INFECTION AND IMMUNITY, Dec. 1994, p. 5227–5233
0019-9567/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 62, No. 12

Superantigenic Properties of the Group A Streptococcal Exotoxin SpeF (MF)

ANNA NORRBY-TEGLUND,[1]* DUANE NEWTON,[2,3] MALAK KOTB,[2,3,4] STIG E. HOLM,[1] AND MARI NORGREN[1]

Department of Clinical Bacteriology, Umeå University, S-901 85 Umeå, Sweden,[1] and Veterans Administration Medical Center,[2] and Departments of Microbiology and Immunology[3] and Surgery,[4] University of Tennessee, Memphis, Tennessee 38104

Received 20 June 1994/Returned for modification 9 August 1994/Accepted 6 September 1994

Streptococcal pyrogenic exotoxin F (SpeF), previously referred to as mitogenic factor, is a newly described potent mitogen produced by group A streptococci. To investigate whether this protein belongs to the family of microbial superantigens, we analyzed the cellular and molecular requirements for its presentation to T cells and compared it with the known streptococcal superantigen pyrogenic exotoxin A (SpeA) and the nonspecific polyclonal T-cell mitogen phytohemagglutinin (PHA). SpeF and SpeA were efficiently presented by autologous antigen-presenting cells (APCs) and an allogeneic B lymphoma cell line, Raji. In contrast, the monocytic cell line U937, which does not express major histocompatibility complex (MHC) class II molecules, failed to present SpeF as well as SpeA but supported the response to PHA. Thus, the presentation of SpeF by APCs was class II dependent but not MHC restricted. The requirement for HLA class II was further supported by the ability of anti-HLA-DQ monoclonal antibody to block the SpeF-induced proliferative response by 75 to 100%. Paraformaldehyde (PFA) fixation of autologous APCs resulted in an impaired ability of SpeF and SpeA to induce optimal T-cell proliferation. In contrast, fixation of Raji cells did not affect the induced proliferation. The stimulatory effect of PHA remained unaffected by both the use of PFA-fixed APCs and the addition of the HLA class II-specific monoclonal antibodies. The addition of a supernatant enriched in interleukin 1 and interleukin 6 to fixed autologous APCs resulted in an increased SpeF-induced response; thus, the impairment was not due to a requirement for processing, but, rather, costimulatory factors produced by metabolically active APCs were needed. SpeF was found to preferentially activate T cells bearing Vβ 2, 4, 8, 15, and 19, as determined by quantitative PCR. The data presented clearly show that SpeF is a superantigen. We also studied the prevalence of the *speF* gene in clinical isolates by Southern blot analyses, and the gene could be detected in 42 group A streptococcal strains, which represented 14 serotypes.

Superantigens are a group of microbial proteins which are very powerful immunomodulators. The bacterial superantigens include, among others, the *Staphylococcus aureus* enterotoxins (1, 33) and toxic shock syndrome toxin 1 (1, 33) as well as the streptococcal pyrogenic exotoxins A, B, and C (SpeA, -B, and -C) (1), the cell surface M protein (49), and the recently identified streptococcal superantigen (SSa) (36), all produced by group A streptococci (GAS).

Superantigens induce massive T-cell proliferation; 5 to 40% of the cells become activated, which can be compared to approximately 0.001% for a conventional antigen (30). Their capacity to activate such a large T-cell population is due to their ability to cross-link the relatively invariable parts of the major histocompatibility complex (MHC) molecule on antigen-presenting cells (APCs) and the T-cell receptor (TCR) of the T cells without prior internalization and processing by the APC (10, 11, 23, 33, 35, 44, 51). Superantigens are presented to T cells by APCs that express MHC class II molecules in an MHC-unrestricted manner. Superantigens interact with the variable region of the T-cell receptor β chain, and each superantigen has affinity for a set of Vβ elements (9, 23, 32, 33). This results in a preferential expansion of all T cells bearing those specific Vβ elements, which is followed by a high production of cytokines (2, 8, 16, 17, 26, 40, 52). The superantigen-induced overproduction of cytokines is believed to strongly influence the development of toxic shock syndrome (16, 17, 30, 33, 40, 43, 46).

SpeA and SpeC show high structural homology with each other but no significant homology with SpeB (14). Furthermore, SpeA and SpeC have been shown to be encoded by bacteriophages, while SpeB is chromosomally encoded (15, 24, 25). SpeB also has cysteine proteinase activity and can as such convert precursor interleukin 1β (IL-1β) to mature IL-1β (18, 28). The Spes share many biological activities, such as pyrogenicity, mitogenicity, enhancement of host susceptibility to endotoxin shock, and superantigenicity (1, 29, 53, 55). A novel mitogenic factor, called MF, expressed by GAS has been reported recently (22). The nucleotide sequence of MF did not reveal any significant homology with the Spes or any other known proteins. However, the MF protein induced a cytokine production profile similar to that of the Spes, and nanogram concentrations were sufficient for induction of a massive T-cell response, which indicated that MF might exhibit superantigenic activity (40). Even though pyrogenicity tests of MF have not been performed, we argue that the protein is a pyrogenic exotoxin on the basis of the massive induction of pyrogenic cytokines noted in vitro, and therefore the protein is renamed SpeF in accordance with the other streptococcal exotoxins. The clinical relevance of SpeF has been shown by analyses of neutralizing antibodies in sera from patients with streptococcal toxic shock syndrome (STSS) or uncomplicated infections (41). The study showed a correlation between the low neutralizing capacity of SpeB and SpeF mitogenicity and severe infections (41). In the present report, we have analyzed the superantigenicity of SpeF on the basis of its interaction with APCs and T cells.

* Corresponding author. Mailing address: Department of Clinical Bacteriology, Umeå University, S-901 85 Umeå, Sweden. Phone: 46-90 101121. Fax: 46-90 101121.

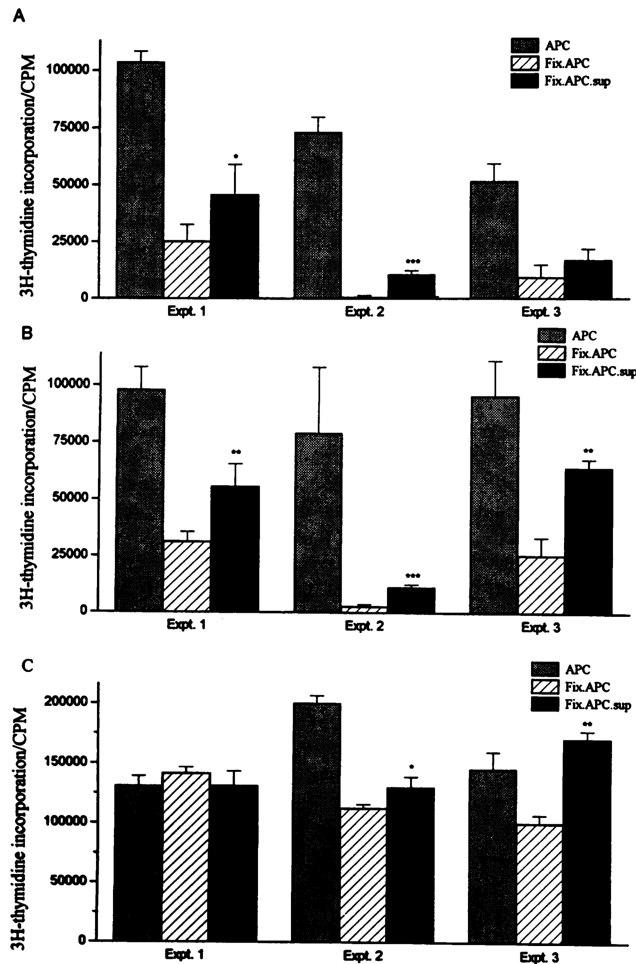

FIG. 1. Proliferative response induced by SpeF, SpeA, and PHA under various cell culture conditions. Purified human T cells (1×10^5 cells) were incubated with either autologous APCs (5×10^4) cells, paraformaldehyde-fixed APCs (Fix.APC), or fixed APCs plus the addition of an IL-1- and IL-6-enriched supernatant (Fix.APC.sup). The cells were stimulated with 7.5 ng of SpeF per ml (A), 7.5 ng of SpeA per ml (B), or 5 μg of PHA per ml (C) for 3 days, after which [^{3}H]thymidine was added for 6 h and the incorporation was measured. The values are presented as: the mean counts per minute (cpm) of toxin-stimulated cells minus the mean cpm of unstimulated cells ± standard deviation. Statistically significant higher values for fixed APCs plus supernatant as compared with those of fixed APCs are indicated at the top of relevant bars: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The mean cpm values ± standard deviations for unstimulated cells in experiments 1, 2, and 3 were 1,324 ± 327, 1,638 ± 972, and 7,016 ± 118, respectively.

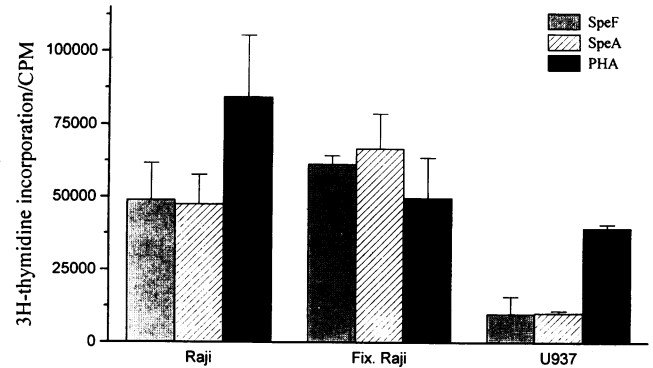

FIG. 2. Analyses of the requirements for accessory cells of SpeF. The Burkitt lymphoma cell line Raji and the monocytic cell line U937 were tested as accessory cells for SpeF stimulation. Purified T cells (1×10^5) were incubated with either Raji (2×10^4), fixed Raji (2×10^4), or U937 (2×10^4) cells. The cells were stimulated with 7.5 ng of SpeF per ml, 7.5 ng of SpeA per ml, or 5 μg of PHA per ml for 3 days, after which [^{3}H]thymidine was added for 6 h and the incorporation was measured. The values are presented as the mean counts per minute (cpm) of toxin-stimulated cells minus the mean cpm of unstimulated cells ± standard deviation. The mean cpm values ± standard deviations for unstimulated cells in Raji cell cultures, fixed Raji cell cultures, and U937 cell cultures were 2,760 ± 327, 7,016 ± 838, and 689 ± 111, respectively.

MATERIALS AND METHODS

Bacterial isolates. T1M1 and T12M12 GAS strains isolated from patients with uncomplicated pharyngotonsillitis in Sweden during 1976, 1980–1981, 1984–1985, and 1988–1989 (38), two of each serotype per year, were tested for the presence of the gene encoding the SpeF protein. Four additional T1M1 GAS strains isolated in Sweden during 1988–1989 from patients with STSS (38) as well as 16 strains of various serotypes isolated from patients with erysipelas during 1988–1990 were tested (39). NY5 of serotype T8/25, SF130 of T1M1 serotype, J17 of serotype T19, K56 of serotype T12M12, and D471 of serotype M6 were used as reference strains.

Serotyping of GAS strains. T typing was performed by agglutination with T type-specific antisera from the Institute of Sera and Vaccines, Prague, The Czech Republic (13). To further discriminate between the T types, a serum opacity reaction test was performed as described previously (34).

Antigens. SpeF was purified from a clinical GAS isolate, Oslo 85, kindly provided by A. Höiby, Department of Bacteriology, National Institute of Public Health, Oslo, Norway. Oslo 85 was found to be a high producer of SpeF by Western blot (immunoblot) analyses using a polyclonal monospecific SpeF antisera. Ethanol-precipitated overnight culture supernatant of Oslo 85 was subjected to preparative isoelectric focusing in 2% Biolyte solution (pH range, 3–10; Bio-Rad Laboratories, Upplands Väsby, Sweden) with a Rotofor (Bio-Rad). The purified protein was analyzed by Western blot as well as sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining and determined to be 99% pure. SpeA was kindly provided by D. Gerlach and W. Köhler, Institute für Experimentelle Mikrobiologie, Jena, Germany (12). Phytohemagglutinin (PHA) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

DNA techniques. Chromosomal DNA was prepared essentially by the method of B. M. Chassy (5) after growth in Todd-Hewitt broth supplemented with 20 mM glycine. The restriction enzyme *Hin*dIII and *Taq* DNA polymerase were used as described in the manufacturer's recommendations (Boehringer-Mannheim, Bromma, Sweden). DNA fragments were isolated from agarose gels by the Gene Clean method (Bio 101, La Jolla, Calif.).

PCR. The gene encoding SpeF was amplified by PCR (42) using chromosomal DNA from GAS strain NY5 as a template. The oligonucleotides used were nucleotides 789 to 808 (5′-CGAAATTAGAAAAGAGGAC-3′) as the 5′ primer and nucleotides 1963 to 1982 (5′-GGCTGAGCAAAAGTGT GTG-3′) as the 3′ primer. The reaction mixture contained 200 μM each dATP, dCTP, dGTP, and dTTP, 170 μg of bovine serum albumin per ml, 20 pmol of each primer, PCR buffer

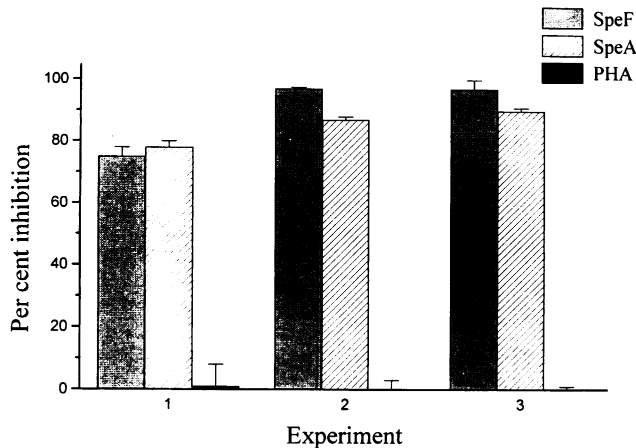

FIG. 3. Inhibition of toxin-induced proliferation by an HLA class II DQ-specific monoclonal antibody. Autologous APCs were preincubated for 15 min at room temperature with 0.1 μg of class II DQ-specific monoclonal antibody per ml and then cultured with purified T cells and 7.5 ng of SpeF per ml, 7.5 ng of SpeA per ml, or 5 μg of PHA per ml for 3 days. The data are presented as percent inhibition ± standard deviation of three separate experiments as compared with cell cultures with untreated APCs.

(Boehringer-Mannheim), and 2.5 U of *Taq* DNA polymerase (Boehringer-Mannheim). The gene was amplified by one denaturation cycle of 94°C for 1 min and then 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and one cycle of 72°C for 3 min. This PCR product (1 μl) was used as the template in a second PCR, which was run for 25 cycles at an annealing temperature of 53°C but otherwise as described above.

speA and *speB* were also amplified by using chromosomal DNA from SF130 and from a clinical Swedish isolate, T1BW (38), as templates in the respective PCRs. The oligonucleotides and reaction conditions for *speA* and *speB* amplification have been described previously in detail (38). A booster PCR was performed for detection of the *speA* gene in strains that were negative for *speA* by Southern blot analyses. The gene was first amplified as described previously (38), and 1 μl of this PCR product was used as a template in a booster PCR which was run for 25 cycles at an annealing temperature of 50°C but otherwise as described previously (38).

Southern blot analyses. *Hin*dIII-digested chromosomal DNA was separated on 0.7% agarose gels and transferred to Hybond N filters (Amersham, Solna, Sweden) by the method of Southern (45). The filters were probed with PCR-amplified gene fragments of the *speF*, *speA*, and *speB* genes. For labelling of the probe and detection of DNA hybrids, the nonradioactive digoxigenin DNA labelling and detection kit (Boehringer-Mannheim) was used. Hybridization and washing were performed at 65°C under high-stringency conditions in buffers recommended by Boehringer-Mannheim.

Preparation of PBMCs and cell culture conditions. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood obtained from healthy adults by centrifugation on Ficoll-Metriozate density gradients (Lymphoprep; Nycomed AS, Oslo, Norway) (4). T cells were separated from purified PBMCs by two rounds of erythrocyte rosetting (27) and then by overnight adherence to plastic. The erythrocyte rosette-negative populations enriched in B cells and monocytes were used as APCs. Raji cells and U937 cells were treated with mitomycin C (Boehringer-Mannheim) before being used as APCs. The cells were cultured in RPMI 1640 with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (RPMI-HEPES; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 0.1 mg of gentamicin (Schering-Plough Int., Kenilworth, N.J.) per ml, 2 mM L-glutamine (GIBCO BRL, Life Technologies Ltd., Paisley, Scotland), and 10% heat-inactivated fetal calf serum. For the Vβ analysis, T cells (1×10^7) and APCs (5×10^4) were stimulated with 7.5 ng of SpeF per ml or with 10 μg of anti-CD3 per ml and cultured for 3 days. The viable cells were recovered on a Ficoll density gradient and recultured for 24 h with 10 U of recombinant IL-2 per ml (Collaborative Research, Cambridge, Mass.) (32).

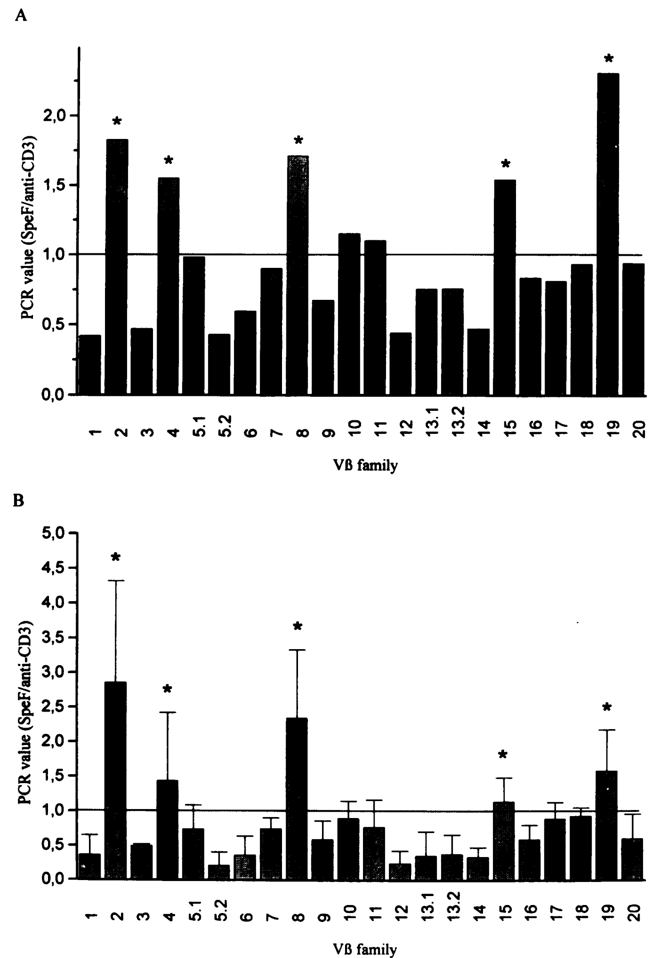

FIG. 4. Human T-cell receptor Vβ specificity of SpeF. Erythrocyte rosette-purified T cells (10^6/ml) were stimulated with either 7.5 ng of SpeF per ml or 2 μg of anti-CD3 (OKT3) per ml for 3 days. IL-2 (20 U/ml) was added to the cultures 24 h prior to harvesting. RNA was extracted from the cells, and cDNA was prepared and analyzed with a reverse transcription-PCR-based method. The PCR value for each Vβ was compared with the value obtained after OKT3 stimulation. The PCR value equals (area Vβn/area Cα)$_{\text{SpeF}}$ divided by (area Vβn/area Cα)$_{\text{OKT3}}$, where n equals Vβ family number 1 to 20. (A) Vβ panel of cells from one donor, which is representative of data obtained from three donors repeated at least six times; (B) average PCR ratios of Vβ-specific expression in cells obtained from five donors. The data are mean values ± standard deviations of each Vβ.

In the proliferation assays, T cells (1×10^5) and APCs ($5 \times$

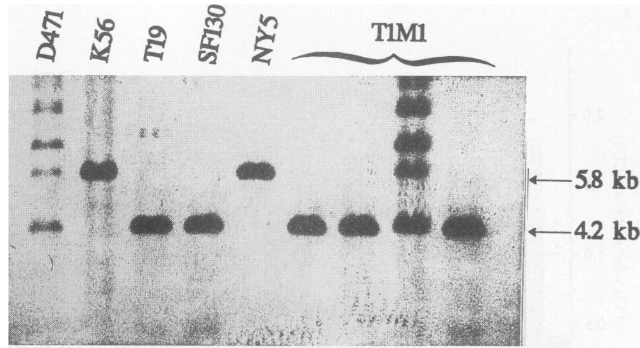

FIG. 5. Southern blot of *Hin*dIII-digested chromosomal DNA using a SpeF-specific probe. Chromosomal DNA was restricted with *Hin*dIII, separated on a 0.7% agarose gel, and transferred to a Hybond N filter. The filter was probed with a PCR-amplified *speF* gene fragment. For detection and labelling of DNA, the nonradioactive digoxigenin DNA labelling kit from Boehringer-Mannheim was used. The sizes of the two major fragments are indicated on the right. The four T1M1 strains indicated by a bracket were all isolated during 1988–1989.

10^4), paraformaldehyde fixed or unfixed, were stimulated with 7.5 ng of SpeF per ml, 7.5 ng of SpeA per ml, or 5 μg of PHA (Sigma) per ml. After 3 days of culture, the cells were pulsed with 1 μCi of [^{3}H]thymidine (specific activity, 16.0 Ci/mmol) per well and harvested after 6 h.

Blocking of HLA class II molecules by monoclonal antibodies. Autologous APCs were preincubated for 15 min at room temperature with 0.1 μg of monoclonal antibodies per ml, after which the cells were stimulated and cultured as described above. The monoclonal antibodies used were anti-human HLA class II DP+DQ+DR (Bu25), anti-human class II DQ (Bu46; The Binding Site, Birmingham, Ala.), and anti-human class II DR (B8.12.2; Immunotech, Marseilles, France).

Paraformaldehyde fixation of APCs. Purified APCs were fixed with 0.5% (wt/vol) paraformaldehyde for 15 min at room temperature, and 8 volumes of 0.15 M glycine (pH 7.2) were added immediately after the incubation. The cells were washed once with 0.15 M glycine buffer, incubated with culture media for 30 min, washed three times in Hanks balanced salt solution, and resuspended in culture medium, as described previously (50). The fixed cells were stimulated with SpeF, SpeA, and PHA for three days. The cells were also stimulated with 10 μg of purified protein derivative (PPD) per ml for 6 days, and the lack of proliferative response seen after PPD stimulation confirmed that the fixation procedure had been successful.

Assessment of monocyte culture supernatants. IL 1- and IL 6-enriched culture supernatant was obtained by stimulating PBMCs (5×10^6 cells per ml) with 1 μg of indomethacin (Sigma) per ml for 24 h in RPMI complete medium containing 10% fetal calf serum as described previously (31). The cell-free supernatant was filter sterilized and stored at −20°C. Fifty microliters of supernatant was added per well in the cell culture experiments.

Analysis of T-cell receptor Vβ gene usage by PCR. Total RNA was extracted from stimulated cell cultures by using RNAzol-B (Tel-Test, Inc., Friendswood, Tex.). First-strand cDNA was synthesized from total RNA by using a random hexanucleotide primer and reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and used as a template in the PCR amplification. The methods are described in detail by Kotb et al. (32). Quantification of the PCR products were accomplished by use of 5′-^{32}P-labelled primers for the Cβ and the Cα chains. The Cα cDNA was coamplified with the Vβ cDNA and used as an internal control. Radiolabelled PCR products were separated on 2% agarose gels and exposed on X-ray films. The relative amounts of Cα and Vβ were determined by scanning the autoradiograms and integrating the relevant peaks. The PCR values were normalized by dividing the area of the Vβ peak by the area of the Cα peak. To determine the specific expansion, the normalized Vβ value for SpeF-stimulated cells was then divided by the corresponding Vβ value obtained after anti-CD3 stimulation. The PCR value is described by the following equation: PCR value = $(\text{area of V}\beta n/\text{area of C}\alpha)_{\text{SpeF}}/(\text{area of V}\beta n/\text{area of C}\alpha)_{\text{anti-CD3}}$, where n equals Vβ family number 1 to 20. Ratios greater than one indicate specific expansion of particular Vβ elements.

TABLE 1. Occurrence of *speF*, *speA*, and *speB* in clinical GAS isolates[a]

Serotype[b]	Yr of isolation	Occurrence[c] of:		
		speF	*speA*	*speB*
M6 (D471)	1971	+	+	+
M12 (K56)	1956	+	−	+
T19 (J17)	Before 1930s	+	+	+
T1M1 (SF130)	Before 1930s	+	+	+
T8/25(NY5)	Before 1930s	+	+	+
T1M1	1976	2/2	2/2	2/2
T1M1	1980–1981	2/2	2/2	2/2
T1M1	1984–1985	2/2	2/2	2/2
T1M1	1988–1989	6/6	6/6	6/6
T12M12	1976	2/2	2/2	2/2
T12M12	1980–1981	2/2	2/2	2/2
T12M12	1984–1985	2/2	2/2	2/2
T12M12	1988–1989	2/2	2/2	2/2
T2	1992	2/2	0/2	2/2
T3	1992	2/2	1/2	2/2
T4	1992	2/2	1/2	2/2
T8	1992	2/2	0/2	2/2
T11	1992	1/1	1/1	1/1
T12M22	1992	2/2	1/2	2/2
T14	1992	2/2	0/2	2/2
T28	1992	2/2	0/2	2/2
PolyY	1992	2/2	2/2	2/2

[a] The genes were detected by Southern blot analyses of *Hin*dIII-digested chromosomal DNA with PCR-amplified probes specific for each toxin gene.
[b] The names of the reference strains are given in parentheses.
[c] Symbols: +, gene found in serotype; −, gene not found. In the case of numerical values, the number of isolates with the gene relative to the total number of isolates is given.

RESULTS AND DISCUSSION

Superantigens have generated a lot of attention during recent years, in part because of their potential role in both autoimmunity and manifestations in acute infections. The toxic shock syndrome caused by *S. aureus* has been shown to be mediated by the superantigen toxic shock syndrome toxin 1 (6, 48) and the Spes have been implicated in the development of STSS caused by GAS (19, 20, 37, 47, 54).

Although the biological activities of SpeF strongly suggested superantigenic properties of the protein, further studies were required to verify this. To determine if SpeF is a superantigen, we investigated whether SpeF was HLA class II dependent in

its T-cell stimulation and showed affinity for a unique Vβ repertoire, which are the major criteria for defining superantigens. We analyzed the interaction of SpeF with autologous human APCs, Raji cells, U937 cells, and autologous human T cells.

APC requirement for induction of T-cell proliferation by SpeF. The requirement of SpeF for APCs to induce T-cell proliferation was analyzed by cell culture experiments using autologous APCs and Raji cells. Both cell types functioned as accessory cells for SpeF-induced T-cell proliferation (Fig. 1A and 2), which indicated a lack of MHC restriction in presentation of this molecule. However, paraformaldehyde fixation of autologous APCs resulted in an impaired presentation of both SpeF and SpeA (Fig. 1A and B). The proliferative response to SpeF was decreased by 75 to 100%, and that to SpeA was decreased by 67 to 96%. In comparison, when APCs were treated with paraformaldehyde, a mild decrease in PHA-induced proliferation was noted in two of the three experiments (Fig. 1C). However, fixation of Raji cells did not affect their ability to function as accessory cells. This is in accordance with other studies reporting an impairment of superantigen activity by fixation of autologous APCs (50), while fixed Raji cells were functional as accessory cells (9, 50). Raji cells express class II molecules on their surface as well as constitutive high levels of the costimulatory molecule B7 and membrane-bound cytokines (3); as proposed by Tomai et al. (50), this could be the reason for their function as accessory cells even when they are metabolically inactive. Tomai et al. (50) also reported that the impairment of superantigen stimulation could be overcome by the addition of recombinant IL-1 and recombinant IL-6; this argued for a role of costimulatory factors normally produced by active APCs. We obtained similar results with cell culture experiments with fixed autologous APCs where both the SpeA- and SpeF-induced proliferative responses were increased by the addition of a cell supernatant enriched in IL-1 and IL-6. Thus, the impairment in SpeF ability to induce T-cell proliferation when fixed APCs were used was not due to the requirement for cellular processing but rather to the need for costimulatory factors.

SpeF requires HLA class II molecules for T-cell activation. The monocytic cell line U937, which lacks class II molecules on its cell surface, was also tested as accessory cells and was found to be nonfunctional for SpeF or SpeA presentation but functional for PHA stimulation (Fig. 2). This was indicative of a requirement for class II molecules in SpeF-induced T-cell activation. Further support for class II dependence was obtained by blocking HLA class II molecules on APCs with class II-specific monoclonal antibodies. The experiment showed an almost complete inhibition of SpeF-induced proliferation (75 to 100%) by a class II DQ-specific monoclonal antibody as compared with that of untreated APCs (Fig. 3). Two other monoclonal antibodies, one specific for HLA class II DR and one for class II DR+DP+DQ, were tested, but only a slight effect was noted for the DR-specific antibody (9 to 34%; data not shown). The HLA class II DQ-specific monoclonal antibody inhibited the SpeA mitogenicity by 78 to 90%, while PHA stimulation was not affected by the antibody (Fig. 3). The lack of inhibition by the DR and DR+DP+DQ antibodies might be a result of binding of the antibodies to parts of the class II molecules other than the superantigen-interactive parts. On the other hand, both SpeA and SpeB as well as a streptococcus-derived protein have been shown previously to bind preferentially to DQ molecules (7, 21). Thus, preferential binding to HLA class II DQ might be a common motif among streptococcal superantigens.

SpeF induces preferential expansion of specific Vβ-bearing cells. The Vβ repertoire was analyzed by using blood from five different individuals, each stimulated with either SpeF or anti-CD3 monoclonal antibody for 3 days. Analysis of the Vβ gene expression was performed by use of a reverse transcription-PCR-based method. Comparison of Vβ expression in SpeF-stimulated cultures with that in anti-CD3-stimulated cultures revealed that SpeF induced a consistent pattern of preferential Vβ expansion which involved Vβ 2, 4, 8, 15, and 19 (Fig. 4). This is a unique Vβ specificity which is not shared with any of the staphylococcal enterotoxins or other Spes. Taken together with the HLA class II dependence, but not restriction, this clearly shows that SpeF is a novel group A streptococcal superantigen.

Prevalence of the SpeF gene in GAS isolates. GAS strains of 14 different serotypes, isolated from patients with different disease manifestations from 1930 to 1992, were tested for the presence of the *speF*, *speA*, and *speB* genes. The Southern blot technique using PCR-amplified probes specific for each toxin revealed that all 42 strains harbored the *speF* gene as well as the *speB* gene, while 12 (29%) strains lacked the *speA* gene (Table 1). These frequencies are in agreement with previous findings that SpeA is phage encoded, while SpeB is chromosomally encoded (24). That *speF* could be found in all strains tested suggested that the gene was chromosomally encoded. Figure 5 shows a Southern blot of *Hin*dIII-digested chromosomal DNA probed with a *speF*-specific gene fragment. A difference in fragment size could be detected between different serotypes, while in T1M1 and T12M12 strains, the *speF* fragment was constant in size (Fig. 5 and data not shown). Thus, there was no extensive polymorphism in the *speF* gene region of the tested strains, which further supports the assumption that *speF* is chromosomally encoded.

An argument commonly used when discussing the clinical importance of the Spes in STSS has been that if the gene is found in all strains, including those isolated from bacteremia patients and patients with uncomplicated infections, the toxin cannot be of any clinical importance. However, we have previously reported that SpeB and SpeF are of clinical relevance since sera from septic patients had a significantly lower neutralizing capacity of the toxin mitogenicity than that of sera from patients with uncomplicated infections (41). Our theory is that the specificity of the host humoral response as well as the reactivity of the immune cells of the patient will determine which particular toxin(s) produced by the infecting strain will be decisive for the clinical manifestation. Thus, we think that SpeB and SpeF can be equally as important in the development of STSS as other streptococcal superantigens.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council to S.E.H. (10844) and M.N. (8876). A.N. was supported by grants from the Kempe Foundation and the Lennanders Foundation, and M.N. was supported by a grant from the Medical Faculty, University of Umeå. M.K. was supported by funds from the Veterans Administration and the National Institutes of Health (GM-38530).

REFERENCES

1. **Alouf, J. E., H. Knöll, and W. Köhler.** 1991. The family of mitogenic, shock-inducing and superantigenic toxins from staphylococci and streptococci, p. 367–414. *In* J. E. Alouf and J. H. Freer (ed.), Sourcebook of bacterial protein toxins. Academic Press, Inc., San Diego, Calif.
2. **Andersson, J., S. Nagy, L. Björk, J. Abrams, S. Holm, and U. Andersson.** 1992. Bacterial toxin-induced cytokine production studied at the single-cell level. Immunol. Rev. **127:**69–96.
3. **Arnold, A., S. Lipkowitz, M. Suthanthiran, A. Novogrodsky, and**

K. H. Stenzel. 1985. Human B lymphoblastoid cell lines provide an interleukin 1-like signal for mitogen-treated T lymphocytes via direct cell contact. J. Immunol. 134:3876–3881.

4. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21(Suppl.):77–89.
5. Chassy, B. M. 1976. A gentle method for the lysis of oral streptococci. Biochem. Biophys. Res. Commun. 68:603–608.
6. Choi, Y., J. A. Lafferty, J. R. Clements, J. K. Todd, E. Gelfand, J. Kappler, P. Marrack, and B. L. Kotzin. 1990. Selective expansion of T cells expressing Vβ2 in toxic shock syndrome. J. Exp. Med. 172:981–984.
7. Esaki, Y., Y. Fukui, T. Sudo, K. Yamamoto, T. Inamitsu, Y. Nishimura, K. Hirokawa, A. Kimura, and T. Sasazuki. 1994. Role of human major histocompatibility complex DQ molecules in superantigenicity of streptococcus-derived protein. Infect. Immun. 62:1228–1235.
8. 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.
9. Fleischer, B., R. Gerardy-Schahn, B. Metzroth, S. Carrel, D. Gerlach, and W. Köhler. 1991. An evolutionary conserved mechanism of T cell activation by microbial toxins. Evidence for different affinities of T cell receptor-toxin interaction. J. Immunol. 146:11–17.
10. Fleischer, B., H. Schrezenmeizer, and P. Conradt. 1989. T lymphocyte activation by staphylococcal enterotoxins: role of class II molecules and T cell surface structures. Cell. Immunol. 120:92–101.
11. Fraser, J. D. 1989. High affinity binding of staphylococcal enterotoxins A and B to HLA-DR. Nature (London) 339:221–223.
12. Gerlach, D., and W. Köhler. 1980. Isolierung und Characterisierung von erythrogenen Toxinen. I. Untersuchung des von Streptococcus pyogenes Stamm NY5, gebildeten Toxin A. Zentralbl. Bakteriol. Hyg. I. Abt. Orig. A 247:177–191.
13. Griffith, G. 1934. The serological classification of Streptococcus pyogenes. J. Hyg. 34:542–584.
14. Goshorn, S. C., and P. M. Schlievert. 1988. Nucleotide sequence of streptococcal pyrogenic exotoxin type C. Infect. Immun. 56:2518–2520.
15. Goshorn, S. C., and P. M. Schlievert. 1989. Bacteriophage association of streptococcal pyrogenic exotoxin type C. J. Bacteriol. 171:3068–3073.
16. Hackett, S., and D. Stevens. 1992. Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. J. Infect. Dis. 165:879–885.
17. 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.
18. Hauser, A. R., and P. M. Schlievert. 1990. Nucleotide sequence of the streptococcal pyrogenic exotoxin type B and relationship between the toxin and the streptococcal proteinase precursor. J. Bacteriol. 172:4536–4542.
19. Hauser, A. R., D. L. Stevens, E. L. Kaplan, and P. M. Schlievert. 1991. Molecular analysis of pyrogenic exotoxins from *Streptococcus pyogenes* isolates associated with toxic shock-like syndrome. J. Clin. Microbiol. 29:1562–1567.
20. Holm, S. E., A. Norrby, A.-M. Bergholm, and M. Norgren. 1992. Aspects of pathogenesis in serious group A streptococcal infections in Sweden, 1988–1989. J. Infect. Dis. 166:31–37.
21. Imanishi, K., H. Igarashi, and T. Uchiyama. 1992. Relative abilities of distinct isotypes of human major histocompatibility complex class II molecules to bind streptococcal pyrogenic exotoxin types A and B. Infect. Immun. 60:5025–5029.
22. 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.
23. Janeway, C. A., J. Yagi, Jr., P. J. Conrad, M. E. Katz, B. Jones, S. Vroegop, and S. Buxser. 1989. T-cell responses to Ms and to bacterial proteins that mimic its behaviour. Immunol. Rev. 107:61–88.
24. Johnson, L. P., and P. M. Schlievert. 1984. Group A streptococcal phage T12 carries the structural gene for pyrogenic exotoxin A. Mol. Gen. Genet. 194:52–56.
25. Johnson, L. P., M. A. Tomai, and P. M. Schlievert. 1986. Bacteriophage involvement in group A streptococcal pyrogenic exotoxin A production. J. Bacteriol. 166:623–627.
26. Jupin, C., S. Andersson, C. Damais, J. E. Alouf, and M. Parant. 1988. Toxic shock syndrome toxin 1 as an inducer of human tumor necrosis factors and γ interferon. J. Exp. Med. 167:752–761.
27. Kaplan, E., and C. Clark. 1974. Improved rosetting assay for detection of human T lymphocytes. J. Immunol. Methods 5:131–135.
28. Kapur, V., M. W. Majesky, L.-L. Li, R. A. Black, and J. M. Musser. 1993. Cleavage of interleukin 1β (IL-1β) precursor to produce active IL-1β by a conserved extracellular cysteine protease from *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA 90:7676–7680.
29. Kim, Y. B., and D. W. Watson. 1972. Streptococcal exotoxins: biological and pathological properties, p. 33–50. *In* L. W. Wannamaker and J. H. Matsen (ed.), Streptococci and streptococcal diseases. Recognition, understanding and management. Academic Press, Inc., New York.
30. Kotb, M. 1992. Role of superantigens in the pathogenesis of infectious diseases and their sequelae. Curr. Opin. Infect. Dis. 5:364–374.
31. Kotb, M., G. Majumdar, M. Tomai, and E. H. Beachey. 1990. Accessory cell-independent stimulation of human T cells by streptococcal M protein superantigen. J. Immunol. 145:1332–1336.
32. Kotb, M., R. Watanabe-Onishi, B. Wang, M. A. Tomai, L. Le Gros, P. M. Schlievert, M. El Demellawy, and A. M. Geller. 1993. Analysis of the TCR Vβ specificities of bacterial superantigens using PCR. Immunomethods 33:33–40.
33. Marrack, P., and J. Kappler. 1990. The staphylococcal enterotoxins and their relatives. Science 248:705–711.
34. Maxted, W. R., and J. P. Widdowson. 1972. The protein antigens of group A streptococci, p. 256–261. *In* L. W. Wannamaker and J. M. Matsen (ed.), Streptococci and streptococcal diseases. Recognition, understanding, and management. Academic Press, Inc., New York.
35. Mollick, J. A., R. G. Cook, and R. R. Rich. 1989. Class II MHC molecules are specific receptors for staphylococcus enterotoxin A. Science 244:817–820.
36. 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 aminoterminal homology to staphylococcal enterotoxins B and C. J. Clin. Invest. 92:710–719.
37. Musser, J. M., A. R. Hauser, M. H. Kim, P. M. Schlievert, K. Nelson, and R. K. Selander. 1991. *Streptococcus pyogenes* causing toxic-shock-like syndrome and other invasive disease: clonal diversity and pyrogenic exotoxin expression. Proc. Natl. Acad. Sci. USA 88:2668–2672.
38. Norgren, M., A. Norrby, and S. E. Holm. 1992. Genetic diversity in T1M1 group A streptococci in relation to clinical outcome of infection. J. Infect. Dis. 166:1014–1020.
39. Norrby, A., B. Eriksson, M. Norgren, C. Jorup Rönström, A. C. Sjöblom, K. Karkkonen, and S. E. Holm. 1992. Virulence properties of erysipelas associated group A streptococci. Eur. J. Clin. Microbiol. Infect. Dis. 11:1136–1143.
40. Norrby-Teglund, A., M. Norgren, S. E. Holm, U. Andersson, and J. Andersson. 1994. Similar cytokine induction profiles of a novel streptococcal mitogenic factor, MF, and pyrogenic exotoxins A and B. Infect. Immun. 62:3731–3738.
41. Norrby-Teglund, A., K. Pauksens, S. E. Holm, and M. Norgren. 1994. Relation between low capacity of human sera to inhibit streptococcal mitogens and serious manifestation of streptococcal disease. J. Infect. Dis. 170:585–591.
42. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.
43. Schlievert, P. M. 1993. Role of superantigens in human disease. J. Infect. Dis. 167:997–1002.
44. Scholl, P., A. Diez, W. Mourad, J. Parsonnet, R. S. Geha, and T.

Chatila. 1989. Toxic shock syndrome toxin 1 binds to major histocompatibility complex class II molecules. Proc. Natl. Acad. Sci. USA **86:**4210–4214.

45. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **94:**503–517.
46. **Stevens, D., A. E. Bryant, and S. P. Hackett.** 1993. Sepsis syndromes and toxic shock syndromes: concepts in pathogenesis and a perspective of future treatment strategies. Curr. Opin. Infect. Dis. **6:**374–383.
47. **Stevens, D. L., M. H. Tanner, J. Winship, R. Swarts, K. M. Ries, P. M. Schlievert, and E. Kaplan.** 1989. Severe group A streptococcal infections associated with a toxic shock-like syndrome and scarlet fever toxin A. N. Engl. J. Med. **321:**1–7.
48. **Todd, J. K.** 1988. Toxic shock syndrome. Clin. Microbiol. Rev. **1:**432–436.
49. **Tomai, M., M. Kotb, G. Majumdar, and E. H. Beachey.** 1990. Superantigenicity of streptococcal M protein. J. Exp. Med. **172:** 359–362.
50. **Tomai, M. A., E. H. Beachey, G. Majumdar, and M. Kotb.** 1992. Metabolically active antigen presenting cells are required for human T cell proliferation in response to the superantigen streptococcal M protein. FEMS Microbiol. Immunol. **89:**155–164.
51. **Uchiyama, T., K. Imanishi, S. Saito, M. Araake, X.-J. Yan, H. Fujikawa, H. Igarashi, H. Kato, F. Obata, N. Kashiwagi, and H. Inoko.** 1989. Activation of human T cells by toxic shock syndrome toxin-1: the toxin-binding structures expressed on human lymphoid cells acting as accessory cells are HLA class II molecules. Eur. J. Immunol. **19:**1803–1809.
52. **Uchiyama, T., Y. Kamagata, X.-J. Yan, M. Kohno, M. Yoshioko, M. Fujikawa, H. Igarashi, M. Okubo, F. Awano, T. Saito-Taki, and M. Nakano.** 1987. Study on the biological activities of toxic shock syndrome toxin-1. II. Induction of the proliferative response and the interleukin-2 production by T cells from human peripheral blood mononuclear cells stimulated with the toxin. Clin. Exp. Immunol. **68:**638–647.
53. **Wannamaker, L. W., and P. M. Schlievert.** 1988. Exotoxins of group A streptococci, p. 267–296. *In* M. C. Hardegree and A. T. Tu (ed.), Bacterial toxins. Marcel Dekker, Inc., New York.
54. **Watanabe-Ohnishi, R., D. E. Low, A. McGeer, D. L. Stevens, P. M. Schlievert, D. Newton, B. Schwartz, B. Kreiswirth, Ontario Streptococcal Study Project, and M. Kotb.** Selective depletion of Vβ-bearing T cells in patients with severe group A streptococcal toxic shock. J. Infect. Dis., in press.
55. **Watson, D. W.** 1960. Host-parasite factors in group A streptococcal infections: pyrogenic and other effects of immunological distinct exotoxins related to scarlet fever toxins. J. Exp. Med. **111:**255–284.