MAJOR ARTICLE

Selective Modulation of Superantigen-Induced Responses by Streptococcal Cysteine Protease

Rita G. Kansal,[1,2] Victor Nizet,[3] Arthur Jeng,[4] Woei-Jer Chuang,[5] and Malak Kotb[1,2]

[1]Veterans Affairs Medical Center, Research Service, and [2]Departments of Surgery and of Microbiology and Immunology, University of Tennessee Health Science Center, Memphis; Departments of [3]Pediatrics and [4]Medicine, University of California, San Diego School of Medicine, La Jolla; [5]Department of Biochemistry, National Cheng Kung University, College of Medicine, Tainan, Taiwan

Streptococcal pyrogenic exotoxin (Spe) B, a streptococcal cysteine protease, is believed to be important in group A streptococcal (GAS) pathogenesis. The present study examined the effect of SpeB on the activity of superantigenic exotoxins secreted by M1T1 GAS isolates. The proliferative response of human lymphocytes to culture supernatant (SUP) from an SpeB$^+$ isolate increased significantly ($P < .05$) when the isolate was grown with N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, a cysteine protease inhibitor. The lymphocyte-stimulating activity of SUP from a spontaneous SpeB$^-$ variant or SpeB$^-$ knockout (ΔSpeB) mutant was also significantly higher than that of SUP from the SpeB$^+$ parent isolate ($P < .001$). The addition of recombinant SpeB to the ΔSpeB mutant reduced the lymphocyte response to a level comparable to that with the SpeB$^+$ isolate. SpeB affected superantigens that stimulate cells expressing T cell receptor Vβ (TCRBV)–4, TCRBV7, and TCRBV8 but not those that stimulate TCRBV2. SpeB has a selective proteolytic effect on GAS superantigens.

Group A streptococci (GAS) are important human pathogens responsible for a wide range of infections, ranging from uncomplicated pharyngitis to necrotizing fasciitis and streptococcal toxic shock syndrome (STSS) [1]. A vast repertoire of virulence factors participate in the pathogenesis of GAS [2–4]. The streptococcal superantigens, including several streptococcal pyrogenic exotoxins (Spes), elicit potent inflammatory responses that play major roles in the pathogenesis of invasive GAS infections. These responses can be exacerbated by SpeB (the streptococcal cysteine protease), lipoteichoic acid, streptolysin-O, and other virulence components that, together, contribute to tissue damage, hypotension, and organ failure [2, 5, 6]. Recent studies have shown that invasive streptococcal infections with varying degrees of clinical severity can be caused by genetically related M1T1 isolates [7]. However, the expression of Spe genes was notably variable among these genetically related isolates [7]. Although there was no correlation between the expression of SpeA and severity of disease, there was a significant inverse relation between expression of SpeB and severity of the invasive infection [8]. Our attempts to manipulate the Spe phenotype of these isolates in vitro by varying environmental and culture conditions failed [9]. By contrast, in vivo passage of SpeA$^-$SpeB$^+$isolates induced expression of SpeA and repressed that of SpeB [9], a finding that suggested that expression of Spe might be altered by host-pathogen interactions in vivo.

SpeB is the major proteolytic enzyme produced by GAS and plays a very complex role in pathogenesis. This protease exerts multiple effects on both the host

Received 28 June 2002; revised 12 September 2002; electronically published 24 January 2003.

Presented in part: 41st annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 16–19 December 2001 (abstract 961).

Financial support: American Heart Association (grant 0160234B to R.G.K.); National Institutes of Health (NIH; grants AI 48694 to V.N. and AI 40198 to M.K.); Research and Development Office, Medical Research Service, Department of Veterans Affairs (Merit Award to M.K.); University of California San Diego (institutional NIH training award; grant AI 107036 to A.J.); Edward J. Mallinckrodt, Jr. Foundation (to V.N.).

Reprints and correspondence: Dr. Malak Kotb, University of Tennessee Health Science Center, 956 Court Ave., Suite A-202, Memphis, TN 38163 (mkotb@utmem.edu).

The Journal of Infectious Diseases 2003;187:398–407
© 2003 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2003/18703-0007$15.00

and the bacteria itself. SpeB has been shown to degrade matrix proteins, generate active interleukin (IL)–1β and kinin from their precursors, and activate human matrix metalloproteases that induce tumor necrosis factor–α production [10–14]. These effects are believed to promote bacterial invasiveness by destroying tissue and promoting inflammatory responses. In addition, SpeB abrogates the fibronectin-dependent internalization of GAS into cultured mammalian cells by remodeling the bacterial surface. This decreases bacterial adherence and internalization and may increase dissemination [15]. Although the proteolytic remodeling of GAS surface proteins is advantageous to the bacteria, several studies have proposed that overexpression of SpeB would result in nonspecific degradation of key protective virulence proteins and the loss of important bacterial defenses [5, 16, 17]. For example, M and M-like proteins confer resistance to phagocytosis by inhibiting complement deposition, but the degradation of M protein by SpeB renders the bacterium more susceptible to phagocytosis, even in the presence of type-specific antibodies against this protein. SpeB has also been shown to solubilize a 116-kDa fragment of bacterial cell surface–bound C5a peptidase that is capable of inhibiting phagocytic cells from reaching the site of infection by degrading C5a peptidase, one of the most potent chemotactic factors, and thus interferes with recruitment of neutrophils at the site of infection [5, 16]. Thus, the expression and proteolytic effects of SpeB may have to be differentially regulated by the bacteria, depending on the site and context of the infection, as well as on a temporal sequence of events in the host immune response [18].

Although several studies have suggested that a lack of SpeB expression is associated with reduced virulence in animal models [19–21], recent studies have suggested that these effects might be attributed to an unintentional selection of GAS with decreased capsule expression during the electroporation of recombinant DNA for the creation of targeted SpeB$^-$ mutants. A reduction in capsule synthesis would render the bacteria more susceptible to phagocytosis [22]. These studies, however, do not rule out the importance of SpeB in GAS pathogenesis. The role of this protease may vary depending on when, where, and how much SpeB protein is produced. The recent observation that SpeB$^-$ isolates may have a survival advantage in vivo [17], together with our finding of an inverse relation between disease severity and expression of SpeB [8], prompted us to hypothesize that, under certain conditions, increased expression of SpeB may actually attenuate certain virulence phenotypes of the bacteria. This hypothesis is supported by the finding that the bacterial surface protein G–related α2-macroglobulin–binding protein protects the bacterial surface proteins against the proteolytic actions of SpeB and other host proteinases by binding to plasma α2-macroglobulin, a potent protease inhibitor [18]. In the present study, we examined the effect of expression of SpeB on the superantigenic activity of GAS exotoxins, which are major virulence factors produced by the bacteria. We provide evidence for the selective proteolytic degradation of superantigenic activity by native, as well as recombinant, SpeB.

MATERIALS AND METHODS

Characterization of GAS isolates. Clinical M1T1 isolates were identified as described elsewhere [8]. Seven of these isolates (5448, 5449, 5459, 5628, 5836, 8004, and 8143) were included in the present study. These isolates were genetically related, as determined by a panel of molecular tests [7]. The *spe* genotype was performed by polymerase chain reaction (PCR), as described elsewhere [7]. All isolates had *speA, speB, speG, smez,* and *speF* genes. Isolates 5448, 5449, and 5836 were selected because they produced high levels of the active form of SpeB, whereas isolates 5459, 5628, and 8004 produced neither the zymogen nor the active form of SpeB. Isolate 8143 was chosen because it produced both the zymogen and the active forms of SpeB, although the zymogen was the predominant form produced. Expression of SpeA, SpeB, and SpeF was detected by Western blots, as described elsewhere [8]. More-detailed studies were conducted with isolate 5448 SpeB$^+$ wild type *(wt)* and its spontaneous in vivo–derived variant (5448 SpeB$^-$ vrt). The 5448 SpeB$^-$ vrt was obtained by in vivo passage of 5448 SpeB$^+$ *wt* in mice, as described elsewhere [9].

Creation of* spe*B in-frame allelic exchange knockout in M1 GAS strain 5448. PCR was used to amplify an 1800-bp GAS chromosomal DNA fragment that contained *spe*B along with upstream and downstream sequence. The specific primers used were 5′-GTCAAGCACAACCTATTGTT-3′ (*spe*B forward) and 5′-GTAAGCTTCTATCATACTCC-3′ (*spe*B reverse). The PCR product was T-A cloned in pCR2.1 (Invitrogen) to create pSpeB-TV. This vector served as a template for an inverse PCR using a reverse primer immediately upstream of the start codon and a forward primer immediately after the stop codon of *spe*B; these primers were designed with 25-bp 5′ extensions corresponding to the start and end of the chloramphenicol acetalytransferase gene *(cat),* respectively. The resultant linearized PCR product, containing an in-frame deletion of *spe*B, was used to transform *Escherichia coli* Top10 together with an ~650-bp PCR amplicon of the complete *cat* gene from pACYC184. In vivo recombination events were identified by screening for Top10 exhibiting ampicillin resistance and chloramphenicol resistance and verified by PCR and restriction analysis to contain an in-frame substitution of *spe*B with *cat.* The mutated *spe*BΔ*cat* gene and flanking DNA was subcloned as a *Bam*HI/*Xba*I fragment to the temperature-sensitive erythromycin resistance vector pHY304 to produce knockout vector pSpeBΔ*cat*-KO. GAS strain 5448 (serotype M1) was rendered competent for electrophoretic transformation by growth in Todd Hewitt broth (THB; Difco) plus 0.3% glycine [23] and transformed with pSpeBΔ*cat*-KO, and single recom-

bination events were identified at 37°C under erythromycin selection. Selection was relaxed by serial passage at 30°C without antibiotics, and double crossover events were identified as GAS 5448 mutants exhibiting chloramphenicol resistance but erythromycin susceptibility. Precise in-frame allelic exchange of *spe*B in the GAS chromosome was verified by PCR using *cat* primers with upstream and downstream primers and the absence of amplification of the *wt* gene(s). The confirmed mutant was designated 5448 ΔSpeB.

Expression and purification of recombinant SpeB (r-SpeB). The structural gene of SpeB was amplified by PCR from genomic DNA of GAS and then cloned into the pET-21a vector. The recombinant plasmid was transformed into the *E. coli* BL21(DE3)pLyS strain, and the system was inducibly expressed under control of a strong T7 promoter. Recombinant SpeB was produced by growing cells at 37°C in Luria Bertani medium for 24 h and purified by Ni^{2+}-chelating chromatography (Pharmacia Biotech). The protein was then concentrated by amicon ultrafiltration using a 10-kDa cutoff membrane and exchanged with PBS buffer. The enzymatically active form of SpeB was achieved by incubating the 40-kDa SpeB precursor for 10 min at 37°C in the presence of 5 m*M* dithiothreitol and EDTA.

Preparation of bacterial culture supernatant (SUP). Three representative M1T1 $SpeB^+$ isolates producing ⩾200 μg/mL SpeB (5448, 5449, and 5836) and 4 genetically related M1T1 $SpeB^-$ isolates producing very small or undetectable amounts of SpeB (5459, 5628, 8004, and 8143) were included in the study. Bacterial isolates were streaked on blood agar plates, and the isolated colonies were cultured overnight in 12.5 mL of THB, which was supplemented with 1.5% yeast extract (Difco). The bacteria were grown under microaerophilic conditions without shaking at 37°C. The overnight culture SUPs were filter sterilized, aliquoted, and stored at −20°C. For some experiments, SUP was concentrated 10 times using ethanol, dialyzed extensively, and stored at −20°C. In other experiments, to study the effect of proteolytic activity of SpeB on other superantigens, GAS isolates were also grown in the presence of a specific inhibitor of cysteine protease, N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine (E-64), at a final concentration of 28 μ*M*. The SUPs were processed the same way, and expression of various Spes was tested and their superantigenic activity evaluated as detailed below.

Detection of Spe proteins. Expression of SpeA, SpeB, and SpeF was detected by Western blot, as described elsewhere [8]. The specificity of mouse monoclonal antibody (MAb) to SpeB was checked by probing all Western blots used for SpeB detection with a paired isotype-matched antibody of irrelevant specificity (mouse anti–His-Abs of isotype G1, same as the mouse MAb to SpeB). The proteolytic activity of SpeB was detected using the EnzChek protease assay kit (Molecular Probes), as described elsewhere [8]. In brief, the SUPs were diluted 1:1 in Tris buffer (pH 7.4), and 100 μL was added to the microplate wells in duplicate. To control for specificity, parallel wells containing 28 μ*M* E-64, a cysteine protease–specific inhibitor, were included in the assay. The fluorescent substrate was added to the test and control wells, and the plates were incubated according to the manufacturer's instructions at room temperature for 24 h in the dark, and then the excitation/emission was read at wavelengths of 590/640. The protease activity was determined by subtracting the amount of fluorescence obtained in the presence of E-64 from the total fluorescence obtained without the inhibitor.

Superantigenic activity of culture SUPs. The proliferation-inducing activity of GAS SUPs was determined using peripheral blood mononuclear cells (PBMC) from healthy individuals as detailed elsewhere [24]. In brief, the PBMC were isolated from the blood of healthy individuals by ficoll-hypaque gradient centrifugation. PBMC (10^5 cells/200 μL) were stimulated with serial 2-fold dilutions of either SUP or the polyclonal mitogen phytohemagglutinin A (PHA) at a concentration of 1 μg/mL. After 72 h of culture, the cells were pulsed for 6 h with 1 μCi of [^{3}H]thymidine, harvested onto glass-fiber filters, and counted in a β-scintillation counter (Packard). Each experiment was performed in triplicate and repeated at least 3 times. THB (with or without E-64) was always included as a negative control.

Toxicity of SpeB for the cultured PBMC. The possibility that the decrease in the proliferation-inducing activity of SUP from the 5448 $SpeB^+$ *wt* versus that from the $SpeB^-$ mutant (5448 ΔSpeB) was not due to a direct toxic effect of SpeB on the lymphocytes was addressed. The PBMC were cultured for 72 h with no stimulus or with SUP from either the 5448 $SpeB^+$ *wt* or 5448 ΔSpeB. After 72 h, these cells were harvested, and their viability was checked by Trypan blue exclusion. The number of apoptotic cells was determined by flow cytometry using the annexin V-PE apoptosis detection kit (BD PharMingen), according to the manufacturer's instructions. Furthermore, the viability of cells cultured with $SpeB^+$ and $SpeB^-$ SUP was checked in functional assays. The harvested cells were washed twice with fresh medium and then transferred to fresh wells and incubated with either medium alone or medium containing 1 μg/mL PHA or 20 ng/mL SpeA. The proliferative response of the recultured cells was assayed 72 h later, as described above.

Analysis of the T cell receptor Vβ (TCRBV) repertoire by flow cytometry. A quantitative analysis of the preferential expansion of lymphocytes with specific TCRBV was conducted by flow cytometry using the IO Test Beta Mark TCRBV Repertoire kit (Beckman Coulter). A CD3-PC5 conjugate was used as an additional marker to enable proper gating on T lymphocytes only. PBMC were isolated from the blood of a healthy

individual by ficoll-hypaque gradient centrifugation and incubated at 10^7cells/5 mL of RPMI 1640 medium with 10% fetal calf serum and stimulated with 1:2000 diluted GAS SUP (with or without E-64) or PHA at 1 μg/mL. After 72 h, the cells were cultured for additional 24 h in the presence of 10 U/mL recombinant human IL-2, to allow for the regeneration of modulated T cell receptors. The cells were then harvested, washed extensively with PBS containing 1% bovine serum albumin, and stained with different antibodies, according to the manufacturer's instructions. The CD3-PC5 blastogenic cells were gated on, and simultaneous analysis of 3 TCRBV per tube was performed using a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using Cell Quest software (Becton Dickinson). A minimum of 30,000 cell events was acquired for the analysis.

RESULTS

Proteolytic degradation of GAS superantigens by SpeB. In initial experiments, we observed that SUP from GAS isolates producing high levels of the active form of SpeB had greatly decreased levels of other proteins on Coomassie blue–stained SDS-PAGE gels. With this in mind, the expression levels of SpeF and SpeA were examined from GAS isolates grown with or without E-64. In the presence of E-64, SpeB protein was accumulated in several inactive zymogen forms ranging from 40 to 32 kDa (figure 1, *top right*). SpeF protein was only detected in the $SpeB^-$ SUP and in SUP from the $SpeB^+$ isolates grown with E-64 (figure 1, *middle*). SpeA expression was undetectable or ⩽20 ng/mL in the majority of M1T1 isolates in our series. However, in the isolates that expressed both SpeA and SpeB, there was no indication of SpeA degradation, because SpeA was readily detectable in the absence or presence of E-64 (figure 1, *lane 8143*). In addition, we sequenced the entire SpeB and SpeA genes in all the isolates we used and found no mutations. Furthermore, incubation of r-SpeF and r-SpeA with $SpeB^+$ SUP or enzymatically active r-SpeB confirmed the resistance of SpeA to the proteolytic effects of SpeB (data not shown). These results suggest that SpeB degrades SpeF but not SpeA.

Effect of SpeB on the proliferation-inducing activity of GAS supernatants. We next determined whether SpeB expression affects the proliferation-inducing activity of GAS culture SUP containing a mixture of GAS superantigens. The 5448 $SpeB^+$ *wt* and 8004 $SpeB^-$ *wt* isolates were grown for 4, 12, and 24 h with or without E-64. The SUPs were recovered and tested for expression of SpeB, cysteine protease activity, and lymphocyte proliferation–inducing activity. Analysis of SUPs from the 5448 $SpeB^+$ *wt* isolate showed that the SpeB protein, as well as cysteine protease activity, were undetectable in early log phase, increased gradually by midlog phase, and reached a maximum during the stationary phase (figure 2*A* and 2*B*). The proliferation-inducing activity of SUP from the 5448 $SpeB^+$ *wt* collected at different growth phases was inversely related to the amount of SpeB expressed, with the lowest activity seen in the stationary-phase SUPs. The inhibition of cysteine protease activity by E-64 restored the proliferation-inducing activity of the 24-h SUPs from the $SpeB^+$ isolates. The proliferation-inducing activity of the SUPs obtained at 24 h from the $SpeB^+$ isolate

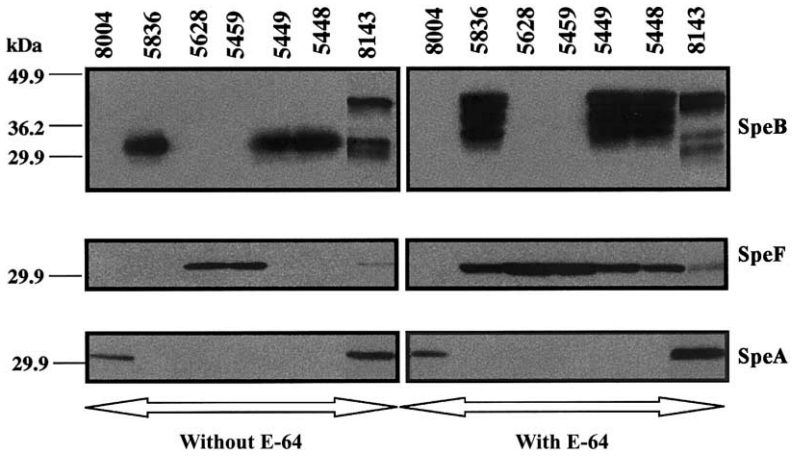


Figure 1. Effect of streptococcal pyrogenic exotoxin (Spe) B, the streptococcal cysteine protease, on the expression of SpeF and SpeA in partially purified culture supernatants of group A streptococci (GAS) isolates. Seven genetically related GAS isolates were grown with or without N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine (E-64), and the expression of SpeB, SpeF, and SpeA in partially purified culture supernatants was determined by Western blots using rabbit polyclonal (anti-SpeA and anti-SpeF) or mouse monoclonal (anti-SpeB) antibodies, as described in Materials and Methods. No cross-reactivity was observed when the blots were probed with a paired isotype-matched antibody of irrevlevant specificity. In the presence of E-64, SpeB protein was accumulated in several inactive zymogen forms ranging from ~40 to 32 kDa *(top panel).* Lane with supernatant from strain 8143 was added from a different experiment.

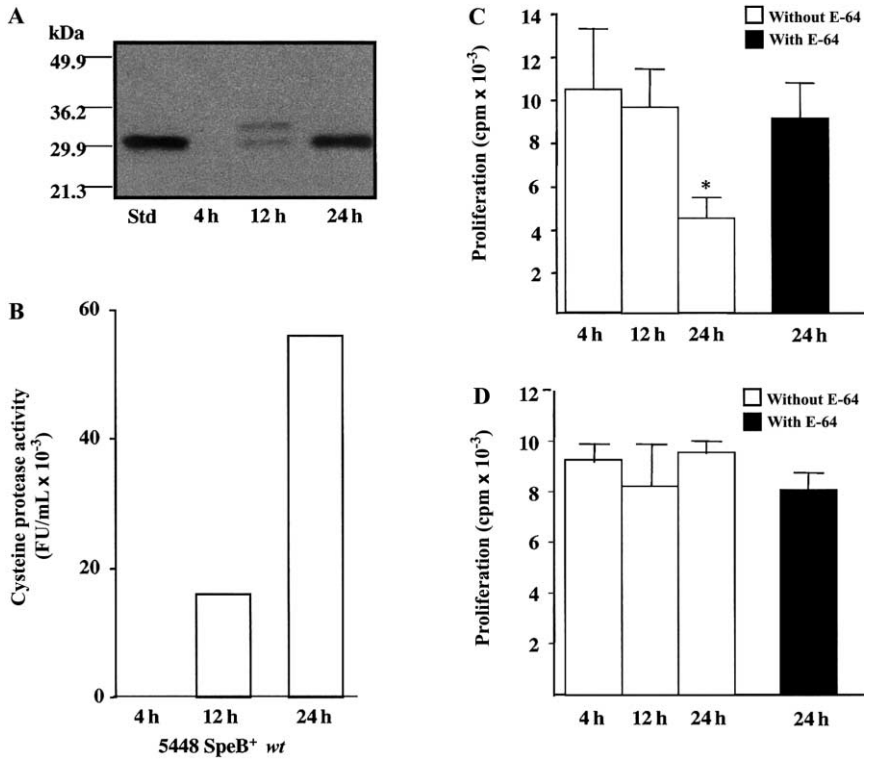


Figure 2. Effect of streptococcal pyrogenic exotoxin (Spe) B, the streptococcal cysteine protease, on the proliferation-inducing activity of group A streptococci (GAS) supernatants (SUPs). A SpeB$^+$ isolate (5448 wild type) and a SpeB$^-$ isolate (8004) was grown in Todd Hewitt broth for 4, 12, and 24 h with or without N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine (E-64). The SUPs were recovered, centrifuged to remove cells and debris, filter sterilized, and tested for expression of SpeB *(A),* cysteine protease activity *(B),* and lymphocyte proliferative activity *(C* and *D),* as described in Materials and Methods. For proliferation-inducing activity, each SUP was diluted appropriately in RPMI 1640 medium and tested against PBMC from a healthy individual. PBMC (10^6 cells/mL) were stimulated with 1:2000 dilutions of the SUP. Proliferation was assessed after 3 days of culture, and the mean ($\pm$SD) cpm [^{3}H]thymidine uptake for triplicate cultures were calculated. The data are representative of 3 experiments. Statistical significance of the differences in the proliferation-inducing responses after stimulation with SUP from the same isolate grown with or without E-64 were determined by Student's *t* test. $^*P \leq .05$. FU, fluorescence units; Std, standard.

was significantly lower ($P \leq .05$) than that from the same isolate grown with E-64 (figure 2*C*). By contrast, there was no difference in the proliferation-inducing activity of SUPs from the SpeB$^-$ isolate when tested at different growth phases or when grown with or without E-64 (figure 2*D*).

To further confirm that the decrease in proliferation-inducing activity resulted from expression of SpeB, an isogenic SpeB$^-$ mutant (5448 ΔSpeB) was generated from 5448 *wt* by an in-frame allelic exchange, as described in Materials and Methods. SpeB protein was readily detected in the 24-h SUP from the 5448 SpeB$^+$ *wt* strain but not the 5448 ΔSpeB mutant (figure 3*A*). Cysteine protease activity was undetectable in 5448 ΔSpeB SUP (figure 3*B*). The proliferative response to the stationary phase SUP from 5448 ΔSpeB was significantly ($P \leq .001$) higher than that of 5448 SpeB$^+$ *wt* at all the 4 dilutions tested (figure 3*C*). Similar results were observed when SUP from 5448 SpeB$^+$ *wt* was compared with the in vivo derived 5448 SpeB$^-$ vrt (data not shown).

Reduction of proliferative response is not due to direct toxicity of SpeB on lymphocytes. The possibility that the decrease in proliferation-inducing activity of SUP from the 5448 SpeB$^+$ *wt* versus that from 5448 ΔSpeB was due to the toxic effects of SpeB toward the cultured PBMC was ruled out in several assays. The viability and percentage of apoptotic cells cultured with SpeB$^+$ and SpeB$^-$ SUP were not significantly different (data not shown). Of importance, when cells that had been cultured with SpeB$^+$ and SpeB$^-$ SUPs were harvested, washed, and restimulated with PHA or SpeA, the extent of proliferation was quite comparable (figure 3*D*). These results support the conclusion that the decrease in the proliferation-inducing activity of SUPs from the 5448 SpeB$^+$ *wt* versus that from 5448 ΔSpeB is not due to the toxic effects of SpeB on the PBMC.

Effect of r-SpeB on the proliferation-inducing activity of SUP from 5448 ΔSpeB. The enzyme activity of r-SpeB was confirmed by the protease assay, as explained in Materials and Methods, and was found to be 700 fluorescence units/μg of protein. The proliferation-inducing activity of the SUP from 5448 ΔSpeB incubated with 200 μg/mL enzymatically active r-SpeB for over-

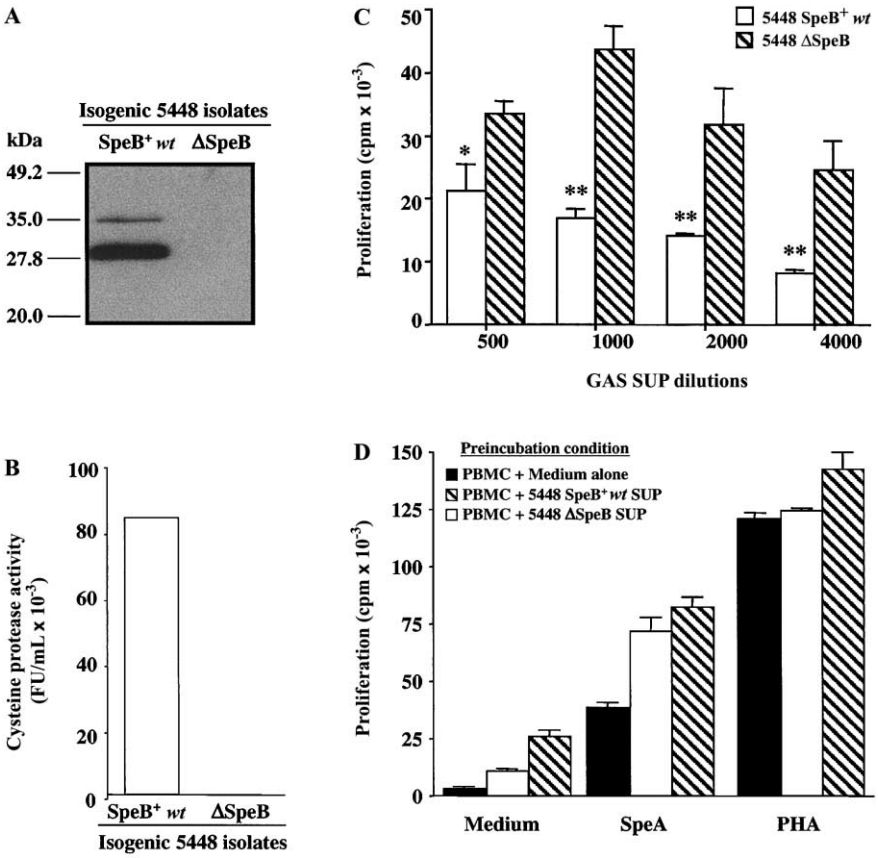


Figure 3. The proliferation-inducing activity of the supernatants (SUPs) from 5448 streptococcal pyrogenic exotoxin (Spe) B^+ wild-type *(wt)* and its isogenic $SpeB^-$ mutant 5448 ΔSpeB. The SUP from 5448 $SpeB^+$ *wt* and 5448 ΔSpeB were collected at 24 h after incubation. The SUPs were recovered, centrifuged to remove cells and debris, and filter sterilized. These SUPs were tested for expression of SpeB *(A),* cysteine protease activity *(B),* and lymphocyte proliferation–inducing activity *(C)* using serial double dilutions of the SUP ranging from 1:500 to 1:4000, as described in Materials and Methods. Statistical significance of the differences in the proliferation-inducing responses after stimulation with these SUP was determined by Student's *t* test. $*P \leq .05$; $**P \leq .001$. *D,* The proliferation-inducing activity of peripheral blood mononuclear cells (PBMC) preincubated with no stimulus or 1:2000 dilutions of SUP from the 5448 $SpeB^+$ *wt* and 5448 ΔSpeB. After 72 h of preincubation, cells were harvested, washed, and restimulated with phytohemagglutinin A (PHA) or SpeA. FU, fluorescence units; GAS, group A streptococcus.

night was significantly ($P < .001$) lower than the SUP from 5448 ΔSpeB. Furthermore, the proliferation-inducing activity of the SUP from 5448 ΔSpeB incubated with r-SpeB was quite comparable to the SUP from 5448 $SpeB^+$ *wt* (figure 4). Similarly, when the 5448 ΔSpeB bacteria were grown in THB containing the enzymatically active form of r-SpeB, the proliferation-inducing activity of the recovered SUP was significantly lower than that of SUP from 5448 ΔSpeB without added r-SpeB (data not shown). Together, these experiments support the conclusion that the decrease in the proliferation-inducing activity of SUP from 5448 $SpeB^+$ *wt* is indeed due to the proteolytic effects of SpeB on at least a fraction of GAS superantigens.

Analysis of the TCRBV repertoire. The finding that the SpeB induces the degradation of SpeF but not SpeA, together with the fact that residual proliferative activity is seen with SUP expressing high levels of SpeB or SUP with r-SpeB added, indicated that the various Spes may be differentially affected by SpeB proteolytic activity. The detection of many GAS Spe proteins is not possible by conventional methods because they are produced in very low concentrations [25]. To overcome this difficulty and to address the issue of differential effects of SpeB on other Spes, we exploited the fact that superantigens cause the expansion of T lymphocytes with specific TCRBV and analyzed the effect of SpeB expression on the TCRBV repertoire of T cells stimulated with GAS SUP from $SpeB^+$ and $SpeB^-$ isolates grown with or without E-64 as well as the SUP from 5448 ΔSpeB. The TCRBV expansion was compared in unstimulated, PHA-stimulated, and GAS SUP–stimulated PBMC. In all, 24 TCRBV were analyzed. SUP from the 5448 $SpeB^+$ *wt* induced only the expansion of TCRBV2 (figure 5, *inset table*). The expansion of TCRBV2 in response to the SUP from 5448 $SpeB^+$ *wt* was 10 times higher than that seen with the polyclonal mitogen PHA (64% vs. 7% of the activated lymphocytes). In contrast, the SUP from the same isolate grown with E-64 induced the expansion

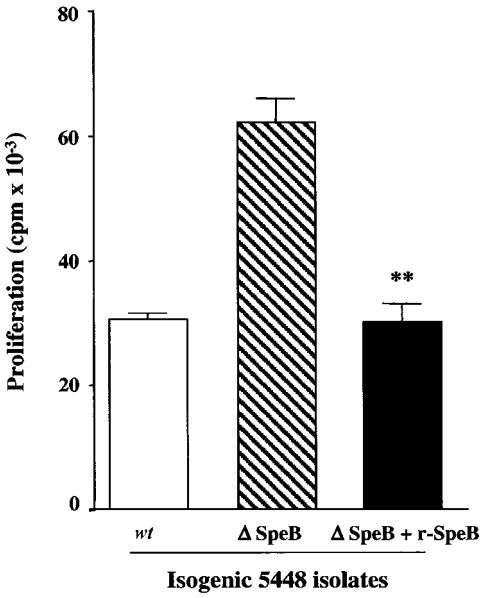


Figure 4. Effect of recombinant streptococcal pyrogenic exotoxin B (r-SpeB) on the proliferation-inducing activity of supernatant (SUP) from 5448 ΔSpeB. The SUP from 5448 ΔSpeB was incubated with r-SpeB overnight at a concentration of 200 μg/mL, and the proliferation-inducing activity was compared with the SUP without r-SpeB using serial double dilutions of the SUP ranging from 1:500 to 1:4000, as described in Materials and Methods. The data presented are from a 1:500 dilution. The statistical significance of the differences in the proliferation-inducing responses after stimulation with these SUPs was determined by Student's *t* test. $^{**}P \leq .001$. *wt,* wild type.

of TCRBV4 (×5), TCRBV7 (×2), and TCRBV8 (×8) as well. The same TCRBVs were also expanded in T cells stimulated with SUP from another SpeB$^+$ isolate (5449) grown with E-64, as well as from an SpeB$^-$ isolate (8004) (figure 5, *inset table*). The percentage of increase in TCRBV2 T cell expansion in response to the SUP from 5448 SpeB$^+$ *wt* grown without E-64 (~66%) versus the same isolate grown with E-64 (~34%) or 5448 ΔSpeB (~34%) can be attributed to the lower total number of activated lymphocytes in response to SUP from 5448 SpeB$^+$ *wt,* compared with the number of those activated in the other 2 groups, and to the apparent lack of proteolytic effect of SpeB on superantigens causing TCRBV2 expansion. Of importance, the TCRBV repertoire expansion profile of SUP from the 5448 ΔSpeB mutant was identical to that of the SpeB$^-$ isolate and of the SpeB$^+$ isolate grown with E-64.

TheTCRBV repertoire of T cells stimulated with GAS SUP from SpeB$^+$ *wt* and 5448 ΔSpeB and the SUP from 5448 ΔSpeB incubated with r-SpeB was also analyzed. Incubation of SUP from 5448 ΔSpeB with r-SpeB resulted in no significant expansion of TCRBV4, TCRBV7, and TCRBV8. However, the expansion of TCRBV2 was unaffected (data not shown). The data indicate that the superantigens that caused expansion of cells with TCRBV2 are resistant to SpeB proteolytic activity, whereas the superantigens responsible for the expansion of TCRBV4, TCRBV7, and TCRBV8 are degraded by this streptococcal protease.

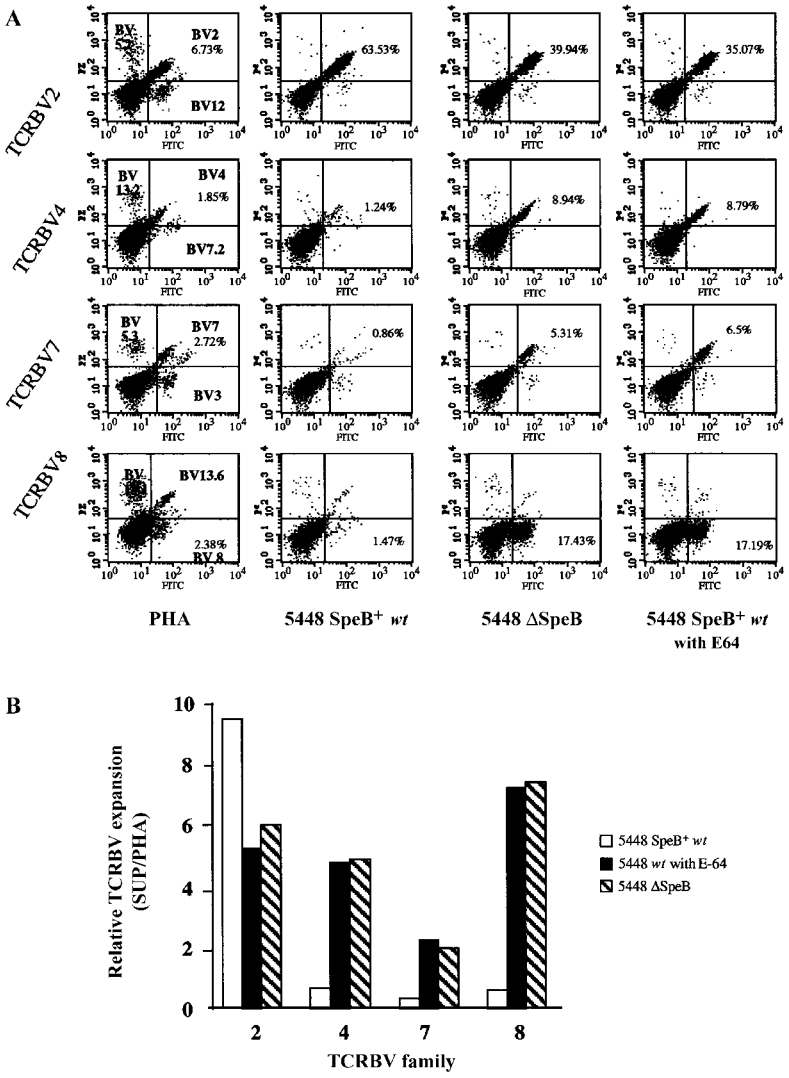
DISCUSSION

SpeB is an important virulence factor of GAS. SpeB has been shown to modify mammalian host receptors, activate cytokine production, and stimulate metalloproteases [10, 12, 13]. These activities are thought to enhance bacterial virulence. Conversely, SpeB has also been shown to modify several streptococcal proteins that are important for bacterial survival in the host [5, 16]. Recent studies have suggested that GAS may need to differentially regulate SpeB expression, depending on the site of infection and, possibly, the temporal events occurring during the infection [18]. Our recent studies showed that an inverse relationship exists between SpeB expression and the severity of invasive GAS infections [8]. These observations raised the possibility that downregulation of SpeB may be necessary for the persistence of certain GAS virulence factors that mediate the severe responses seen in STSS. The Spes are thought to be the major mediators of STSS. These exotoxins have been shown to play an important role in disease pathogenesis because of their pyrogenicity, enhancement of endotoxic shock, and superantigenic effects on the immune response [2, 26]. Recently, a direct correlation between the magnitude of the superantigen-induced potent inflammatory cytokine response and the severity of the systemic manifestations of disease was demonstrated [27].

We hypothesize that SpeB, a cysteine protease secreted by many GAS isolates, may degrade superantigens produced by these isolates. GAS isolates are known to produce a long list of superantigens, some of which are very effective at infinitely low concentrations [25, 28]. We specifically demonstrate direct degradation of mitogenic factor SpeF but not of SpeA. In addition to providing the evidence that SpeB proteolytically degrades this important factor, we also show that expression of SpeB is concomitant with a significant decrease in the lymphocyte-activating capacity of the bacterial SUP and that these changes can be inhibited by the addition of the cysteine protease inhibitor E-64. Although GAS isolates secrete another cysteine proteinase (IdeS), this proteinase degrades only human IgG and is not inhibited by E-64 [29].

An isogenic SpeB$^-$ mutant demonstrated the same increase in proliferation-inducing activity as that observed with the E-64 inhibition of *wt* SpeB proteolysis. The addition of r-SpeB to the isogenic SpeB$^-$ mutant restores the *wt* phenotype, with a significantly reduced lymphocyte-activating capacity of the GAS SUP. Together, these results definitively identify SpeB as the factor responsible for the observed effects on superantigen activity.

The degradation of other superantigens by SpeB is likely, because the magnitude of decreased proliferation-inducing ac-



TCRBV profile of human PBMC stimulated with SpeB⁺ and SpeB⁻ GAS isolates

	TCRBV in the activated lymphocytes, %							
TCRBV	PHA	5448 *wt*	5448 *wt*+E-64	5448 ΔSpeB	5449 *wt*	5449 + E-64	8004	8004 + E-64
1	3.65	2.71	2.08	2.60	3.93	2.39	2.08	3.78
2	6.73	**63.53**	**35.07**	**39.94**	**70.67**	**39.51**	**41.53**	**46.61**
3	2.82	1.96	1.34	1.39	1.74	0.83	0.91	1.21
4	1.85	1.24	**8.79**	**8.94**	1.11	**10.13**	**7.88**	**8.07**
5.1	4.93	1.54	1.25	1.29	1.61	1.03	1.12	1.18
5.2	1.99	0.34	0.39	0.49	0.64	0.32	0.33	0.50
5.3	1.48	0.19	0.28	0.23	0.29	0.24	0.35	0.19
7	2.72	0.86	**6.05**	**5.31**	1.06	**6.17**	**5.87**	**4.69**
7.2	0.92	0.44	0.25	0.34	0.30	0.23	0.29	0.31
8	2.38	1.47	**17.19**	**17.43**	1.69	**20.08**	**19.03**	**16.22**
9	6.93	2.92	1.79	1.84	1.74	1.48	1.41	1.65
11	0.98	0.72	0.44	0.47	0.69	0.44	0.37	0.44
12	2.74	0.95	0.55	0.93	1.30	0.61	1.49	2.01
13.1	4.05	0.56	0.33	0.69	1.29	0.51	0.55	0.39
13.2	1.18	1.03	0.72	0.96	1.23	0.73	0.94	1.08
13.6	2.53	0.64	0.39	0.35	1.61	0.28	0.59	0.27
14	3.58	1.45	0.66	1.07	1.28	0.76	2.76	3.59
16	1.55	0.40	0.45	0.59	0.52	0.35	0.31	0.65
17	5.43	2.29	0.93	1.42	2.23	1.13	1.10	1.14
18	0.53	0.28	0.10	0.07	0.15	0.12	0.05	0.05
20	3.46	1.33	0.66	1.22	0.87	0.74	0.62	0.80
21.3	3.69	1.19	0.82	0.81	1.06	0.87	0.71	0.81
22	2.72	1.84	1.15	1.15	1.87	1.29	1.08	1.32
23	1.41	0.45	0.19	0.43	0.53	0.43	0.30	0.56

Bold and underlined nos. represent the most affected TCRBV

Figure 5. Superantigen-induced expansions of T lymphocytes expressing specific T cell receptor Vβ (TCRBV) of the human peripheral blood mononuclear cells (PMBC) stimulated with supernatant (SUP) from 5448 SpeB$^+$ wild type *(wt)* isolate with or without N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine (E-64) and its isogenic streptococcal pyrogenic exotoxin (Spe) B$^-$ mutant (5448 ΔSpeB). Quantitative analysis of preferential TCRBV expansion was conducted by flow cytometry using IO Test Beta Mark TCRBV Repertoire kit (Beckman Coulter), as detailed in Materials and Methods. PBMC were isolated from the blood of a healthy individual by ficoll-hypaque gradient centrifugation and incubated at 10^7 cells/5 mL of RPMI 1640 with 10% fetal calf serum and stimulated with 1:2000 diluted group A streptococci (GAS) SUP or phytohemagglutinin A (PHA) at 1 μg/mL. After 72 h of culture, the cells were cultured for additional 24 h in the presence of 10 U/mL recombinant human interleukin-2, to allow the regeneration of modulated T cell receptors. Cells were then harvested, washed extensively with PBS containing 1% bovine serum albumin, and stained with different antibodies according to the manufacturer's instructions. The CD3-PC5 blastogenic cells were gated on and simultaneous analysis of 3 TCRBVs per tube was performed using a FACSCalibur flow cytometer (Becton Dickinson). *A,* First-row histograms show the distribution of TCRBV5.2 (upper left [UL]), TCRBV2 (upper right [UR]), and TCRBV12 (lower right [LR]). The second, third, and fourth rows show the distribution of TCRBV13.2 (UL), TCRBV4 (UR), and TCRBV7.2 (LR); TCRBV5.3 (UL), TCRBV7 (UR), and TCRBV3 (LR); and TCRBV13.1 (UL), TCRBV13.6 (UR), and TCRBV8 (LR), respectively. Percentages shown in some regions represent the most commonly affected T cells with respective TCRBV specificity. *B,* TCRBV most affected after stimulation with various GAS SUPs. The superantigen-specific expansion of certain TCRBV was calculated by dividing the percentage of lymphocytes with a particular TCRBV specificity after stimulation with particular GAS SUP by the percentage of lymphocytes with the same TCRBV specificity after stimulation with PHA. Inset table shows all the 24 TCRBV analyzed in the study. FITC, fluorescein isothiocyanate.

tivity cannot be attributed to SpeF degradation alone. Therefore, the effect of SpeB on low-concentration but potent superantigens such as SpeG and SMEZ was addressed by studying specific changes in the TCRBV repertoire that may reflect the footprint of these superantigens. An expansion of TCRBV2-bearing T cells is expected in response to 4 of 5 superantigens present in our strain—SpeA, SpeF, SpeG, and SMEZ. SpeF, SpeG, and SMEZ have been shown to stimulate TCRBV4,

whereas SpeB, SpeF, SpeG, and SMEZ stimulate TCRBV8 [25]. SMEZ is considered to be the most potent streptococcal superantigen, and it is likely responsible for the majority of TCRBV7 and TCRBV8 stimulating activity [25, 30]. In the present study, we have successfully shown that the superantigens responsible for the expansion of TCRBV4, TCRBV7, and TCRBV8 are more susceptible to proteolytic degradation by SpeB than the superantigens responsible for the expansion of TCRBV2. This was confirmed when the SUP obtained from $SpeB^+$ isolates (5448 *wt* and 5449 *wt*) grown with E-64 showed greater proliferation-inducing activity and caused a significant expansion of TCRBV2, TCRBV4, TCRBV 7, and TCRBV8. The isogenic knockout mutant 5448 ΔSpeB, as well as a spontaneous 5448 $SpeB^-$ variant, reproduced the higher proliferation-inducing activity and TCRBV expansion profile found with SpeB inhibition by E-64. Of interest, no significant change in the TCRBV2 expansion was identified. In fact, the percentage of TCRBV2 cells in activated T cells from cultures stimulated with $SpeB^+$ SUP was higher than that in cultures stimulated with $SpeB^-$ SUP, because the TCRBV2 cells represented majority of activated T cells, inasmuch as no other TCRBV subsets were stimulated in the presence of active SpeB. Incubation of SUP from 5448 ΔSpeB with the enzymatically active r-SpeB resulted in a significant decrease in the proliferation-inducing activity and the TCRBV expansion profile, with TCRBV2 cells being the only apparent stimulated cells in this population.

In summary, the results presented here provide evidence that the expression of SpeB by GAS can result in proteolytic degradation of its own superantigens. This critical observation may, in large part, explain the observed inverse correlation of SpeB expression with disease severity among genetically related M1T1 GAS isolates associated with invasive infection [8]. Down-regulation of SpeB in vivo, as reported in other studies [8, 17], may itself represent an important virulence phenotype in the pathogenesis of STSS. Future investigations will shed light on how the proteolytic effects of SpeB are regulated by GAS, depending on the site and extent of infection, as well as the impact of these changes on the host inflammatory response and disease outcome.

Acknowledgments

We thank Allison McGeer and Don E. Low for providing the isolates used in the study, James Musser for providing the anti–streptococcal pyrogenic exotoxin (Spe) B monoclonal antibody, and Carleen Collins for providing us with the SpeA expression vector, which we used to prepare recombinant SpeA.

References

1. Low DE, Schwartz B, McGeer A. The re-emergence of severe group A streptococcal disease: an evolutionary perspective. Emerg Pathog **1997**; 1:93–123.
2. Kotb M. Bacterial pyrogenic exotoxins as superantigens. Clin Microbiol Rev **1995**; 8:411–26.
3. Cunningham MW. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev **2000**; 13:470–511.
4. Norrby-Teglund A, Kotb M. Host-microbe interactions in the pathogenesis of invasive group A streptococcal infections. J Med Microbiol **2000**; 49:849–52.
5. Berge A, Bjorck L. Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. J Biol Chem **1995**; 270:9862–7.
6. Schlievert PM, Assimacopoulos AP, Cleary PP. Severe invasive group A streptococcal disease: clinical description and mechanisms of pathogenesis. J Lab Clin Med **1996**; 127:13–22.
7. Chatellier S, Ihendyane N, Kansal RG, et al. Genetic relatedness and superantigen expression in group A streptococcus serotype M1 isolates from patients with severe and nonsevere invasive diseases. Infect Immun **2000**; 68:3523–34.
8. Kansal RG, McGeer A, Low DE, Norrby-Teglund A, Kotb M. Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases. Infect Immun **2000**; 68: 6362–9.
9. Kazmi SU, Kansal R, Aziz RK, et al. Reciprocal, temporal expression of SpeA and SpeB by invasive M1T1 group a streptococcal isolates in vivo. Infect Immun **2001**; 69:4988–95.
10. Burns EH Jr, Marciel AM, Musser JM. Activation of 66-kilodalton human endothelial cell matrix metalloprotease by *Streptococcus pyogenes* extracellular cysteine protease. Infect Immun **1996**; 64:4744–50.
11. Herwald H, Collin M, Muller-Esterl W, Bjorck L. Streptococcal cysteine proteinase releases kinins: a virulence mechanism. J Exp Med **1996**; 184:665–73.
12. Kapur V, Majesky MW, Li L-L, Black RA, Musser JM. Cleavage of interleukin 1β (IL-1β) precursor to produce active IL1-β by a conserved extracellular cysteine protease from *Streptococcus pyogenes.* Proc Natl Acad Sci USA **1993**; 90:7676–80.
13. Kapur V, Topouzis S, Majesky MW, et al. A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. Microb Pathog **1993**; 15:327–46.
14. Wolf BB, Gibson CA, Kapur V, Hussaini IM, Musser JM, Gonias SL. Proteolytically active streptococcal pyrogenic exotoxin B cleaves monocytic cell urokinase receptor and releases an active fragment of the receptor from the cell surface. J Biol Chem **1994**; 269:30682–7.
15. Chaussee MS, Cole RL, van Putten JP. Streptococcal erythrogenic toxin B abrogates fibronectin-dependent internalization of *Streptococcus pyogenes* by cultured mammalian cells. Infect Immun **2000**; 68:3226–32.
16. Raeder R, Woischnik M, Podbielski A, Boyle MD. A secreted streptococcal cysteine protease can cleave a surface-expressed M1 protein and alter the immunoglobulin binding properties. Res Microbiol **1998**; 149:539–48.
17. Raeder R, Harokopakis E, Hollingshead S, Boyle MD. Absence of SpeB production in virulent large capsular forms of group A streptococcal strain 64. Infect Immun **2000**; 68:744–51.
18. Rasmussen M, Bjorck L. Proteolysis and its regulation at the surface of *Streptococcus pyogenes.* Mol Microbiol **2002**; 43:537–44.
19. Lukomski S, Montgomery CA, Rurangirwa J, et al. Extracellular cysteine protease produced by *Streptococcus pyogenes* participates in the pathogenesis of invasive skin infection and dissemination in mice. Infect Immun **1999**; 67:1779–88.
20. Lukomski S, Sreevatsan S, Amberg C, et al. Inactivation of *Streptococcus pyogenes* extracellular cysteine protease significantly decreases mouse lethality of serotype M3 and M49 strains. J Clin Invest **1997**; 99:2574–80.
21. Lukomski S, Burns EH Jr, Wyde PR, et al. Genetic inactivation of an extracellular cysteine protease (SpeB) expressed by *Streptococcus pyogenes* decreases resistance to phagocytosis and dissemination to organs. Infect Immun **1998**; 66:771–6.

22. Ashbaugh CD, Wessels MR. Absence of a cysteine protease effect on bacterial virulence in two murine models of human invasive group A streptococcal infection. Infect Immun **2001**; 69:6683–8.
23. Framson PE, Nittayajarn A, Merry J, Youngman P, Rubens CE. New genetic techniques for group B streptococci: high-efficiency transformation, maintenance of temperature-sensitive pWV01 plasmids, and mutagenesis with Tn917. Appl Environ Microbiol **1997**; 63:3539–47.
24. Norrby-Teglund A, Kaul R, Low DE, et al. Plasma from patients with severe invasive group A streptococcal infections treated with normal polyspecific IgG inhibits streptococcal superantigen–induced T cell proliferation and cytokine production. J Immunol **1996**; 156:3057–64.
25. Proft T, Moffatt SL, Berkahn CJ, Fraser JD. Identification and characterization of novel superantigens from *Streptococcus pyogenes.* J Exp Med **1999**; 189:89–102.
26. Kotb M. Role of superantigens in the pathogenesis of infectious diseases and their sequelae. Curr Opin Infect Dis **1992**; 5:364–74.
27. Norrby-Teglund A, Chatellier S, Low DE, McGeer A, Green K, Kotb M. Host variation in cytokine responses to superantigens determine the severity of invasive group A streptococcal infection. Eur J Immunol **2000**; 30:3247–55.
28. Proft T, Moffatt SL, Weller KD, Paterson A, Martin D, Fraser JD. The streptococcal superantigen SMEZ exhibits wide allelic variation, mosaic structure, and significant antigenic variation. J Exp Med **2000**; 191: 1765–76.
29. von Pawel–Rammingen U, Johansson BP, Bjorck L. IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. EMBO J **2002**; 21:1607–15.
30. Unnikrishnan M, Altmann DM, Proft T, et al. The bacterial superantigen streptococcal mitogenic exotoxin Z is the major immunoactive agent of *Streptococcus pyogenes.* J Immunol **2002**; 169:2561–9.