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The Bacterial Superantigen Streptococcal Mitogenic Exotoxin Z Is the Major Immunoactive Agent of Streptococcus pyogenes

Meera Unnikrishnan,* Daniel M. Altmann,* Thomas Proft,† Faisal Wahid,† Jonathan Cohen,* John D. Fraser,† and Shiranee Sriskandan2*  

The gene encoding streptococcal mitogenic exotoxin Z (SMEZ) was disrupted in Streptococcus pyogenes. Despite the presence of other superantigen genes, mitogenic responses in human and murine HLA-DQ transgenic cells were abrogated when cells were stimulated with supernatant from the smez- mutant compared with the parent strain. Remarkably, disruption of smez led to a complete inability to elicit cytokine production (TNF-α, lymphotoxin-α, IFN-γ, IL-1 and -8) from human cells, when cocultured with streptococcal supernatants. The potent effects of SMEZ were apparent even though transcription and expression of SMEZ were barely detectable. Human Vβ8+ T cell proliferation in response to S. pyogenes was SMEZ-dependent. Cells from HLA-DQ8 transgenic mice were 3 logs more sensitive to SMEZ-13 than cells from HLA-DR1 transgenic or wild-type mice. In the mouse, SMEZ targeted the human Vβ8+ TCR homologue, murine Vβ11, at the expense of other TCR T cell subsets. Expression of SMEZ did not affect bacterial clearance or survival from peritoneal streptococcal infection in HLA-DQ8 mice, though effects of SMEZ on pharyngeal infection are unknown. Infection did lead to a rise in Vβ11+ T cells in the spleen which was partly reversed by disruption of the smez gene. Most strikingly, a clear rise in murine Vβ4+ cells was seen in mice infected with the smez- mutant S. pyogenes strain, indicating a potential role for SMEZ as a repressor of cognate anti-streptococcal responses. The Journal of Immunology, 2002, 169: 2561–2569.  

The purpose of the current study was to address the contribution made by SMEZ, in a physiological context, to the immunological effects of GAS both in vitro and in vivo; the study was made possible by use of isogenic streptococcal strains which differed only in ability to produce SMEZ.  

Materials and Methods  
Bacterial strains and media  
An M89 Streptococcus pyogenes clinical necrotizing fasciitis isolate (H293) with toxin genotype–streptococcal pyrogenic exotoxin (speA, speC, streptococcal superantigen (ssa), speI, speG, speH, speJ, mitogenic factor (mff), smez)–was used in all experiments. Genotypes of strains were determined by PCR and sequencing confirmed that the strain carried the smez-13 allele as predicted by M serotype. M typing was performed by the Central Public Health Laboratory (Colindale, U.K.). Streptococci were cultured in Todd Hewitt broth with 0.2% yeast (THY) (Oxoid, Basingstoke, U.K.), or RPMI 1640 containing 10% FCS, 2 mM glutamine (Life Technologies, Paisley, U.K.), or on horse blood agar (Oxoid) at 37°C, aerobically. S. pyogenes strain H305 is an M1 serotype organism and was used to derive probes for smez.  

Supercompetent Escherichia coli-SCS1 cells (Strategene, La Jolla, CA) were grown in Luria Bertrani broth aerobically at 37°C. Kanamycin (Sigma-Aldrich, Poole, U.K.) was used at a concentration of 50 μg/ml for the selection of E. coli transformants. Erythromycin (Ery) lactobionate (Abbott Laboratories, Dublin, Ireland) was used at 1 μg/ml for streptococci.  

Reagents  
All chemical reagents were purchased from Sigma-Aldrich. Restriction enzymes were from Life Technologies. rSMEZ alleles were prepared as previously described (3); anti-SMEZ serum was raised in rabbits immunized with rSMEZ-1 and -2.  

Transformations  
A 331-bp internal fragment corresponding to bases 9–340 of the 630-bp smez-1 coding sequence was amplified from genomic DNA extracted from H305 using primers smez1 and smez2 (Table 1). Primers were designed using the published smez-2 sequence aligned to the M1 genome database.

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to allow recognition between different smez alleles (2, 4). The temperature-sensitive vector pGhostaph1 was created to facilitate cloning in E. coli by ligating of the kanamycin resistance gene 3′-aminoglycoside phosphotransferase type 1 (Aph1) from pUC4K to the BamHI site of pGhost5′-dig (Appligene, Illkirch, France). The 331-bp smez fragment was then cloned into the EcoRI site of pGhostaph1 in SCSI supercompetent E. coli. Restriction enzyme analysis of plasmid DNA from one kanamycin-resistant transformant revealed that three copies of the 330-bp smez fragment had ligated to pGhostaph1; this plasmid was designated pGhostsmez and was used to transform the S. pyogenes strain, H293. pGhostsmez transformants were cultured on Ery-containing THY agar plates at 30°C for 36–48 h; pGhostsmez is replicative in Gram-positive bacteria at 30°C. Transformed colonies were then inoculated into THY broth (+ Ery) and cultured at 30°C for 24–36 h. The cultures were then transferred to 37°C, and incubated for 12–14 h. At 37°C, the plasmid is nonreplicative and may integrate into the chromosome at the smez locus. Cultures were then streaked onto THY + Ery plates. The replicative plasmid, pDL413, was used to control for transformation efficiency as before (5); transformation efficiencies of 10^3–10^6 μg plasmid DNA were typically observed.

**Southern hybridization and PCR**

Southern analysis was performed on Xhol-cut genomic DNA extracted from the parent strain H293 and an Ery-resistant transformant, H377, using a 331-bp digoxigenin (DIG)-labeled smez probe (amplion prepared using primers smez1 and smez2). Blots were stripped and reprobed as described previously with a 673-bp DIG-labeled aph1 probe (corresponding to nucleotides 838-1531 of the pUC4K sequence, amplified using primers Aph1 and Aph2) (6). PCR analysis of parent strain H293 and mutant H377 was performed using the primers smezP (which corresponds to a nucleotide sequence 69-bp upstream of the coding sequence for smez on the M1 genome database) and smezR (which anneals to nucleotides 601–618 of the smez-2 gene) in addition to primer pair smez1/smezR.

**Growth analysis of bacterial isolates**

The ability of the parent strain H293 and mutant strain H377 to grow in both broth and whole blood was compared in standard conditions. Growth in THY was monitored over 12 h by measurement of OD_{600}, while growth in heparinized murine blood was monitored over 3 h in a rolling whole blood osmoprophagocytosis assay as described previously (5). Growth in blood was expressed as fold increase in CFU (i.e., total CFU after 3 h/CFU inoculated). Quantitation was performed by plating out dilutions onto blood agar plates.

**RNA and Western analysis**

Northern analysis of RNA obtained at mid-log, late-log, and stationary growth phases from parent strain H293 and mutant strain H377 was performed as described previously using the DIG-labeled 330-bp smez PCR product as a probe; control hybridizations were performed using DIG-labeled probes for rIF (or DNaseB) (7). For RT-PCRs, 10 μg of streptococcal RNA, prepared from each strain at each growth phase, was treated with DNase I, Amplification Grade (Sigma-Aldrich) for 15 min at room temperature. DNase I was inactivated by heating for 10 min at 65°C. This RNA was used for reverse transcription (RT) using the Superscript II Reverse Transcript Kit (Life Technologies) as per the supplier’s protocol, either in the presence or absence of reverse transcriptase (5 μg DNAse I-treated RNA per tube). A single 20-μl RT reaction was performed for each strain at each growth phase; 2 μl of each RT reaction was then used as template DNA for amplification using primers smez1/smezR, MF1/MF2, and SPEG1/SPEG2 (Table I). Amplification (28 cycles) was performed using an annealing temperature of 55°C.

For Western analysis, 10 μl of cell-free THY bacterial culture supernatant was subjected to SDS-PAGE under reducing conditions, electroblotted onto nitrocellulose, and incubated with a 1/10,000 dilution of rabbit polyclonal anti-SMEZ serum, before development using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ), rSMEZ-13, 10 ng, was run as a positive control.

**Preparation of human PBMC**

Blood (20 ml) was drawn from healthy donors and diluted 1:2 in 0.9% saline. The diluted blood was overlaid on equal volumes of Ficoll-Paque (Amersham Pharmacia Biotech) and centrifuged at 800 × g for 35 min. The interface, rich in lymphocytes, was removed and washed once in HBSS at 800 × g for 10 min. The cells were finally resuspended in 10 ml of RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 50 μM penicillin, and 50 μg/ml streptomycin (Life Technologies).

**Preparation of culture supernatants**

Cell-free culture supernatants used for the PBMC or splenocyte stimulation assays were obtained from overnight cultures of H293 or H377 in RPMI 1640 containing 10% FCS, 2 mM glutamine without antibiotics. Bacterial counts of the H293 and H377 broths as estimated by viable counts were similar (H293 = 2.4 × 10^7 CFU/ml; H377 = 3.6 × 10^7 CFU/ml). Cultures were filter-sterilized and supernatants were stored at −20°C. No endotoxin could be detected in RPMI supernatants using the Limulus assay.

**Proliferation and cell stimulation**

Human PBMC (2 × 10^5 cells/well) or murine spleen cells (4 × 10^5 cells/well) in a volume of 200 μl were stimulated with culture supernatants (diluted to 1% and 0.1%) or rSMEZ-13 (0.1–10 ng/ml) at 37°C, 5% CO_2, Control supernatants included Con A (10 μg/ml), rSMEZ-1 (0.1–10,000 ng/ml), and staphylococcal enterotoxin B (SEB, 10–1000 pg/ml). Tritiated thymidine (1 μCi/ well) was added to each well after 60 h and cells were harvested at 72 h onto filter mats in a 1295-004 Betaplate 96-well cell harvester (Wallac and Berthold, Milton Keynes, U.K.). cpm were measured in a 1205 Betaplate liquid scintillation counter (Wallac and Berthold). For cytokine assays, human PBMCs were stimulated with 1% bacterial supernatant or 0.1 ng/ml rSMEZ-13. Cell-free tissue culture supernatants from stimulated human PBMCs were collected and frozen at −20°C before cytokine analysis.

**TCR-Vβ repertoire analysis in vitro**

Human PBMC (2 × 10^6 cell/ml) or murine spleen cells (1–10 × 10^6 cell/ml) were stimulated with streptococcal culture supernatants (1% dilution) or rSMEZ-13 (10 ng/ml) for three days at 37°C in the presence of 5% CO_2 in 0.5 ml RPMI 1640. After 3 days, 0.5 ml fresh RPMI was added to each well along with 20 ng/ml rIL-2 (R&D Systems, Abingdon, U.K.), and incubated for a further 3 days. Human PBMC or murine spleen cells were washed once and resuspended in 1× PBS, 1% BSA (Sigma-Aldrich) buffer. Cells (4 × 10^5 to 1 × 10^6) were stained with FITC or PE-labeled Ab and incubated for 45 min at 4°C in the dark. Human cells were double-stained with PE-conjugated anti-human TCR-Vβ2, Vβ8 and Vβ12 (BD Biosciences) together with either FITC-conjugated anti-human CD4 or anti-human CD8 (Sigma-Aldrich) and analyzed by flow cytometry. Murine spleen cells were double-stained with PE-conjugated Abs specific to murine Vβ2, 3, 4, 5.1, 7, 8.1, 10, 11, and 13 (BD Biosciences), along with FITC-conjugated Abs to either murine CD4 or CD8 (Sigma-Aldrich). Cells were washed thoroughly with 2 ml PBS-BSA buffer and used for FACS analysis. FACS analysis was performed in a FACS Calibur (BD Biosciences). Gated events (20,000) were counted for each sample.

**Animals**

C57BL/10.DQ8 transgenic mice carrying genomic constructs for DQA1*0301 and DQB1*0302 and FVB/N.DRI transgenic mice carrying genomic constructs for DRA1*0101 and DBRI*0101 have been previously described (8, 9). For in vitro comparison of responsiveness to SMEZ,
C57/BL10.DQ8 and FVB/N.DR1 transgenic mice both were used on a matched syngeneic (C57BL10 × FVB/NJ) background. Transgenic mice were genotyped as previously described. Age and weight-matched HLA transgene-negative littermates were used as controls. Groups of transgenic mice used in in vivo experiments were age-, sex-, and weight-matched. All animals received food and water ad libitum. All animal procedures were conducted within local and home office ethical guidelines.

**Immunological effects of SMEZ during infection**

Parent strain H293 and mutant strain H377 were grown overnight in 100 ml THY at 37°C, in the absence of any antibiotic. Bacteria were washed once and resuspended in 3–4 ml of 0.9% saline such that the OD600 was identical between strains. Suspensions were adjusted to 10⁷ CFU/ml. Bacterial suspension (0.2 ml) was administered i.p. to groups of C57BL/10.DQ8 transgenic mice identical in sex, age, and weight. To address whether SMEZ affected survival in this model of peritoneal infection, groups of nine C57BL/10.DQ8 transgenic mice were infected i.p. with either H293 or H377 and survival was monitored over 7 days.

To investigate the immunological effects of SMEZ during sepsis, groups of five C57BL/10.DQ8 mice were infected i.p. with either H293 or H377; 48 h after infection, blood was drawn by cardiac puncture for estimation of blood bacterial counts. In separate experiments, serum was obtained for cytokine estimation (48 and 60 h after infection) and SMEZ quantification (60 h only) by centrifugation of cardiac puncture blood samples at 5000 × g for 10 min. Peritoneal aspirates were also collected from killed mice for bacterial quantification (48 h), cytokine analysis (48 and 60 h), and SMEZ quantification (60 h only); 2 ml sterile saline was injected into the peritoneum, massaged for 1 min, and then 0.5–1 ml fluid was withdrawn from the peritoneal cavity. The aspirate was centrifuged at 5000 × g for 10 min and the supernatant was frozen at –20°C. Bacterial counts were quantified by plating of dilutions of serum or lavage fluid onto blood agar plates. Peritoneal SMEZ mutagenic mouse isolates subcultured from the H377-infected mice; isolates were initially cultured on blood agar without antibiotic, then replica-plated onto THY + Ery. Genomic DNA was isolated from each isolate and analyzed by PCR, using primer pairs smezP/smezR and smez1/smez2.

**Proliferation bioassay for SMEZ production in vivo**

Sera and peritoneal aspirates from H293- and H377-infected mice were coinoculated at 1/10, 1/100, and 1/1000 dilutions with human PBMCs from a single donor in standard proliferation assays. Results were compared with parallel studies using rSMEZ-13 (0.1 fg/ml to 100 ng/ml) as the stimulant. The relationship between rSMEZ-13 concentration and proliferation was plotted and used to semiquantitatively assess the concentrations of SMEZ in serum and peritoneal aspirate.

**TCR-Vβ repertoire analysis in vivo**

For analysis of the TCR-Vβ T cell subsets expanded during infection, groups of five C57BL/10.DQ8 transgenic mice were infected i.p. with H293 (1.52 × 10⁷ CFU/mouse) or H377 (5.3 × 10⁷ CFU/mouse). Mice were killed 48 h after infection and spleens from groups of infected transgenic mice were dissected out and dissociated into single cell suspensions in RPMI 1640. Spleen cells were stained immediately with Abs against murine Vβ4, Vβ8, or Vβ11 and murine CD4 and the proportion of stained cells was quantitated by flow cytometry as described above.

**Cytokine measurements**

Both human and murine cytokine quantitation was done by ELISA, using paired Abs purchased from R&D Systems. Standard curves were set up with the respective recombinant cytokines (R&D Systems); all cytokine measurements were made within sensitivity ranges of individual assays.

**Statistics**

The Mann-Whitney U or ANOVA tests were used for comparison between groups in all in vitro experiments. Values of p ≤ 0.05 were considered significant. Survival was compared using the log rank test.

**Results**

**Genotypic analysis of the smez mutant, H377: Southern and PCR analysis**

A single Ery transformant was obtained after culture at 37°C and this was designated strain H377. Southern analysis demonstrated a difference between XbaI-cut genomic DNA from the parent strain H293 and H377, when probed with smez (Fig. 1B). The single 2.7-kb smez band seen in XbaI-cut genomic DNA from H293 was replaced by two bands of 5.5 and 6.5 kb in H377 DNA, confirming that pGhostsmez had integrated at the smez locus as shown in Fig. 1A, causing an insertional duplication of the smez gene. All further work was performed using this strain. Probing with the aph1 probe further confirmed the insertion of the plasmid at the smez locus. Aph1 cohybridized with smez to the 6.5-kb band as expected (data not shown).

Southern hybridization results were confirmed by PCR: primer pair smezP and smezR yield a 678-bp fragment from H293 genomic DNA, which was not seen when using H377 DNA as a template. Primer pairs smez1 and aph1R did not produce any ampiclon from the wild-type DNA as expected, but showed a 2.1-kb ampiclon with the H377 DNA. Interestingly, primers smez1 and smez2 amplified a product of 331 bp in the wild-type strain; three bands of sizes 331, 662, and 993 bp were amplified from the H377 DNA, confirming that the 331-bp smez triplet insertion seen in pGhostsmez persisted in the mutant H377. Hence, the PCR results further confirmed the insertion of pGhostsmez at the smez locus.

**Analysis of bacterial growth in vitro**

There was no difference between the growth rates of H293 and H377 when cultured in THY (not shown). Furthermore, the two strains did not differ significantly in their ability to grow in whole murine blood when cultured over 3 h at 37°C (H293: 31.2-fold increase; H377: 41.1-fold increase). In addition, there were no differences in DNase production, hemolysis, or capsule production when colonies were observed on solid medium.

**Transcription and expression of smez is highly restricted in vitro**

Northern analysis failed to detect a smez transcript from either strain at any phase of growth. This was in contrast to mf transcripts which were easily detected at late-log and stationary phases in RNA from both strains (not shown). Transcripts for smez could only be detected by RT-PCR, using primers smez1 and smezR (which anneal to the 5′ and 3′ ends of the smez coding sequence). This yielded a faint, but distinct, 608-bp band in H293 total RNA from the late-log phase of growth only, which was not seen for H377 (Fig. 1C). Transcripts for spec and mf did not differ between H293 and H377.

Although rSMEZ-13 yielded a clearly defined band, Western blotting using polyclonal anti-SMEZ antiserum failed to detect a specific SMEZ band in 10 µl supernatant samples from H293, even when supernatants were concentrated 10-fold.

SMEZ is the major mitogen secreted by S. pyogenes

H293 culture supernatants induced a dose-dependent mitogenic response in human PBMC from three donors. The H377 culture supernatant demonstrated a marked reduction in mitogenicity (Fig. 2A). Hence, the mitogenic activity of H293 in human PBMC is largely attributable to SMEZ.

Human PBMC extracted from blood obtained from three donors were stimulated with a range of concentrations of pure rSMEZ-13. A dose-dependent mitogenic response was observed; human PBMC responded to rSMEZ-13 concentrations as low as 0.1 fg/ml (Fig. 2B). To estimate the concentration range of SMEZ-13 produced in vitro in broth culture by H293, the mitogenic activity of the SMEΖ” culture supernatant was compared with that of pure rSMEZ-13. It was concluded that the concentration of SMEZ in H293 broth could be as low as 4–5 pg/ml.
SMEZ is responsible for S. pyogenes-induced expansion of human V8-positive T cells in vitro

SMEZ is known to stimulate V8+ human cells (1, 2, 10), though V8+ human T cell stimulation has also been attributed to a number of other streptococcal products (11). To investigate the contribution of SMEZ to the overall V8-stimulatory effect of GAS, human PBMC were stimulated with culture supernatants from H293 (SMEZ+) and H377 (SMEZ-). H293 supernatant induced expansion of V8+ both in the CD4+ and CD8+ T cell populations (Table II). The increase in V8+ T cells was eliminated when using H377 supernatant. This effect was specific to V8+ T cells, as there were no differences observed in proportions of V8+ or V812+ T cells between H293- and H377-stimulated PBMC cultures (data not shown). Furthermore, there were no differences observed in proportions of V8+ or V812+ T cells between supernatant-stimulated PBMC cultures and cultures incubated in medium alone.

SMEZ accounts for cytokine production by streptococcal supernatant-stimulated human PBMCs

Human PBMC from three donors were stimulated with H293, H377, rSMEZ-13 or medium alone for 6, 24, 48, and 72 h and the production of IFN-γ, TNF-α, TNF-β, IL-1β, and IL-8 was measured by ELISA. There was a time-dependent increase in production of all the cytokines, except TNF-β, up to 72 h from H293-stimulated PBMCs. For TNF-β, there was an increase up to 48 h, followed by a subsequent decrease. For all the cytokines studied, H293 supernatant stimulated production of significant levels of cytokines compared with both the H377 and medium controls (Fig. 3, left panels). Surprisingly, cytokine release from PBMCs stimulated with supernatant from H377 was no different to cells incubated with medium alone. rSMEZ induced a time-dependent increase in production for all cytokines, similar to that observed for the culture supernatants, except for IFN-γ, which decreased after a 48-h incubation (Fig. 3, right panels).

Cells from transgenic mice expressing HLA-DQ8 are highly sensitive to SMEZ-13, compared with mice expressing HLA-DR1, or wild-type mice

To study the immunological effects of SMEZ in vivo, it was essential to establish the murine cell types that were most sensitive to SMEZ by examination of the mitogenic activity of rSMEZ-13 in vitro. HLA-DQ8 transgenic murine spleen cells were at least 3 logs more sensitive to rSMEZ-13 than wild-type murine cells, yielding a detectable mitogenic response even at 0.1 pg/ml SMEZ (Fig. 4A). Spleen cells from HLA-DR1 transgenic mice were less sensitive to
SMEZ than HLA-DQ8 cells (requiring 10 pg/ml of rSMEZ-13 for proliferation); these marked differences persisted even when cells were coincubated with rSMEZ-1 instead of rSMEZ-13 (data not shown). In contrast, HLA-DQ8 and HLA-DR1 transgenic spleen cells demonstrated no significant differences in responsiveness to the staphylococcal superantigen SEB. SEB concentrations of 100 pg/ml produced significant proliferation in transgenic cells, while wild-type cells required 3 logs more SEB to proliferate (not shown). Overall, spleen cells from HLA-DQ8 transgenic mice were more sensitive to rSMEZ-13 than HLA-DR1 transgenic or wild-type murine spleen cells.

Consistent with this observation, H293 culture supernatant was able to induce a clear mitogenic response in HLA-DQ8 splenocytes, but not at all in cells from HLA-DR1 transgenic or wild-type mice. Mitogenic activity induced by the H293 supernatant in the HLA-DQ8 murine splenocytes was abrogated when cells were coincubated with supernatant from H377 (Fig. 4B). Hence, the mitogenic activity of H293 was specifically detectable in murine cells expressing HLA-DQ8 and the activity was wholly attributable to SMEZ. Therefore, all further murine experiments were performed using C57BL/10.DQ8 mice.

SMEZ-13 specifically expands the murine T cell V\^β11^ CD4^+^ subset; V\^β11^ T cell expansion in S. pyogenes-stimulated murine spleen cells is wholly attributable to SMEZ

To study the murine V\^β-T cell subsets expanded by SMEZ-13, spleen cells from C57BL/10.DQ8 mice were stimulated with pure rSMEZ-13 (10 ng/ml). Significant expansion of V\^β11^ CD4^+^ T cells was seen, while there was a marked reduction in all the other V\^β-bearing T cell subsets studied, compared with unstimulated controls (Table III).
cell subsets compared with the unstimulated control, consistent with the lack of mitogenicity of H377 in murine spleen cell culture.

**Role of SMEZ in peritoneal streptococcal infection**

Peritoneal bacterial clearance 48 h after the onset of i.p. infection did not differ between mice infected with H293 (median 1.4 × 10^3 CFU/ml, range 30–1.33 × 10^4 CFU/ml) and mice infected with H377 (median 1.1 × 10^5 CFU/ml, range 0–4.0 × 10^4 CFU/ml) (Fig. 5A). Colonies (25 of 25) cultured from H377-infected mice were Ery-resistant and PCR analysis confirmed that the smez mutation was stable in all isolates.

There was no difference in 7-day survival between mice infected i.p. with H293 and mice infected with H377 (Fig. 5B). At 48 h, only one of five mice in each group was bacteremic, consistent with previous data using this bacterial strain.

**Cytokine levels in serum and peritoneal aspirates from H293- and H377-infected mice**

TNF-α, IFN-γ, and IL-6 levels were measured in peritoneal aspirates obtained from H293- and H377-infected C57BL/10.DQ8 mice, 48 or 60 h after infection, in separate experiments. TNF-α and IFN-γ levels were higher in the H293-infected groups compared with the H377-infected group in both the experiments (at 48 and 60 h), though these differences were not significant. IL-6 levels were higher in the H293-infected group compared with the H377-infected group at both time points; differences in IL-6 levels were statistically significant (Mann-Whitney U test) (Fig. 6). Levels of cytokines in serum were at the lower limits of detection; there were no detectable differences between the two groups.

**SMEZ is produced in vivo during acute infection**

Sera and peritoneal aspirates obtained at 60 h from C57BL/10.DQ8 mice infected with H293 or H377 were used to stimulate single donor human PBMC proliferation. Peritoneal aspirates from four of five H293-infected mice stimulated significant proliferation of human PBMCs, whereas aspirates from H377-infected mice and control peritoneal lavage fluid did not. Thus, the mitogenic activity of peritoneal lavage fluid from H293-infected mice could be attributed to SMEZ (Fig. 7A). Serum from three of five H293-infected mice induced significant proliferation of human PBMCs compared with H377-infected mice and control mouse serum, though this difference was not significant as not all H293-infected mice had detectable mitogenic activity in serum (Fig. 7B). The data confirmed that SMEZ was produced at the site of infection in H293-infected mice. The relation between SMEZ-13 concentration and donor PBMC proliferation, measured as cpm tritiated thymidine incorporation, was plotted (not shown) and was used to

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**Table III.** *Murine spleen cell TCR Vβ repertoire alteration in vitro by SMEZ and H293 (WT) or H377 (smez⁻) streptococcal supernatants*¹

<table>
<thead>
<tr>
<th></th>
<th>% CD4⁺ Cells Expressing Specified TCR Vβ (Mean of 3 ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>Vβ2</td>
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<tr>
<td><strong>Pure toxin</strong></td>
<td></td>
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<tr>
<td>Medium</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>SMEZ-13</td>
<td>0.06 ± 0.05</td>
</tr>
<tr>
<td><strong>Supernatants</strong></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>nd</td>
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<td>H293</td>
<td>nd</td>
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<td>H377</td>
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¹ SMEZ-13 was used at 10 ng/ml. Supernatants or RPMI were added at 1% dilution. Values in bold indicate those which are significantly different from values for cells incubated with medium alone. Underlined values represent significant increases compared with values for cells incubated with medium alone (p ≤ 0.05). nd, Not done.
derive rough estimates for SMEZ concentrations in peritoneal lavage fluid (median 600 fg/ml, range 0–200 pg/ml) and serum (median 1 pg/ml, range 0–7 ng/ml). As SMEZ in the peritoneum had been diluted by lavage, concentrations of SMEZ at the serosal surface of i.p. organs may be 40- to 50-fold higher than those found in lavage fluid. Body fluids contain several cytokines which may alter the SMEZ-induced proliferative response in vitro, and these figures must be regarded as estimates only.

**In vivo analysis of the Vβ-bearing T cell subsets expanded during infection**

Intraperitoneal infection with *S. pyogenes* H293 led to an increase in the proportion of Vβ11 T cells in murine spleen. There was a small but statistically significant decrease in the proportion of Vβ11 T cell population of H377-infected mice, compared with those of the H293-infected mice. There were no significant differences in the proportions of Vβ8 T cells in spleens from both groups of mice. Interestingly, a small but statistically significant increase in Vβ4 T cells in H293-infected mice was dramatically enhanced when mice were infected with the SMEZ-negative strain H377 (Table IV).

**Discussion**

The family of recognized streptococcal superantigens has expanded considerably since completion of the University of Oklahoma M1 streptococcal genome sequence database (4). From the array of toxin genes identified to date, SMEZ appears to be the most potent. As the *smez* gene is present in all serotypes studied, displays allelic and antigenic variation, and is in linkage equilibrium with *emm* type (3), we hypothesized that SMEZ production is of importance to bacterial survival in the host population. To fully address the role played by SMEZ in streptococcal pathogenesis, we have developed an isogenic *smez* mutant from a clinically invasive *S. pyogenes* isolate that did not have the genes for the phage-encoded toxins, SPEA and SPEC, and which was known to produce SMEZ-13, one of the most potent SMEZ alleles.

In stark contrast to other secreted proteins made by *S. pyogenes*, transcripts for *smez* were almost undetectable, in keeping with the extremely low concentrations of SMEZ protein produced in broth culture. Whereas transcripts for other secreted proteins are maximally detected at stationary phases of growth (7), transcripts for *smez* were only detected by RT-PCR at late-log phase; whether this reflects mRNA instability or constitutive repression of the
that a native preparation of the SMEZ-16 allele was 10-fold more active than a native preparation of SPEA when cytokine production from human mononuclear cells was quantified (20). The cytokine-stimulating role of a range of streptococcal secreted proteins has been extensively reported (21, 22); data from the current study underpin the value of examining protein function in a physiological context, using supernatants from isogenic strains which are free of endotoxin contamination. We and others have shown that levels of cytokines such as TNF-β (lymphotoxin-α) and IFN-γ are produced by superantigen-stimulated human mononuclear cells in a time-dependent manner, peaking at 72–96 h after stimulation, consistent with the pattern of superantigen-induced T cell mitogenesis (23–25). Despite the potent promitogenic activity of SMEZ, the rapidity of SMEZ-dependent cytokine production, seen at 24 h, and the SMEZ-dependent induction of monokines such as IL-1 and IL-8 was unexpected. The possibility that the smez mutant had sustained a nonspecific phenotypic change during genetic manipulation was considered but discounted, because expression of other virulence factors was unchanged, and because the strains grew equally well in both broth and whole blood. It was felt unlikely that expression of recognized superantigens such as SPEG would be affected by the mutation, as the gene encoding SMEZ is distant from genes encoding other secreted toxins. Indeed, transcription of mf and speg was unaffected by the mutation. Although smez is adjacent to dpp and a cluster of genes regulated by mga, none of these latter virulence factors is associated with T cell mitogenesis. Transcripts for the mga-regulated virulence factor emm89 are detectable by Northern blotting to equal degrees in both H293 and H377 while transcription of dpp is not detectable in the M89 strain described in this study (M. Unnikrishnan, unpublished observations). Furthermore, the abrogation of cytokine induction was specific to secreted proteins made by the smez mutant, as the smez mutation did not affect TNF-α or IFN-γ induction by whole washed heat-killed streptococci (S. Sriskandan, not shown).

Investigation of the role played by superantigens in bacterial pathogenesis has been hampered by a lack of disease models which are superantigen-sensitive and which can be investigated using readily available immunological tools. We recently demonstrated that mice expressing human HLA-DQ transgenes were highly sensitive to SPEA, both in vitro and in vivo, compared with wild-type mice. Although HLA-DQ-dependent immune activation during invasive infection with a SPEA-producing M1 S. pyogenes was largely dependent on SPEA production in this artificial system, mortality was unaffected by disruption of the gene encoding SPEA (26). From an evolutionary standpoint, it is likely that expression of superantigens favors bacterial survival or dissemination within the host range, rather than destruction of the host and consequent abortive infection. In this study, HLA transgenic mice were used to investigate the role played by the ubiquitous streptococcal superantigen SMEZ in pathogen-host interactions. SMEZ binds exclusively to the MHC class II β-chain in a zinc-dependent manner (27). APCs which are transfected with HLA-DQ-B1*0302 or HLA-DQ-B1*0401 present rSMEZ-13 to Jurkat cells more efficiently than cells which express HLA-DQ-B1*0302, as the smez mutation did not affect TNF-α or IFN-γ induction by whole washed heat-killed streptococci (S. Sriskandan, not shown).

Investigation of the role played by superantigens in bacterial pathogenesis has been hampered by a lack of disease models which are superantigen-sensitive and which can be investigated using readily available immunological tools. We recently demonstrated that mice expressing human HLA-DQ transgenes were highly sensitive to SPEA, both in vitro and in vivo, compared with wild-type mice. Although HLA-DQ-dependent immune activation during invasive infection with a SPEA-producing M1 S. pyogenes was largely dependent on SPEA production in this artificial system, mortality was unaffected by disruption of the gene encoding SPEA (26). From an evolutionary standpoint, it is likely that expression of superantigens favors bacterial survival or dissemination within the host range, rather than destruction of the host and consequent abortive infection. In this study, HLA transgenic mice were used to investigate the role played by the ubiquitous streptococcal superantigen SMEZ in pathogen-host interactions. SMEZ binds exclusively to the MHC class II β-chain in a zinc-dependent manner (27). APCs which are transfected with HLA-DQ-B1*0302 or HLA-DQ-B1*0401 present rSMEZ-13 to Jurkat cells more efficiently than cells which express HLA-DR1 (T. Proft, unpublished observations). Spleen cells from HLA-DQ transgenic mice (expressing HLA-DQ-B1*0302) were clearly more responsive to rSMEZ-13 than HLA-DR1 transgenics, and subsequent in vivo work was therefore conducted in this murine strain.

In vivo experiments using an i.p. model of infection demonstrated that SMEZ did not contribute to mortality or impede bacterial clearance in this site. However, the peritoneum is an unusual setting for GAS infection and one cannot exclude a role for SMEZ in the pathogenesis of mucosal or skin surface infection, carriage, or reactivation. In particular, assessment of the role of SMEZ in

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Table IV.  Comparison of proportions of Vβ-bearing CD4+ T cell subsets expanded in the spleen in H293 (WT) and H377 (SMEZ-negative)-infected DQ8 mice

<table>
<thead>
<tr>
<th>Subset</th>
<th>% Vβ4</th>
<th>% Vβ8</th>
<th>% Vβ11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>7.326 ± 0.41</td>
<td>18.29 ± 1.531</td>
<td>4.45 ± 0.362</td>
</tr>
</tbody>
</table>

b Values in bold indicate statistically significant differences compared to the uninfected control.
c Significant differences between H293- and H377-infected mice.
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smez promoter is unknown. This feature was not unique to the M89 serotype studied, as transcripts for smez were also not detected in the M1 serotype strain H305 used in previous studies. In both cases, mf and emm transcripts were easily detected by Northern hybridization. As smez is less than 20 kb from the mga virulence regulon which regulates expression of genes such as emm, it is possible that regulation of this superantigen is interconnected with regulation of virulence factors involved in bacterial survival in the host. Despite the low levels of SMEZ protein present in supernatant from H293, this study demonstrates that the mitogenic activity of streptococcal superantigen in human PBMC culture is largely attributable to SMEZ. Indeed, proliferation of Vβ8-expressing Jurkat cells is the most reliable and sensitive method of detecting SMEZ production by streptococci (3). These findings contrast with our previous studies, which demonstrated that MF (shown to be DNaseB) did not contribute to the mitogenic activity of the same M1 strain (6). In an earlier study, we demonstrated that SPEA contributed very little to the overall mitogenic activity of an M1 strain in human PBMC culture, despite SPEA concentrations in excess of 500 ng/ml in culture supernatants from the parent strain (5). We conclude that the tiny amounts of SMEZ present in culture supernatants from H293, calculated by bioassay to be ~5 pg/ml, are largely responsible for the mitogenic activity of this strain.

Native preparations of a wide range of streptococcal proteins, including M protein, SPEA, SPEB, and MF were previously reported to selectively stimulate human T cells bearing TCR Vβ8 (11–15). More recently a previously unknown streptococcal superantigen, SPEX (identical to SMEZ) was shown to underlie the potent stimulation of human Vβ8+ T cells attributed to SPEB and MF (16, 17). As the quantities of SMEZ required for stimulation of human T cells are 2–3 log-fold less than other streptococcal toxins and as SMEZ copurifies with other streptococcal proteins (10), we speculated that all the TCR Vβ8-stimulatory activity of S. pyogenes could be attributed to SMEZ, or contamination with SMEZ. Using an isogenic mutant streptococcal strain, deficient in SMEZ production, this work confirms that SMEZ is responsible for all detectable S. pyogenes superantigen-induced TCR Vβ8 human T cell proliferation. Streptococcal superantigen from both the parent and smez mutant strain failed to induce detectable expansion of human T cells bearing TCR Vβ2, the subset targeted by the recently characterized superantigen SPEJ (18, 19), consistent with the low level of residual mitogenicity in the smez mutant strain.

The studies described demonstrate unequivocally that SMEZ is the single most important stimulus to cytokine production from S. pyogenes-stimulated human mononuclear cells. Indeed, supernatant from the smez– mutant did not elicit detectable cytokine production from human cells, when compared with medium alone. This may be because SMEZ is the only significant stimulus present in supernatant which can lead to cytokine production, or, more likely, because SMEZ is an essential cofactor which synergizes with other streptococcal proteins. Very recently, it was reported...
nasopharyngeal streptococcal infection is the focus of future work. One major aim of the experiments described was to investigate whether SMEZ was produced during infection. Data obtained using peritonal lavage fluid and serum from mice infected with isogenic streptococcal strains differing only in SMEZ production demonstrated that mitogenic activity in these fluids during infection is attributable to SMEZ. In separate work, we have detected a highly potent mitogen in the serum of some patients with streptococcal toxic shock and mice infected with M1 S. pyogenes; mitogenic activity was inhibited by polyclonal anti-SMEZ serum, but not by polyclonal anti- sera to other streptococcal superantigens, confirming that the dominant promitogenic toxin produced during invasive infection is SMEZ (T. Proft, M. Unnikrishnan, S. Sriskanad, manuscript in preparation).

IL-6 levels were measured in S. pyogenes-infected mice as a parameter of severity of sepsis, as levels are known to correlate with mortality (28). TNF-α and IFN-γ were measured, as these proinflammatory cytokines can mediate outcome in superantigen-exposed mice (29). Consistent with previous studies of streptococcal sepsis, significant elevation of cytokines was not seen in serum of infected mice. In contrast, it was possible to detect local production of cytokines in the peritoneum of mice infected with the parent streptococcal strain; this was reduced in those infected with the smeZ mutant strain.

Although the entire murine T cell repertoire could not be investigated in this study, it is likely that murine TCR Vβ11 is the principal target of SMEZ. Murine Vβ11 is the closest homologue of human Vβ8 and demonstrates 71–77% identity at the nucleotide level (30). Infection with S. pyogenes led to an increase in Vβ11+ T cells in murine spleen, which was reduced in mice infected with the smeZ mutant. Coupled with the cytokine data, the results are consistent with an in vivo superantigen response. However, remarkably, mice infected with the smeZ mutant demonstrated a clear increase in the proportion of CD4+Vβ4+ cells at 48 h. Scheming of the T cell repertoire was not seen in spleen cells cultured in vitro with supernatants from the smeZ mutant strain. Thus, the in vivo change in T cell repertoire is unlikely to be a residual effect of other superantigens expressed by the smeZ mutant. CD4+ Vβ-specific Ag-specific immune responses can be detected early in the course of an infection (31). We speculate that exposure to SMEZ may suppress expression of TCR Vβ subsets which are of importance in the development of cognate responses to streptococcal Ags. The development of isogenic streptococcal mutants which differ only in toxin production and HLA class II transgenic murine models which are superantigen-sensitive are critical steps toward understanding the role played by superantigens in evasion of the host immune response.

References


