A relative lack of neutrophils around *Streptococcus pyogenes* is observed in streptococcal toxic shock syndrome (STSS). Because the bacteria spread rapidly into various organs in STSS, we speculated that *S. pyogenes* is equipped with molecules to evade the host innate immune system. Complement C3b opsonizes the pathogen to facilitate phagocytosis, and a complex of C3b converts C5 into anaphylatoxin. Because we found that C3 (C3b) is degraded in sera from patients with STSS, we investigated the mechanism of C3 (C3b) degradation by *S. pyogenes*. We incubated human C3b or serum with recombinant SpeB (rSpeB), a wild-type *S. pyogenes* strain isolated from an STSS patient or its isogenic ΔspeB mutant and examined the supernatant by Western blotting with anti-human C3b. Western blot and Biacore analyses revealed that rSpeB and wild-type *S. pyogenes* rapidly degrade C3b. Additionally, C3 (C3b) was not detected in sera collected from infected areas of STSS patients. Furthermore, the survival rate in human blood and in mice was lower for the ΔspeB mutant than the wild-type strain. Histopathological observations demonstrated that neutrophils were recruited to and phagocytosed the ΔspeB mutant, whereas with the wild-type strain, few neutrophils migrated to the site of infection, and the bacteria spread along the fascia. We observed the degradation of C3 (C3b) in sera from STSS patients and the degradation of C3 (C3b) by rSpeB. This suggests that SpeB contributes to the escape of *S. pyogenes* from phagocytosis at the site of initial infection, allowing it to invade host tissues during severe infections.

*S. pyogenes* is a Gram-positive bacterium that often causes throat and skin infections such as pharyngitis and impetigo. During the past decade, it was reported that *S. pyogenes* causes severe infectious diseases, including acute rheumatic fever, necrotizing fasciitis, and streptococcal toxic shock syndrome (STSS)² (1, 2). The death toll from severe infections was estimated to be at least 500,000 each year (2). Therefore, many researchers have focused on *S. pyogenes*, and some aspects of the infection have been elucidated.

The initial step of *S. pyogenes* infection is bacterial adhesion to host epithelial cells through extracellular matrix proteins, for example, fibronectin (3–5). Fibronectin-binding proteins of *S. pyogenes* have been identified as adhesins and invasins, and their role in the invasion of epithelial cells by *S. pyogenes* has been examined in several studies (6, 7); however, how the invading bacteria escape the immune system and grow in host tissues is not understood. To cause systemic and septic infections, *S. pyogenes* must evade the immune system of the host and survive in plasma, after which the organisms may spread into various organs via the blood stream. Histopathological studies have demonstrated that there are few or no inflammatory cells (e.g. neutrophils) at the site of infection in patients with STSS and severe streptococcal infections (8, 9).

The complement system plays an important role in innate immunity, which acts as a protective shield at early phases of infection. In addition, the complement system is also an effector in the acquired immune system. Complement fragments C3a, C3b, and C5a are produced during activation of the complement system (10). C3a and C5a are anaphylatoxins that attract neutrophils. C5a and interleukin-8 are key neutrophil chemoattractants. It has been reported that C5a and interleukin-8 are neutralized by *S. pyogenes* (9, 11, 12). C3b binds strongly to the surface of bacteria, mediating their opsonization (10, 13, 14). In addition, surface-bound C3b forms a complex with C2a and C4b and functions as a C5a convertase, generating the most powerful anaphylatoxin, C5a (10, 13, 14).

Pandiripally *et al.* (15) reported that the surface protein FbaA of *S. pyogenes* recruits complement regulatory protein factor H (FH) and factor H-like protein 1 (FHL-1). Based on this, we postulated that C3b is inactivated on the surface of *S. pyogenes*. To date, however, there have been few reports on C3 (C3b)-degrading molecules in *S. pyogenes* or other bacteria.

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2 The abbreviations used are: are used, STSS, streptococcal toxic shock syndrome; PBS, phosphate-buffered saline; cfu, colony forming units; HE, hematoxylin and eosin.
SpeB Degrades C3 (C3b) and Inhibits Innate Immunity

We recently found that C3 (C3b) is degraded in sera from infected areas in patients with STSS but not in sera from healthy volunteers. Additionally, genetic inactivation of streptococcal pyrogenic exotoxin B (SpeB), a cysteine protease, in S. pyogenes decreases the bacterium resistance to phagocytosis and reduces its ability to spread into various organs in mice (16, 17). These studies, however, did not examine how SpeB contributes to bacterial propagation in vivo. On the basis of the previous findings, we hypothesized that S. pyogenes produces one or more C3 (C3b)-degrading proteases, one of which is SpeB, allowing the bacterium to evade the host immune system.

EXPERIMENTAL PROCEDURES

Human Sera and Streptococcal Strains—All of the studies on patients and volunteers were performed with approval of the ethics committee Osaka University Graduate School of Dentistry and Asahi General Hospital, and all patients gave informed consent. Human blood samples were collected from the veins of eight healthy volunteers (age 31.3 ± 4.9 years) and during surgery in 13 patients with STSS (age 35.2 ± 23.8 years) from an infected area in the acute phase at the surgical site (supplemental Fig. S1). S. pyogenes strain SSI-9 (serotype M1) was isolated from a Japanese patient with STSS (4), and its isogenic ΔspeB mutant was constructed with a suicide vector as described previously (18). Briefly, we amplified an internal portion of the speB gene by PCR and ligated it into pSF151 (19). Strain SSI-9 was transformed with the resulting plasmid by electroporation, and the transformant was selected on a spectinomycin-containing agar plate. Following verification of targeted mutagenesis by colony-directed PCR (4), we designated the ΔspeB mutant as strain TR-11. There was no growth difference on laboratory medium between strains SSI-9 and TR-11 (18).

Construction of Recombinant SpeB—rSpeB was prepared as follows. Chromosomal DNA was extracted from S. pyogenes strain SSI-9 as a template for PCR. The entire length of the speB gene was amplified by PCR using primers 5'-CATGCCATGGATAAAAAGAAATTAGGTATCAG-3' (sense, containing an NcoI recognition site) and 5'-CCCCCGGGAGGTTGATGCCATACACAGCATTGG-3' (antisense, containing a SmaI recognition site). To produce rSpeB with a C-terminal histidine tag, the PCR fragment was digested with NcoI and SmaI and then cloned into NcoI/SmaI-digested pIVEX2.3-MCS vector (Roche Applied Science). The scpA (streptococcal C5a peptidase) gene was provided by Dr. P. P. Cleary (University of Minnesota) (11) and was ligated into expression vector pGEX-6P-1 (GE Healthcare Bio-Sciences KK). The resulting plasmids, which harbored the speB or the scpA gene, were introduced into Escherichia coli strain BL21 (DE3) pLysE (Novagen), and the transformant was induced with 0.1 mM isopropyl-1-thio-β-d-galactopyranoside at 20 °C for 24 h. The rSpeB protein was produced as a 43-kDa zymogen and converted to 28-kDa active form by self-processing during purification by nickel-nitrilotriacetic acid-agarose (Qiagen) and diethylaminoethyl column chromatography (Bio-Rad). The rScpA protein was purified by glutathione-Sepharose 4B (GE Healthcare Bio-Sciences KK) and diethylaminoethyl column chromatography. The purified rSpeB and rScpA (C5a peptidase) proteins were dialyzed against PBS.

Western Blot Analysis—Western blotting was performed as described previously (5). Sera from patients with STSS or healthy volunteers were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with a solution of 5% skim milk and 10% goat serum (Tissue Culture Biologicals), the membrane was incubated with rabbit anti-human C3b serum (Nordic Immunology). Immunoreactive bands were detected with alkaline phosphatase-labeled goat anti-rabbit IgG (Cell Signaling) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium alkaline phosphatase substrate solution (Moss). To examine the proteolytic activity of SpeB, 5 μl of C3b (Calbiochem, 200

![FIGURE 1. A patient with STSS (59 years old, female, deceased). HE staining of necrotic fascia and subcutaneous tissues is shown. S. pyogenes was distributed along the fascia (arrows), and neutrophils (arrowheads) did not migrate around the bacteria.](Image)

![FIGURE 2. Analysis of C3 (C3b) in human serum samples.](Image)
µg/ml) or human sera were incubated with an equal volume of rSpeB (0–10 µM), wild-type strain SSI-9 (2 × 10⁶ cfu/ml), or the ΔspeB mutant strain TR-11 (2 × 10⁶ cfu/ml). Following a 1-h incubation at 37 °C, each mixture was examined by Western blot analysis with anti-human C3b serum.

N-terminal Amino Acid Sequencing—The targeted protein was separated using 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was stained with 0.1% Coomassie Brilliant Blue R-250 (CBB) for 1 h and then destained with 7.5% acetic acid containing 40% methanol and washed with distilled water for 24 h. N-terminal amino acid sequencing was performed using the Edman degradation method with an ABI protein sequencer model 491HT (Applied Biosystems).

Biomolecular Interaction Analysis (Biacore)—The direct surface plasmon resonance was measured using the Biacore X biomolecular interaction monitoring system (Biacore). Biacore analysis was performed as described previously (20). Human C3b was diluted and adjusted to 100 µg/ml in 10 mM sodium acetate (pH 4.0) and then immobilized on the surface of a sensor chip CM5 (Biacore International AB) using an Amine Coupling Kit (Biacore International AB). Lyophilized rSpeB was suspended in HBSP buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, pH 7.4) and adjusted to 2.5, 5, and 10 µM. For binding analysis, rSpeB was injected at a flow rate of 5 µl/min at 37 °C. C3b degrading activity was quantified as the decrease in resonance units on the sensorogram and analyzed by BIA evaluation software version 4.02 (Biacore International AB) as described previously (21).

Bactericidal Test—In vitro whole blood bactericidal assays were performed with a modified version of the bactericidal test described by Lancefield (22). S. pyogenes strains from patients were cultured until mid-log phase (optical density at 600 nm = 0.5–0.6) and adjusted with PBS or culture supernatant to 2–5 × 10⁵ cfu/ml. Human whole blood was collected from a healthy donor, who was asked to confirm that the donor did not have an infection. The blood was also examined by ASO-latex (Seiken) to confirm that it was not infected with S. pyogenes. The blood was heparinized, and 90-µl samples of it were mixed with 10 µl of each bacterial suspension. After a 3-h incubation at 37 °C on a rotary mixer, the mixtures were grown on blood-agar plates, and the number of colonies were counted. For the C3b inhibition assay, rabbit anti-C3b or preimmune F(ab′)₂ fragment was prepared using an ImmunoPure F(ab′)₂ preparation kit (Pierce) and, prior to the bactericidal assay, 10 µg of the F(ab′)₂ fragment was incubated with 100 µl of human blood at 37 °C for 1 h.

Infection of Mice—All of the animal procedures complied with the Osaka University Graduate School of Dentistry guidelines and were approved by the institutional animal care and ethics committee. BALB/c mice (6-week-old, female) were purchased from Charles River Japan. S. pyogenes strains were grown at 37 °C to mid-log phase and washed twice with PBS. The bacteria were suspended in PBS, and the suspensions were...
on days 0, 2, 7, and 14. Whole blood was centrifuged, and the serum was analyzed using a complement activity measuring kit, CH50 (Seiken). Two weeks after infection, each quadriceps section was incised, shredded, and frozen at −80 °C. After 10 min, the tissues were centrifuged at 4 °C, and the supernatants were immediately applied to a 7.5% SDS-PAGE gel. C3 (C3b) degradation at the site of infection was examined by Western blotting using anti-C3b serum.

Statistical Analysis—The significance of differences between the means of groups was evaluated using a nonparametric Mann-Whitney U test. All of the tests were analyzed using Statistical View J-5.0 software (SAS Institute Inc.). Differences were considered significant at \( p < 0.05 \).

RESULTS

C3 (C3b) Was Not Detected in Sera Collected from Infected Areas of STSS Patients—In most cases of STSS, histopathological examination showed little or no infiltration of neutrophils around S. pyogenes at early stages of infection, and the bacterial organisms are found along the fascia (Fig. 1). To investigate the interaction between the host and S. pyogenes in STSS, during operations, sera were collected from the infected area of STSS patients. We then examined whether S. pyogenes isolated from an STSS patient can degrade complement C3 (C3b), a complement fragment that plays an important role in innate immunity by activating the alternative pathway and by binding to the bacterial surfaces to mediate opsonization. In the alternative pathway, C3b acts with factor B to form the alternative pathway C3 convertase. Subsequently, the C3 convertase may act with an additional C3b molecule to form the alternative pathway C5 convertase. During activation via classical pathway, C4 acts with C2 to form the classical pathway C5 convertase upon addition of a C3b molecule, and then the C5 convertase generates C5a. Therefore, elimination of C3 (C3b) by S. pyogenes is expected to prevent the accumulation of C5a, the most powerful anaphylatoxin, reducing neutrophil migration and bacterial opsonization. As shown in Fig. 2, Western blotting with anti-C3b showed that C3b (C3) was not present in sera from patients with STSS collected from the site of infection, whereas C3 (C3b) was clearly detected in sera from all of the healthy volunteers. In contrast, C5 in blood were very low level. There was not a significant difference among all samples from the healthy volunteers and patients with STSS by Western blotting (supplemental Fig. S2). Therefore, we speculated that, in STSS, C3 (C3b) is degraded into fragments at sites of infection by S. pyogenes proteases.

Streptococcal Cysteine Protease SpeB Degrades Complement C3 (C3b)—S. pyogenes expresses two major proteases, one of which is an extracellular cysteine protease named SpeB (24), and the other is a cell-associated serine protease named C5a peptidase (11). We tested the ability of recombinant SpeB (rSpeB) and C5a peptidase to degrade purified C3b in healthy human serum. Fig. 3A shows that the purified human C3b (>116-kDa bands; black arrows) was degraded by rSpeB into ~85-kDa fragments (gray arrowheads). In contrast, recombinant C5a peptidase, ScpA, could not degrade C3b (data not shown). Furthermore, rSpeB degraded C3b in serum, even though it contains a variety of ions and inhibitors (Fig. 3A, lanes 6 and 7).

To further investigate the role of native SpeB in C3b degradation, we compared its degradation in serum by wild-type S.
**SpeB Degrades C3 (C3b) and Inhibits Innate Immunity**

*S. pyogenes* strain SSI-9, which was isolated from a patient with STSS, and its isogenic ΔspeB mutant strain TR-11, which was constructed by integrating the spectinomycin resistance gene into the speB gene. Homologous recombination in TR-11 was confirmed by colony-directed PCR (data not shown) and by the loss of the proteolytic activity on skim milk agar (Fig. 3B). Western blotting showed that SSI-9 but not TR-11 could cleave serum C3 (C3b) (Fig. 3A, lanes 8 and 9). Reverse transcription-PCR analysis was used to investigate the transcriptional levels of other virulence genes in strain TR-11, which confirmed that the major virulence factors were not affected by deletion of the speB gene, as compared with the wild-type strain SSI-9 (Fig. 3C). We also extracted the cleaved fragment of C3b from a 15% SDS-PAGE gel and determined the N-terminal amino acid sequence to be GQREVVAD, which was identical to the N terminus of the β-chain (25). In addition, the other SpeB-cleavage sites were determined by using a partial cleavage condition (15 min, 37 °C). Identical sites were used, as follows: LLPVG (156 amino acid residues from the N terminus of C3), SNLDEDIIA (749 amino acid residues), DPERLREG (948 amino acid residues), and IQPGA (1472 amino acid residues). These results showed that SpeB was able to cleave one or more sites of the C3 (C3b) molecule.

**Recombinant SpeB Rapidly Degrades C3b**—These results might support the hypothesis that SpeB contributes to the inhibition of the complement system by degrading C3b; however, there was a problem that required further investigation. To evade opsonization or inhibit other immunological functions of C3b, it is necessary that *S. pyogenes* cannot only degrade C3b but also carry out the degradation rapidly when the two proteins come into contact. Biacore analysis confirmed that rSpeB can rapidly cleave immobilized C3b (Fig. 4A). The arrow in Fig. 4A indicates that the C3b in contact with SpeB shifted to a hydrolysis product. The initial rates of hydrolysis of C3b can be obtained from the initial slopes (first 30 s) in the sensorgram (Fig. 4A, lines a–c). In this system, 1000 resonance units correspond to a mass shift of 1 ng/mm² (26). A plot of the initial rate of C3b cleavage as a function of the rSpeB concentration was linear (Fig. 4B). Biacore analysis further demonstrated that 10 μM rSpeB degrades C3b at an initial rate of 12 pg/s (Fig. 4B).

**The Survival Rate in Human Blood Was Lower for the ΔspeB Mutant than the Wild-type Strain**—We next examined the survival of wild-type strain SSI-9 and ΔspeB strain TR-11 in the blood of healthy, uninfected volunteers. A 3-h bactericidal test (Fig. 5A) showed that strain SSI-9 survived significantly at a higher rate than strain TR-11 (p < 0.05), but this difference was not observed for the mutant strain. Furthermore, as shown in Fig. 5B, inhibition of C3 (C3b) with anti-C3b F(ab')₂ fragment increased the survival of the ΔspeB mutant strain in blood. Collectively, these results suggest that the extracellular SpeB acts as an inhibitor of innate immunity by degrading C3 (C3b) in blood.

**The ΔspeB Mutant Did Not Survive in Mice**—We subsequently investigated the roles of SpeB in vivo using a murine model of infection. Bacterial dissemination was determined by measuring the number of *S. pyogenes* cells in blood following subcutaneous injection with equal colony-forming units (cfu) of SSI-9 or TR-11. The two strains showed significantly different abilities to disseminate in mice (Fig. 6). For strain SSI-9, the bacterial numbers increased from 48 to 72 h after inoculation, and bacteria were recovered from 8 of 10 mice. In contrast, strain TR-11 was recovered from only 1 of 10 mice at 48 h, and the number of colonies formed was lower than for the wild-type strain.

**SpeB Degrades C3b in S. pyogenes-infected Sites in Mice**—Our *in vitro* assay results demonstrated that C3b was cleaved in the serum component (Fig. 4A); therefore, we also examined whether native SpeB from *S. pyogenes* could degrade C3 (C3b) in vivo. First, we collected and examined whole blood from infected mice. There were no systemic differences between the wild-type-injected and ΔspeB mutant strain infected mice in regard to total complement activities (Fig. 7A). C3 (C3b) was found to be degraded at the site of infection by the wild-type strain (Fig. 7B). In contrast, there was no C3 (C3b) degradation observed in tissues infected with the ΔspeB mutant strain. These results suggest that SpeB degraded C3 (C3b) in mice, and there is also the possibility that the ΔspeB mutant strain might be rapidly cleared by mice immune systems.

**Histopathological Analysis of Interactions between SpeB and Neutrophils**—Next, we injected each strain into the quadriceps and, after 24 h, collected tissue samples including the areas surrounding the site of infection and examined them by HE
staining and immunohistochemistry using an antisemur against S. pyogenes. We observed that many wild-type SSI-9 cells remained under the epidermis and accumulated along the fascia (Fig. 8, A–C). Immunostaining of the same area confirmed that the SSI-9 strain spread on the fascia (Fig. 8, panels a–c, brown staining). Additionally, even at the site of infection, only a few neutrophils were detected by HE staining in SSI-9-infected mice. These histopathological findings in mice were the same as those obtained from human STSS tissue samples (supplemental Fig. S1). In contrast, in mice infected with the ΔspeB TR-11 strain, there were a large number of neutrophils at the site of infection (Fig. 8, panels D and d), and the infiltrating inflammatory cells engulfed the bacteria, so that few free bacteria could be found inside in the neutrophil-surrounded area (Fig. 8, panels E, F, e, and f). Similar findings were obtained in three mice for each strain.

**DISCUSSION**

We frequently found a relative lack of neutrophil migration to sites of infection in the early phase of STSS (supplemental Fig. S1). Cockerill et al. (8) and Hidalgo-Grass et al. (9, 27) reported similar findings in severe streptococcal infections, including necrotizing faciitis and sepsis. These two groups postulated that the lack of neutrophil accumulation is due to a streptococcal protease that degrades interleukin-8 or its mouse homologue, macrophage inflammatory protein 2; however, other anaphylatoxins, including C5a and C3a, also help attract neutrophils to the site of infection (10). Chen and Cleary (11) identified a C5a peptidase from the surface fraction of S. pyogenes and showed that it can inactivate C5a. Nevertheless, as long as C3b is present, C5a is supplied continuously through a C3b-dependent positive feedback mechanism (10). Because the complexes of C3b generate C5a and C3a and because C3b binding to pathogen leads to ingestion by neutrophils (10), we suspected that S. pyogenes can also inactivate C3 (C3b), reducing the production of anaphylatoxins.

*In vitro* analysis of proteolysis by Western blotting and Bia-core revealed that both rSpeB and native SpeB released from S. pyogenes degrade purified and serum C3 (C3b) (Figs. 3A and 4A). Steinruecke et al. (21) reported that, under the same conditions, 20 μg IgA protease from Neisseria gonorrhoeae degrades IgA at 0.45 pg/s; therefore, it appears that SpeB rapidly cleaves C3b (12 pg/s). Additionally, the ΔspeB mutant TR-11 was opsonized and killed in human blood more efficiently than the wild-type SSI-9 strain (Fig. 5A). Also, SSI-9 survived longer in mice than TR-11 (Fig. 6). Histopathological analysis revealed an absence of neutrophils around the bacteria in SSI-9-infected mice (Fig. 8, A–C), whereas bacteria were engulfed by neutrophils, and their numbers were lower in TR-11-infected mice (Fig. 8, D–F). Unfortunately, mouse models do not completely reflect human infectious diseases. Nevertheless, in studies of S. pyogenes, most researchers have used mice (4, 9, 27); hence we selected the present mouse model. We found no difference between the wild-type and ΔspeB mutant strain TR-11 in regard to the levels of ScpA activities (supplemental Fig. S3). On the basis of these results, we speculated that SpeB released from S. pyogenes degrades and inactivates C3 (C3b), inhibiting the complement system around the area of infection. The inability of neutrophils in the early stage of infection may allow the bacteria to colonize tissues, resulting in severe infections.

Lukomski et al. (28, 29) reported that the ΔspeB mutant was effectively cleared by neutrophils in mice and not recovered from their blood after subcutaneous infection. In further investigations, they demonstrated that SpeB is required for dissemination of S. pyogenes from the initial infection site, resulting in severe infectious diseases including necrosis; however, how SpeB contributes to evasion of the host innate immunity has not been clear. In a previous study, SpeB was shown to convert...
M protein into its mature form and to enhance its immunoglobulin binding activity (30). Because the M protein inhibits phagocytosis by neutrophils (30, 31), it has been thought that SpeB indirectly allows \textit{S. pyogenes} to evade neutrophils by modifying M protein, whereas Nyberg et al. (32) reported that the cleavage of M protein did not occur in the presence of plasma. This hypothesis will be tested in further experiments. In addition, \textit{S. pyogenes} has been shown to indirectly inactivate C3 (C3b) via the fibronectin-binding protein, FbaA (4), which binds FH and FHL-1 cofactors that function to convert C3b into an inactive form (15, 33, 34). Pandiripally et al. (34) speculated that C3 (C3b) is inactivated on the bacterial surface by FbaA. Finally, in the current study, we showed that SpeB can directly degrade and inhibit the function of C3 (C3b). C3 (C3b) degradation is very important to decrease innate immunity (35). Interestingly, inhibition of the Fc region of anti-M antibodies revealed low inhibition to eliminate \textit{S. pyogenes} by neutrophils. In contrast, blocking of iC3b or the major iC3b receptor CD11b/CD18 led to growth of \textit{S. pyogenes} (35). Also both iC3b and its receptor demonstrated critical roles in killing \textit{S. pyogenes} by neutrophils in the innate immune system. Our results do not conflict with those findings. However, in a study of C1q-dependent innate immunity, Yuste et al. (36) suggested that some strains of \textit{S. pyogenes} were eliminated by C1q-mediated innate immunity and killed independent of C3b-deposition on the bacterial surface (36). Thus, we speculated that development of STSS correlates with the phenotypes of \textit{S. pyogenes} and concentration of C1q in the host plasma.

Recently, it has been shown how \textit{S. pyogenes} inhibits and evades the complement system by inhibiting its cofactors (37–39). SpeB protease was indeed powerful to degrade C3 (C3b);
however, it has been revealed that SpeB is produced in the early stationary phase (37). Further, Wei et al. (15, 37) demonstrated that S. pyogenes could bind FH and FHL-1 on the bacterial surface through FbaA and M protein during the log phase. Although FbaA was cleaved and then denied the FH and FHL-1 binding activities of SpeB in the early stationary phase, FbaA may contribute to inhibit the complement system in that phase. S. pyogenes may be able to adjust SpeB and FbaA to be available when and where they are needed. Indeed, André et al. (38) reported that M protein on the bacterial surface could bind and retain C4b-binding protein, which inhibits complement activation in plasma. It was also shown that a reduction in C4b-binding protein in plasma led to a decrease in complement deposition on the surface of S. pyogenes and helped to resist phagocytosis. In addition, it has been suggested that the surface M protein binds to CD46 molecules on host cells to adhere to C3 and block the activation ability of CD46 (39).

Therefore, it appears that S. pyogenes is equipped with several molecules that allow it to escape from the host immune system (9, 11, 12, 20, 27, 35–39). Our data suggest that SpeB is one of the most important virulence factors for allowing spread of S. pyogenes to various tissues from site of initial infection. For this hypothesis to be valid, sufficient levels of SpeB must be produced in vivo. It is likely that this occurs because Loughman and Caparon (23) demonstrated that in vivo conditions are the most suitable for SpeB expression; SpeB mRNA levels are at least 1000-fold higher in S. pyogenes recovered from infected mice than in bacteria grown in vitro. The present results using an in vivo mouse infection model also suggest that S. pyogenes from an STSS patient may produce and release a sufficient level of SpeB to inhibit the C3 (C3b) function in innate immunity.

When we suspended the wild-type strain in anti-SpeB serum and injected it into the quadriceps muscle of mice, the bacteria were engulfed and cleared by neutrophils (Fig. 9), and necrotic regions were not observed even at the initial site of infection. Because SpeB is not only secreted but also expressed on the bacterial surface, anti-SpeB serum might neutralize SpeB and inhibit the C3 (C3b) function in innate immunity.

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