Degradation of Complement 3 by Streptococcal Pyrogenic Exotoxin B Inhibits Complement Activation and Neutrophil Opsonophagocytosis[▽]

Chih-Feng Kuo,¹ Yee-Shin Lin,² Woei-Jer Chuang,³ Jiunn-Jong Wu,⁴ and Nina Tsao^{5*}

Departments of Nursing¹ and Biological Science and Technology,⁵ I-Shou University, Kaohsiung County, and Departments of Microbiology and Immunology,² Biochemistry,³ and Medical Technology,⁴ College of Medicine, National Cheng Kung University, Tainan, Taiwan

Received 10 August 2007/Returned for modification 13 October 2007/Accepted 16 December 2007

Streptococcal pyrogenic exotoxin B (SPE B), a cysteine protease, is an important virulence factor in group A streptococcus (GAS) infection. The inhibition of phagocytic activity by SPE B may help prevent bacteria from being ingested. In this study, we examined the mechanism SPE B uses to enable bacteria to resist opsonophagocytosis. Using an enzyme-linked immunosorbent assay, we found that SPE B-treated serum impaired the activation of the classical, the lectin, and the alternative complement pathways. In contrast, C192S, a SPE B mutant lacking protease activity, had no effect on complement activation. Further study showed that cleavage of serum C3 by SPE B, but not C192S, blocked zymosan-induced production of reactive oxygen species in neutrophils as a result of decreased deposition of C3 fragments on the zymosan surface. Reconstitution of C3 into SPE B-treated serum unblocked zymosan-mediated neutrophil activation dose dependently. SPE B-treated, but not C192S-treated, serum also impaired opsonization of C3 fragments on the surface of GAS strain A20. Moreover, the amount of C3 fragments on the A20 cell surface, a SPE B-producing strain, was less than that on its isogenic mutant strain, SW507, after opsonization with normal serum. A20 opsonized with SPE B-treated serum was more resistant to neutrophil killing than A20 opsonized with normal serum, and SPE B-mediated resistance was C3 dependent. These results suggest a novel SPE B mechanism, one which degrades serum C3 and enables GAS to resist complement damage and opsonophagocytosis.

Group A streptococcus (GAS; *Streptococcus pyogenes*) is an important human pathogen that causes a variety of infections, including pharyngitis, impetigo, cellulitis, necrotizing fasciitis, puerperal sepsis, and streptococcal toxic shock syndrome (11, 41, 42). Despite intensive care with antimicrobial therapy, the mortality has remained high for these infections and postinfection sequelae, such as acute rheumatic fever (47). Several mechanisms by which GAS evades the innate immunity have been described (21, 47). The surface M and M-like proteins of GAS avoid opsonization by complement- and phagocytosis-mediated killing, in part by binding to complement regulatory proteins, such as C4b-binding protein, factor H, and factor H-like protein (2, 23). Unlike M protein, both the hyaluronic acid capsule and collagen recruitment of GAS form the physical barrier on the bacterial surface to avoid complement opsonization and phagocytosis by neutrophils (47). Also, GAS expresses C5a peptidase to digest C5a and inhibit recruitment of neutrophils to sites of infection (22). Another GAS protective mechanism involves membrane attack complex inhibition (16) and resistance to neutrophil phagocytosis (47) through secretion of streptococcal inhibitor of complement. Moreover, GAS also secretes several exotoxins and enzymes, such as IdeS (immunoglobulin G [IgG]-degrading enzyme of *S. pyogenes*)/Mac (group A streptococcal Mac-1-like protein), streptococcal pyrogenic exotoxin B (SPE B), and endo- β -N-acetylglucosaminidase (EndoS), which are able to bind or cleave IgG

and inhibit opsonophagocytosis (8, 9, 10, 12, 14, 29, 45, 46). Furthermore, GAS secretes DNases to degrade the neutrophil extracellular trap, which functions as an extracellular bactericidal weapon of neutrophils, thereby evading the innate immune response (43).

Several reports suggest that SPE B, a cysteine protease, may be a critical virulence factor in streptococcal infections. Clinical investigations indicate that high levels of SPE B protease activity are significantly associated with signs of streptococcal toxic shock syndrome and with mortality (19). Patients with lower antibody levels against SPE B are more likely to succumb to invasive GAS disease (15). SPE B digests a number of host proteins, including immunoglobulin (8, 10, 14, 45), kininogen (17), matrix metalloprotease (3), urokinase receptor (51), fibrinogen (33), and fibronectin as well as vitronectin (25) and interleukin-1 precursor (24). A *speB* mutant strain has decreased resistance to neutrophil phagocytosis, dissemination to organs, and mortality in the mouse model (26, 28, 30, 31, 32). The exact mechanism by which SPE B inhibits neutrophil phagocytosis has yet to be clearly defined.

The complement system, an important arm of innate immunity, plays an essential role in the early defense of bacterial infection. The human complement system consists of more than 30 proteins in plasma and on host cells. Based on different recognition molecules to sense a foreign substance, three different complement pathways are the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). Even though the initiation stages are diverse in these three complement pathways, they converge in the generation of C3 convertase, which cleaves the central component C3 (48, 49). Following C3 cleavage, the attachment of C3b to the bacterial surface is necessary to initiate opsonophagocytosis by phago-

* Corresponding author. Mailing address: Department of Biological Science and Technology, I-Shou University, Kaohsiung County, Taiwan. Phone: 886-7-6151100, ext. 7314. Fax: 886-7-2252051. E-mail: ninatsao@isu.edu.tw.

[▽] Published ahead of print on 3 January 2008.

cytes. Moreover, the C3 convertase is changed into a C5 convertase, which mediates membrane attack complex formation on the bacterial surface, thereby eliminating bacteria effectively (36, 48, 49). In our previous study, we indicated that SPE B degraded serum properdin, the only known positive regulator of AP activation, leading to a decrease in bactericidal activity by neutrophils toward GAS (44). In the present study, we further show that SPE B impairs all three complement pathways through degradation of the central component C3, thereby decreasing the deposition of C3 fragments on the bacterial surface and further inhibiting opsonophagocytosis-mediated killing of neutrophils.

MATERIALS AND METHODS

Preparation of recombinant SPE B and its mutant, C192S. The recombinant SPE B and the C192S mutant lacking protease activity were prepared as described previously (6). Briefly, the genomic DNA of GAS was extracted and the structural gene of pro-SPE B was amplified by PCR with the sense primer 5'-GGATCCGGATCCCATCATCATCATCATGATCAAACTTTGCTCGTAACGAA-3' (with a His₆ tag and BamHI recognition) and antisense primer 5'-GGATCCGGATCCCTAAGGTTTGTGCTGCTACAACAG-3' (with BamHI recognition). The PCR product was purified and then cloned into the BamHI site of a pET-21a vector. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) pLyS, and the system was under the control of a strong T7 promoter. The wild-type construct was further used to produce the C192S mutation using overlap extension PCR. The recombinant protein was produced by growing cells at 28°C overnight in LB medium containing 10 g of Bacto tryptone, 5 g of Bacto yeast extract, and 10 g of NaCl in 1 liter and purified using Ni²⁺-chelating chromatography (Amersham Biosciences, Uppsala, Sweden). Recombinant wild-type SPE B was converted into a 28-kDa active form during the course of purification. The protein was concentrated using Amicon ultrafiltration with a 10-kDa cutoff membrane and exchanged with phosphate-buffered saline (PBS) (6, 44).

Enzyme-linked immunosorbent assay (ELISA) for assessment of complement functional activity. Complement activation of the CP, the LP, or the AP pathway by human serum was detected by using a WiaLisa COMPL300 total complement functional screen kit (Wieslab AB, Lund, Sweden) (37, 40). The kit provides coated strips as follows: strips of wells for CP evaluation are precoated with IgM, strips for AP evaluation are coated with lipopolysaccharide from *Salmonella typhosa*, and LP pathway strips are coated with mannan. Following the kit instructions, the serum of a healthy individual, provided in the kit, was diluted 100-fold for the CP and LP assays and 18-fold for the AP assay in specific buffers, which ensured that activation of only one of the pathways occurred (40). The diluted serum was then incubated with different concentrations (5, 10, or 20 µg/ml) of SPE B or C192S for 15 min in a 37°C water bath. Thereafter, SPE B- or C192S-treated human serum was added to strips and incubated for 1 h at 37°C. Alkaline phosphatase-conjugated anti-human C5b-9 antibody, provided in the kit, was added, and the mixture was incubated for another 30 min at room temperature. Finally, the substrate was added and absorbance values were read at 405 nm. For each assay, standard positive and negative control sera were provided in the kit. Complement activity was calculated as follows: activity = $100\% \times (A_{405} \text{ of sample} - A_{405} \text{ of negative control}) / (A_{405} \text{ of standard serum} - A_{405} \text{ of negative control})$ (40). In each assay, samples, standard serum, and negative control serum were tested in duplicate at a fixed dilution. The results of one of three experiments are presented.

Identifying C192S-binding ligands in human serum. One gram of CNBr-activated Sepharose 4B gel (Pharmacia Biotech, Uppsala, Sweden) was suspended in 250 ml of 1 M HCl for 30 min at 4°C, and then the gel was washed with alternating 750 ml of 1 M HCl, 100 ml of distilled water, and 300 ml of reaction buffer containing 0.1 M NaHCO₃ and 0.5 M NaCl (pH 8.0). The gel suspended in 15 ml of the reaction buffer (pH 8.0) was incubated with 10 mg of C192S at 4°C overnight, and then the coupled gel was washed with 250 ml of 1 M glycine-reaction buffer (pH 8.0) to block unused activated sites. Thereafter, the coupled gel was washed three times with alternating 200 ml of borate buffer containing 0.1 M boric acid and 1 M NaCl (pH 8.5), 100 ml of distilled water, 200 ml of acetate buffer containing 0.1 M sodium acetate and 1 M NaCl (pH 4.0), and 100 ml of distilled water and then was suspended in PBS.

One milliliter of normal human serum was passed through a CNBr-activated Sepharose 4B-packed affinity column that was immobilized with C192S. Buffer

containing 50 mM Tris (pH 7.0) was used to wash nonbinding materials. After several washes, binding ligands of C192S were eluted with 0.1 M glycine (pH 3.0), and different fractions were collected from the point when the signal appeared on the recording graph by measuring the absorbance at 280 nm (44). The contents of different fractions were verified using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After being blocked, blots were developed with goat anti-human C3 polyclonal antibody (Calbiochem, San Diego, CA) that was diluted 7,000-fold with PBS. Blots were then hybridized using horseradish peroxidase-conjugated rabbit anti-goat IgG (Calbiochem), and the protein bands were visualized using enhanced chemiluminescence (Amersham Biosciences).

Detection of cleavage of C3 by SPE B. The purified C3 (200 µg/ml; Calbiochem) was incubated with 20 µg/ml of SPE B or C192S at 37°C for 30 min. In another experiment, an equal volume of human serum, diluted 20-fold with PBS, was incubated with PBS, 40 µg/ml of SPE B, or 40 µg/ml of C192S at 37°C for 15 min with 5 mM dithiothreitol (DTT)-0.1 mM EDTA, which was used to activate SPE B protease activity (44). The reaction mixtures mentioned above were separated using 8% SDS-PAGE and then detected by Western blotting with goat anti-human C3 polyclonal antibody (Calbiochem), as described above.

Preparation of opsonized zymosan. Zymosan (Sigma, St. Louis, MO) was boiled in PBS for 30 min, washed three times with PBS, and resuspended at 20 mg/ml in Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY) containing 14 mg/ml of CaCl₂ and 14 mg/ml of MgCl₂ (52). For opsonization, 0.5 ml of zymosan particles (20 mg/ml) was mixed with 0.5 ml of normal human serum and incubated for 30 min at 37°C (52). The opsonized zymosan particles were then washed and resuspended in DPBS at a concentration of 10 mg/ml. In some experiments, normal human serum was incubated with 20 µg/ml of either SPE B or C192S at 37°C for 30 min, and different concentrations of C3 (10, 20, or 40 µg/ml) were then added to the reaction mixture. Thereafter, the pretreated sera were incubated with 0.5 ml of zymosan particles (20 mg/ml) for 30 min at 37°C.

Luminol-dependent CL detection in neutrophils. Human neutrophils were collected from healthy donors and purified using Ficoll-Paque centrifugation. Human neutrophils (10⁶ cells in 80 µl of DPBS) were incubated with opsonized zymosan particles (10 µg in 10 µl of DPBS), 5 µl of luminol (superoxide anion assay kit; Sigma), and 5 µl of enhancer (40 mM; Sigma). The reaction was monitored with a multidetection microplate reader (Synergy 2 model; Bio-Tek Instruments, Inc., Winooski, VT) for 15 min and recorded in millivolts. The results are expressed as the kinetic profiles of chemiluminescence (CL) production or the integrated area under the curves of the CL profile (13, 38). The results of one of three experiments are presented.

Detection of opsonized C3 fragments on zymosan. Opsonized zymosan was mixed with sample dye, boiled, and then separated using 8% SDS-PAGE and blotted with goat anti-human C3 polyclonal antibody (Calbiochem) that was diluted 7,000-fold with PBS as described above.

Analysis of complement deposition on GAS by flow cytometry. *S. pyogenes* A20 (type M1, T1; opacity factor negative) was isolated from the blood of a patient with necrotizing fasciitis at the National Cheng Kung University Hospital. SW507, a *speB* isogenic mutant of A20, was generated by J. J. Wu, Department of Medical Technology, National Cheng Kung University (26). *S. pyogenes* was cultured in tryptic soy broth containing 0.5% yeast extract (Difco Laboratories, Detroit, MI) overnight at 37°C and then subcultured in fresh broth (1:50, vol/vol) for another 16 h. The concentration of bacteria was determined with a spectrophotometer (Beckman Instruments, Somerset, NJ) by measuring the optical density at 600 nm.

Washed A20 strain cells were resuspended at 5×10^7 CFU in 0.5 ml of PBS containing 0.1% gelatin (PBS-G), and human serum (0.25 ml) pretreated either with 20 µg/ml of SPE B or C192S at 37°C for 15 min was added and incubated for another 30 min. The reaction mixture was washed twice with 1 ml of PBS-G at 8,000 ×g for 5 min. In another experiment, washed A20 or SW507 cells (5×10^7 CFU in 0.5 ml of PBS-G) were incubated with 0.25 ml of human serum at 37°C for 30 min and then washed twice with 1 ml of PBS-G. Opsonized bacteria, as indicated above, were then fixed with 0.2 ml of 1% paraformaldehyde at room temperature for 10 min. Goat anti-human C3 polyclonal antibody (Calbiochem; 20 µg/ml in PBS-G containing 0.3% bovine serum albumin) was added and incubated for another 30 min at 4°C. Thereafter, 0.2 ml of fluorescein isothiocyanate-conjugated anti-goat IgG (Calbiochem) that had been diluted 1:100 in 0.3% bovine serum albumin-PBS-G was added and incubated for 30 min at 4°C. Finally, the bacteria were washed and resuspended in 1 ml of PBS for flow cytometric analysis (BD Biosciences, San Jose, CA) (5). Each assay was performed at least three times, and the results are expressed as the mean ± standard deviation.

FIG. 1. Effects of SPE B and C192S on the classical pathway (A), lectin pathway (B), or alternative pathway (C) of complement activation. The human sera diluted with specific buffers were incubated with different concentrations of SPE B or C192S, and the complement activation by human serum was detected in an ELISA. The complement activity was calculated as described in Materials and Methods. The results of one of three experiments are presented.

Detection of opsonized C3 fragments on the GAS surface. Opsonized GAS, as described above, was washed twice with PBS. The pellet was suspended in 1 M hydroxylamine (Sigma)–1% SDS (pH 9.0 in 5 mM NaHCO₃–Na₂CO₃) at 37°C for 20 min to disrupt ester bonds between the complement fragments and the bacterial surface. Centrifugation (13,000 × g) was used to separate the bacterial pellet from the C3 fragment suspension. Two hundred microliters of the C3 fragment suspension was reduced with 10 mM DTT (Sigma)–1% SDS at 37°C for 60 min and then alkylated with 20 µl of 22 mM iodoacetamide (Sigma) (pH 8.0 in 100 mM of Tris–10 mM of EDTA) at 37°C for 60 min (4). The reaction mixture was separated using 8% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore). After being blocked, blots were developed with goat anti-human C3 polyclonal antibody (Calbiochem) as described above.

Neutrophil killing assay. Human serum was diluted twofold with sterile normal saline and then treated with 20 µg/ml of SPE B for 30 min at 37°C in the presence of 5 mM DTT–0.1 mM EDTA. Then, the purified C3 (4 or 10 µg/ml) was reconstituted to the reaction mixture. A20 cells (1.5×10^3 CFU) were opsonized with pretreated sera for 5 min at 37°C before adding 3×10^6 neutrophils. Incubation was continued at 37°C during end-over-end rotation for 2.5 h ($T_{2.5}$). Portions of 20 µl were removed and diluted in 10 ml of PBS containing 0.025% Triton X-100. Viable bacteria were determined by colony counting using the pour plate method (44). The time point of neutrophil addition was indicated as T_0 . The relative bacterial amount in each group was calculated as follows: (CFU at $T_{2.5}$)/(CFU at T_0). The relative bacterial ratio was calculated as follows: (relative bacterial amount in each group)/(relative bacterial amount in serum control group). The results of three experiments are presented and expressed as means $\pm$ standard deviations.

FIG. 2. Effects of SPE B and C192S on C3. (A) The reaction mixture of fraction 13 (F13) from the elution, purified C3, purified C3 plus SPE B, or purified C3 plus C192S was separated using 8% SDS-PAGE and blotted using goat anti-human C3 antibody. (B) Diluted human serum was incubated with either 40 µg/ml of C192S or SPE B for 15 min with 5 mM DTT–0.1 mM EDTA, and then the reaction mixture was separated using 8% SDS-PAGE and blotted using goat anti-human C3 antibody, as described in Materials and Methods. Untreated serum was used as a control.

Statistics. Statistical analysis was performed using analysis of variance. Differences were considered significant at a P level of <0.05 .

RESULTS

SPE B inhibits three complement pathways. To examine the effect of SPE B on complement activation, a simple ELISA-based procedure for the classical, alternative, and lectin pathways was used in this study (37, 40). The human sera treated with either SPE B or C192S were used to activate complement pathways. SPE B, but not C192S, was able to impair the activation of both the classical and the lectin pathways in a dose-dependent manner compared with the PBS-treated group (Fig. 1A and B). As complement activation of the alternative pathway detected by the ELISA requires a serum concentration that is 10-fold higher than that required for the classical and lectin pathways (37, 40), the impairment of the alternative pathway by SPE B was notably detectable at 20 µg/ml but not at 5 or 10 µg/ml (Fig. 1C). These results suggested that SPE B could effectively damage the three pathways of complement activation.

SPE B but not C192S cleaves C3. To examine the effect of SPE B on C3, a convergent point for the classical, alternative, and lectin complement pathways (48, 49), the interaction of SPE B and C3 was studied next. Because of the protease activity of SPE B and its autocatalysis reaction (26, 27), human sera were passed through an affinity column packed with C192S, a SPE B mutant, and immobilized on Sepharose 4B.

FIG. 3. Effect of C3 on SPE B-mediated inhibition of neutrophil activation by zymosan. Human neutrophils were incubated with zymosan that was opsonized with SPE B-treated serum and different concentrations of C3, and the CL production of neutrophils was then detected as described in Materials and Methods. The CL production of neutrophils is expressed as the kinetic profile and presented in millivolts per minute (A) or as the integrated area under the curves (AUCs) (B). The CL production of neutrophils that was induced by zymosan, which was opsonized either with untreated or C192S-treated serum, was used as a control. The results of one of three different experiments are presented.

Different fractions were collected when the elution proceeded and their contents were analyzed using SDS-PAGE followed by Western blotting with anti-C3 antibody. The Western blot result showed that fraction 13 contained β -chain and some protein fragments, ranging from 41 to 67 kDa, that are likely to be the autolytic fragments of the C3 α -chain (20) compared with the blot results of purified C3 (Fig. 2A), suggesting that C192S could bind C3 in the human serum. For further analysis of the interaction of SPE B and C3, purified C3 was either incubated with SPE B or C192S for 30 min, and the reaction mixture was verified using SDS-PAGE and blotted with anti-C3 antibody. After the 30-min incubation, SPE B but not C192S cleaved purified C3 effectively (Fig. 2A). The similar cleaving results were found with SPE B-treated, but not C192S-treated, human sera (Fig. 2B). These results suggested that SPE B was able to bind serum C3 and cleave it effectively.

Inhibition of zymosan-induced neutrophil activation by SPE B was C3 dependent. The complement-mediated neutrophil activation through opsonophagocytosis was further examined. Zymosan was opsonized with PBS-, SPE B-, or C192S-treated

FIG. 4. SPE B but not C192S inhibits C3 opsonization on the A20 surface. A20 cells were opsonized with either SPE B- or C192S-treated serum, and C3 fragments on the A20 surface were detected in a flow cytometric assay with anti-C3 antibody. The results are expressed as a histogram of fluorescence (A) and mean fluorescence (B). The histogram of fluorescence in panel A indicates, in order from left to right, the blank, SPE B, C192S, and PBS groups. The results collected from three different experiments are expressed as means $\pm$ standard deviations. *, $P < 0.05$ compared with values determined for the PBS-treated group.

human sera for 30 min. The neutrophil oxidative burst stimulated by opsonized zymosan was detected in a CL assay (13, 38). SPE B effectively decreased the production of CL in human neutrophils at the concentration of 20 μ g per ml compared with the PBS-treated group. In contrast, C192S had no significant effect on CL production of neutrophils (Fig. 3A). Reconstitution of purified C3 restored CL production of neutrophils in a dose-dependent manner (Fig. 3A and B). Further analysis of C3 deposition on the zymosan surface indicated that the amount of C3 fragments on the zymosan surface was significantly decreased after opsonization with SPE B-treated, but not PBS- or C192S-treated, human sera (data not shown). Reconstitution of purified C3 restored the C3 deposition amount on the zymosan surface dose dependently, even in the presence of SPE B (data not shown). These results suggested that SPE B inhibited zymosan-mediated human neutrophil activation by decreasing C3 deposition on the zymosan surface.

Degrading C3 by SPE B inhibits bacterial killing by human neutrophils. To examine whether the C3 opsonization on the GAS surface was affected by SPE B, GAS strain A20 was incubated with either SPE B- or C192S-treated human serum. The C3 amount deposited on the A20 surface was detected using anti-C3 antibodies and flow cytometric analysis. The results indicated that SPE B, but not C192S, significantly decreased C3 deposition on the A20 surface (Fig. 4A and B). C3 fragments released from the bacterial surface after opsonization of the A20 strain with SPE B- or C192S-treated serum were also confirmed by Western blotting (data not shown), indicating a result similar to that shown in the flow cytometric

FIG. 5. Degradation of C3 by SPE B inhibits the bactericidal activity of neutrophils. (A) Detection of C3 fragments on the GAS surface. A20 and SW507 cells were opsonized with normal serum, and C3 fragments on the surface of A20 or SW507 cells were detected in a flow cytometric assay with anti-C3 antibody. The results are presented as mean fluorescence $\pm$ the standard deviation. *, $P < 0.05$ compared with values determined for SW507. (B) Effect of C3 on SPE B-mediated inhibition of neutrophil killing. A20 cells (1.5×10^3 CFU) were opsonized using serum pretreated with either 20 μ g/ml of SPE B alone or SPE B plus C3 (4 or 10 μ g/ml) for 30 min at 37°C in the presence of 5 mM DTT–0.1 mM EDTA, and then neutrophils (3×10^6) were added for the bactericidal assay. The results are presented as the relative bacterial ratio as described in Materials and Methods. The results of three experiments are presented and are expressed as means $\pm$ standard deviations. *, $P < 0.05$ compared with values determined for the SPE B-treated group.

assay. Further comparison of C3 deposition on the surface of a SPE B-producing strain, A20, and its isogenic mutant, SW507, after opsonization with human serum in a flow cytometric assay indicated that the amount of C3 fragments on the A20 surface was less than that of SW507 (Fig. 5A). These results suggested that SPE B impairs C3 opsonization on the GAS surface.

The effects of degradation of C3 by SPE B on the killing ability of neutrophils were examined further. A GAS strain, A20, opsonized with SPE B-treated serum, was incubated with human neutrophils from a healthy donor, and the bactericidal activity of neutrophils was assayed after 2.5 h. A20 opsonized with SPE B-treated serum was more resistant than A20 opsonized with normal serum to neutrophil killing (Fig. 5B). SPE B-mediated A20 resistance to neutrophil killing was inhibited after reconstitution of purified C3 to SPE B-treated serum (Fig. 5B). These results suggested that degradation of C3 by SPE B reduced both serum opsonization and neutrophil killing activity.

DISCUSSION

Several lines of evidence suggest that SPE B contributes to increased GAS invasion (28, 30, 31), degradation of fibronectin, vitronectin, and fibrinogen (25, 33), cleavage of the urokinase plasminogen activator receptor (51), activation of matrix metalloprotease (3), generation of active interleukin-1 β from its precursor (24), damage to endothelial cells (28), and reduced phagocytic activity of U937 monocytic cells (27). In addition, SPE B cleaves the Fc portion of antigen-bound IgG, thereby contributing to bacterial evasion of antibody-mediated opsonophagocytosis (10, 14). In contrast, SPE B has been shown to degrade a bacterial surface protein, Fba, which contributes to phagocytosis resistance through binding of factor H and factor H-like protein 1 in in vitro experiments (50). However, these in vitro experimental findings seem to not directly reflect in vivo infections, where streptococci are likely to be coated with host proteins that could protect bacterial surface proteins from SPE B degradation (50).

Our previous studies using an animal model showed a correlation of SPE B with the pathogenicity of GAS diseases (26, 28). Wild-type strain NZ131 multiplied in the air pouch, secreted SPE B (approximately 30 to 40 μ g per ml), and resulted in bacteremia and dissemination to various organs within 48 h, whereas *speB* mutant SW510 bacteria were confined to the air pouch and eliminated. Coinoculation with SW510 and SPE B caused bacterial growth and dissemination, indicating SPE B helped bacteria to resist the host defense mechanism and continue to multiply (28).

SPE B produced from GAS is released extracellularly as a zymogen (pro-SPE B) with a molecular mass of 40 kDa, and the active form is a 28-kDa mature protease that plays an important role in GAS pathogenesis. The maturation of pro-SPE B can be achieved through proteolysis by autoprocessing of the 28-kDa active form of SPE B under physiological conditions (6). Analysis of the cleavage sites by the active form of SPE B reveals trends, with a lysine residue at the P1 site and a preference for a hydrophobic residue, such as isoleucine, tyrosine, methionine, or valine, at the P2 site (6). Using a SPE B concentration (20 μ g per ml) with physiological relevance to an animal model (28), we found that SPE B could degrade both α , α' (41-kDa fragment of the α -chain), and β -chains of C3 effectively (Fig. 2). However, the detailed cutting sites by SPE B on α - and β -chains of C3 need further investigation. The similar protease mechanisms by which other pathogens act on C3 are also demonstrated by a chymotrypsin-like surface protease, dentilisins, of *Treponema denticola* (53), a trypsin-like protease of *Porphyromonas gingivalis* (39), the elastase and alkaline protease of *Pseudomonas aeruginosa* (18), and a gelatinase of *Enterococcus faecalis* (35).

Even though the initiation stages are diverse in the CP, LP, and AP complement pathways, activation in all of them leads to opsonization of the target by C3b and its cleavage fragment, iC3b. Complement receptor 1 (CR1) on phagocytes binds C3b or C4b, and CR3 or CR4 binds iC3b, resulting in phagocytosis of the target and phagocyte activation that is characterized by the generation of reactive oxygen species (7). Phagocytic cells require either complement deposition or antibodies—or preferably both—to recognize and eliminate bacteria efficiently. Recently, the functions of complement receptors and antibody

receptors in phagocyte killing have been examined (34). Blocking Fc receptors had little effect on the killing by neutrophils. However, inhibiting C3b/iC3b generation or blocking CR3 (CD11b/CD18) enabled GAS to grow efficiently in immune human serum (34). This observation indicates that the function of complement receptors on phagocytes is most important to effectively eliminate GAS.

Several mechanisms specific to complement attack have been reported in GAS (1, 2, 5, 16, 21, 22, 23). In our previous study, we demonstrated that a virulent GAS mechanism inhibited the alternative pathway by degrading properdin with SPE B (44). Reconstitution of a high dose of properdin (40 µg per ml) into SPE B-treated serum increased neutrophil killing activity of GAS to a limited degree (unpublished observation), indicating that other factors by which SPE B inhibits neutrophil killing are involved. We suggest here a novel mechanism through which SPE B resists neutrophil killing: degradation of C3 and impairment of all complement pathways. Therefore, in addition to cleaving IgG and inhibiting antibody-mediated opsonophagocytosis, our findings indicate that SPE B may help GAS evade innate immunity by inhibiting complement-mediated opsonophagocytosis and further neutrophil activation.

ACKNOWLEDGMENTS

This work was supported by grants ISU96-04-13 from I-Shou University, NHRI-EX96-9429SP from the National Health Research Institutes, and NSC96-2320-B214-001 from the National Science Council, Taiwan.

We acknowledge the editorial assistance of Jonathan Courtenay.

REFERENCES

- Berggard, K., E. Johnsson, E. Morfeldt, J. Persson, M. Stalhammar-Carlsson, and G. Lindahl. 2001. Binding of human C4BP to the hypervariable region of M protein: a molecular mechanism of phagocytosis resistance in *Streptococcus pyogenes*. *Mol. Microbiol.* **42**:539–551.
- Bisno, A. L., M. O. Brito, and C. M. Collins. 2003. Molecular basis of group A streptococcal virulence. *Lancet Infect. Dis.* **3**:191–200.
- Burns E. H., Jr., A. M. Marciel, and J. M. Musser. 1996. Activation of a 66-kilodalton human endothelial cell matrix metalloprotease by *Streptococcus pyogenes* extracellular cysteine protease. *Infect. Immun.* **64**:4744–4750.
- Campbell, J. R., C. J. Baker, and M. S. Edwards. 1991. Deposition and degradation of C3 on type III group B streptococci. *Infect. Immun.* **59**:1978–1983.
- Carlsson, F., C. Sandin, and G. Lindahl. 2005. Human fibrinogen bound to *Streptococcus pyogenes* M protein inhibits complement deposition via the classical pathway. *Mol. Microbiol.* **56**:28–39.
- Chen, C. Y., S. C. Luo, C. F. Kuo, Y. S. Lin, J. J. Wu, M. T. Lin, C. C. Liu, W. Y. Jeng, and W. J. Chuang. 2003. Maturation processing and characterization of streptolysin. *J. Biol. Chem.* **278**:17336–17343.
- Cheung, K., A. C. Archibald, and M. F. Robinson. 1983. The origin of chemiluminescence produced by neutrophils stimulated by opsonized zymosan. *J. Immunol.* **130**:2324–2329.
- Collin, M., and A. Olsen. 2001. Effect of SpeB and EndoS from *Streptococcus pyogenes* on human immunoglobulins. *Infect. Immun.* **69**:7187–7189.
- Collin, M., and A. Olsen. 2003. Extracellular enzymes with immunomodulating activities: variations on a theme in *Streptococcus pyogenes*. *Infect. Immun.* **71**:2983–2992.
- Collin, M., M. D. Svensson, A. G. Sjöholm, J. C. Jensenius, U. Sjöbring, and A. Olsen. 2002. EndoS and SpeB from *Streptococcus pyogenes* inhibit immunoglobulin-mediated opsonophagocytosis. *Infect. Immun.* **70**:6646–6651.
- Cone, L. A., D. R. Woodard, P. M. Schlievert, and G. S. Tomory. 1987. Clinical and bacteriologic observations of a toxic shock-like syndrome due to *Streptococcus pyogenes*. *N. Engl. J. Med.* **317**:146–149.
- Cunningham, M. W. 2000. Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.* **13**:470–511.
- Dahlgren, C., and A. Carlsson. 1999. Respiratory burst in human neutrophils. *J. Immunol. Methods* **232**:3–14.
- Eriksson, A., and M. Norgren. 2003. Cleavage of antigen-bound immunoglobulin G by SpeB contributes to streptococcal persistence in opsonizing blood. *Infect. Immun.* **71**:211–217.
- Eriksson, B. K. G., J. Andersson, S. E. Holm, and M. Norgren. 1999. Invasive group A streptococcal infections: T1M1 isolates expressing pyrogenic exotoxins A and B in combination with selective lack of toxin-neutralizing antibodies are associated with increased risk of streptococcal toxic shock syndrome. *J. Infect. Dis.* **180**:410–418.
- Fernie-King, B. A., D. J. Seilly, C. Willers, R. Wurzner, A. Davies, and P. J. Lachmann. 2001. Streptococcal inhibitor of complement (SIC) inhibits the membrane attack complex by preventing uptake of C5b7 onto cell membranes. *Immunology* **103**:390–398.
- Herwald, H., M. Collin, W. Muller-Esterl, and L. Björck. 1996. Streptococcal cysteine proteinase releases kinins: a novel virulence mechanism. *J. Exp. Med.* **184**:665–673.
- Hong, Y., and B. Ghebrehewet. 1992. Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. *Clin. Immunol. Immunopathol.* **62**:133–138.
- Hsueh, P. R., J. J. Wu, P. J. Tsai, J. W. Liu, Y. C. Chuang, and K. T. Luh. 1998. Invasive group A streptococcal disease in Taiwan is not associated with the presence of streptococcal pyrogenic exotoxin genes. *Clin. Infect. Dis.* **26**:584–589.
- Isaac, L., D. Aivazian, A. Taniguchi-Sidle, R. O. Ebanks, C. S. Farah, M. P. C. Florido, M. K. Pangburn, and D. E. Isenman. 1998. Native conformations of human complement components C3 and C4 show different dependencies on thioester formation. *Biochem. J.* **329**:705–712.
- Jarva, H., T. S. Jokiranta, R. Wurzner, and S. Meri. 2003. Complement resistance mechanisms of streptococci. *Mol. Immunol.* **40**:95–107.
- Ji, Y., L. McLandsborough, A. Kondagunta, and P. P. Cleary. 1996. C5a peptidase alters clearance and trafficking of group A streptococci by infected mice. *Infect. Immun.* **64**:503–510.
- Johnsson, E., K. Berggård, H. Kotarsky, J. Hellwage, P. F. Zipfel, U. Sjöbring, and G. Lindahl. 1998. Role of the hypervariable region in streptococcal M proteins: binding of a human complement inhibitor. *J. Immunol.* **161**:4894–4901.
- Kapur, V., M. W. Majesky, L. L. Li, R. A. Black, and J. M. Musser. 1993. Cleavage of interleukin 1β (IL-1β) precursor to produce active IL-1β by a conserved extracellular cysteine protease from *Streptococcus pyogenes*. *Proc. Natl. Acad. Sci. USA* **90**:7676–7680.
- Kapur, V., S. Topouzis, M. W. Majesky, L. L. Li, M. R. Hamrick, R. J. Hamill, J. M. Patti, and J. M. Musser. 1993. A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. *Microb. Pathog.* **15**:327–346.
- Kuo, C. F., J. J. Wu, K. Y. Lin, P. J. Tsai, S. C. Lee, Y. T. Jin, H. Y. Lei, and Y. S. Lin. 1998. Role of streptococcal pyrogenic exotoxin B in the mouse model of group A streptococcal infection. *Infect. Immun.* **66**:3931–3935.
- Kuo, C. F., J. J. Wu, P. J. Tsai, F. J. Kao, H. Y. Lei, M. T. Lin, and Y. S. Lin. 1999. Streptococcal pyrogenic exotoxin B induces apoptosis and reduces phagocytic activity in U937 cells. *Infect. Immun.* **67**:126–130.
- Kuo, C. F., Y. H. Luo, H. Y. Lin, K. J. Huang, J. J. Wu, H. Y. Lei, M. T. Lin, W. J. Chuang, C. C. Liu, Y. T. Jin, and Y. S. Lin. 2004. Histopathologic changes in kidney and liver correlate with streptococcal pyrogenic exotoxin B production in the mouse model of group A streptococcal infection. *Microb. Pathog.* **36**:273–285.
- Lei, B., F. R. DeLeo, N. P. Hoe, M. R. Graham, S. M. Mackie, R. L. Cole, M. Liu, H. R. Hill, D. E. Low, M. J. Federle, J. R. Scott, and J. M. Musser. 2001. Evasion of human innate and acquired immunity by a bacterial homolog of CD11b that inhibits opsonophagocytosis. *Nat. Med.* **7**:1298–1305.
- Lukomski, S., C. A. Montgomery, J. Rurangirwa, R. S. Geske, J. P. Barrish, G. J. Adams, and J. M. Musser. 1999. Extracellular cysteine protease produced by *Streptococcus pyogenes* participates in the pathogenesis of invasive skin infection and dissemination in mice. *Infect. Immun.* **67**:1779–1788.
- Lukomski, S., E. H. Burns, Jr., P. R. Wyde, A. Podbielski, J. Rurangirwa, D. K. Moore-Poveda, and J. M. Musser. 1998. Genetic inactivation of an extracellular cysteine protease (SpeB) expressed by *Streptococcus pyogenes* decreases resistance to phagocytosis and dissemination to organs. *Infect. Immun.* **66**:771–776.
- Lukomski, S., S. Sreevatsan, C. Amberg, W. Reichardt, M. Woischnik, A. Podbielski, and J. M. Musser. 1997. Inactivation of *Streptococcus pyogenes* extracellular cysteine protease significantly decreases mouse lethality of serotype M3 and M49 strains. *J. Clin. Invest.* **99**:2574–2580.
- Matsuka, Y. V., S. Pillai, S. Gubba, J. M. Musser, and S. B. Olmsted. 1999. Fibrinogen cleavage by the *Streptococcus pyogenes* extracellular cysteine protease and generation of antibodies that inhibit enzyme proteolytic activity. *Infect. Immun.* **67**:4326–4333.
- Nilsson, M., M. Weineisen, T. Andersson, L. Truedsson, and U. Sjöbring. 2005. Critical role for complement receptor 3 (CD11b/CD18), but not for Fc receptors, in killing of *Streptococcus pyogenes* by neutrophils in human immune serum. *Eur. J. Immunol.* **35**:1472–1481.
- Park, S. Y., K. M. Kim, J. H. Lee, S. J. Seo, and I. H. Lee. 2007. Extracellular gelatinase of *Enterococcus faecalis* destroys a defense system in insect hemolymph and human serum. *Infect. Immun.* **75**:1861–1869.
- Rooijackers, S. H. M., and J. A. G. van Strijp. 2007. Bacterial complement evasion. *Mol. Immunol.* **44**:23–32.
- Roos, A., L. H. Bouwman, J. Munoz, T. Zuiverloon, M. C. Faber-Krol, F. C. Fallaux-van den Houten, N. Klar-Mohamad, C. E. Hack, M. G. Tilanus, and

- M. R. Daha. 2003. Functional characterization of the lectin pathway of complement in human serum. *Mol. Immunol.* **39**:655–668.
38. Russo-Carbolante, E. M., A. E. Azzolini, A. C. Polizello, A. I. De Assis-Pandochi, and Y. M. Lucisano-Valim. 2005. Propylthiouracil and thiamazole do not alter in vitro neutrophil oxidative burst. *Metabolism* **54**:300–305.
 39. Schenkein, H. A., H. M. Fletcher, M. Bodnar, and F. L. Macrina. 1995. Increased opsonization of a *prtH*-defective mutant of *Porphyromonas gingivalis* W83 is caused by reduced degradation of complement-derived opsonins. *J. Immunol.* **154**:5331–5337.
 40. Seelen, M. A., A. Roos, J. Wieslander, T. E. Mollnes, A. G. Sjöholm, R. Wurzner, M. Loos, F. Tedesco, R. B. Sim, P. Garred, E. Alexopoulos, M. W. Turner, and M. R. Daha. 2005. Functional analysis of the classical, alternative, and MBL pathways of the complement system: standardization and validation of a simple ELISA. *J. Immunol. Methods* **296**:187–198.
 41. Stevens, D. L. 1992. Invasive group A streptococcus infections. *Clin. Infect. Dis.* **14**:2–11.
 42. Stevens, D. L. 1999. The flesh-eating bacterium: what's next? *J. Infect. Dis.* **179**:S366–S374.
 43. Sumby, P., K. D. Barbican, D. J. Gardner, A. R. Whitney, D. M. Welty, R. D. Long, J. R. Bailey, M. J. Parnell, N. P. Hoe, G. G. Adams, F. R. DeLeo, and J. M. Musser. 2005. Extracellular deoxyribonuclease made by group A *Streptococcus* assists pathogenesis by enhancing evasion of the innate immune response. *Proc. Natl. Acad. Sci. USA* **102**:1679–1684.
 44. Tsao, N., W. H. Tsai, Y. S. Lin, W. J. Chuang, C. H. Wang, and C. F. Kuo. 2006. Streptococcal pyrogenic exotoxin B cleaves properdin and inhibits complement-mediated opsonophagocytosis. *Biochem. Biophys. Res. Commun.* **339**:779–784.
 45. von Pawel-Rammingen, U., and L. Björck. 2003. IdeS and SpeB: immunoglobulin-degrading cysteine proteinases of *Streptococcus pyogenes*. *Curr. Opin. Microbiol.* **6**:50–55.
 46. von Pawel-Rammingen, U., B. P. Johansson, and L. Björck. 2002. IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J.* **21**:1607–1615.
 47. Voyich, J. M., J. M. Musser, and F. R. DeLeo. 2004. *Streptococcus pyogenes* and human neutrophils: a paradigm for evasion of innate host defense by bacterial pathogens. *Microbes Infect.* **6**:1117–1123.
 48. Walport, M. J. 2001. Complement. First of two parts. *N. Engl. J. Med.* **344**:1058–1066.
 49. Walport, M. J. 2001. Complement. Second of two parts. *N. Engl. J. Med.* **344**:1140–1144.
 50. Wei, L., V. Pandiripally, E. Gregory, M. Clymer, and D. Cue. 2005. Impact of the SpeB protease on binding of the complement regulatory proteins factor H and factor H-like protein 1 by *Streptococcus pyogenes*. *Infect. Immun.* **73**:2040–2050.
 51. Wolf, B. B., C. A. Gibson, V. Kapur, I. M. Hussaini, J. M. Musser, and S. L. Gonias. 1994. Proteolytically active streptococcal pyrogenic exotoxin B cleaves monocytic cell urokinase receptor and releases an active fragment of the receptor from the cell surface. *J. Biol. Chem.* **269**:30682–30687.
 52. Wolf, H. M., J. W. Mannhalter, H. C. Salzmann, J. Göttlicher, R. Ahnrad, and M. M. Eibl. 1988. Phagocytosis of serum-opsonized zymosan down-regulates the expression of CR3 and FcRI in the membrane of human monocytes. *J. Immunol.* **141**:3537–3543.
 53. Yamazaki, T., M. Miyamoto, S. Yamada, K. Okuda, and K. Ishihara. 2006. Surface protease of *Treponema denticola* hydrolyzes C3 and influences function of polymorphonuclear leukocytes. *Microbes Infect.* **8**:1758–1763.

Editor: A. Camilli