Interaction between Group A Streptococci and the Plasmin(ogen) System Promotes Virulence in a Mouse Skin Infection Model

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Group A streptococci are capable of acquiring a surface-associated, unregulatable plasminogen-like enzymatic activity when incubated in human plasma. The effect of this enzymatic activity on virulence of group A isolate CS101 was examined in a mouse skin infection model. Initial studies demonstrated enhanced virulence for bacteria preincubated in human plasma but not in plasminogen-depleted plasma. A direct correlation between surface-associated enzymatic activity and virulence was not observed; however, an association between virulence and the assembly of a surface-associated plasminogen activator that could activate mouse plasminogen was noted. This activity enhanced virulence in wild type but not in pfg− mice. These results support the hypothesis that acquisition of a surface-associated plasminogen-dependent enzymatic activity can contribute to the virulence of group A streptococcal invasive infections.

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Materials and Methods

Bacteria. An M49 serotype group A streptococcal isolate, CS101, was used for all studies. This isolate is opacity factor-positive and has been extensively studied for the key gene products required for acquisition of plasminogen-dependent protease activity when incubated in human plasma [15]. Bacteria were grown overnight in Todd-Hewitt broth at 37°C as stationary cultures.

General reagents. Mouse plasma was purchased from Calbiochem (La Jolla, CA). Human plasma was obtained from the American Red Cross (Toledo, OH), and units from 3 separate donors were pooled. Mouse and human plasminogen were purified by passing an appropriate source of plasma over a lysine-Sepharose column (Pharmacia, Piscataway, NJ) and eluting bound plasminogen with 0.2 M 1-arginine caproic acid as described previously [22]. Plasminogen-free fibrinogen (Calbiochem) was further purified by passage over a lysine-Sepharose column to remove any trace of contaminating plasminogen. The synthetic plasmin-selective substrate, H-D-Val-Leu-Lys-nitroanilide (S-2251), was obtained from
Preparation of streptokinase. Recombinant group A streptokinase was cloned from group A streptococcal isolate CS101 and expressed in *Escherichia coli* DH5α as previously described [15]. The corresponding wild type form of streptokinase was purified from the media of an overnight culture of isolate CS101 grown in chemically defined medium (Hazleton Research Products, Lenexa, KS) at 37°C for 6–8 h as described previously [15]. The bacteria were then pelleted by centrifugation (9000 g for 10 min), and the bacteria-free supernatant was concentrated ~50-fold by use of a concentrator with a molecular cutoff of ~10,000 (Amicon, Beverly, MA). The supernatants were then filtered through 0.2-μm membranes and stored at −20°C for later use. A control preparation obtained by incubation of an isogenic mutant of isolate CS101, in which the *ska* gene was inactivated, was treated in an identical way and used as a control [15]. Streptokinase isolated from the culture fluid of a group C streptococcal isolate was purchased from Sigma.

Characterization of streptokinase preparations. Both recombinant and wild type group A streptokinase preparations were characterized functionally and antigenically. The functional assay measured the ability to generate plasmin from plasminogen in the fluid phase (see below). Antigenic analysis was done by separating preparations of streptokinase on 10% SDS-polyacrylamide gels, transferring to a nitrocellulose membrane by electroblotting, and then probing the blot with a human antiserum that contained specific antibodies to streptokinase. Any resulting specific antigen-antibody complexes were detected by enhanced chemiluminescent assay (Pierce, Rockford, IL) by use of a protein G–horseradish peroxidase probe.

Measurement of plasminogen activation. The activation of mouse or human plasminogen by streptokinase or urokinase was carried out in the fluid phase as described previously [23, 24]. Briefly, 100-μL aliquots of different concentrations of purified human or mouse plasminogen were incubated with differing concentrations of streptokinase or urokinase (200 Plough units) and mixed with 100 μL of S-2251 in PBS (0.01 M, pH 7.6) to yield a final concentration of 400 μM substrate. The conversion of plasminogen to plasmin was measured in a microplate reader (Bio-Kinetics EL-312; Bio-Tek, Winooski, VT) by the hydrolysis of S-2251 as measured by change in absorbance at 405 nm (A405) over time at 37°C. This assay demonstrates linearity for both human and mouse plasmin to an A405 of at least 1.25; by use of urokinase as the plasminogen activator, no significant differences in the ability to quantify human and mouse plasmin were noted.

Assay for acquisition of bacterial surface-associated protease activity. Surface-associated plasmin-like protease activity was measured as previously described [25]. Briefly, 10⁷ cfu of bacteria were incubated with different combinations of human plasma or purified fibrinogen or plasminogen with or without exogenous streptokinase at 37°C for the times stated. The bacteria were pelleted by centrifugation and washed three times with PBS before the bacterial
pellet was resuspended in 400 μL of the plasmin-selective substrate S-2251 (400 μM) and incubated for a fixed time at 37°C. The cleavage of S-2251 was then measured by measuring the A405 of 100 μL of the bacterium-free supernatant in a microplate reader.

Measurement of bacteria-associated plasminogen-activator activity. Bacteria were pretreated with different combinations of reagents as described above to generate a surface-associated protease activity. The cells were pelleted, washed three times with PBS, and then incubated with either mouse or human plasma (10%) or mouse or human plasminogen (10 μg/mL) in 400 μL of PBS buffer at 37°C for 30 min. The bacteria were then pelleted, 100 μL of the bacteria-free supernatants were incubated with S-2251 at 37°C, and A405 was monitored over time. Plasminogen activation was determined by hydrolysis of the plasmin-selective substrate, S-2251, as described above.

Mouse model of skin infection. The mouse model of skin infection was as described previously [20, 21]. Female CD1 outbred mice (6–8 weeks old, 20–24 g in weight) were obtained from Charles River Laboratories (Portage, MI). For these experiments, bacteria were pretreated before injection into the mice as follows: Isolate CS101, grown in 300 mL of Todd-Hewitt broth, was incubated at 37°C as a stationary culture for 14–16 h. Bacteria were then pelleted (9000 g for 20 min at 4°C) and washed twice with sterile PBS (0.01 M, pH 7.6). The bacteria were then resuspended in PBS and adjusted to a concentration equivalent to 10^8 cfu/mL by adjusting the A405 with a previously determined nonagram. The bacterial suspension was aliquoted, and samples were incubated with different combinations of reagents for 30 min at 37°C. After this pretreatment, bacteria were pelleted by centrifugation at 5000 g for 15 min at 4°C, washed three times with PBS buffer, and then resuspended to the original volume in PBS. The pretreated bacteria were injected into a mouse skin air sac as described previously [21]. Essentially, each mouse was injected intradermally with 0.1 mL of the bacterium-free supernatant in a microplate reader.

In the initial experiments, isolate CS101 was incubated at 37°C for 30 min with either buffer, 30% human plasma, or 30% plasminogen-depleted human plasma with or without exogenous streptokinase (100 U/mL). At that time, the bacteria were extensively washed and resuspended in sterile PBS buffer (0.01 M, pH 7.6), and a 10^6 cfu aliquot was injected into a skin air sac. Each experimental group consisted of 10 outbred CD1 mice. An aliquot of bacteria (10^6 cfu) from each pretreatment group was also tested for surface-associated plasmin-like enzymatic activity by measurement of hydrolysis of the plasmin-selective synthetic substrate, S-2251, as described in Materials and Methods.

As shown in figure 2A, there were marked differences in the amount of surface-associated enzymatic activity observed among the bacteria pretreated under varying conditions. After 30 min of incubation at 37°C, streptococci incubated in human plasma plus exogenous streptokinase had acquired the highest surface-associated enzymatic activity, determined by cleavage of substrate S-2251, whereas other groups showed dramatically lower or nondetectable surface-associated enzymatic activity.

The effect of the in vitro pretreatment on the virulence of group A isolate CS101 injected into a skin air sac is shown for a representative experiment in figure 2B. Analysis of the data indicates that a statistically significant difference (P < .05) was observed between bacteria preincubated in plasma versus bacteria preincubated with either PBS or plasminogen-depleted plasma. These results suggested that a plasminogen-dependent activity acquired during the in vitro preincubation phase could enhance the virulence potential of the bacteria. If exogenous streptokinase was added to the preincubation mixture, a significant enhancement of virulence was observed for streptococci preincubated either with human plasma plus streptokinase (P < .01) or, surprisingly, with plasminogen-depleted plasma plus streptokinase (P < .01) compared with virulence of bacteria preincubated with either PBS alone, streptokinase alone, or plasminogen-depleted plasma alone (figure 2B).

A comparison between surface-associated enzymatic activity (figure 2A) and virulence failed to demonstrate any clear correlation. Significant enzymatic activity was observed only for the experimental group preincubated with plasma and exogenous streptokinase. A low level of activity was observed for the group incubated in plasminogen-depleted plasma plus strep-
Figure 2. Effect of pretreating bacteria with different combinations of buffer, human plasma, or plasminogen-depleted plasma with or without streptokinase on their ability to cleave plasmin-selective synthetic substrate S2251 (A) or to establish lethal infection in mouse skin infection model (B). A: Measurement of cell-associated plasmin-like enzymatic activity for bacteria pretreated with either human plasma, plasminogen-depleted human plasma, or PBS, with or without exogenous streptokinase (100 U/mL). Bacteria were washed and cell-associated enzymatic activity was determined by measuring hydrolysis of plasmin-selective synthetic substrate, H-D-Val-Leu-Lys-para-nitroanilide (S-2251). SK, streptokinase; PLM, human plasma; PLG-D, plasminogen-depleted human plasma; OD, optical density. All experiments were done in duplicate, and <5% variation was noted between samples. B: Comparison of virulence of bacteria pretreated with either human plasma, plasminogen-depleted human plasma, or PBS, with or without exogenous streptokinase (100 U/mL) before injection into skin air sac. SK, streptokinase; PLM, human plasma; PLG-D, plasminogen-depleted human plasma. Bacteria were pretreated, washed, and injected at 10⁸ cfu into mouse skin air sac in each of 10 outbred CD1 mice. Mice were monitored for time to death. Comparison of data for significant differences between groups was done with StatView SE+ Graphics. Statistically significant difference was observed between groups injected with bacteria preincubated in human plasma (P < .05) and bacteria preincubated in either PBS, streptokinase alone, or plasminogen-depleted plasma. Bacteria preincubated with either human plasma containing exogenous streptokinase or human plasminogen-depleted plasma containing exogenous streptokinase were more virulent (P < .01) than were bacteria pretreated with PBS alone, streptokinase alone, or plasminogen-depleted plasma alone.

tokinase, indicating that the plasminogen depletion process was not 100% efficient.

This experiment has been repeated, and a similar pattern of enhanced virulence was noted for bacteria preincubated in human plasma with or without added streptokinase or for bacteria preincubated with human plasminogen-depleted plasma plus streptokinase, but not in the absence of the plasminogen activator (data not shown).

The failure to demonstrate a direct association between enzymatic activity acquired during the in vitro pretreatment step and the pattern of infection suggests that either the property was not important or that a low threshold level was sufficient either to initiate invasion or to facilitate cooperation with factors present at the site of infection to potentiate the virulence of the treated organisms.

Our laboratory has demonstrated that S. pyogenes incubated in human plasma can acquire a surface plasminogen-activator activity [16, 17]. It is possible that this surface-associated enzymatic complex could activate mouse plasminogen and that the secondary activation of the mouse plasminogen system could influence the course of infection. To test this hypothesis, bacterial isolate CS101 was first incubated with human plasma or plasminogen-depleted human plasma with or without exogenous streptokinase at 37°C for 30 min under the conditions described above. The bacteria were then washed extensively to remove fluid-phase plasma proteins and streptokinase. The washed bacterial pellet was resuspended in buffer containing mouse plasminogen (10 μg/mL) and incubated for 30 min at 37°C. The bacteria-free supernatant was harvested and incubated with the plasmin-selective synthetic substrate S-2251 to monitor activation of mouse plasminogen.

The results presented in figure 3 demonstrate that bacteria preincubated in human plasma with exogenous streptokinase acquired a surface plasminogen-activator activity capable of converting mouse plasminogen to plasmin, whereas bacteria preincubated in plasminogen-depleted plasma without streptokinase or bacteria incubated with streptokinase alone failed to acquire a surface mouse plasminogen-activator activity. Interestingly, bacteria incubated in plasminogen-depleted plasma with streptokinase did acquire a surface plasminogen-activator activity that was capable of activating mouse plasminogen (figure 3). A low level of plasminogen-activator activity associated with bacteria preincubated with human plasma but not plasminogen-depleted plasma in the absence of exogenous streptokinase was detectable when the assay described in figure 3 was continued over a 5-h incubation period (data not shown).
Figure 3. Kinetics of activation of mouse plasminogen by surface-associated plasminogen-activator complex. Bacteria were incubated with various combinations of human plasma or plasminogen-depleted plasma with or without added streptokinase. After incubation, bacteria were washed and tested for their ability to convert mouse plasminogen (10 μg/mL) to plasmin. Plasmin activity was quantified by hydrolysis of plasmin-selective synthetic substrate H-D-Val-Leu-Lys-paranitroanilide (S-2251). SK, streptokinase; PLM, human plasma; PLG-D, plasminogen-depleted human plasma.

If mouse plasminogen-activator activity was important in enhancing virulence and this activator could be assembled when a low level of human plasminogen (<1 μg/mL) was present, these findings would explain the unexpected results (figure 2) obtained using plasminogen-depleted plasma. To test this hypothesis, the ability to assemble a surface mouse plasminogen-activator complex using purified human proteins was tested. In preliminary studies, a surface complex capable of activating mouse plasminogen could be assembled and demonstrated an absolute requirement for fibrinogen, plasminogen, and streptokinase (data not shown).

In the next series of studies, bacteria incubated with different combinations of human plasminogen, fibrinogen, and/or streptokinase were tested for their ability to enhance virulence in the mouse skin air sac model. Bacteria preincubated with either 50 μg/mL fibrinogen or 10 μg/mL plasminogen with or without 5 U of streptokinase for 30 min at 37°C were compared for virulence when injected into a skin air sac on groups of 10 outbred CD1 mice. These conditions were selected from preliminary studies to determine the minimal quantity of streptokinase required to assemble a mouse plasminogen-activator complex on the pretreated bacteria. The results presented in figure 4A demonstrate that bacteria preincubated with human fibrinogen, plasminogen, and streptokinase were significantly more invasive than untreated bacteria (P < .01). Preincubation of bacteria with any combination of two of the three reactants failed to result in any significant change in virulence when tested in the mouse skin infection model (figure 4A). Aliquots of bacteria, pretreated with each combination of reactants, were also tested for their ability to activate mouse plasminogen. As shown in figure 4B, only bacteria incubated with fibrinogen, plasminogen, and streptokinase demonstrated a surface-associated plasminogen-activator activity capable of generating plasmin from mouse plasminogen.

These experiments were done with wild type streptokinase isolated from a group C streptococcal isolate as well as recombinant streptokinase cloned from group A isolate CS101 and expressed in E. coli. Similar results were obtained in all experiments, and a significant effect on virulence was observed only.
for organisms pretreated with fibrinogen, streptokinase, and plasminogen. This was also the only combination of reagents that resulted in formation of a cell-associated complex capable of activating mouse plasminogen (data not shown).

Taken together, all of the experiments presented thus far suggest that the ability of group A streptococci to activate plasminogen in the skin of an infected host would enhance their ability to disseminate and cause an invasive infection. These results would predict that the ability of group A streptococcal isolate CS101 with surface plasminogen-activator activity would be significantly less virulent in plg\(^{-/-}\) transgenic mice lacking plasminogen than it would be in their wild type plg\(^{+/+}\) littermates. In preliminary studies, significant differences in LD\(_{50}\)s of isolate CS101 for plg\(^{-/-}\) and plg\(^{+/+}\) littermates were found. For plg\(^{-/-}\) mice, the LD\(_{50}\) was 10\(^6\) cfu, whereas the wild type littermates showed an LD\(_{50}\) of \(\sim 10^7\) cfu (data not shown). This difference was not unexpected, since age-matched plg\(^{-/-}\) mice were smaller and appeared runted compared with their wild type littermates, and plg\(^{-/-}\) mice are also known to demonstrate differences in inflammatory responses [26, 29].

Consequently, to test the effects of surface plasminogen-activator activity on invasive potential, we compared the virulence of a 10\(^7\)-cfu inoculum of CS101 preincubated with human plasma and exogenous streptokinase in virulence in either wild type or plg\(^{-/-}\) transgenic mice. This challenge dose was the LD\(_{50}\) for plg\(^{+/+}\) mice, whereas this inoculum failed to kill any wild type mice in preliminary studies. The rationale for this experiment was that if plasminogen activation contributed to invasive potential, then there would be no expected enhancement of virulence in plg\(^{-/-}\) mice, whereas a more invasive infection in plg\(^{+/+}\) mice would be predicted. The results presented in table 1 demonstrate that the presence of plasminogen-activator activity on the surface of isolate CS101 resulting in 3 of 6 plg\(^{-/-}\) mice dying during the time course of the experiment. This result was expected on the basis of using a challenge dose at the LD\(_{50}\) for the plg\(^{-/-}\) mice. When these pretreated bacteria were injected into the skin of normal wild type littermates, death of 4 of 6 was observed (table 1). This result is in marked contrast to injection of untreated bacteria, which failed to kill any wild type mice at this dose. The pretreated bacteria were capable of activating mouse plasminogen (data not shown).

At the end of the experiment, all surviving mice were euthanized and their spleens were cultured. Viable streptococci were recovered from each of the 2 surviving plg\(^{+/+}\) mice, whereas none of the surviving plg\(^{-/-}\) mice had detectable bacteria in their spleens. These results indicate that the presence of an intact plasminogen system in the infected host can influence the pattern of infection.

**Discussion**

The ability of a number of invasive human pathogens to interact with the host plasminogen system has been suggested as a potential common virulence strategy to enable organisms to penetrate tissue barriers [12, 13]. Group A streptococci have been shown to be capable of a highly sophisticated interaction with the human plasminogen system. Group A organisms incubated in human plasma acquire a surface plasminogen-activator activity in a multistep process (see figure 1). Initially, fibrinogen binds to a surface fibrinogen-binding protein to provide a site for anchoring a streptokinase-plasminogen complex. This can be achieved with either endogenously produced or exogenously provided streptokinase [11, 14, 15, 17].

The surface enzymatic complex assembled in human plasma has intrinsic enzymatic activity as well as plasminogen-activator potential for both human and mouse plasminogen. Furthermore, the surface plasminogen-activator complex is stable in human plasma at 37°C and can also convert plasminogen in plasma to plasmin, which can bind to surface plasmin-binding structures, despite the presence of α2-antiplasmin and other serpins in the reaction mixture [14, 16, 17]. This highly sophisticated mechanism for acquiring an unregulatable host enzymatic activity with broad substrate specificity has been postulated as a potential mechanism by which group A streptococci could acquire invasive potential [12, 13].

To test this prediction, we have used an established mouse model of group A streptococcal skin infection [20, 21]. Since, however, streptokinase from *S. pyogenes* isolates fails to activate mouse plasminogen directly in the fluid phase, the predictions of this hypothesis cannot be tested directly in a mouse model. Consequently, in the initial experiments, a two-stage strategy was used. In the first in vitro stage, bacteria were preincubated with human plasma or plasminogen-depleted plasma in the presence or absence of exogenous streptokinase to enable assembly of a surface enzymatic complex. The treated bacteria were then washed free of unbound human proteins and tested for effects on virulence in a second in vivo stage.

The initial studies demonstrated that the presence of plasminogen in the preincubation reaction had a significant effect on virulence of group A streptococci when injected into a skin air sac (figure 2). Bacteria preincubated in plasma were signif-
icantly more virulent than were those preincubated in plasminogen-depleted plasma ($P < .05$) or buffer alone ($P < .05$). In parallel studies, significantly enhanced virulence compared with that of untreated bacteria was noted for bacteria preincubated with either plasma ($P < .01$) or plasminogen-depleted plasma ($P < .01$) to which exogenous streptokinase was added. Bacteria preincubated with streptokinase alone had no significant effect ($P > .05$).

Initial attempts to correlate the absolute quantity of surface enzymatic activity acquired in vitro with virulence measured in vivo failed to demonstrate a direct association. In addition, the results obtained with bacteria incubated with plasminogen-depleted plasma to which exogenous streptokinase was added were particularly interesting. These bacteria demonstrate significant enhanced virulence ($P < .01$) compared with untreated bacteria and also a low but detectable level of enzymatic activity (figure 3). Because of the low levels of enzymatic activity detected under the experimental conditions used, a potential cooperation between the enzymatic activity and host factors in the mouse was tested.

Although streptokinase from *S. pyogenes* isolates incubated in the fluid phase with mouse plasminogen failed to generate plasmin, we found that bacteria preincubated with human plasma could assemble a surface plasminogen-activator complex that could activate mouse plasminogen (figure 3). This activator activity was detected for bacteria incubated with either human plasma or plasminogen-depleted plasma to which exogenous streptokinase was added. In studies using purified fibrinogen, plasminogen, and exogenous streptokinase, we have confirmed that all three reactants are required to assemble a bacteria-associated plasminogen-activator activity that can activate mouse plasminogen. These studies have also demonstrated that this activity can be assembled when trace levels of plasminogen (0.1 µg/mL) are present, provided a source of fibrinogen and exogenous streptokinase is also added (data not shown). Bacteria with a surface plasminogen-activator activity generated by incubation with purified fibrinogen, plasminogen, and streptokinase were also significantly more virulent in the mouse skin infection model.

Since the activator complex can be assembled with trace levels of plasminogen, this could occur at many sites of infection. In addition, the studies of bacteria preincubated in human plasma without added streptokinase for 30 min at 37°C demonstrated that a low level of mouse plasminogen–activator activity could be assembled, which correlated with enhanced virulence under circumstances in which only low levels of streptokinase would be produced. These findings suggest that group A streptococci are highly efficient in acquiring plasminogen-dependent activities that contribute to virulence in the mouse.

Recently, transgenic mice in which the plasminogen gene was inactivated [26, 29] have been developed. These mice have proved to be of great value in analyzing the roles of plasminogen in various biologic processes [29–33]. There are significant differences in the size and general health of $\text{plg}^{-/-}$ mice and their wild type littermates. In general, $\text{plg}^{-/-}$ mice appear runted, and in this study we found a significant difference in their susceptibility to group A isolate CS101. The LD$_{50}$ for the wild type littermates was 10$^5$ cfu, whereas the LD$_{50}$ for the $\text{plg}^{-/-}$ mice was 10$^7$ cfu. This difference makes direct comparisons between wild type and $\text{plg}^{-/-}$ mice for susceptibility to infection complex. However, it is possible to assess whether the ability to activate mouse plasminogen enhances virulence. In this study, we have found that the presence of a mouse plasminogen–activator complex on the surface of isolate CS101 enhances the virulence of the organism in wild type mice, while having no detectable effect on the $\text{plg}^{-/-}$ group (table 1).

Evidence for a role for the plasminogen system in invasive infections has also been suggested in other infectious models. In a mouse model for Lyme disease, Coleman et al. [34] have shown the importance of plasminogen in the dissemination of the parasite from the tick vector. In related studies, Sodeinde et al. [35] and Gougen et al. [36], using a skin model of *Yersinia pestis* infection, have also provided evidence for the importance of plasminogen activation in invasion. Other studies by Gyetko et al. [37], using a model of *Cryptococcus neoformans* lung infection, have also suggested a role for the urokinase plasminogen-activator system for successful host defense against this pathogen in a lung model. Taken together, these studies suggest that pathogens capable of activating plasminogen in the infected host or capable of acquiring host plasmin by some other mechanism may have a selective advantage for crossing tissue barriers and causing disseminated infections.

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