequences of amino-terminal and other regions of streptococcal superantigens already reported. Searches of SwissProt (Amos Bairoch, Switzerland) and PIR (Protein Identification Resource, National Biomedical Research Foundation, USA) databases for homology of this sequence failed to find any identical or significantly homologous proteins. The amino acid composition of SPM-2 (molecule ratio percentage of amino acid) was also different from those of SPE (data not shown).

Immunological or antigenic homology between SPM-2 and CAP

The results in Fig. 6 show that T-cell subsets induced by SPM-2 or CAP stimulation were preferentially $CD4^+$ T cells, whereas rSPE-A and -C stimulated equally both $CD4^+$ and $CD8^+$ T cells. The treatment of SPM-2 or CAP-stimulated PBL with anti-SPM-2 antiserum (1:80 dilution) almost inhibited the appearance of $V\beta8^+$ T cells, indicating antigenic homology between SPM-2 and CAP molecules (Table 2). On the other hand, no inhibitory effects of this antiserum were observed on activation of T cells by SEB, rSPE-A, -C or Con A stimulation.

Distribution of SPM-2 activity among group A streptococci

Finally, we investigated the distribution of SPM-2 activity in the culture supernatants of various laboratory strains of *S. pyogenes* and other streptococci. As a result of the measurement of proliferative responses of PBL by incubation with respective culture supernatants (1:10 dilution), all strains of *S. pyogenes* tested could stimulate PBL, but the supernatants of streptococci other than group A streptococci (*S. agalactiae*, *S. mitis*, *S. faecalis*, *S. sanguis*, and *S. mutans*) did not stimulate PBL from several donors (data not shown). One of the major populations induced by stimulation of PBL with *S. pyogenes* culture supernatants was $V\beta8^+$ T cells (Table 3). On the other hand, these supernatants of *S. pyogenes* strains (except for the T19 strain) also contained SPE-C activity with stimulation of $V\beta2^+$ T cells, although no SPE-A activity with stimulation of $V\beta12^+$ T cells was detected in any culture supernatants of *S. pyogenes* in our experimental systems. Culture supernatants of many clinical isolates of *S. pyogenes* also activated both $V\beta2^+$ and 8^+ T cells (depicting one of the clinical isolates in Table 3). T-cell activation by SPM-2 stimulation required a 10-fold higher concentration than needed when using SPE-C. Moreover, anti-SPM-2 antiserum substantially inhibited the induction of $V\beta8^+$ T cells by these supernatants (Table 4), like the T12 strain, strongly suggesting that the substance with properties of SPM-2 for induction of $V\beta8^+$ T cells from PBL may be produced by almost all strains of *S. pyogenes*.

DISCUSSION

In this study we have isolated SPM-2 as a novel superantigen with a molecular weight of 29 000 and a pI of 6·0 from the culture supernatant of *S. pyogenes* strain T12. The SPM-2, unlike SPM recently isolated from the culture supernatant of strain T12,[18] was mitogenic only to human T cells, but did not stimulate murine lymphocytes. SPM-2, with a unique sequence of amino-terminal amino acids, was found to activate preferentially helper T cells bearing TCR Vβ4, 7 and 8 in the presence of APC that expressed HLA-DR molecules, as determined by PCR amplification of cDNA prepared from the stimulated cells (Figs 3, 4 and 5). It was also indicated that SPM-2 and CAP might belong to a family of streptococcal mitogenic proteins by the findings that (a) both SPM-2 and CAP activated $V\beta8^+$ helper T cells, and that (b) both activities were inhibited by anti-SPM-2 antiserum (Tables 1, 2 and Fig. 6). Furthermore, we showed that the same activities as SPM-2 could be detected in the culture supernatants of almost all laboratory and clinical strains of *S. pyogenes* (Table 3), indicating the extensive distribution of SPM-2 activity among group A streptococci.

In addition to our reports,[16–18] streptococcal superantigens that stimulate human T cells, SPE (A, B, and C), streptococcal M proteins, SSA and SPE-F have also been reported.[10–14] However, it has become apparent that SPE-B is not a superantigen, because purified SPE-B preparations contain trace amounts of the uncharacterized superantigen responsible for $V\beta8^+$ T-cell stimulation.[26] In addition to the description of each physicochemical property, it has also been reported that SPE-A stimulated $V\beta2^+$, 8^+ and 12^+ T cells, SPE-C stimulated

Table 2. Inhibition of Vβ8 T-cell-stimulating ability of SPM-2 and CAP by anti-SPM-2 antiserum

	% induction of Vβ8⁺T cells	
Serum (1:80)	SPM-2	CAP
Normal rabbit serum	17·3	22·1
Anti-SPM-2 serum	1·8	1·5

PBL were stimulated with SPM-2 (50 ng/ml) and CAP (5 μg/ml) in the medium containing anti-SPM-2 or normal rabbit serum for 4 days, followed by addition of rIL-2 (100 IU/ml) for 1 day. Analysis of Vβ8 expression of T cells was carried out by flow cytometry.

Vβ2⁺ and 8⁺ T cells, and pep M5 stimulated Vβ2⁺, 4⁺ and 8⁺ T cells, respectively.[26,27] SSA stimulated Vβ1⁺, 3⁺ and 15⁺ T cells, and SPE-F stimulated Vβ2⁺, 4⁺, 8⁺, 15⁺ and 19⁺ T cells.[13,14] Although each of the streptococcal superantigens had a unique pattern of Vβ specificity, they shared an affinity to the Vβ8 element. It is, however, a serious problem that the molecule responsible for Vβ8⁺ T-cell stimulation existed in these preparations, because recombinant superantigens (SPE-A or SPE-C) could not stimulate Vβ8⁺ T cells.[26] This is consistent with the interpretation that the ability to stimulate the Vβ8⁺ T-cell subset depends on a novel superantigen distinct from SPE, probably from M proteins or SPE-F, and is extensively distributed in supernatants of *S. pyogenes*.[26]

Table 3. Vβ expression of T cells stimulated with culture supernatants of *S. pyogenes* strains

	% positive cells			
Strain	CD3	Vβ2	Vβ8	Vβ12
T12	97·1	39·3	16·7	0·2
T18	94·8	26·9	13·1	0·3
T19	95·2	3·1	21·1	0·2
C203	90·6	33·3	15·2	0.8
B220	89·8	26·7	12·8	1.8
OSLO85	95·1	22·4	7·1	0·7
SF130	94·4	23·1	25·5	2·8
Y378*	87·5	16·1	17·3	2·2
Con A	96·5	4·2	3·9	0·4

PBL were stimulated with culture supernatants (dilution of 1:10) of *S. pyogenes* strains for 4 days, followed by addition of rIL-2 (100 IU/ml) for 1 day. Analysis of Vβ expression of T cells stimulated by supernatants was carried out by flow cytometry.

*Clinical isolate.

From the results obtained in the present study (e.g. extensive neutralization of Vβ8⁺ T cell-stimulating activity by anti-SPM-2 antiserum), it is considered possible that the Vβ8⁺ T-cell-stimulating superantigen that contaminated SPE or other preparations may be a derivative of SPM-2, although we have no data about the Vβ8 stimulator called SPE-X by

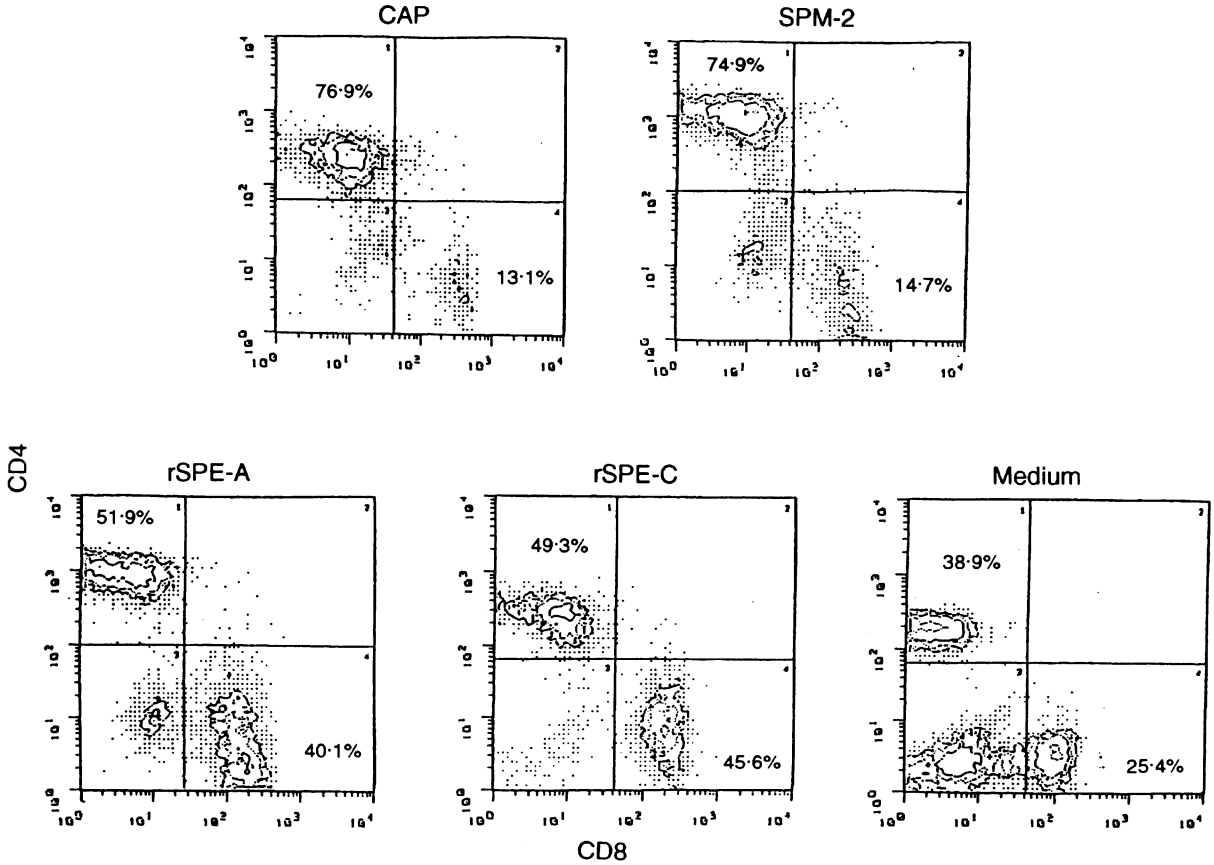

Figure 6. Profiles of human PBL stimulated by SPM-2 and CAP. PBL were cultured with SPM-2, CAP, rSPE-A or rSPE-C at 1 μg/ml for 4 days, respectively. PBL were stained with PE–anti-CD4 and FITC–anti-CD8 mAb, and analysed by flow cytometry.

Table 4. Inhibition of Vβ8 T-cell-stimulating ability of culture supernatants of *S. pyogenes* strains by anti-SPM-2 antiserum

	% induction of Vβ8$^+$ T cells			
Serum (1:80)	T12	T19	B220	C203
Normal rabbit serum	13·8	12·7	9·3	15·2
Anti-SPM-2 serum	4·8	6·7	5·2	5·8

PBL were stimulated with culture supernatants (dilution of 1:1000) of *S. pyogenes* strains in medium containing anti-SPM-2 or normal rabbit serum for 4 days, followed by addition of rIL-2 (100 IU/ml) for 1 day. Analysis of Vβ8 expression of T cells was carried out by flow cytometry.

Braun *et al.*[26] Since database searching for the amino acid sequence highlighted the difference of amino-terminal sequences between SPM-2 and other streptococcal superantigens reported previously (Fig. 5), the determination of the full sequence for the SPM-2 protein is necessary for investigation of fine structures of the molecule and binding sites to the MHC class II molecule and TCR Vβ region. Experiments are now under way to isolate genomic clones coding the SPM-2 molecule from *S. pyogenes* strain T12.

As an approach for research of the *in vivo* mode of action of SPM-2, which produced no erythematous reaction in the skin of rabbits at 24 h after injection, we have also clarified increased levels of anti-SPM-2 antibodies in patients with rheumatoid arthritis or post-streptococcal glomerulonephritis, and return to normal levels in the inactive stage of the diseases (data not shown). Thus, the SPM-2 is also a target antigen for host antibodies, and the determination of circulating anti-SPM-2 antibody titres at least provides an important method for diagnosing streptococcal infection in clinical practice. If a close association between autoimmune sequelae and increase in anti-SPM-2 antibody levels exists, it should be considered a possibility that SPM-2 with antigenic similarity to structural proteins (CAP) localized to bacterial cytoplasmic membranes may have antigenic properties cross-reactive with structural proteins localized to host cell membranes. Conceivably, the association between *S. pyogenes* infection and autoimmune sequelae may offer a useful model for the role of superantigens in the pathogenesis of autoimmune disease.

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