Streptococcal Pyrogenic Exotoxin B Enhances Tissue Damage Initiated by Other \textit{Streptococcus pyogenes} Products

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This study compared the pathology and infection pattern of streptococcal pyrogenic exotoxin B–positive (SpeB\textsuperscript{+}) and SpeB-negative (SpeB\textsuperscript{−}) isogenic variants of an M1 isolate of \textit{Streptococcus pyogenes} in a mouse skin air sac model. SpeB\textsuperscript{+} strains resulted in severe local tissue damage that extended from the epidermis through the subcutaneous layers, whereas isogenic SpeB\textsuperscript{−} variants had reduced gross pathology. At the histologic level, differences in necrosis and host responses to each variant were apparent. Injection of purified SpeB alone into a skin air sac failed to induce any significant tissue damage; however, coinjection of the enzyme with either the wild-type or the \textit{speB} mutant resulted in increased and accelerated tissue necrosis. Surprisingly, coinjection of the enzyme with the spleen-recovered SpeB\textsuperscript{−} variant failed to induce a lesion.

The role of streptococcal pyrogenic exotoxin B (SpeB) in the pathogenesis of group A streptococci is confusing and controversial. Lukomski et al. \cite{1,2,3} and others \cite{4} have shown that insertional inactivation of the \textit{speB} gene reduces the virulence of the organism, when injected subcutaneously or intraperitoneally in a mouse model. Svensson et al. \cite{5}, who used a humanized skin SCID mouse infection model, reported that SpeB-negative (SpeB\textsuperscript{−}) mutants were less virulent than the wild-type parent strains. In addition, Kapur et al. \cite{6} reported that vaccination with SpeB protected mice from a subsequent lethal challenge with group A streptococci.

In contrast, Asbaugh et al. \cite{7} found that isogenic gene replacement mutants deficient in SpeB were equally virulent to their parent isolate in a murine model of invasive soft tissue infection. In this model, hyaluronic acid capsule appeared to be a more predictive phenotype for virulence. Our laboratory showed that passage of SpeB-positive (SpeB\textsuperscript{+}) M1 isolates in mice can result in the selection of stable SpeB\textsuperscript{−} variants that are associated with increased invasive potential \cite{8}.

In studies of isolate 64 passaged sequentially in human blood, an inverse correlation between SpeB expression and capsule size was observed \cite{9}. After \textasciitilde 9 sequential passages in human blood, isolates had increased surface M protein expression and enhanced invasive potential in a skin air sac model \cite{10,11}. Kansal et al. \cite{12} also found an inverse correlation between SpeB expression and invasive isolates recovered from patients with streptococcal infections.

To understand the role of SpeB in group A streptococcal skin infection, we analyzed the changes in skin pathology observed after injection with the wild-type M1 isolate 1881 (WT1881), an isogenic \textit{speB} mutant, or a stable SpeB\textsuperscript{−} variant of isolate 1881 selected by biologic pressure in mouse skin (SR1881) \cite{8}. The effect of complementing the SpeB\textsuperscript{−} phenotype by the addition of the purified active enzyme was also evaluated.

Materials and Methods

\textbf{Bacteria.} \textit{Streptococcus pyogenes} 1881 (serotype M1, opacity factor negative), obtained from the Centers for Disease Control and Prevention, has been characterized elsewhere \cite{13}. The parent isolate (WT1881) secretes SpeB in culture and expresses a surface M1 protein that binds only human IgG3 \cite{8}. A stable SpeB\textsuperscript{−} variant of isolate 1881 (SR1881) was isolated from the spleen of mice that died after injection with this isolate in a skin air sac model, as described elsewhere \cite{8}. Despite the difference in IgG-binding profile of the expressed M1 protein, the sequence of the \textit{emm1} gene in the SpeB\textsuperscript{−} wild-type and the spleen recovered SpeB\textsuperscript{−} variant were identical \cite{8}. The difference in IgG-binding phenotype is attributable to posttranslational processing of the M1 protein by SpeB \cite{14}. An isogenic isolate of 1881 in which the \textit{speB} gene was inactivated by insertion of a spectinomycin-resistance cassette was generated as described elsewhere \cite{14}.

In all the studies, a single colony of WT1881, SR1881, or the \textit{speB} mutant was inoculated into Todd-Hewitt broth containing 0.3% yeast extract (THY) and was cultured for 16 h at 37°C in an atmosphere containing 10% CO\textsubscript{2}, without agitation. All bacterial variants had equivalent growth rates and reached stationary phase under the culture conditions used. After overnight incubation, the bacteria were harvested by centrifugation, were washed, and were resuspended in sterile saline (10 mM PBS [pH 7.4]). Bacterial density was determined by measuring the absorbance at 550 nm (\textit{A}\textsubscript{550}). The bacterial suspension was diluted to 10\textsuperscript{9} cfu/mL by use of a previously determined optical density standard nomogram \cite{15}. When appropriate, spectinomycin was added to the media at a concentration of 100 \textmu g/mL.
Mice. Outbred CD1 mice were purchased from Charles River Laboratories and were maintained in our animal center. Food and water were provided ad libitum.

Skin air sac procedure. The air sac procedure was a modification of a procedure described elsewhere [16]. An air connective tissue pouch was generated on the back of the mouse by slow subdermal injection of 0.9 mL of air via a 27-gauge needle on a 1.0-mL syringe. The syringe containing the air also contained 0.1 mL of a suspension of S. pyogenes containing 10^9 cfu. Groups of 3 mice were killed by cervical dislocation 1, 8, and 24 h after infection. Tissues around the injection site were excised and immersed in 10% neutral buffered formalin. After fixation, selected portions of tissue were embedded in paraffin, and 4 μm sections were cut and stained with hematoxylin-eosin for histopathologic review.

The stained sections were independently evaluated by 2 pathologists who examined each slide without any knowledge of which S. pyogenes isolate had been injected. Three sections per mouse per time point were analyzed for each isolate that was injected. One section was chosen from the center of the lesion, and one was chosen from each edge. All sections contained normal tissue at the periphery. Each experimental series was repeated at least twice and, consequently, a minimum of 18 pathology sections were evaluated for each variant. On the basis of this analysis, a representative picture for each treatment condition was selected.

Photographs of the skin infection site were taken at a constant focal length from the same distance and angle and were printed to the same enlargement. The area of a lesion was determined from a photograph by direct measurement of the dimensions (length × width) and was scored as follows: 0, no detectable lesion; ±, 0–1.5 cm^2; +, 1.5–2.5 cm^2; ++, 2.5–3.5 cm^2; and ++++, >3.5 cm^2.

In addition, 2 independent observers scored lesions on all experimental animals at 24 h by the following subjective scoring system: 0, no lesion; +, small lesion; ++, intermediate lesion; or ++++, severe lesion. In all cases, each observer independently gave each mouse an identical score. The subjective scoring system paralleled the score on the basis of the lesion area determined from photographs of the same animals, as described above.

Purification of SpeB. The cysteine protease SpeB was purified from culture supernatant of S. pyogenes isolate 1881, according to the protocol of Berge and Bjorck [17]. In brief, the bacteria were grown to stationary phase in THY media at 37°C in 10% CO₂. Bacteria were removed by centrifugation, and the culture supernatant was filtered through a 0.22-μm pore-size filter. The filtered supernatant was adjusted to 80% saturation with ammonium sulfate, and the precipitated material was recovered by centrifugation at 4000 g for 20 min at 4°C. The pellet was resuspended in 50 mL of distilled water and was dialyzed against 5 mM MES (2-[N-morpholino] ethanol sulfonic acid; pH 6.0). The sample was applied to an S-Sepharose column in this buffer, and proteins were eluted using a buffer gradient (5–250 mM MES [pH 6.0]). One milliliter fractions were collected, were monitored for absorbance at 280 nm, and were analyzed for SpeB by SDS-PAGE and Western blotting analysis.

Determination of cysteine protease production. Cysteine protease activity present in the ammonium sulfate–precipitated culture supernatants was assayed as described elsewhere [14]. In brief, 50 μL of the concentrated culture supernatant, with or without the addition of 0.1 mM dithiothreitol, was added to wells of a microtiter plate. After incubation for 30 min at 37°C, to allow for the reduction/activation of the enzyme, 150 μL of substrate buffer (1 mM Benz-Pro-Phe-Arg-paranitroanilide [Sigma] in 60 mM sodium phosphate [pH 6.0]) was added to each well. Cleavage of the substrate was monitored by measuring product generation at 405 nm over time in a microtiter plate reader (Biotek).

The cleavage of substrate and the generation of product were determined to be linear with time to an A₄₀₅ of 1.5. The cysteine protease–specific inhibitor E64 (Sigma) was included in parallel assays at a concentration of 10 μM, to determine whether all the enzyme activity being measured could be attributed to the presence of a cysteine protease [14].

PAGE and Western blotting. We analyzed culture supernatants and purified active SpeB by SDS-PAGE and Western blotting analysis, as described elsewhere [14]. In brief, proteins were denatured by boiling for 5 min in 0.5 M Tris-HCl (pH 6.8) containing 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue. Denatured proteins were separated by electrophoresis in 12% polyacrylamide minigels. After electrophoresis, separated polypeptides were transferred to nitrocellulose (Bio-Rad) by electroblotting, as described elsewhere [14]. The nitrocellulose membranes were blocked and incubated overnight at room temperature in 5 mL of 0.15 M veronal-buffered saline containing 1% Tween 20 and then were probed with a 1:5000 dilution of a monospecific anti-SpeB antiserum (Toxin Technologies).

After incubation with specific antibody overnight at room temperature with shaking, nitrocellulose membranes were washed and incubated with a protein G alkaline phosphatase conjugate (1:250) for 4 h and then were washed and developed with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium phosphate substrate (KPL), as instructed by the manufacturer. Low-range prestained molecular weight markers (Bio-Rad) were included on each gel.

Northern blot analysis. Analysis of speB gene expression was done by Northern blotting techniques. RNA was harvested from WT1881 or SpeB variants grown overnight (37°C, 10% CO₂) in 10 mL of THY media. Cells were harvested by centrifugation (5 min, 4000 g, 4°C) and were resuspended in 500 μL of lysis buffer I (25% glucose, 10 mM EDTA, and 100 mM Tris [pH 7.0]). An equal volume of lysis buffer II (2 mg/mL lysosyme and 1 mg/mL mutanolysin) was added, and the suspensions were incubated at 37°C for 20 min. Cells then were sedimented and resuspended in 3 mL of Trizol reagent (Gibco). Total RNA was isolated according to the manufacturer’s instruction. RNA concentrations were determined at A₂₆₀, and RNA from each strain was subjected to electrophoresis on a 1.2% agarose (0.66 M formaldehyde) gel in 1× 3-(N-morpholino) propane sulfonic acid buffer.

After electrophoresis, RNA was transferred to a nylon membrane (Hybond-N+), according to the manufacturer’s instructions (Millipore), and was hybridized with digoxigenin-dUTP–labeled probe, as described elsewhere [18]. The speB-specific probe used in this study was generated by polymerase chain reaction (PCR), using primers SpeB forward (GGGGGGGAAATTCCAATGACCTACCCTAAAAGC) and SpeB reverse (GGGGGGGACCTCGCCTACTTATACCGA-CACC). The rgg-specific probe used in this study was generated by PCR by using primers Rgg 1 (GCGGTATCATCATATAAA) and Rgg 2 (ATAAGGATCGTATAGGGTC).
Table 1. *Streptococcus pyogenes* strains used in the study of streptococcal pyrogenic exotoxin B (SpeB) tissue damage in mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Wild type (WT1881)</td>
<td>OF(+) emm1; speB (wild type)</td>
<td>Emm11, SpeB⁺</td>
<td>[14, 19, 21]</td>
</tr>
<tr>
<td>Spleen recovered (SR1881)</td>
<td>OF(+) emm1; speB (wild type)</td>
<td>Emm17, SpeB⁺</td>
<td>[14, 19, 21]</td>
</tr>
<tr>
<td>speB mutant 1881</td>
<td>OF(+) emm1; speB mutant</td>
<td>Emm17, SpeB⁺</td>
<td>[14, 19, 21]</td>
</tr>
</tbody>
</table>

NOTE: SpeB⁺, SpeB positive; SpeB⁻, SpeB negative.

Solubilization and analysis of IgG-binding surface proteins. Proteins reactive in a nonimmune fashion with human immunoglobulins were extracted from the bacterial surface by treatment with CNBr, as described elsewhere [19]. This process is efficient for solubilizing IgG-binding protein from group A streptococci [19]. CNBr-extracted surface proteins were electrophoresed in 12% polyacrylamide gels, were transferred to nitrocellulose (as described above), and were incubated with an appropriate dilution of each labeled probe. Human IgG1, IgG2, and IgG4 were labeled with horseradish peroxidase (HRP) by HRP-labeling kit (Zymed Laboratories), as described elsewhere [19]. Human IgG3 cryoglobulin was labeled with biotin by using biotin-N-hydroxysuccinimide ester (BOSu; Calbiochem), according to the method of Bayer and Wilchek [20].

Nitrocellulose membranes probed with biotinylated probes were washed and reprobed with HRP-labeled streptavidin (Amersham Life Sciences). The membranes were developed using an enhanced chemiluminescence Western blotting kit (Amersham Life Sciences), as instructed by the manufacturer, and were exposed to Kodak XAR-5 film for 5–60 s at room temperature.

Results

Phenotypic characteristics of the isogenic strains used in this study. Three phenotypic variants of *S. pyogenes* M1 serotype isolate 1881 were selected for study (table 1). The production and activity of SpeB in each isolate was analyzed by functional activity assay (data not shown) and Western immunoblotting (figure 1). In the parent isolate (WT1881), a significant level of active SpeB enzyme (Mr ~28,000) was detectable in the culture supernatant. A variant of this isolate, selected by injection into the skin of mice and recovery from the spleen after a lethal infection (SR1881), failed to secrete either the zymogen or active form of SpeB (figure 1). The speB mutant generated by insertional inactivation of the speB gene in isolate 1881 had the expected SpeB⁻ phenotype (figure 1).

CNBr extracts of each isolate also were analyzed for expression of the surface M1 protein (table 1). The WT1881 isolate expressed low levels of an IgG3-binding M1 protein. The speB mutant expressed significant levels of an M1 protein that bound all 4 human IgG subclasses, and the SR1881 variant expressed the highest level of IgG-binding protein that bound to all 4 human IgG subclasses (table 1).

When the WT1881 isolate was grown in the presence of the cysteine protease inhibitor, E64, the CNBr extract contained levels of M1 protein equivalent to the speB mutant, and this form of the M1 protein was reactive with all 4 human IgG subclasses (data not shown). Growth of the isogenic speB mutant or SR1881 variant in E64 did not alter the level of M1 protein present in CNBr extracts.

These results confirm our earlier studies that SpeB can posttranslationally modify the M1 protein, changing both its quantitative and qualitative expression [14]. The increase in surface M and M-related protein expression after passage through the skin is a consistent finding for both SpeB⁺ and SpeB⁻ group A isolates and was independent of the effects of SpeB [19, 21]. The results in figure 1 and table 1 indicate that isolate SpeB⁺ 1881 can be converted to a stable SpeB⁻ variant by biologic pressures in the mouse.

The changes in SpeB protein expression could be due to a failure to secrete the protein or to an effect on transcription or translation of the speB gene. To determine the nature of speB regulation in the SR1881 variant, quantitative Northern blotting analysis was performed. The results (figure 2) demonstrate that speB mRNA is down-regulated ~10-fold in the SR1881 isolate, compared with the WT1881 isolates.

To determine whether the regulation of speB message in the

![Figure 1](https://academic.oup.com/jid/article-abstract/184/6/723/843733/186572340349213)
The gross pathology data in figure 4 show that distinct infection patterns can be distinguished in mice. The gross pathology at the infection site. Groups of outbred CD1 mice were injected subcutaneously with SpeB+ and SpeB− isogenic variants of isolate 1881 (~10⁶ cfu/mouse). The pattern of infection was observed for a 96-h period. All 3 isogenic variants had identical growth rates and colony morphologies in vitro. Mice infected subdermally with the WT1881 isolate developed a necrotic skin lesion within 24 h (figure 4A). Mice injected with the SpeB− SR1881 isogenic strain demonstrated no detectable skin lesions at 24 h (figure 4B) or at any time during the 96-h observation period, despite 100% mortality of mice during this time (data not shown). In contrast to the severe necrotic lesion observed in mice injected with isolate WT1881, mice injected with the speB isogenic mutant developed small discrete abscesses 24 h after inoculation (figure 4C). No mice injected with the saline control (PBS) developed visible lesions (figure 4D).

The gross pathology data in figure 4 show that distinct infection patterns can be distinguished in mice. The histopathology of the underlying skin was analyzed at different times after infection with each SpeB+ or SpeB− isogenic variant. A control group of mice injected with sterile PBS was included as a negative control. Three mice from each group were euthanized 1, 8, and 24 h after infection, and sections of skin and underlying tissues from the infection site were prepared for histopathologic examination, as described in Materials and Methods. Each section was examined, and the degree of necrosis and infiltration of host polymorphonuclear leukocytes and/or monocytes in the major layers of the mouse skin were evaluated. Figure 5 shows the typical pathology of mouse skin for reference. Low magnification fields (×100) were used to evaluate the overall extent of necrosis and inflammation in skin sections from mice infected with different bacterial variants (figure 6). The high magnification fields (×400) were used to evaluate necrosis and inflammation around the panniculus carnosus muscle.

Analysis of skin tissue samples harvested 1 h after infection with either the WT1881 or SR1881 isolates showed a very mild inflammatory response concentrated around the air sac without noticeable necrosis of the panniculus carnosus (figure 6B, 6E). This response was not significantly different from that observed after injection of sterile PBS (figure 6A and 6E). In contrast, injection of the speB mutant isolate yielded patchy necrosis of the panniculus carnosus and an admixture of neutrophils and mononuclear inflammatory cells (figure 6C and 6G). This inflammatory response was not observed in sections

**Figure 2.** Northern hybridization analysis of streptococcal pyrogenic exotoxin B (speB) gene message in wild-type (WT1881) and spleen-recovered (SR1881) isolates. Total cellular RNA was isolated from each isolate at stationary phase and was separated by 3-(N-morpholino) propane sulfonic acid–formaldehyde agarose gel electrophoresis. RNA source is shown above lanes; amount of loaded RNA is shown below corresponding lanes. A, RNA hybridized with digoxigenin-labeled polymerase chain reaction–generated DNA probe specific for speB. B, Ethidium-bromide stained RNA before transfer to nylon membrane, showing the levels of 16S and 23S rRNA in each sample.

SR1881 variant is due to an effect on rgg message, which encodes a transcriptional regulator of speB [22, 23]. Northern blotting analysis was done using a probe specific for rgg. The results (figure 3) indicate that rgg mRNA was down-regulated >10-fold in SR1881, compared with WT1881. Insertional inactivation of speB does not down-regulate rgg expression, as expected, because rgg is located upstream of speB and is transcribed in the opposite direction [22, 23]. Taken together, these results indicate that the SpeB+ phenotype of the SR1881 isolate is due to regulation at the transcriptional level.

**Gross pathology and histopathology at the infection site.** Groups of outbred CD1 mice were injected subcutaneously with SpeB+ and SpeB− isogenic variants of isolate 1881 (~10⁶ cfu/mouse). The pattern of infection was observed for a 96-h period. All 3 isogenic variants had identical growth rates and colony morphologies in vitro. Mice infected subdermally with the WT1881 isolate developed a necrotic skin lesion within 24 h (figure 4A). Mice injected with the SpeB− SR1881 isogenic strain demonstrated no detectable skin lesions at 24 h (figure 4B) or at any time during the 96-h observation period, despite 100% mortality of mice during this time (data not shown). In contrast to the severe necrotic lesion observed in mice injected with isolate WT1881, mice injected with the speB isogenic mutant developed small discrete abscesses 24 h after inoculation (figure 4C). No mice injected with the saline control (PBS) developed visible lesions (figure 4D).

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**Figure 3.** Northern hybridization analysis of rgg message in wild-type (WT1881), spleen-recovered (SR1881), and speB mutant isolates. Total cellular RNA was isolated from each isolate at stationary phase and was separated by 3-(N-morpholino) propane sulfonic acid–formaldehyde agarose gel electrophoresis. RNA source is shown above lanes; amount of loaded RNA is shown below corresponding lanes. A, RNA hybridized with digoxigenin-labeled polymerase chain reaction–generated DNA probe specific for rgg. B, Ethidium-bromide stained RNA before transfer to nylon membrane, showing the levels of 16S and 23S rRNA in each sample.
from mice infected with either the WT1881 or SR1881 variants and was consistent and unique to skin examined 1 h after injection with the speB mutant.

At 8 h after infection, tissue from mice injected with the WT1881, SR1881, or the speB mutant demonstrated an inflammatory response characterized by a significant number of neutrophils and mononuclear cells within the dermis and subcutis (figure 6N, 6O, and 6P). This process extended beyond the air sac to the underlying fat and muscle tissue. There was necrosis of the panniculus carnosus and subcutaneous soft tissue but not of the epidermis (figure 6J, 6K, and 6L). The combined necrosis and inflammation appeared to be most severe in mice infected with the WT1881 isolate (figure 6J and 6N), followed by the SR1881 isolate (figure 6L and 6P). The response in the speB mutant was the least severe (figure 6K and 6O). Skin sections from the PBS control group showed a mild inflammatory response similar to that observed in the 1-h specimens (compare figure 6E and 6M).

At 24 h after infection with the SpeB WT1881 isolate, we observed an extensive skin necrosis that involved the epidermis, dermis, and underlying subcutaneous soft tissues (figure 6R and 6V). Tissue damage was severe and extended throughout the subcutaneous fat and muscle layers. By contrast, skin sections from animals injected with the speB mutant harvested at 24 h demonstrated multifocal skin necrosis (figure 6S and 6W). Necrosis and inflammation showed a similar pattern that involved the epidermis, dermis, and underlying subcutaneous soft tissues. The extent of inflammation and necrosis was less than that in mice infected with the SpeB WT1881 (compare figure 6V and 6W).

A similar pattern of less extensive necrosis and inflammatory response was observed in animals infected with the SpeB SR1881 isolate (figure 6T and 6X). Although there was no gross evidence of necrosis of the skin after infection with the SR1881 isolate (figure 4B), focal areas of necrosis were observed microscopically. The PBS control group did not differ remarkably in histopathology from the earlier time periods (figure 6Q and 6U).

Analysis of the contribution of SpeB to the pathology of skin infection. Figure 4 and 6 show subtle differences in patterns of pathology for infection with SpeB WT1881, the speB mutant, and the SpeB SR1881 variant. To investigate the role of SpeB in contribution to these differences, 5 µg of

Figure 4. Gross appearance of infection site 24 h after infection in outbred CD1 mice injected in a skin air sac with wild-type Streptococcus pyogenes (WT1881; A), spleen-recovered S. pyogenes (SR1881; B), speB mutant of 1881 S. pyogenes (C), and PBS (D).

Figure 5. Hematoxylin-eosin–stained formalin-fixed normal mouse skin tissue biopsy specimens. Epidermal, dermal, and muscular panniculus carnosus layers and subcutis are readily distinguished in panel A (original magnification, ×100). At high power (B; original magnification, ×400), tissue surrounding the defined cellular structure of the unique mouse panniculus carnosus muscle layer is seen. Bars, 100 µm.
Figure 6. Histopathologic analysis of skin sections from mice 1, 8, and 24 h after infection with wild-type 1881 (WT1881), spleen-recovered variant (SR1881), or isogenic speB mutant of 1881. Sections were fixed in formalin and were stained with hematoxylin-eosin. Three sections from each mouse and 3 mice per time point per infectious agent were evaluated. Photomicrographs are representative of pathology observed at each time point for each experimental condition. Two magnifications for each sample are shown. Experimental groups are identified at tops of figures. A–H, 1-h postinfection time-point samples; I–P, 8-h postinfection samples, Q–X, 24-h postinfection samples. Bars, 100 μm.
purified active SpeB was injected subcutaneously into an air sac on groups of outbred CD1 mice. Animals injected with SpeB alone did not develop any superficial lesions and were indistinguishable from mice injected with PBS during a 4-day observation period.

Tissue samples also were harvested from mice injected with the enzyme at 1, 8, and 24 h after infection. At each of these time points, the epidermis and underlying tissues were intact, with no sign of damage or necrosis. Mild inflammation was noted in the 8-h and 24-h postinfection sections, which was equivalent in its extent to the inflammatory response observed in the mice injected with PBS (data not shown). Similar studies that used a higher concentration of SpeB (25 μg/mouse) also failed to cause any significant pathology in the air sac model (data not shown).

The results of these studies suggest that SpeB alone is not sufficient to cause the pathologic damage associated with infection by a SpeB- isolate. Because the SpeB- isolates cause more damage at the infection site than the isogenic SpeB+ strains, a potential role for SpeB in concert with other streptococcal factors was anticipated. Consequently, we hypothesize that the role of SpeB is to enhance tissue injury that is initiated by other bacterial or host factors. Precedent for this type of synergistic interaction between SpeB and streptolysin O (SLO) has been reported elsewhere [24].

To test this possibility, the effect of mixing 5 μg of purified active SpeB with either the SpeB+ WT1881 organism, the speB mutant, or the SpeB- SR1881 variant before injection into a skin air sac was tested. When the speB mutant isolate and the purified enzyme were coinjected in the mouse, necrotic lesions similar in gross appearance to those observed in animals challenged with the WT1881 isolate were observed (table 2). These results suggested that purified SpeB could complement the phenotype of the mutant.

When the WT1881 isolate was coinjected with the purified enzyme, mice developed necrotic lesions that encompassed a larger area of skin than those present in animals challenged with WT1881 alone. In addition, the kinetics of lesion formation changed. After injection of WT1881 admixed with SpeB, lesions were observed by 16 h after inoculation, whereas, in the absence of added SpeB, a lesion was not visible before 20–24 h. The results summarized in table 2 indicate that, in the presence of SpeB, tissue damage is enhanced and accelerated at the infection site. Of interest, coinjection of the SpeB- SR1881 variant with purified SpeB failed to elicit lesions at any postinfection time (table 2). Taken together, these findings suggest that SpeB enhances tissue injury induced by other factors present at the infection site.

### Table 2.
Characteristics of superficial lesions in mice 24 h after infection with wild-type Streptococcus pyogenes isolate or the isogenic streptococcal pyrogenic exotoxin B (SpeB)-negative variants coinjected with or without 5 μg of purified SpeB.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lesion sizea</th>
<th>Subjective scoreb</th>
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<tbody>
<tr>
<td>WT1881</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WT1881 + SpeB (5 μg)</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>speB mutant</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>speB mutant + SpeB (5 μg)</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>SR1881</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SR1881 + SpeB (5 μg)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**NOTE.** Results shown are representative of 2 independent experiments (6 mice/group evaluated). Lesions were scored as follows: 0, no detectable lesion; ±, 0–1.5 cm²; +, 1.5–2.5 cm²; ++, 2.5–3.5 cm²; and ++++, >3.5 cm².

a Lesion size was estimated from photographs (see Materials and Methods).

b Lesions were scored by independent observers.

The cysteine protease, SpeB, is an attractive candidate for a role in the virulence of S. pyogenes. The chromosomally encoded enzyme, once activated, has the ability to modify both human and bacterial proteins, which could contribute to the observed pathology [25–27]. The enzyme converts pro interleukin (IL)–1β to the active cytokine [26] and activates human matrix metalloproteases [27], which could contribute to tissue damage. In addition, posttranslational modification of bacterial proteins (e.g., M proteins [14] and SLO [28]) has been documented. The expression of the speB gene occurs maximally in the late logarithmic phase [29] and is regulated at the transcriptional level by an upstream gene, rgg [22, 23].

Once translated, the protease is first secreted as a zymogen molecule that must undergo autocatalysis, to yield an active functional enzyme [30–33]. These properties make analysis of the role of SpeB in group A streptococcal pathogenesis very complicated, since it is not clear whether the active enzyme can be generated at different infection sites in the host. Furthermore, the presence of specific host protease inhibitors and/or nonspecific protein substrates may prevent selective modification of bacterial surface proteins or key host proteins, such as IL-1. The potential for variation among strains and between in vivo and in vitro models may in part account for the conflicting evidence for an association between SpeB and virulence.

In this study, we used a well-characterized mouse skin air sac model to compare the gross pathology and micropathology of a streptococcal infection with a SpeB+ wild-type M1 serotype (WT1881), an isogenic speB mutant, and a biologically selected SpeB- variant (SR1881). In agreement with previous reports [3, 4], the WT1881 organism caused a major gross lesion in the epidermis after injection into a skin air sac. Infection with the speB mutant produced only a minor lesion under the same experimental conditions (figure 4).

At the microscopic level, the difference between the SpeB+ WT1881 isolate and the paired isogenic speB mutant appears

1.5–2.5 cm²; and 3.5 cm².

1.5–2.5 cm²; and 0.5–1.5 cm².

mice injected with PBS (data not shown). Similar studies that used a higher concentration of SpeB (25 μg/mouse) also failed to cause any significant pathology in the air sac model (data not shown).

The results of these studies suggest that SpeB alone is not sufficient to cause the pathologic damage associated with infection by a SpeB- isolate. Because the SpeB- isolates cause more damage at the infection site than the isogenic SpeB+ strains, a potential role for SpeB in concert with other streptococcal factors was anticipated. Consequently, we hypothesize that the role of SpeB is to enhance tissue injury that is initiated by other bacterial or host factors. Precedent for this type of synergistic interaction between SpeB and streptolysin O (SLO) has been reported elsewhere [24].

To test this possibility, the effect of mixing 5 μg of purified active SpeB with either the SpeB+ WT1881 organism, the speB mutant, or the SpeB- SR1881 variant before injection into a skin air sac was tested. When the speB mutant isolate and the purified enzyme were coinjected in the mouse, necrotic lesions similar in gross appearance to those observed in animals challenged with the WT1881 isolate were observed (table 2). These results suggested that purified SpeB could complement the phenotype of the mutant.

When the WT1881 isolate was coinjected with the purified enzyme, mice developed necrotic lesions that encompassed a larger area of skin than those present in animals challenged with WT1881 alone. In addition, the kinetics of lesion formation changed. After injection of WT1881 admixed with SpeB, lesions were observed by 16 h after inoculation, whereas, in the absence of added SpeB, a lesion was not visible before 20–24 h. The results summarized in table 2 indicate that, in the presence of SpeB, tissue damage is enhanced and accelerated at the infection site. Of interest, coinjection of the SpeB- SR1881 variant with purified SpeB failed to elicit lesions at any postinfection time (table 2). Taken together, these findings suggest that SpeB enhances tissue injury induced by other factors present at the infection site.

### Discussion

The cysteine protease, SpeB, is an attractive candidate for a role in the virulence of S. pyogenes. The chromosomally encoded enzyme, once activated, has the ability to modify both human and bacterial proteins, which could contribute to the observed pathology [25–27]. The enzyme converts pro interleukin (IL)–1β to the active cytokine [26] and activates human matrix metalloproteases [27], which could contribute to tissue damage. In addition, posttranslational modification of bacterial proteins (e.g., M proteins [14] and SLO [28]) has been documented. The expression of the speB gene occurs maximally in the late logarithmic phase [29] and is regulated at the transcriptional level by an upstream gene, rgg [22, 23].

Once translated, the protease is first secreted as a zymogen molecule that must undergo autocatalysis, to yield an active functional enzyme [30–33]. These properties make analysis of the role of SpeB in group A streptococcal pathogenesis very complicated, since it is not clear whether the active enzyme can be generated at different infection sites in the host. Furthermore, the presence of specific host protease inhibitors and/or nonspecific protein substrates may prevent selective modification of bacterial surface proteins or key host proteins, such as IL-1. The potential for variation among strains and between in vivo and in vitro models may in part account for the conflicting evidence for an association between SpeB and virulence.

In this study, we used a well-characterized mouse skin air sac model to compare the gross pathology and micropathology of a streptococcal infection with a SpeB+ wild-type M1 serotype (WT1881), an isogenic speB mutant, and a biologically selected SpeB- variant (SR1881). In agreement with previous reports [3, 4], the WT1881 organism caused a major gross lesion in the epidermis after injection into a skin air sac. Infection with the speB mutant produced only a minor lesion under the same experimental conditions (figure 4).

At the microscopic level, the difference between the SpeB+ WT1881 isolate and the paired isogenic speB mutant appears
to be more quantitative than qualitative. These differences, however, could not be attributed solely to SpeB, since injection of the purified enzyme induced no significant pathology.

When the speB mutant was coinjected with purified active SpeB, the pathology at the infection site was similar to that observed with the wild-type organism, and a skin lesion was apparent within 24 h. Formation of the lesion was dependent on the enzymatic activity of SpeB, since coinjection of heat-inactivated SpeB had no significant effect on the gross pathology, compared with injection of the speB mutant alone. Coinjection of SpeB with the WT1881 organism accelerated the kinetics of tissue damage (significant superficial lesion within 16 h), again providing evidence for a role for the enzyme in the tissue-damaging process associated with skin infection in this model.

Of interest, the SpeB SR1881 variant differed in a number of respects from the speB mutant. This isolate was recovered from the spleen of a mouse lethally infected with an SpeB’ population of isolate 1881 grown from an individual colony. The SR1881 variant demonstrated a stable SpeB’ phenotype. Although, as noted in figure 4, this isolate differed from WT1881 in its ability to cause a gross skin lesion, necrosis and destruction of underlying tissue was observed (figure 6). In agreement with earlier studies, this isolate was more invasive and caused a lethal infection in all mice within 48–96 h [8]. By contrast, <40% of mice injected with either the wild-type or speB mutant died over the same time period.

Further differences between the SpeB’ phenotypic variants (speB mutant and SR1881) were observed in their ability, when coinjected with purified SpeB, to induce a superficial skin lesion. In the case of the SpeB’ SR1881 isolate, no enhancement of tissue damage was noted. These results suggest that the ability of SpeB to enhance tissue damage requires the expression of additional bacterial cofactors. Analysis of the speB and rgg gene expression in the SR1881 isolate indicated that neither message for the positive regulator, rgg, nor the enzyme, SpeB, was present in this isolate. A recent study reported that rgg controls expression of a wide array of streptococcal products in addition to SpeB [34], and our results are consistent with SpeB modifying a second rgg-controlled gene product.

The difference in kinetics and extent of tissue damage observed microscopically for each isolate was not as marked as anticipated from the superficial lesions observed in this model. Analysis of the host response indicated subtle differences in the inflammatory response to the different organisms. At the earliest time studied, 1 h after infection, there was a significant inflammatory response only in mice infected with the speB mutant. This may explain the reduced severity of tissue damage associated with this organism. By 8 h, the inflammatory responses to SpeB’ and SpeB’ variants were nearly equivalent, and, by 24 h, the speB mutant had fewer inflammatory cells than did the SpeB’ SR1881 variant or the WT1881 organism. Kinetics of the inflammatory response may differ, depending on the phenotype of the isolate and the strain of mouse studied. This may explain some of the apparently contradictory results reported in inflammatory responses between wild-type and speB mutants in other models [3, 4].

The results of our study indicate that SpeB alone is not sufficient to induce soft tissue injury or inflammation; however, SpeB is effective in enhancing tissue necrosis initiated by other bacterial factors. Precedent for this type of enhancing role for SpeB was reported by Shanley et al. [24], who found that SpeB augments lung tissue injury induced by other products of S. pyogenes, such as SLO and cell wall antigens.

It is clear that critical bacterial factors, in addition to SpeB, must be expressed, to result in severe necrotic tissue damage in the skin. Not all group A isolates can produce these key substrate(s), bacterial or host, that SpeB modifies to exacerbate tissue damage. Thus, the extent of soft damage would be expected to be influenced by the isolate and by host responses to key bacterial products. Identification of the factors (bacterial or host) that contribute to severe tissue damage and the mechanism by which SpeB enhances their tissue-damaging potential may help to identify the unique host-pathogen environment that is critical for the pathogenesis of necrotizing fasciitis.

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References

9. Raeder R, Harokopakis E, Hollingshead S, Boyle MDP. Absence of SpeB

Addendum. Two recent articles demonstrated that an in vivo selected variant of a CsrS/CsrR mutant was associated with larger necrotic lesions [35], and a novel secreted protein, MspA, was identified and found to be regulated by CsrS/CsrR [36]. In addition, MspA is coregulated with other factors, such as streptococcal pyrogenic exotoxin B (SpeB), that were involved in necrotic lesion formation. Consequently, MspA is a candidate product for the unidentified bacterial cofactor predicted in this study to be processed by SpeB as part of the mechanism for generating necrotic skin lesions.