Inactivation of *Streptococcus pyogenes* Extracellular Cysteine Protease Significantly Decreases Mouse Lethality of Serotype M3 and M49 Strains

Slawomir Lukomski,* Srinand Sreevatsan,* Cornelia Amberg,‡ Werner Reichardt,‡ Markus Woischnik,§ Andreas Podbielski,§ and James M. Musser*

**Section of Molecular Pathobiology, Department of Pathology, Baylor College of Medicine, Houston, Texas 77030; ‡Institute of Experimental Microbiology, University of Jena; and §Department of Medical Microbiology, University of Ulm, Germany*

Abstract

Cysteine proteases have been implicated as important virulence factors in a wide range of prokaryotic and eukaryotic pathogens, but little direct evidence has been presented to support this notion. Virtually all strains of the human bacterial pathogen *Streptococcus pyogenes* express a highly conserved extracellular cysteine protease known as streptococcal pyrogenic exotoxin B (SpeB). Two sets of isogenic strains deficient in SpeB cysteine protease activity were constructed by integrational mutagenesis using nonreplicating recombinant plasmids containing a truncated segment of the *speB* gene. Immunoblot analyses and enzyme assays confirmed that the mutant derivatives were deficient in expression of enzymatically active SpeB cysteine protease. To test the hypothesis that the cysteine protease participates in host mortality, we assessed the ability of serotype M3 and M49 wild-type strains and isogenic protease-negative mutants to cause death in outbred mice after intraperitoneal inoculation. Compared to wild-type parental organisms, the serotype M3 *speB* mutant lost virtually all ability to cause mouse death ($P < 0.00001$), and similarly, the virulence of the M49 mutant was detrimentally altered ($P < 0.005$). The data unambiguously demonstrate that the streptococcal enzyme is a virulence factor, and thereby provide additional evidence that microbial cysteine proteases are critical in host–pathogen interactions. (*J. Clin. Invest.* 1997. 99:2574–2580.) Key words: group A *Streptococcus* • virulence • toxin • genetics • streptococcal pyrogenic exotoxin B

Introduction

Streptococcus pyogenes is a human pathogen which causes a wide spectrum of diseases, ranging from pharyngitis and impetigo to necrotizing fasciitis and streptococcal toxic shock syndrome. Despite years of study, we are far from a full understanding of the molecular mechanisms mediating group A *Streptococcus* (GAS)[1] host–pathogen interactions. Among the many putative virulence factors expressed by GAS is a potent extracellular cysteine protease known as streptococcal pyrogenic exotoxin B (SpeB) (1–3). This enzyme is synthesized as a 40-kD zymogen, and in appropriate environmental conditions undergoes autocatalytic truncation to produce a 28-kD mature, active cysteine protease. Structure-function studies have documented that residues Cys192 and His340 are required for appropriate autocatalytic zymogen processing and enzymatic activity (4, 5). The *speB* structural gene is chromosomally located, highly conserved, and with a single exception found in all strains (6–8). Virtually all GAS strains express immunoreactive SpeB (8), and the level of production by some organisms increases in stationary-phase cultures (9, 10).

The potential role of SpeB in host–pathogen interaction has been explored extensively by in vitro studies and animal model experiments (3, 8, 11–19). SpeB has been shown to process, activate, or otherwise alter various biologically important proteins. In vitro, the enzyme cleaves human fibronectin and degrades vitronectin (8), cleaves human interleukin 1β precursor to form bioactive IL-1β (12), processes monocytic cell urokinase receptor (13), releases M protein and C5a peptidase from the streptococcal cell surface (1, 15), and releases biologically active kinins from H-kininogen, their purified precursor protein (16). In addition, recent studies have discovered that the cysteine protease activates a 66-kD human matrix metalloprotease, a process hypothesized to participate in the extensive soft tissue destruction observed in some patients with invasive streptococcal disease (17). In vivo studies conducted with animal models have shown that purified SpeB is lethal to mice (10) and causes cardiac necrosis in rabbits (18). Rat experiments found that the cysteine protease acts synergistically with either streptococcal cell wall antigen or streptolysin O to augment lung injury (19). Active immunization of mice with purified 28-kD cysteine protease elicits a protective response in a model of invasive disease (20), an observation consistent with human studies suggesting an important role for SpeB in host–pathogen interactions (21–24).

Despite these observations, elucidation of the exact role of SpeB in mediating GAS disease has been hindered by the lack of animal model experiments using isogenic strains in which the streptococcal cysteine protease has been inactivated by molecular genetic strategies. This lack extends to virtually all

Address correspondence to James M. Musser, M.D., Ph.D., Section of Molecular Pathobiology, Department of Pathology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030. Phone: 713-798-4198; FAX: 713-798-4595; E-mail: jmusser@path.bcm.tmc.edu

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1. *Abbreviations used in this paper:* BHI, brain-heart infusion; Em, erythromycin; GAS, group A *Streptococcus*; Sp, spectinomycin; SpeB, streptococcal pyrogenic exotoxin B.

other bacterial and eukaryotic microbial cysteine proteases which have been implicated as participants in human infections (25, 26). The goal of the present study was to formally assess the contribution of SpeB to GAS virulence by use of isogenic mutant strains in which the *SpeB* gene had been inactivated.

Methods

Bacteria. GAS serotype M3 strain AM3 (also known as strain Richards; reference 27) was obtained from the National Collection of Type Cultures (Public Health Laboratory Services, London, UK). This organism was recovered from a patient with puerperal sepsis (27). Serotype M49 strain CS101 was provided by P.P. Cleary (University of Minnesota, Minneapolis, MN). GAS strains were grown at 37°C in a 5% CO_2/20% O_2 atmosphere. Before conducting the mouse challenge experiments, these two strains were characterized at the molecular level for several genes of interest. A 147-basepair segment of the *emm* gene encoding the hypervariable amino terminus of the M protein was sequenced in both strains (28). The *emm* gene segment in strain CS101 differs from a previously characterized M49 gene (GenBank accession number M23689) by an A→T substitution located at position 203, resulting in a Phe68Tyr alteration. The *emm* sequence in strain AM3 was identical to a previously characterized M3 sequence deposited under GenBank accession number U11945 (28). The *speB* gene was also sequenced in both strains. AM3 and CS101 have allele *speB3* and *speB65*, respectively. Allele *speB3* occurs in M3 strains commonly recovered from patients with invasive streptococcal disease and has been described previously (8, 29; GenBank accession number L26126). Allele *speB65*, which has not been described previously, differs from a reference *speB* sequence deposited in GenBank (accession number M35110) by the occurrence of five nucleotide changes that would result in two amino acid substitutions (Ala111Val and Ala317Ser) in the 40-kD extracellular zymogen. PCR analysis (30) found that strain CS101 contains the gene (*speA*) encoding streptococcal pyrogenic exotoxin A, whereas strain AM3 lacks this gene. In addition, AM3 has the *speC* gene encoding streptococcal pyrogenic exotoxin C, but CS101 lacks this gene. *Escherichia coli* strain DH5α (GIBCO BRL, Gaithersburg, MD) was used as the host for recombinant plasmids.

Construction of the chromosomal speB mutants. To perform insertional inactivation mutagenesis of the *speB* gene in the M3 strain, the *speB* gene of AM3 was amplified by PCR and a 0.6-kb HindIII–PstI internal fragment coding for amino acids 63 to 283 of the SpeB zymogen was subsequently cloned into nonreplicating vector pVA8911 harboring an erythromycin (Em) resistance gene (31). The resulting plasmid pVA8912 was electroporated into parental strain AM3 and recombinant organisms were selected on solid media supplemented with Em (2 μg/ml) (Fig. 1). Similarly, insertional mutagenesis of the M49 strain followed the strategy and protocols described previously (32, 33). In brief, a fragment of the *speB* gene corresponding to nucleotide 100 to 1157 of a sequence deposited as GenBank accession number M35110 (2) was amplified from genomic DNA of M49 strain CS101 by PCR and cloned into the *E. coli* vector pSF152 (34). This gene fragment includes 60 bp of upstream noncoding sequence lacking a promoter region (2, 35), and also contains DNA encoding amino acids 1 through 338 of the SpeB zymogen. Plasmid pSF152 carries a spectinomycin (Sp) resistance marker. The recombinant plasmid was electroporated into parental strain CS101 and recombinant organisms were selected on solid media supplemented with Sp (60 μg/ml). The design of these plasmids is such that active cysteine protease would not be produced by the recombinant mutant strains, because the SpeB molecules would be truncated and lack a His340 residue important in enzyme function (5).

Proteolytic activity assays. Initially, colonies putatively expressing mutant SpeB molecules were screened for decreased proteolytic activity on calcium-caseinate agar (Merck, Rahway, NJ). Next, the proteolytic activity present in the culture supernatants of the parental wild-type and *speB* mutant strains was assayed as described previously (33). This assay measures degradation of fluorescein-labeled casein essentially according to the protocol described by Twining (36).

Immunoblot detection of SpeB. SpeB production by the wild-type and mutant strains was assessed by Western blot analysis of culture supernatants. Strains were grown in Chemically Defined Medium (37) (JRH Biosciences, Lenexa, KS) at 37°C for 35–40 h and harvested by centrifugation. The proteins present in the culture supernatants were precipitated by treatment with 0.7 volume ethanol at 4°C for 5–6 h, separated by electrophoresis with 12% SDS-PAGE, and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Immunodetection of SpeB was performed with rabbit polyclonal antisera raised against recombinant purified mutant zymogen containing the Cys192Ser substitution, or murine monoclonal antibody 2A3-B2-C12 (4). Based on data derived from linear B-cell epitope mapping, this antibody is known to recognize amino acid residues 302 to 308 in SpeB (4). Horseradish peroxidase–conjugated goat anti–rabbit IgG or goat anti–mouse IgG (Bio-Rad) was used as the secondary antibody, and detection was done using a chromogenic substrate (Bio-Rad). Purified wild-type mature SpeB protease (28 kD), and recombinant purified 40-kD Cys192Ser mutant zymogen were used as positive controls. Prestained SeeBlue marker proteins (Novex, San Diego, CA) were used as molecular mass standards.

Mouse intraperitoneal inoculations. Mouse lethality studies were conducted with adult (18–20 g) male outbred CD-1 Swiss mice (Harland, Houston, TX), using protocols similar to those described previously (20). Bacteria were grown in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) overnight at 37°C. Cells were harvested and washed once with ice cold sterile PBS. The A_{600} was adjusted to 0.8 (representing $\sim 10^7$ CFU/ml) with PBS. Inocula (10^6 CFU in 0.25 ml) were prepared in PBS. The number of CFU injected per mouse was verified for each experiment by colony counts with BHI agar. Medium used for cultivation of mutant GAS strains was supplemented with Em or Sp.

Bacteria were injected intraperitoneally into groups of 15 or 30 mice. For each experiment, equal numbers of animals were injected with the wild-type strain, *speB* mutant, or sterile PBS. The mice were observed for 5 d (120 h) after challenge, and survival was assessed at intervals of roughly 1 h. Blood was collected from dead animals by cardiac puncture and cultured on blood agar plates and in BHI medium. The presence of the chromosomal insertions in *speB* mutant strains recovered from the dead mice was confirmed by growing the organisms on media with appropriate antibiotic.

Determination of bacterial minimum lethal dose. Before conducting the mouse mortality studies, the minimum lethal dose of bacteria was determined for the wild-type parental M3 and M49 strains used for mutant construction. The strategy used has been previously described (20). The minimum lethal dose for each parental organism was identified as 10^6 CFU.

Statistical analysis. Kaplan-Meier survival curves were plotted for the mouse mortality experiments using the paired wild-type and *speB* mutant derivatives, and examined for statistical significance with the Mantel-Haenszel summary χ^2 test (38).

Results

Construction of the chromosomal speB mutants. Isogenic *speB* mutants of the serotype M3 and M49 bacterial isolates were engineered to address the role of SpeB in GAS-mediated mouse death after intraperitoneal inoculation. Suicide plasmids harboring a truncated copy of the *speB* gene were used to achieve allelic exchange (Fig. 1). Homologous recombination occurred at a single site within the *speB* sequence in each parental strain, resulting in chromosomal integration of the plasmids containing a nonfunctional copy of *speB*. Integration of the mutated *speB* gene into the genomes of the parental M3

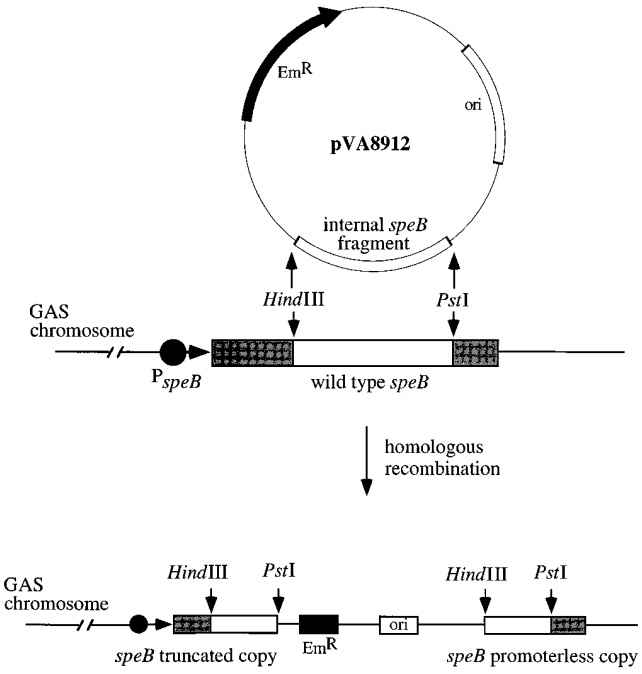

Figure 1. Insertional inactivation of *speB* in serotype M3 strain AM3. The *speB* gene of AM3 was amplified by PCR and a 0.6-kb HindIII-PstI internal fragment of *speB* coding for amino acids 63 to 283 of the SpeB zymogen was subsequently cloned into nonreplicating vector pVA8911 (31), to produce pVA8912. The HindIII-PstI fragment of *speB* lacks the gene promoter (P_{speB}), and portions of the 3′ and 5′ ends of the gene. Electroporation of wild-type strain AM3 with the recombinant suicide plasmid, and subsequent chromosomal recombination, resulted in generation of the isogenic mutant derivative strain that lacks expression of the active SpeB cysteine protease. A similar strategy was used to obtain an isogenic derivative of M49 strain CS101. To produce the M49 mutant, a ~ 1,100-bp fragment of *speB* containing 60 bp of upstream noncoding sequence lacking a promoter region, and DNA encoding amino acids 1 through 338 of SpeB zymogen, was cloned into the *E. coli* vector pSF152 (34), which carries a spectinomycin resistance marker. In each case, the wild-type *speB* gene was insertionally inactivated by a single homologous recombination event, thereby ablating SpeB cysteine protease activity.

and M49 strains was confirmed using Southern hybridization with *speB*-specific and antibiotic resistance marker-specific DNA probes. Growth rates of the parental strains were compared to those of the mutants cultured both with and without antibiotic selection (Em for the M3 *speB* or Sp for the M49 *speB*). There was no difference in the growth of the mutant and wild-type parental strains in BHI medium (data not shown).

Mutant derivatives are deficient in cysteine protease production. The mutant strains were initially tested for residual proteolytic activity on calcium-caseinate indicator agar. The wild-type parental strains produced typical clear zones around their colonies. In contrast, the mutant derivatives lacked virtually all proteolytic activity on the medium. For more detailed quantitative investigation, an enzymatic assay (33, 36) with fluorescein-labeled casein was used. The wild-type parental strains had abundant proteolytic activity, whereas the mutant derivatives were deficient in proteolytic activity (Table I). The proteolytic activity of the wild-type strains in this assay was diminished by the cysteine protease inhibitor E-64, a result indicating that the activity was due to SpeB.

Table I. Proteolytic Activity of Wild-type and Isogenic speB Mutant Strains of S. pyogenes

Strain	Proteolytic activity*
	$U \times h^{-1} \times mg^{-1}$
AM3 (M3)	126±17
AM3 *speB*$^-$	46±11
CS101 (M49)	2319±150
CS101 *speB*$^-$	37±10

*Activity values are the mean of triplicate determinations, with standard deviations of the mean values.

To confirm that the cysteine protease deficiency was caused by production of mutant SpeB molecules, we studied the SpeB immunoreactive material found in culture supernatants by Western blot analysis (Fig. 2). Under the conditions tested, the wild-type M3 and M49 strains expressed the expected 40-kD zymogen form of SpeB, as assessed by rabbit polyclonal antisera raised against purified recombinant mutant zymogen, and murine monoclonal antibody directed against mature 28-kD protease. In contrast, the derivative strains expressed truncated mutant molecules with the apparent molecular mass (~ 30.5 and 36.4 kD for the M3 and M49 strains, respectively) anticipated based on the strategy used to construct these mutants (Fig. 2 *A*). As a further confirmation of the production of truncated SpeB molecules by the mutant derivatives, we also tested culture supernatants with a monoclonal antibody (2A3-B2-C12), which recognizes SpeB amino acid residues 302 to 308 (4). As expected, culture supernatants from the M3 mutant did not contain immunoreactive material because the truncation removes the region of SpeB recognized by this antibody. In contrast, culture supernatants from the M49 mutant, which expresses a truncated SpeB protein containing this epitope, contained immunoreactive material (Fig. 2 *B*). Hence, the monoclonal antibody results effectively differentiate between the 30.5-kD SpeB product lacking the carboxy-terminal end, and the mature 28-kD SpeB molecule with which it comigrates.

Mouse intraperitoneal challenge. Pairs of the isogenic M3 and M49 serotype GAS strains, genetically identical except for the SpeB protease phenotypes, were used in a mouse intraperitoneal infection challenge model to assess the effect of inactivation of this protease on mice mortality (Fig. 3 *A–D*). In these experiments, the parental and derivative strains were always tested simultaneously to minimize variation in experimental conditions. 15 animals were injected intraperitoneally with 10^6 M3 wild-type and M3 *speB* mutant strains. Mortality from systemic infection, assessed at 5 d after challenge, exceeded 90% in the case of wild-type M3 isolate (14 animals dead, 93% of total cohort) (Fig. 3 *A*). In striking contrast, the same inoculum size of the M3 *speB* mutant caused the death of only one animal (14 animals alive, 93% survival). Essentially identical results were obtained when the experiment was repeated with a larger cohort of 30 mice (Fig. 3 *B*). In this second experiment, five animals (17%) survived intraperitoneal injection with the M3 parental strain, whereas the isogenic *speB* mutant caused the death of only two mice (28 animals alive,

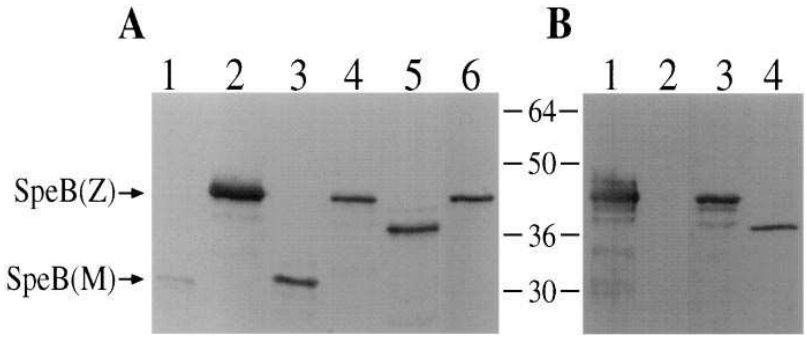

Figure 2. Western blot showing lack of SpeB precursor production by the mutant M3 and M49 strains. Wild-type and mutant bacteria were grown in Chemically Defined Medium (JRH Biosciences) (37) at 37°C for 35–40 h and harvested by centrifugation. The proteins present in the culture supernatants were precipitated by treatment with ethanol, separated by electrophoresis, and transferred to a nitrocellulose membrane. (*A*) Immunodetection of SpeB products was performed with rabbit polyclonal antisera (1:10,000 dilution) raised against recombinant purified mutant zymogen containing the Cys192Ser amino acid substitution that destroys protease activity (4). Horseradish peroxidase–conjugated goat anti–rabbit IgG was used as the secondary antibody, and detection was done by use of a chromogenic substrate. Purified wild-type mature SpeB protease (28 kD), and recombinant 40 kD mutant zymogen were used as positive controls. Lane *1*, purified mature (28 kD) SpeB; lane *2*, concentrated culture supernatant proteins of M3 wild-type parental strain AM3; lane *3*, concentrated culture supernatant proteins of *speB* mutant derivative of AM3; lane *4*, concentrated culture supernatant proteins of M49 wild-type parental strain CS101; lane *5*, concentrated culture supernatant proteins of *speB* mutant derivative of CS101; lane *6*, purified mutant zymogen (40 kD). (*B*) Western blot using monoclonal antibody (2A3-B2-C12), which recognizes SpeB amino acid residues 302 to 308. Horseradish peroxidase–conjugated goat anti–mouse IgG was used as the secondary antibody, and detection was done by use of a chromogenic substrate. Lane *1*, concentrated culture supernatant proteins of M3 wild-type parental strain AM3; lane *2*, concentrated culture supernatant proteins of *speB* mutant derivative of AM3; lane *3*, concentrated culture supernatant proteins of M49 wild-type parental strain CS101; lane *4*, concentrated culture supernatant proteins of *speB* mutant derivative of CS101. Apparent molecular masses (between *A* and *B*) are designated based on standards analyzed and electrophoresed simultaneously, but not shown on the gels. *SpeB(Z)*, 40-kD SpeB zymogen; *SpeB(M)*, 28-kD SpeB mature form.

93% survival). For each experiment, the *speB* mutant derivative was significantly less able to kill mice compared to the isogenic parental organism ($P < 0.00001$, Mantel-Haenszel summary χ^2 test).

Two analogous experiments were then performed with the serotype M49 isogenic strains. 15 animals were used for each strain in both experiments. In the first experiment, 93% (14 of 15) mice infected with the parent M49 strain died within 5 d of

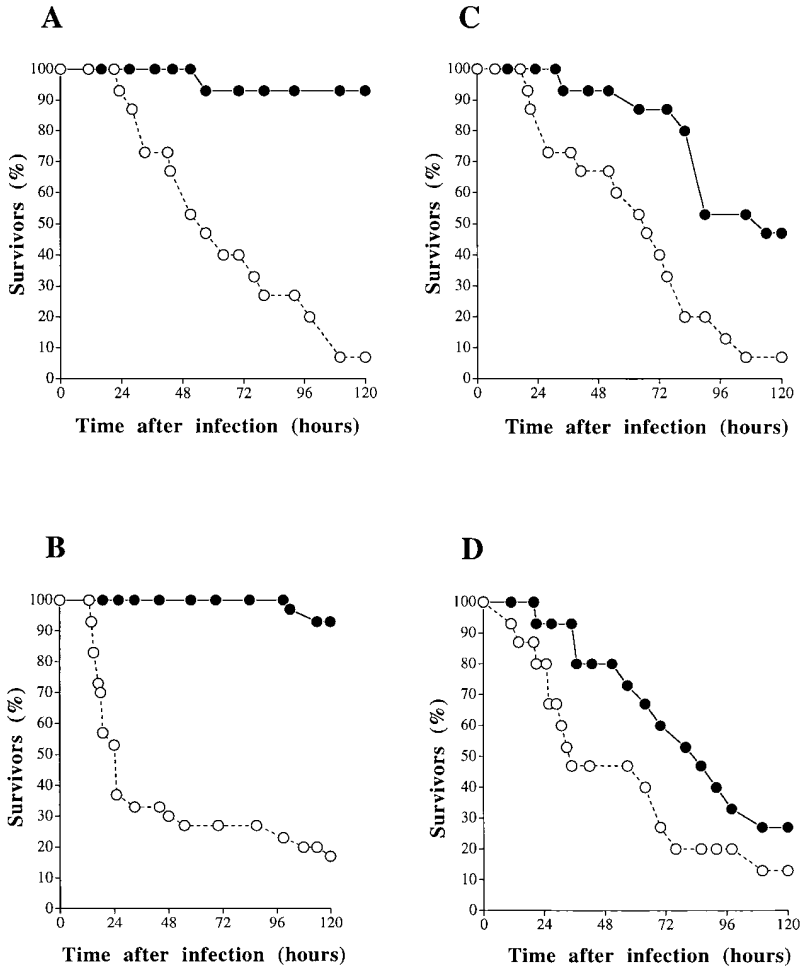

Figure 3. Kaplan-Meier survival plots showing significantly decreased mouse lethality of the cysteine protease-inactivated isogenic group A *Streptococcus* strains. (○, wild-type parental strain; ●, *speB* mutant derivative strain). Lethality studies were conducted with adult (18–20 g) male outbred CD-1 Swiss mice. Bacteria were grown in BHI broth overnight at 37°C, harvested by centrifugation, and washed once with ice-cold sterile PBS. The A_{600} was adjusted to 0.8 (representing $\sim 10^7$ CFU/ml) with PBS. Inocula (10^6 CFU in 0.25 ml) were prepared in PBS and injected intraperitoneally into groups of 15 or 30 mice (time = 0 h). For each experiment, an equal number of control animals was injected with sterile PBS. The number of CFU injected per mouse was verified for each experiment by colony counts with BHI agar. Medium used for cultivation of mutant GAS strains was supplemented with Em or Sp. The mice were observed for 5 d (120 h) after challenge, and survival was assessed at intervals of roughly 1 h. Blood was collected from dead animals by cardiac puncture and cultured on blood agar plates and in BHI medium. The presence of the chromosomal insertions in *speB* mutant strains recovered from the dead mice was confirmed by growing the organisms on media with appropriate antibiotic. All control animals injected with sterile PBS survived (data not shown). As noted above, survival was assessed at roughly 1-h intervals; however, for clarity of figure presentation not all time points are included. (*A*) M3 isogenic strains, experiment 1; $n = 15$ mice/group; $\chi^2 = 70.0$, $P < 0.00001$. (*B*) M3 isogenic strains, experiment 2; $n = 30$ mice/group; $\chi^2 = 57.9$, $P < 0.00001$. (*C*) M49 isogenic strains, experiment 1; $n = 15$ mice/group; $\chi^2 = 24.3$, $P < 0.00001$. (*D*) M49 isogenic strains, experiment 2; $n = 15$ mice/group; $\chi^2 = 9.2$, $P < 0.005$.

challenge (Fig. 3 *C*). In contrast, when mice were inoculated with the isogenic mutant M49 *speB* strain, 47% of the animals survived ($P < 0.00001$). Similar results were obtained when the experiment was repeated (Fig. 3 *D*) ($P < 0.005$). In both experiments, animals injected with the mutant derivatives died at later time points. These results indicated that inactivation of the *speB* gene in this M49 strain significantly decreased mouse mortality.

To rule out the formal albeit unlikely possibility that the results were due to overgrowth of a revertant organism during the in vivo experiment (absence of antibiotic selective pressure), blood obtained from dead animals by cardiac puncture was cultured on blood agar plates or in BHI medium. These cultures yielded β-hemolytic colonies with characteristic GAS morphology. No antibiotic susceptible revertants for either drug were detected when these organisms were tested on solid medium containing Em or Sp. Similarly, cultures grown in BHI liquid medium were serially diluted and plated in duplicate on blood agar plates or BHI plates supplemented with Em or Sp. No differences in colony counts were recorded. Taken together, these results confirmed the stability of the *speB* mutant derivative strains, and ruled out the possibility of revertant overgrowth.

Discussion

Previous studies have provided extensive circumstantial evidence that the streptococcal cysteine protease is involved in the pathogenesis of disease in some GAS infections (reviewed in reference 3). However, because the earlier studies were based largely on results from in vitro enzymologic analyses, the question remained unresolved. Moreover, none of the previous investigations, including the construction of a protease-deficient strain (39), had compared the virulence of isogenic strains differing only in the ability to express enzymatically active SpeB. By showing that the mutant derivatives are significantly less able to cause death after intraperitoneal inoculation in mice, our data demonstrate that SpeB participates in GAS-mediated host death, and thereby unambiguously establish the importance of this enzyme as a streptococcal virulence factor. The data add SpeB to the relatively short list of proven GAS virulence factors which includes M protein, M-related protein, hyaluronic acid capsule, Mga, a fibronectin binding protein, and C5a peptidase (40). Our studies also add direct support to the general concept that microbial cysteine proteases are virulence factors (25, 41, 42).

Although it is a formal possibility that the virulence alteration is due to polarity effects exerted by the *speB* insert, this is highly unlikely for several reasons. First, *speB* transcription is monocistronic, and the transcript length corresponds to the size expected for solely the *speB* gene (35). Second, analysis of 1,600 bp of sequence upstream reveals that the next gene on the same strand ends 880 bp from the *speB* start codon (35). Moreover, based on published data (2), and sequence information contained on contig120 of the most recent streptococcal genome database, there is no open reading frame on either strand for at least 530 bp of sequence downstream from the end of *speB*. Note that only 530 bp are currently available, so the lack of a downstream open reading frame could extend even farther. Third, ~ 100 distinct mutants have been generated and characterized by strategies analogous to those used here, and in no case have we observed polarity effects (our unpublished data). Hence, we believe that taken together, the only reasonable interpretation for all available data bearing on the cysteine protease is that it contributes to streptococcal virulence.

The primary goal of our study was to determine if the streptococcal cysteine protease participated in GAS-mediated host death. The data clearly indicate this is the case, but elucidating the exact cause of the altered virulence will require careful experimental investigation. We presume that the altered virulence in the SpeB-inactivated derivative is due to inability of the cysteine protease to act on one or more proteins expressed by the host or pathogen. In vitro SpeB cleaves or activates a broad range of host proteins such as fibronectin, vitronectin, urokinase plasminogen activator receptor, IL-1β precursor, H-kininogen, and a 66-kD matrix metalloprotease (8, 12, 13, 15, 17). Elucidating the exact cause of the altered virulence will be aided by the availability of transgenic mice in which IL-1β precursor (43, 44) or the urokinase plasminogen activator receptor (45) has been insertionally inactivated. It is also intriguing to speculate that the decreased virulence we observed is caused not by the failure of SpeB to act directly on a host molecule, but by inability to process a protein produced by the pathogen. For example, SpeB cleaves M protein (1, 15), and releases biologically active fragments of M protein and C5a peptidase from the streptococcal cell surface (15). Altered release of these fragments may change host–pathogen interaction sufficiently to result in a less virulent organism.

One unexpected result from our studies was the discovery of a possible difference in the relative contribution of SpeB to host death mediated by the M3 compared to the M49 strain (Fig. 3). The data suggest that in this mouse model system, SpeB plays a greater role in mediating death caused by the M3 strain than by the M49 organism. In most studies conducted with bacterial pathogens, it is relatively uncommon to examine virulence alteration with two distinct sets of isogenic strains such as performed here. This is an especially important issue with GAS because this species contains organisms that are highly divergent in overall chromosomal character (6, 8, 28), horizontal gene transfer processes have generated strains expressing an assortment of presumed virulence factors (46, 47), and GAS can initiate host–pathogen interactions at many different anatomic sites (e.g., posterior pharynx, skin, vagina), each having a distinct set of environment characteristics. We do not know the molecular explanation for the observed difference in SpeB contribution between the M3 and M49 mutants. However, colonies of the M49 strains used were more mucoid compared to those of the M3 organism, an observation suggesting that the mucoid M49 strains had larger capsules than the M3 strains. As shown by the PCR analysis, the M49 strain contains the *speA* gene, whereas the M3 organisms do not. Inasmuch as the capsule is clearly an important streptococcal virulence factor (40, 48), and SpeA also has been implicated in virulence (6), it is possible that these virulence factors are contributing to death caused by the M49 organism. Another formal possibility is that the truncated SpeB protein made by the M49 strain retains a residual, unknown detrimental effect on the host, which the shorter M3 mutant protein does not have. However, injection of mice intraperitoneally with full-length (40 kD) Cys192Ser inactive mutant zymogen does not elicit grossly observable pathology suggesting that this possibility is unlikely (our unpublished observations).

GAS strains expressing M3 protein were the second most

abundant serotype causing invasive infections in the United States in the late 1980s and early 1990s (6, 49). M3 strains also have been responsible for clustered outbreaks characterized by high fatality rates, extensive soft tissue involvement, and streptococcal toxic shock syndrome (50–52). Several observations lead us to speculate that SpeB participates in pathogenesis. First, patients are more likely to die if they have low acute-phase serum antibody levels to SpeB (21). This observation implies that serum antibody directed against SpeB is protective, a concept supported by mouse immunization experiments (20). Second, SpeB activates a 66-kD host matrix metalloprotease expressed by human endothelial cells (17), a process that might account for some of the extensive tissue destruction recorded in certain patients with GAS infections. Third, patients with GAS invasive disease seroconvert to SpeB, which means that the enzyme is expressed in vivo during the course of host–pathogen interactions (53–55, our unpublished data). Fourth, GAS recovered from patients with necrotizing fasciitis and other severe soft tissue involvement produce higher levels of protease than organisms recovered from other infections (23, 24). We believe that these observations, together with the data presented here from our studies with the isogenic M3 and M49 strains, implicate SpeB as a major virulence factor in some human GAS infections.

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