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Streptococcal Mitogenic Exotoxin Z, a Novel Acidic Superantigenic Toxin Produced by a T1 Strain of *Streptococcus pyogenes*

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Streptococcus pyogenes **T1 was previously found to produce an acidic mitogenic exotoxin, designated Aβ, antigenically distinct from erythrogenic toxin type A (ETA) of strains T1 and NY5. Following chemical analysis and biological characterization, we have renamed this toxin streptococcal mitogenic exotoxin Z (SMEZ). Physicochemical separation of SMEZ from ETA was successfully performed on a hydrophobic chromatograph. The isoelectric point was pH 5.3, and the molecular size was estimated to be 28 kDa. These values were similar to those of ETA, but the amino acid composition and the NH_2-terminal sequence of SMEZ were distinct from those of any mitogenic exotoxins hitherto described. Its mitogenic activity was found to be more potent than that of ETA in rabbit lymphocyte cultures. A specific antiserum raised against SMEZ did not cross-react with ETA, ETB, or ETC in the neutralization tests of mitogenic and erythrogenic activities. Its superantigenic nature was evident from the reverse transcriptase PCR findings of the T-cell receptor Vβ profiles of rabbit lymphocytes stimulated in vitro. The Vβ 8 subfamily was unique to SMEZ, while the Vβ 2 and 6 subfamilies were found to be common among lymphocytes stimulated with ETA, ETB, ETC, or SMEZ. The results from this study provide an additional example of the diversity that exists among mitogenic or superantigenic exotoxins of streptococcal origin.**

Streptococcus pyogenes is known to produce at least three antigenically distinct types of erythrogenic toxin (ET) or pyrogenic exotoxin (SPE): A, B, and C. During the 1970s, these toxins were purified and characterized (6, 11, 37). Subsequently, the genes for these three types of ET were cloned and sequenced (7, 19, 22, 40), and the sequences of the purified proteins were shown to be consistent with the deduced amino acid sequences (14, 15, 20, 26, 36, 38). The molecular sizes of the ETs were in the range of 25 to 30 kDa, and their isoelectric points were shown to differ: ETA is an acidic protein with a pI of 5, ETB is a basic protein with a pI range of 8 to 9, and ETC is a neutral protein with a pI of 7. Their biological activities include mitogenicity, pyrogenicity, and enhancement of endotoxin shock (5, 6, 11, 14–16, 20, 26, 36, 37). These exotoxins have also been shown to act as superantigens to stimulate T cells to produce cytokines (5, 9, 12, 20, 21, 27, 31, 34). Recently, Nelson et al. reported that SPE A had four allelic forms (33), indicating that ETs or SPEs are still a diverse group of molecules. In addition, many extracellular mitogens or superantigens other than classical ETs have been described. Mollick et al. found a 28-kDa protein called streptococcal superantigen (SSA) in strains isolated from streptococcal toxic shock syndrome patients (30). Yutsudo et al. purified a 25-kDa mitogen called mitogenic factor (MF) from a NY5 strain product (41) and cloned its gene in *Escherichia coli* (24). Norrby-Teglund et al. described a 27.5-kDa superantigen called SPE F, which they thought to be the same as MF (34, 35). Several authors also have described low-molecular-weight mitogens. Gerlach et al. found two acidic 12-kDa mitogens, pI 4.3 and 4.7 (17), which were thought to be identical to SPE D, previously reported by McMillan et al. (29). Cavaillon et al. separated a basic, pI 10.3, 17-kDa mitogen called ε (9). Geoffroy-Fauvet et al. found a neutral, pI around 7, 8-kDa superantigen (LMWS) in a clinical strain (13). We have recently reported that a T1 strain produces an acidic mitogenic exotoxin, designated Aβ, which is antigenically different from the ETA of strains T1 and NY5 (32). The Aβ toxin was found only after absorption of the T1 strain toxin with an anti-NY5 ETA serum. In the present study we provide evidence that Aβ is not a subtype of ETA but is a unique superantigenic exotoxin, being distinct not only from classical ETs but also from all the other mitogens or superantigens mentioned above. We have renamed this protein streptococcal mitogenic exotoxin Z (SMEZ).

MATERIALS AND METHODS

Bacteria and culture medium. *S. pyogenes* T1 was provided by the Institute of Medical Science, University of Tokyo. A complete synthetic medium (SMS) was designed for optimal toxin production (25). Briefly, the constituents were 100 mg each of DL-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, hydroxy-L-proline, L-serine, and L-valine, 200 mg each of L-glutamine, L-threonine, L-tryptophan, and L-tyrosine, 500 mg of L-cysteine, 30 mg of L-cysteine, 20 mg each of adenine, guanine, and uracil, 0.1 mg of vitamin B_{12}, 0.2 mg each of *p*-aminobenzoic acid and biotin, 0.3 mg of α-tocopherol, 0.5 mg of menadione, 0.8 mg of folic acid, 1.0 mg each of nicotinamide, pyridoxal-HCl, pyridoxamine-2HCl, and thiamine-HCl, 2.0 mg each of pantothenate-Ca and riboflavin, 2.5 mg of β-NAD, 13 mg of choline, 1 mg of $Fe(NO_3)_2$–$9H_2O$, 5 mg each of $FeSO_4$–$7H_2O$ and $MnSO_4$, 10 mg of $CaCl_2$, 200 mg of KH_2PO_4, 450 mg of Na_2HPO_4, 700 mg of $MgSO_4$–$7H_2O$, 4.5 g of CH_3COONa, 2.5 g of $NaHCO_3$, 10 g of glucose, 9.5 g of HEPES, and 20 mg of phenol red per liter of the medium. Culture supernatants of the logarithmic bacterial growth phase were used for toxin isolation.

Toxin purification and chemical analysis. The SMS culture supernatant was filtrated with a Durpore membrane (Millipack-20; pore size, 220 nm; Millipore), concentrated through ultrafiltration with Amicon PM10, and precipitated at 75% saturation with ammonium sulfate (AS). The precipitate was processed by chromatofocusing using PBE 94, pH 6.0 to 4.0 (Pharmacia), and the peak at pH 5.2 was further analyzed on a hydrophobic interaction chromatograph (HIC) (high-pressure liquid chromatography with TSK gel Ether-5PW; Tosoh). Gel permeation chromatography using Superose 12 (Pharmacia) was performed to desalt

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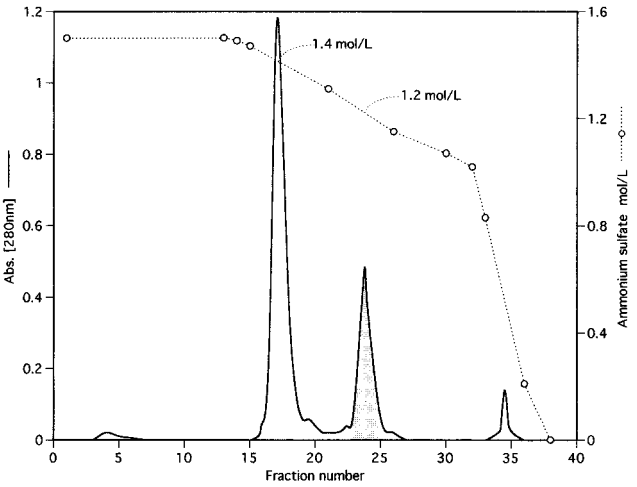

FIG. 1. HIC of the pI 5.2 fraction from the T1 strain product. Starting from a 36-liter culture filtrate, ca. 1.7 mg of protein, contained in the later half of the pH 5.2 peak on pH 6 to 4 chromatofocusing using PBE94 (Pharmacia), was passed through an HIC column (Tosoh), 7.5 mm in inner diameter and 7.5 cm long, containing TSK gel Ether-5PW (Tosoh), under high pressure. The protein was eluted with a gradient of 1.5 to 0 M AS in 0.1 M phosphate buffer, pH 7.0, at a rate of 0.35 ml/min under monitoring with 280-nm UV light. Each fraction of 7 ml or 2 min was collected.

and isolate the peaks on HIC. The molecular size was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (28). The isoelectric point was measured by agarose isoelectric focusing (IEF). The amino acid composition was determined in an L-835 amino acid analyzer (Hitachi). The NH_2-terminal sequence was examined in a gas-phase amino acid sequencer (G1005A; Hewlett-Packard). ETA (26) and ETB (15) of strain NY5, and ETC (36) of strain T18 were purified from culture supernatants as described previously. The T18 and NY5 ETC were gifts from D. Gerlach, Jena, Germany. Immune sera against the HIC-purified products, NY5 ETA, and T18 ETC, respectively, were raised in rabbits by use of Freund (Difco Laboratories, Detroit, Mich.) and RIBI (RIBI ImmunoChem Research, Montreal, Quebec, Canada) adjuvants.

Assay of mitogenic and erythrogenic activities and their inhibition. The mitogenic activity of the purified toxin was measured by tritiated-TdR (ICN Biochemicals) incorporation in a JW-NIBS strain rabbit lymphocyte culture as described elsewhere (2). The 50% effective dose (ED_{50}) was determined from the dose-response curve. The skin test dose was determined in the back skin of rabbits as described elsewhere (26). Various dilutions of rabbit antiserum were employed to neutralize the biological activities. A human serum panel, consisting of sera from 10 healthy adult (male and female) volunteers whose ages ranged from the 20s to the 50s, was used for the neutralization test of the mitogenic activity. The log_2 titer of the 50% inhibition was determined by fourfold successive dilutions of test serum added at a 5% concentration to lymphocyte culture.

ELISA. To determine the antigenic specificity of purified toxin preparations, an amplified enzyme-linked immunosorbent assay (ELISA) was performed by using rabbit antitoxin immune sera as described elsewhere (2).

Analysis of T-cell receptor (TCR) Vβ expression. Total RNA of the cultured lymphocytes was extracted with acid guanidium thiocyanate-phenol-chloroform as described previously (10). An aliquot (0.5 to 5 μg) was reverse transcribed with 20 U of Rous-associated virus-2 reverse transcriptase (RT; Takara Shuzo, Kyoto, Japan) in 20 μl of 50 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 10 mM $MgCl_2$, 3 mM dithiothreitol, 500 μM deoxynucleoside triphosphates, and 4 μg of random nonamers. The reaction proceeded at 42°C for 1 h and then at 95°C for 3 min. Aliquots (0.4 μl) of single-stranded cDNA were amplified by PCR with Vβ subfamily-specific primers and the Cβ primer TBC2R (23). A Vβ 12 subfamily-specific primer (5′-GTGACCCAGAGTCTGGTCACTA-3′) was newly synthesized according to the reported sequence data (DDBJ/EMBL/GenBank accession no. D84651). The PCR mixture (20 μl) consisted of 25 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mg of $MgCl_2$, 1 mM dithiothreitol, 200 μM deoxynucleoside triphosphates, 1.25 U of *Taq* DNA polymerase (Takara Shuzo), and 200 nM each Vβ subfamily-specific primer and TBC2R primer. Amplification was performed with 25 or 30 cycles of denaturation (at 94°C for 1 min), annealing (at 55°C for 2 min), and extension (at 72°C for 3 min). Amplified PCR products were stained with ethidium bromide and analyzed with an Epi-Light UV image analyzer EU-1150II (Aisin Cosmos R&D, Kariya, Japan) to measure the intensity of each band in order to obtain a relative value for the amount of amplification. Each relative value is shown as the percentage of the total density. Concanavalin A (ConA; Miles-Yeda, Rehovot, Israel)-stimulated cultures were used as the control for evaluation of streptococcal toxin-stimulated cultures. Human counterparts of rabbit Vβ subfamilies 1 to 12, except 4, are 7, 2, 9, 3, 4, 5, 8, 10, 18, 22, and 6, respectively. The human counterpart of rabbit Vβ 4 has not been identified yet. Numbers 2 and 8 are common to the human and rabbit subfamilies.

RESULTS

Purification and chemical characterization. The pH 5.2 peak obtained from the chromatofocusing corresponded to the mitogenic activity for rabbit lymphocytes, which was largely but not completely neutralized with an anti-NY5 ETA antiserum, as described previously (32). When the product was applied on an HIC column, three main peaks emerged by elution with an AS gradient (Fig. 1). The first, 1.4-mol peak corresponded to the mitogenic activity completely neutralized with an anti-NY5 ETA serum, and the second, 1.2-mol peak corresponded to a mitogenic activity induced by the toxin designated SMEZ, which was not neutralized with the anti-NY5 ETA serum. These proteins also induced erythematous reactions in the rabbit skin which were respectively neutralized, or not, with the same antiserum. Thus, we successfully separated the two toxins by AS gradient elution on the HIC column. The final yield of SMEZ was 48 μg from a 12-liter culture. SMEZ showed a single band with a molecular size of 28 kDa by SDS-polyacrylamide gel electrophoresis (Fig. 2), and its isoelectric point was 5.3 by agarose IEF. The amino acid composition of SMEZ was unique and differed greatly from any of those previously described for streptococcal exotoxins (Table 1). Lack of cysteine residues was a unique feature of this protein. The sequence of 10 amino acids at the NH_2 terminus was LEVDNNSLLR, which was also not described previously.

Biological activities and their inhibition. The ED_{50} of SMEZ for the rabbit lymphocyte blastogenic response was approximately 0.5 ng/ml. This was 10-fold more potent than T1 ETA and almost comparable with T18 ETC (Fig. 3), while the skin test dose, 10 ng of SMEZ inducing a 10- by 10-mm erythema, was similar to those of T1 ETA and T18 ETC (data not shown). The antiserum raised against the purified toxin neutralized the mitogenic activity in the dose of 0.5 ng/ml by 50%

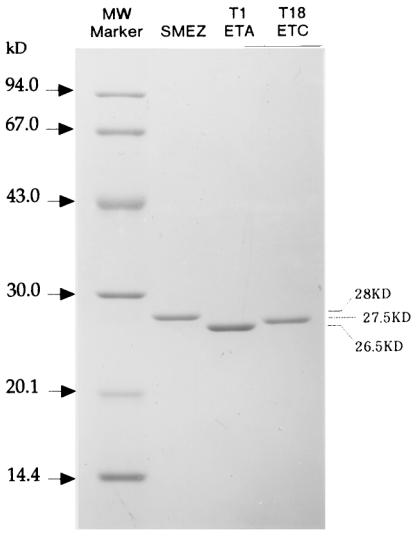

FIG. 2. SDS-polyacrylamide gel electrophoresis of SMEZ, T1 ETA, and T18 ETC. One microgram of SMEZ, 1.5 μg of T1 ETA, and 1 μg of T18 ETC each was run on a 12.5% acrylamide gel according to the method described by Laemmli (28). The LMW kit (Pharmacia) was used as the molecular-size marker. The molecular size (in kilodaltons) of SMEZ was estimated as 28, that of T1 ETA as 26.5, and that of T18 ETC as 27.5. A Coomassie blue stain was used.

TABLE 1. Amino acid composition of streptococcal exotoxins

Amino acid	Percent molecular ratio of toxin[a]					
	SMEZ	ETA	SPE A	SPE B	SPE C	MF
A	3.5	2.1	1.9	7.1	2.4	8.4
R	3.1	1.2	1.4	2.8	3.4	5.4
D/N	18.7	14.5	14.0	13.0	17.3	16.1
C	0	1.1	1.4	0.4	0.5	ND
E/Q	8.6	13.2	12.7	9.1	8.1	9.8
G	5.2	4.2	4.2	11.9	5.3	10.4
H	2.0	2.9	2.8	2.8	2.9	1.6
I	8.4	4.7	5.9	4.3	10.5	3.7
L	7.5	8.9	9.1	5.5	6.2	8.4
K	9.8	9.8	10.0	5.5	10.1	4.8
M	1.8	1.2	1.4	4.0	1.4	0
F	5.6	4.2	4.1	1.6	5.3	2.1
P	3.5	4.2	4.2	4.7	1.9	3.7
S	9.4	6.8	6.9	8.7	7.7	4.8
T	5.6	6.4	6.5	3.6	5.7	8.0
W	ND	0.4	0.5	1.6	0	ND
Y	0.2	6.8	7.7	5.9	7.7	5.8
V	7.0	6.8	6.4	7.5	3.4	6.9

[a] References for toxin composition: 26 (ETA), 40 (SPE A), 22 (SPE B), 19 (SPE C), and 41 (MF). ND, not determined.

at a 1:128 dilution added at a concentration of 5% to the rabbit lymphocyte culture. The antiserum did not cross-react with NY5 ETA, T1 ETA, T18 ETC, or NY5 ETC in the neutralization test of the mitogenic activity or the ELISA (data not shown). The antiserum also completely inhibited the mitogenicity of the previous Aβ preparations obtained by absorption with anti-NY5 ETA serum. Table 2 shows the results of the neutralization test of the mitogenic activities of NY5 ETA, -B, and -C and SMEZ by a panel of human sera from healthy adult volunteers of various ages and sexes. These results again differentiate SMEZ from classical ETs, together with a seroepidemiological variation among humans. Strains producing SMEZ are probably not uncommon, since many persons, 9 of 10 in the panel, had antimitogenic serum antibodies to both SMEZ and ETC.

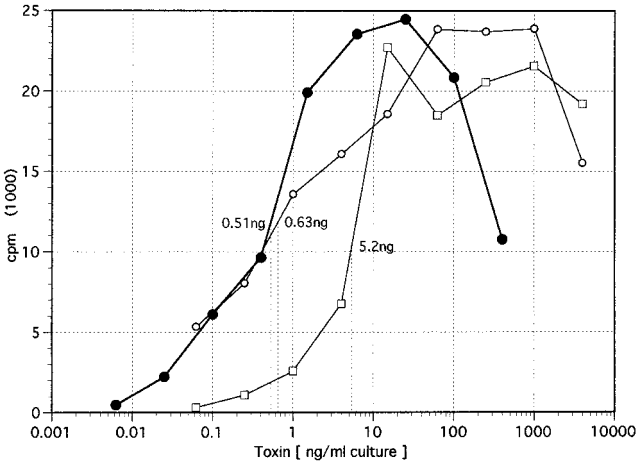

FIG. 3. Dose-response relationship of mitogenic activities of SMEZ, T1 ETA, and NY5 ETC in rabbit lymphocyte culture. Symbols: ●, SMEZ (ED_{50}, 0.51 ng); □, T1 ETA (ED_{50}, 5.2 ng); ○, T18 ETC (ED_{50}, 0.63 ng). The data (in counts per minute) were calculated by subtracting the average counts per minute of control cultures without the toxin from the average counts per minute of triplicate cultures stimulated with the toxin. The counts per minute of the control in this experiment was 236 ± 78. Abscissa, dose of the toxin expressed as the final concentration in culture. Rabbit lymphocyte culture was carried out in a 96-well microplate as described previously (2). Ten microliters of the toxin was added at the start of culture to 200 μl of 4×10^5 rabbit lymphocytes suspended in Dulbecco's modified Eagle medium with 30% autologous inactivated serum. After incubation for 3 days in a CO_2 incubator, tritiated TdR was added to a concentration of 1 μCi/ml and the incubation was continued for a further 17 h. TdR uptake was measured in a liquid scintillator.

TABLE 2. Antimitogen assay[a] of human sera from healthy adult volunteers

Serum no.	Log_2 titer of 50% inhibition of mitogenic activity of:			
	ETA[b]	ETB	ETC	SMEZ
1	6	−[c]	4	6
2	−	2	6	1
3	2	5	3	4
4	−	−	−	−
5	−	9	9	6
6	4	2	5	6
7	−	−	9	6
8	−	−	9	9
9	3	3	6	9
10	7	7	7	9

[a] Ten microliters of fourfold successive dilutions of human sera was added to 200 μl of rabbit lymphocyte culture stimulated with toxins at a dose of 1.0 ng/ml.
[b] ETA, ETB, and ETC are NY5 strain products.
[c] −, negative at a 1:4 dilution.

TCR Vβ profiles. Figure 4 shows the TCR Vβ profiles of ET-, SMEZ-, and ConA-stimulated lymphocytes of two rabbits tested. Table 3 summarizes the TCR Vβ preferences of ET- and SMEZ-stimulated lymphocytes. The Vβ subfamily preference was assessed as significant when the ratio of the incidence of the Vβ in lymphocytes stimulated with a given type of ET or SMEZ to that in ConA-stimulated culture was more than 1.00. Vβ 6 was as common in the responses to ETs, irrespective of their strains or types, as in that to SMEZ. Vβ 1 was common in the responses to both NY5 ETB and -C, with or without Vβ 3. Vβ 2 was common in the responses to NY5 ETC, T1 ETA, and SMEZ, while Vβ 8 was unique to SMEZ. The increase in Vβ 10 may represent an individual skewness, since only one of two rabbits expressed Vβ 10 in response to NY5 ETB, -C, and T1 ETA. No increase in Vβ 10 was seen in the response to SMEZ. Unexpectedly, NY5 ETA seemed to be heterogeneous in the response induced, with Vβ 5 and 10 preference. Furthermore, Vβ 7 and 4 expression was comparable to that in the response to ConA, which may suggest another lectin-like mitogen in the NY5 ETA product.

DISCUSSION

SMEZ, described in this paper, is a superantigenic exotoxin distinct not only from classical ETs but also from all other mitogens or superantigens described previously. SMEZ has erythrogenic activity, but we prefer to use its mitogenic activity in the nomenclature because of the ease and accuracy of the quantitative assay. The amino acid composition and the NH_2-terminal sequence differed from those of any mitogens given in the literature. This toxin was once thought to be a subtype that had deviated minimally from ETA because it was copurified with ETA, was difficult to separate physicochemically, and had the same pI and a similar molecular size. However, the antigenic difference was great enough to separate SMEZ from ETA. Our previous seroepidemiological study of SMEZ by the antimitogen assay revealed that the occurrence of the serum antibody differed from those of antibodies to ETA, -B, and -C among children, including patients with Kawasaki disease and

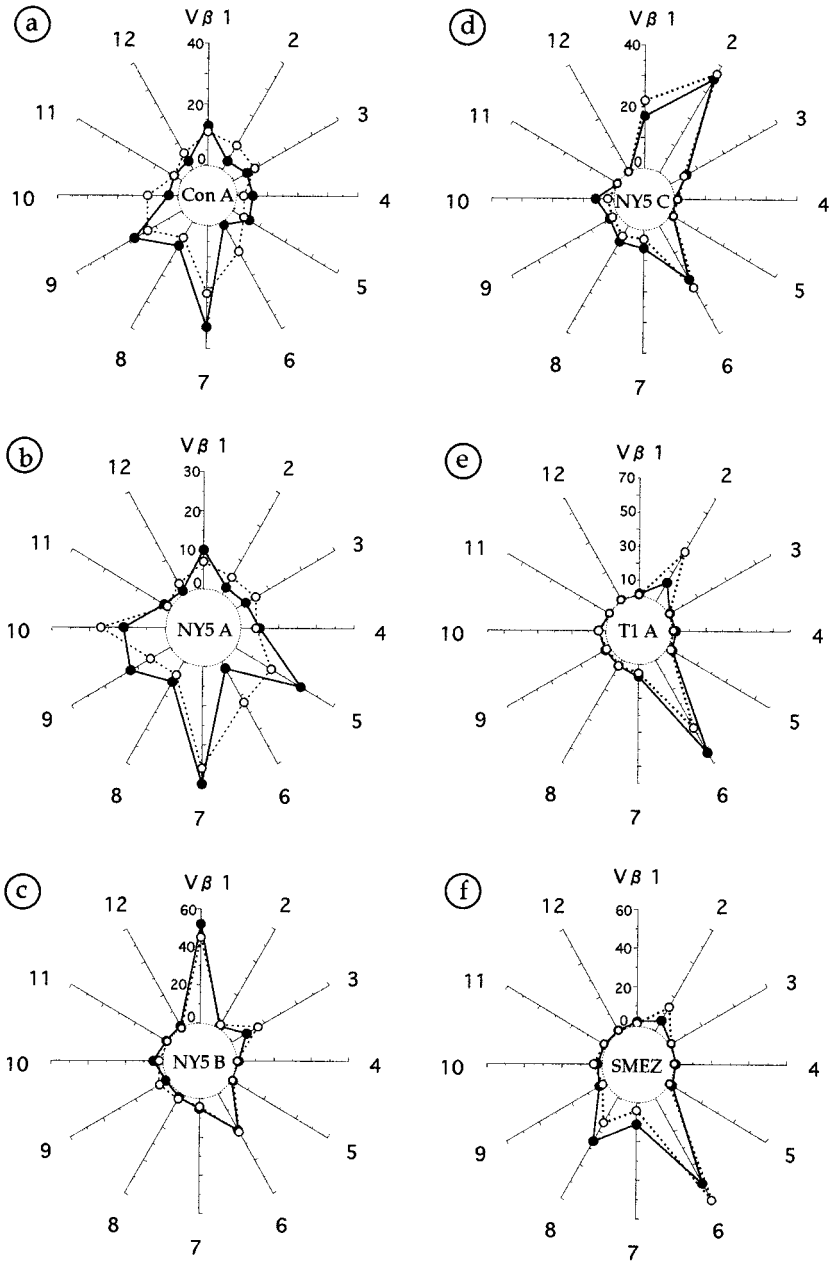

FIG. 4. TCR Vβ profiles of rabbit lymphocytes stimulated with the mitogen ConA (a), NY5 ETA (b), NY5 ETB (c), NY5 ETC (d), T1 ETA (e), or SMEZ (f). Symbols: ●, rabbit 1; ○, rabbit 2. Total RNA of rabbit lymphocytes cultured in an Ambitube (Nunc) for 4 days was extracted after washing in phosphate-buffered saline and processed for RT-PCR assay as described in Materials and Methods. The dose in culture of ConA was 5 μg/ml; that of NY5 ETA, NY5 ETC, T1 ETA, and SMEZ was 1 ng/ml; and that of NY5 ETB was 10 ng/ml. The relative density (as a percentage of total density) of each TCR Vβ subfamily-specific PCR product is shown along the vertical axis of the radar chart.

S. pyogenes infection (3, 4). The study described here again confirms the antigenic difference by use of a human serum panel consisting of 10 healthy adult volunteers' sera. These serological studies also suggest that the distribution of streptococcal strains producing SMEZ is probably not uncommon. The pathobiologic significance of SMEZ awaits further study.

The gene encoding SMEZ was identified in the University of Oklahoma database of *S. pyogenes* DNA (12a). The whole protein amino acid sequence was successfully located, starting with the NH_2-terminal sequence (amino acids 25 through 34, LEVDNNSLLR) described in this paper. The sequence (Fig. 5) correlates with our chemical analysis data describing the amino acid composition and the molecular weight of the mature protein estimated at 27,981 and 25,254 with and without the signal peptide, respectively. Homology to other superantigens was also found to some extent, including staphylococcal enterotoxins A, C1, D, E, H, and SPE A and C.

TCR Vβ profiles of human lymphocytes after stimulation with streptococcal exotoxins were reported with wide variety by some authors. Abe et al. showed the profiles induced with ETA (Vβ 8, 12, and 14) and ETB (Vβ 2 and 8) by RT-PCR and partly by flow cytometry with anti-Vβ monoclonal antibodies

TABLE 3. Preponderant TCR Vβ in rabbit lymphocyte blastogenic response to streptococcal exotoxins

Toxin	Vβ	High ratio[a]	Vβ	Low ratio[b]
NY5 ETA	5	3.00 ± 0.25	4	1.62 ± 0.72
	10	2.97 ± 1.23	6	1.30 ± 0.21
			7	1.06 ± 0.15
NY5 ETB	1	4.23 ± 0.07	10	1.11 ± 0.90
	3	1.97 ± 0.17		
	6	12.05 ± 9.96		
NY5 ETC	1	1.73 ± 0.37	10	1.31 ± 1.10
	2	7.89 ± 3.78		
	6	11.05 ± 8.96		
T1 ETA	2	3.98 ± 0.20	10	0.91 ± 0.49
	6	33.59 ± 29.42		
SMEZ	2	1.70 ± 0.14		
	6	28.03 ± 22.48		
	8	2.87 ± 0.14		

[a] Ratio of incidence in lymphocytes stimulated with the toxin to incidence in ConA-stimulated culture (control). High ratios indicate incidences significantly higher than those in ConA-stimulated culture as determined by *t* test, with *P* values of 0.05 to 0.001.

[b] Except for Vβ 6, low ratios were above 1.00 in only one of two rabbits tested.

(1). Tomai et al. showed by RT-PCR those induced with SPE A (Vβ 2, 12, 14, 15, 8, and 4), SPE B (Vβ 8), and SPE C (Vβ 1, 2, 5.1, 10, and 15) (39). Mollick et al. described the TCR Vβ profile (Vβ 1, 3, 15, and 5.2) induced with SSA (30), which was different from those stimulated with classical ETs described. Braun et al. examined native and recombinant preparations of SPEs by flow cytometry with five reagents of monoclonal antibody (8). Recombinant SPE A and C induced only some of the Vβs (Vβ 12 and 2, respectively) induced with native SPE A (Vβ 8 and 12), B (Vβ 8), and C (Vβ 8 and 2). A Vβ 8-bearing human cell line responded to the three native SPEs but not to the recombinant SPE A and C. Therefore, they postulated a Vβ 8-specific mitogen called SPE X contaminating all three native SPEs. Gerlach et al. purified two proteins called AX and BX (18) and claimed that these represented two varieties of SPE X. Norrby-Teglund et al. described the TCR Vβ profile (Vβ 2, 4, 8, 15, and 19) of human lymphocytes stimulated with SPE F (35). The Vβ profile (Vβ 4, 7, and 8) of human lymphocytes stimulated with LMWS reported by Geoffroy-Fauvet et al. (13) was unique and was shared with those stimulated with NY5 ETA (Vβ 3, 12, 13, 14, 15, 4, 7, and 8) but not with S84 strain ETA (Vβ 3, 12, 13, 14, and 15). They suggested that the NY5 ETA tested may be contaminated with the LMWS.

The TCR Vβ profiles of rabbit lymphocytes stimulated with streptococcal toxins presented here are, to our knowledge, the first appearing in the literature. The results were largely different from those obtained with human lymphocytes mentioned above. Rabbit Vβ 6 was common in the response to ETs and SMEZ, while the human counterpart Vβ 4, has been found only rarely. Rabbit Vβ 1 was common in the response to both NY5 ETB and -C, while the human counterpart, Vβ 7, was not documented in the response to these toxins. Human Vβ 2 and 8, counterparts of the rabbit Vβs with the same numbers, were common in the response to ETs, while rabbit Vβ 2 was found only in the response to NY5 ETC, T1 ETA, and SMEZ, and rabbit Vβ 8 was unique to SMEZ. Rabbit lymphocytes may be expected to show "natural" Vβ profiles without prior sensitization to the streptococcal toxins, since rabbits are not natural hosts for *S. pyogenes*. In contrast, because humans are natural hosts for *S. pyogenes*, the possibility that the profiles of human lymphocytes were somehow modulated by previous sensitization, either documented or not, to the superantigenic exotoxins tested cannot be discounted.

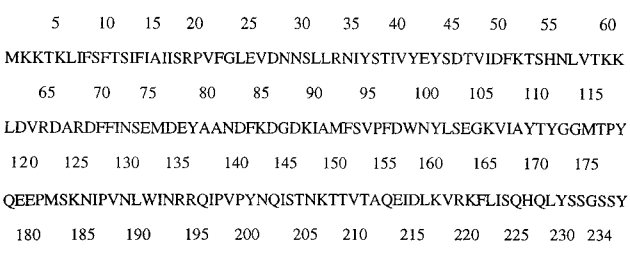

FIG. 5. Complete amino acid sequence of SMEZ from *S. pyogenes* T1.

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REFERENCES

1. **Abe, J., J. Forrester, T. Nakahara, J. A. Lafferty, B. L. Kotzin, and D. Y. M. Leung.** 1991. Selective stimulation of human T cells with streptococcal erythrogenic toxins A and B. J. Immunol. **146:**3747–3750.
2. **Abe, Y., S. Nakano, T. Nakahara, Y. Kamezawa, I. Kato, H. Ushijima, K. Yoshino, S. Ito, S. Noma, S. Okitsu, and M. Tajima.** 1990. Detection of serum antibody by the antimitogen assay against streptococcal erythrogenic toxins. Age distribution in children and the relation to Kawasaki disease. Pediatr. Res. **27:**11–16.
3. **Abe, Y., S. Nakano, T. Nakahara, and Y. Kamezawa.** 1993. Seroepidemiology of a new subtype streptococcal erythrogenic toxin Aβ in children, p. 119–121. *In* M. Takahashi and K. Taubert (ed.), Proceedings of the 4th International Symposium on Kawasaki Disease. American Heart Association, Dallas, Tex.
4. **Abe, Y., S. Nakano, Y. Kamezawa, T. Nakahara, and D. Gerlach.** 1994. Antimitogen assay of human sera against streptococcal erythrogenic toxins A, B and C: relationship to Kawasaki disease and β-hemolytic streptococcal infection, p. 146–147. *In* A. Totolian (ed.), Pathogenic streptococci: present and future. Lancer, St. Petersburg, Russia.
5. **Alouf, J. E., H. Knöll, and W. Köhler.** 1991. The family of mitogenic, shock-inducing and superantigenic toxins from streptococci and staphylococci, p. 367–414. *In* J. E. Alouf and J. H. Freer (ed.), Sourcebook of bacterial protein toxins. Academic Press, London, United Kingdom.
6. **Barsumian, E. L., C. M. Cunningham, P. M. Schlievert, and D. W. Watson.** 1978. Heterogeneity of group A streptococcal pyrogenic exotoxin type B. Infect. Immun. **20:**512–518.
7. **Bohach, G. A., A. R. Hauser, and P. M. Schlievert.** 1988. Cloning of the gene, *speB*, for streptococcal pyrogenic exotoxin type B in *Escherichia coli*. Infect. Immun. **56:**1665–1667.
8. **Braun, M. A., D. Gerlach, V. F. Hartwig, J. H. Ozegowski, F. Romagne, S. Carrel, W. Köhler, and B. Fleischer.** 1993. Stimulation of human T cells by streptococcal "superantigen" erythrogenic toxins (scarlet fever toxins). J. Immunol. **150:**2457–2466.
9. **Cavaillon, J.-M., C. Leclerc, and J. Alouf.** 1983. Polyclonal antibody-forming cell activation and immunomodulation of the in vitro immune response induced by streptococcal extracellular products. Cell. Immunol. **76:**200–206.
10. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
11. **Cunningham, C. M., E. L. Barsumian, and D. W. Watson.** 1976. Further purification of group A streptococcal pyrogenic exotoxin and characterization of the purified toxin. Infect. Immun. **14:**767–775.
12. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. Infect. Immun. **57:**291–294.

12a. **Ferretti, J. J.** Personal communication.

13. **Geoffroy-Fauvet, C., H. Müller-Alouf, E. Champagne, J.-M. Cavaillon, and J. E. Alouf.** 1994. Identification of a new extracellular superantigenic mitogen from group A streptococci. Zentralbl. Bakteriol. Suppl. **24:**90–91.
14. **Gerlach, D., H. Knöll, and W. Köhler.** 1980. Purification and characteriza-

tion of erythrogenic toxins. I. Investigation of erythrogenic toxin A produced by *Streptococcus pyogenes* strain NY-5. Zentralbl. Bakteriol. Hyg. Abt. 1 Orig. A **247:**177–191.
15. **Gerlach, D., H. Knöll, W. Köhler, J. H. Ozegowski, and V. Hribalova.** 1983. Isolation and characterization of erythrogenic toxins. V. Identity of erythrogenic toxin type B and streptococcal proteinase precursor. Zentralbl. Bakteriol. Hyg. Abt. 1 Orig. A **255:**221–233.
16. **Gerlach, D., W. Köhler, H. Knöll, L. Moravek, C. R. Weeks, and J. J. Ferretti.** 1987. Purification and characterization of *Streptococcus pyogenes* erythrogenic toxin type A produced by a cloned gene in *Streptococcus sanguis*. Zentralbl. Bakteriol. Hyg. Abt. 1 Orig. A **266:**347–358.
17. **Gerlach, D., H. Alouf, L. Moravek, M. Pavlik, and W. Köhler.** 1992. The characterization of two new low molecular weight proteins (LMPs) from *Streptococcus pyogenes*. Zentralbl. Bakteriol. **277:**1–9.
18. **Gerlach, D., E. Günther, W. Köhler, S. Vettermann, B. Fleischer, and K.-H. Schmidt.** 1995. Isolation and characterization of a mitogen characteristic of group A streptococci (*Streptococcus pyogenes*). Zentralbl. Bakteriol. **282:**67–82.
19. **Goshorn, S. C., and P. M. Schlievert.** 1988. Nucleotide sequence of streptococcal pyrogenic exotoxin type C. Infect. Immun. **56:**2518–2520.
20. **Gray, E. D.** 1979. Purification and properties of an extracellular blastogen produced by group A streptococci. J. Exp. Med. **149:**1439–1449.
21. **Hackett, S. P., and D. L. Stevens.** 1993. Superantigens associated with staphylococcal and streptococcal toxic shock syndrome are potent inducers of tumor necrosis factor-β synthesis. J. Infect. Dis. **168:**232–235.
22. **Hauser, A. R., and P. M. Schlievert.** 1990. Nucleotide sequence of the streptococcal pyrogenic exotoxin type B gene and relationship between the toxin and the streptococcal proteinase precursor. J. Bacteriol. **172:**4536–4542.
23. **Isono, T., Y. Isegawa, and A. Seto.** 1996. Analysis of T cell receptor Vβ expression in rabbit T lymphocytes induced to proliferate by an HTLV-I-transformed leukemogenic T cell line. J. Cancer Res. Clin. Oncol. **122:**458–464.
24. **Iwasaki, M., H. Igarashi, Y. Hinuma, and T. Yutsudo.** 1993. Cloning, characterization and overexpression of a *Streptococcus pyogenes* gene encoding a new type of mitogenic factor. FEBS Lett. **331:**187–192.
25. **Kamezawa, Y.** 1989. Production and purification of streptococcal erythrogenic toxin type A in a synthetic medium. Med. Biol. **119:**389–394. (In Japanese.)
26. **Kamezawa, Y., and T. Nakahara.** 1989. Purification and characterization of streptococcal erythrogenic toxin type A produced by *Streptococcus pyogenes* strain NY5 cultured in the synthetic medium NCTC 135. Comparison with the dialyzed medium-derived toxin. Microbiol. Immunol. **33:**183–194.
27. **König, B., M. Köller, G. Prevost, Y. Piemont, J. E. Alouf, A. Schreiner, and W. König.** 1994. Activation of human effector cells by different bacterial toxins (leukocidin, alveoysin, and erythrogenic toxin A): generation of interleukin-8. Infect. Immun. **62:**4831–4837.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
29. **McMillan, R. A., T. A. Bloomster, A. M. Saeed, K. L. Henderson, N. E. Zinn, R. Abernathy, D. W. Watson, and R. N. Greenberg.** 1987. Characterization of a fourth streptococcal pyrogenic exotoxin (SPE D). FEMS Microbiol. Lett. **44:**317–322.
30. **Mollick, J. A., G. G. Miller, J. M. Musser, R. G. Cook, D. Grossman, and R. R. Rich.** 1993. A novel superantigen isolated from pathogenic strains of Streptococcus pyogenes with amino terminal homology to staphylococcal enterotoxins B and C. J. Clin. Invest. **92:**710–719.
31. **Müller-Alouf, H., J. A. Alouf, D. Gerlach, J.-H. Ozegowski, C. Fitting, and J.-M. Cavaillon.** 1994. Comparative study of cytokine release by human peripheral blood mononuclear cells stimulated with *Streptococcus pyogenes* superantigenic erythrogenic toxins, heat-killed streptococci, and lipopolysaccharide. Infect. Immun. **62:**4915–4921.
32. **Nakahara, T., Y. Kamezawa, and Y. Abe.** 1992. Immunological heterogeneity of streptococcal erythrogenic toxin type A as revealed by the neutralization test with heterologous combinations of toxin preparations and antisera. Zentralbl. Bakteriol. Suppl. **22:**234–235.
33. **Nelson, K., P. M. Schlievert, R. K. Selander, and J. M. Musser.** 1991. Characterization and clonal distribution of four alleles of the speA gene encoding pyrogenic exotoxin A (scarlet fever toxin) in *Streptococcus pyogenes*. J. Exp. Med. **174:**1271–1274.
34. **Norrby-Teglund, A., M. Norgren, S. E. Holm, U. Andersson, and J. Andersson.** 1994. Similar cytokine induction profiles of a novel streptococcal exotoxin, MF, and pyrogenic exotoxins A and B. Infect. Immun. **62:**3731–3738.
35. **Norrby-Teglund, A., D. Newton, M. Kotb, S. E. Holm, and M. Norgren.** 1994. Superantigenic properties of the group A streptococcal exotoxin SpeF (MF). Infect. Immun. **62:**5227–5233.
36. **Ozegowski, J. H., H. Knöll, D. Gerlach, and W. Köhler.** 1984. Purification and characterization of erythrogenic toxins. VII. Investigation of erythrogenic toxin C produced by *Streptococcus pyogenes*. Zentralbl. Bakteriol. Hyg. Abt. 1 Orig. A **257:**38–50.
37. **Schlievert, P. M., K. M. Bettin, and D. W. Watson.** 1977. Purification and characterization of group A streptococcal pyrogenic exotoxin type C. Infect. Immun. **16:**673–679.
38. **Schlievert, P. M., and E. D. Gray.** 1989. Group A streptococcal pyrogenic exotoxin (scarlet fever toxin) type A and blastogen A are the same protein. Infect. Immun. **57:**1865–1867.
39. **Tomai, M. A., P. M. Schlievert, and M. Kotb.** 1992. Distinct T-cell receptor Vβ gene usage by human T lymphocytes stimulated with the streptococcal pyrogenic exotoxins and pep M5 protein. Infect. Immun. **60:**701–705.
40. **Weeks, C. R., and J. J. Ferretti.** 1986. Nucleotide sequence of the type A streptococcal exotoxin (erythrogenic toxin) gene from *Streptococcus pyogenes* bacteriophage T12. Infect. Immun. **52:**144–150.
41. **Yutsudo, T., H. Murai, J. Gonzalez, T. Takao, Y. Shimonishi, Y. Takeda, H. Igarashi, and Y. Hinuma.** 1992. A new type of mitogenic factor produced by *Streptococcus pyogenes*. FEBS Lett. **308:**30–34.

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