Vascular Dysfunction and Ischemic Destruction of Tissue in *Streptococcus pyogenes* Infection: The Role of Streptolysin O–Induced Platelet/Neutrophil Complexes

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Rapid tissue destruction in group A streptococcal (GAS) necrotizing fasciitis/myonecrosis often necessitates extensive debridement to ensure survival. The mechanisms responsible for this fulminant process remain unknown; we hypothesized that toxin-induced ischemia contributes to necrosis. In a rat model, Doppler flowmetry was used to measure local blood flow at the site of the intramuscular injection of exotoxins from an invasive M-type 1 GAS, which caused a rapid, dose-dependent decrease in perfusion that was irreversible at the highest toxin concentration tested. Videomicroscopic results revealed that blood flow was impeded by occlusive intravascular cellular aggregates. Flow-cytometric results confirmed that GAS toxins induced the coaggregation of platelets and neutrophils, that this activity was attributable to streptolysin O, and that platelet/neutrophil complex formation was largely mediated by platelet P-selectin (CD62P). Strategies that target platelet adherence molecules may prevent vascular occlusion, maintain tissue viability, and reduce the need for amputation in necrotizing GAS infections.

Streptococcal toxic shock syndrome (STSS) is a severe, invasive group A streptococcal (GAS) infection associated with the sudden onset of shock, acute respiratory distress syndrome, renal failure, bacteremia, and death [1, 2]. Despite better clinical recognition of this fulminant infection and intense research on streptococcal virulence factors, morbidity is high, and mortality remains between 30% and 70% [1, 2]. Fifty percent of patients with STSS develop necrotizing fasciitis or myonecrosis, which progress rapidly and require emergency amputation or extensive surgical debridement to ensure survival [1, 2]. In fact, a recent article in the *American Journal of Surgery* suggested that the best therapy remains a single radical debridement in cases of severe GAS soft-tissue infection [3]. This fulminant infection is also characterized by excruciating pain at the site of infection [1, 2], the onset of which occurs well before the manifestation of shock, renal impairment, or acute respiratory distress syndrome.

The mechanisms responsible for the early onset of severe pain and the rapid regional destruction of tissues in these infections have not been elucidated. Many investigators have suggested that these findings are simply the consequence of the digestion of tissues by bacterial proteases. However, we hypothesize that they are the result of the host response to GAS virulence factors. Specifically, we suggest that bacterial toxins subvert the physiological mechanisms controlling hemostasis, such that microvascular thrombosis develops, which leads to reduced tissue perfusion, hypoxia, and subsequent re-
Figure 1. Intravascular thrombosis in streptococcal toxic shock syndrome (STSS). Tissues obtained at autopsy from a patient with STSS and myonecrosis demonstrated numerous platelet/leukocyte thrombi that were not attached to the vascular endothelium (arrow).

Regional tissue necrosis. Clinical and experimental observations support this concept. First, the speed with which skin, subcutaneous tissue, fascia, and muscle are destroyed in these infections is similar to the rate of tissue death following acute arterial thrombosis. Second, intense pain is a prominent feature in clinical conditions that involve occlusion of the arterial blood supply, such as myocardial infarction. Third, tissues destroyed in these infections do not bleed. So characteristic is this latter finding that surgeons routinely extend debridement until bleeding is encountered. Last, histologic examination of necrotic tissues obtained from patients with STSS at biopsy or amputation [1, 2] or from experimental animals challenged with GAS [4, 5] has revealed platelet thrombi and fibrin clots in capillaries, postcapillary venules, and arterioles of the affected musculature and soft tissues.

Recent studies in our laboratory and in others have demonstrated a role of platelet/polymorphonuclear leukocyte (PMNL) complexes in the ischemic necrosis of tissue [6–10]. We hypothesized that extracellular toxins from GAS stimulate the formation of these heterotypic cellular complexes and that, in vivo, these large aggregates contribute to microvascular thrombosis, reduced tissue perfusion, hypoxia, and subsequent regional tissue necrosis. Thus, we investigated the ability of extracellular toxins from GAS to reduce regional skeletal-muscle perfusion in vivo and to elicit platelet/PMNL complex formation in vitro. Results demonstrate that streptococcal exotoxins dose dependently reduce skeletal-muscle blood flow and that streptolysin O (SLO) mediates platelet/PMNL complex formation in a P-selectin–dependent manner.

MATERIALS AND METHODS

Histopathologic examination of human GAS necrotizing infection. Histopathologic slides of tissues from a patient who died of GAS necrotizing myonecrosis and STSS were sent by the patient’s physician to one of the study investigators (D.L.S.) for interpretation and analysis. Sections were photographed by use of a Zeiss Axiophot microscope. For studies involving human subjects, signed, informed consent was obtained from volunteers, and the studies followed the human-experimentation guidelines of the US Department of Health and Human Services and those of the Human Subjects Division, University of Washington, Seattle.

Bacterial toxins and toxin-neutralizing antibody. M-type 1 GAS strain 96-004 was used in the study. This well-characterized strain has been previously reported to produce SLO, streptococcal pyrogenic exotoxins A and B, and nicotinic adenine dinucleotidase [11], and it is genetically representative of...
Figure 2. Reduction in skeletal-muscle perfusion by streptococcal toxins. Skeletal-muscle blood flow in the abdominal musculature of rats was measured by a laser Doppler blood perfusion monitor, as described in Materials and Methods. Normal saline (NS) or toxin from invasive M-type 1 group A streptococcus (strain 96-004) diluted to contain 9, 6, or 3 hemolytic units (HU) of streptolysin O activity \( (\beta = 6/\text{group}) \) was injected intramuscularly, and blood flow was measured for 50 min. Blood-flow readings in a given rat are expressed as the percentage of baseline blood flow for that rat. * \( P < .05 \), analysis of variance with Tukey-Fisher test.

M-type 1 strains that cause serious invasive disease in the United States and elsewhere. Exotoxins from this strain were prepared by 80% ammonium sulfate fractionation of a log-phase culture grown in Todd-Hewitt broth by use of standard techniques. The recovered proteins were exhaustively dialyzed against endotoxin-free water and were frozen at \(-70^\circ C\) until use. This material routinely contained 270 hemolytic units (HU) of SLO activity/mg of protein, as determined by sheep red blood cell hemolysis assays [12]. Similarly, toxins were prepared from an SLO-deficient mutant strain of 96-004 (96004\(\Delta\)slo) constructed in our laboratory by homologous recombination by use of techniques described elsewhere [11]. Recombinant SLO was obtained from Dr. Michael Kehoe (University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom) and had a specific activity of 800 HU/mg [13].

The neutralizing anti-SLO antibody, 3H10 (a murine IgG1) [14] used in the present study was a gift from Dr. Hiroko Sato (National Institutes of Health, Tokyo, Japan). A murine isotype-matched control antibody was obtained from Sigma. F(ab\('\)) fragments of both the control antibody and the anti-SLO antibody 3H10 were obtained by papain digestion of the intact antibodies according to standard techniques. Briefly, immunopurified 3H10 or control IgG was mixed with papain (10 \( \mu \)g of papain/mg of IgG; Sigma) in 0.1 mol/L sodium acetate buffer (pH 5.5) that contained 20 mmol/L EDTA and 100 mmol/L cysteine; this mixture was incubated for 24 h at 37\(^\circ\)C. Iodoacetamide (75 mmol/L) was added to stop the reaction. Fragments of immunoglobulin were separated on immobilized protein A (Pierce) according to the manufacturer’s recommendations and then were dialyzed extensively against Dulbecco’s PBS. F(ab\('\)) fragments prepared in this way yielded a single band at 50 kDa by SDS-PAGE with Coomassie blue staining.

**Blood-flow measurements.** Experiments involving rats were approved by the Animals Subjects Committee, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California. Male Sprague-Dawley rats (weight, 225–250 g) were anesthetized with an intraperitoneal injection of urethane (1.25 g/kg). A carotid artery was cannulated for monitoring blood pressure. A tracheostomy was performed so that a patent airway could be maintained. Skeletal-muscle blood flow in the abdominal musculature was measured by the laser Doppler blood perfusion monitor (LASERFLO BRM; Vasamedics), as described elsewhere [6]. Briefly, an incision was made on the skin, and the flow probe was placed directly on the abdominal skeletal muscle. A period of 20–30 min was allowed for baseline blood flow to stabilize. One hundred microliters of either normal saline or the GAS toxin preparation—diluted to contain 9, 6, or 3 HU of SLO activity \( (\beta = 6/\text{group}) \)—was then injected into the muscle immediately below the center of the probe, where the blood flow was measured for an additional 50 min. In a separate group of rats \( (n = 3) \), the vasoconstrictor phentolamine (10 \( \mu \)mol/L) was used as a positive control. Laser Doppler blood-flow readings in a given rat were expressed as the percentage of baseline blood flow for that rat (i.e., the posttreatment blood-flow measurement at each time point was divided by the pretreatment value and then multiplied by 100).
Figure 3. Induction of heterotypic aggregates of platelets and polymorphonuclear leukocytes (PMNLs) by streptococcal toxins. Platelet/PMNL aggregate formation in whole blood was investigated by dual-color flow cytometry by use of cell-surface markers specific for each cell type. Whole blood was stimulated with PBS, streptococcal toxins from M-type 1 group A streptococcus strain 96004 that contained 2.7 hemolytic units of streptolysin O (SLO) activity (GAS), and streptococcal toxins pretreated with either 10 μg/mL anti-SLO monoclonal antibody (GAS + 3H10) or isotype-matched control antibody (GAS + IgG). PMNLs were gated by characteristic forward and side scatter and by red fluorescent (phycoerythrin [PE]-CD11b) staining (X-axis). Platelet events were followed by green fluorescent (fluorescein isothiocyanate [FITC]–CD42b) staining (Y-axis). PMNLs binding to platelet appear in quadrant 2 as dual-color events. The histograms shown in panel A are from 1 representative experiment of 4, each of which was performed in triplicate, by use of blood from 3 different donors. The quantitative data for these 4 experiments (means ± SDs) are shown in panel B.

Repeated-measures analysis of variance (SAS Procedure MIXED; SAS) was used to compare the means of the different groups of rats. The Tukey-Fisher least significant difference criterion was used to compute post hoc Student’s t tests for pairwise comparisons. P < .05 was considered to be statistically significant. The progression of aggregate formation and vascular occlusion was also visualized in separate groups of rats by videomicroscopy, as described elsewhere [9].

Flow cytometry. Flow-cytometric analyses of platelet/PMNL complex formation was performed as described elsewhere [10]. Briefly, after signed, informed consent was obtained as outlined by the Human Subjects Committee, University of Washington, citrated (or heparinized, where indicated) whole blood was collected by venipuncture from fasting, healthy human volunteers who had not received any medications for the previous 10 days and who had platelet counts of 210–265,000 cells/μL. Blood was drawn with a 19-gauge needle without the aid of a tourniquet, and the first 2 mL was discarded. Whole blood (100 μL) was mixed with 10 μL of fluorescein isothiocyanate–conjugated anti–human CD42b (a pan platelet marker; Pharmingen) and phycoerythrin–conjugated anti–human CD11b (a granulocyte marker; Pharmingen) for 10 min at 37°C. Blood was stimulated with 10 μL of PBS, dilutions of the streptococcal toxin preparations, or recombinant SLO (0.001–4.0 HU/mL). After 10 min at 37°C, samples were immediately prepared for flow-cytometric analysis by use of a commercial formic-acid red cell lysis/formalin cell-fixation procedure (Q-prep; Coulter), according to the manufacturer’s recommendations. Flow-cytometric analysis was performed on a Coulter Epics flow cytometer. The granulocyte gate was drawn on the basis of the
characteristic forward- and side-scatter profiles of this population. CD11b-positive events (i.e., red fluorescence) within this gate (routinely >98%) were analyzed for the percentage of platelet CD42b positivity (i.e., green fluorescence) and the mean fluorescence intensity of the CD42b signal.

To investigate the role of SLO in the induction of platelet/PMNL complexes by crude streptococcal toxin preparations, toxins were preincubated for 10 min at 37°C with F(ab)’ fragments of either control IgG, or the neutralizing monoclonal antibody (MAb) anti–SLO 3H10, at a final concentration of 10 μg/mL or with low-density lipoprotein (LDL) cholesterol (final concentration, 4 mg/mL) or its vehicle (15 mmol/L NaCl and 0.001% EDTA) before their addition to the whole blood specimen. In the latter experiment, recombinant phospholipase C from Clostridium perfringens was used as a cholesterol-insensitive control agonist to stimulate complex formation [9, 10]. Papain-digested antibodies were used in these studies because initial experiments had determined that the use of intact IgG gave highly inconsistent results, likely due to the activation of Fc receptors by streptococcal toxins. Analysis of complex formation was performed as described above.

To investigate the cell-surface receptor/ligand interactions mediating toxin-induced platelet/PMNL complex formation, whole blood was pretreated for 10 min with either a neutralizing antibody against the platelet fibrinogen receptor CD41a (clone HIP-8, murine IgG1, final concentration, 20 μg/mL; Pharmingen) or with a blocking MAb against platelet P-selectin (clone AK-1, murine IgG1, final concentration, 20 μg/mL; Pharmingen). An isotype-matched IgG control antibody at the same concentration was included as a negative control.

RESULTS

Histopathologic analysis of a human case of GAS myonecrosis. Histopathologic slides of tissues obtained at autopsy from a patient meeting the criteria for a diagnosis of STSS with myonecrosis were sent to one of the study investigators (D.L.S.) for interpretation and analysis. Vessels within infected tissues were remarkable for the presence of intravascular thrombi (figure 1, arrow) that consisted of platelets, leukocytes, and fibrin. Many such aggregates were often not attached to the vascular endothelium (figure 1, arrow); when they were of sufficient size, they completely occluded the vessels.

Skeletal-muscle perfusion studies. Intramuscular (im) injection of crude GAS toxins into rats resulted in a dose-dependent reduction in skeletal-muscle blood flow (figure 2). At 10 min after the injection of toxin, perfusion was reduced by ~0%, ~30%, and ~50% in rats injected with toxin that contained 3, 6, and 9 HU of SLO activity, respectively. At the highest concentration of streptococcal toxin tested (9 HU), regional perfusion was reduced by 40% within 6 min. By 10 min, perfusion in this group reached a nadir of 50% of control values; it remained suppressed for the entire 50-min period of study. Similarly, in rats injected with streptococcal toxin that con-
Figure 5. Induction of platelet/polymorphonuclear leukocyte (PMNL) complex formation by recombinant streptolysin O (rSLO). Whole blood was stimulated with increasing concentrations of rSLO. The percentage of PMNL-binding platelets and the surface expression of PMNL CD11b are shown. Data are means (±SDs) from 1 representative experiment of 3 performed in duplicate. HU, hemolytic units.

Streptococcal toxin–induced platelet/PMNL aggregate formation in vitro was investigated by dual-color flow-cytometric analysis. A typical histogram of unstimulated cells is shown in figure 3A (cells in PBS). In this instance, the CD11b-positive PMNL population is clearly distinguished and is largely devoid of adherent platelets, as indicated by the low level of dual-color positivity in this population. In contrast, streptococcal exotoxins stimulated a dramatic shift in the percentage of PMNLs binding platelets (GAS 96004; figure 3A). Pretreatment of the streptococcal toxin preparation with neutralizing MAb against SLO [3H10 F(ab)′2] completely inhibited the toxin-induced platelet/PMNL complex formation (96004 + 3H10; figure 3A), whereas the control antibody was without effect (96004 + IgG; figure 3A). Interestingly, the streptococcal toxin preparation also increased neutrophil CD11b expression—a feature that was only partially inhibited by 3H10. Quantitative measurements of GAS toxin–induced platelet/PMNL complexes and the mean fluorescence intensity of the platelet marker are depicted in figure 3B.

The above results suggested a role of SLO in the induction of platelet/PMNL complex formation. To confirm this, 3 strategies were used. First, because SLO is a cholesterol-binding cytolysin [12], the streptococcal toxin preparation was preincubated with LDL cholesterol before the addition of the toxins to the blood specimen. Cholesterol treatment reduced streptococcal complex formation to background levels but had no effect on complex formation induced by recombinant phospholipase C from C. perfringens (data not shown). Second, platelet/PMNL complex formation induced by toxins from an SLO-deficient mutant of strain 96-004 was investi-
Figure 6. P-selectin mediation of group A streptococcus (GAS) toxin–induced platelet/polymorphonuclear leukocyte (PMNL) complex formation. Whole blood was preincubated with nothing (None), a blocking monoclonal antibody (MAb) against gpIIbIIIa (xCD41a), a blocking MAb against P-selectin (xCD62P), an isotype-matched control antibody (IgG control), or a combination of the anti-gpIIbIIIa and anti–P-selectin autoodies (xCD41a/CD62P). Blood was stimulated with toxins from GAS strain 96-004, and platelet/PMNL complexes were measured by flow cytometry. Data are means (± SDs) of 4 experiments performed in duplicate.

Adherence molecules mediating SLO-induced platelet/PMNL complex formation. Pretreatment of the whole blood with blocking anti–P-selectin antibody (anti-CD62P) dramatically reduced streptococcal toxin–induced complex formation (figure 6), whereas the antibody targeting the platelet fibrinogen receptor gpIIbIIIa (anti-CD41a) had a lesser effect (figure 6). The combination of blocking antibodies did not further reduce complex formation, compared with the anti–P-selectin strategy alone (figure 6).

DISCUSSION

Severe, necrotizing deep soft-tissue infections caused by GAS have reemerged worldwide. In many cases, these life-threatening infections occur in otherwise healthy individuals, and the onset is so swift and the necrosis so extensive that emergency surgery, which often includes multiple amputations, is necessary to ensure survival. Survivors require prolonged hospitalization and physical rehabilitation. Yet, despite intensive research on streptococcal virulence factors and their roles in the pathogenesis of STSS, the mechanisms responsible for the rapid destruction of viable tissue in this infection have received little attention.

We hypothesized that tissue destruction in this infection is the result of ischemic necrosis following thrombosis of the microvasculature. The present work conclusively demonstrates that GAS toxins from an invasive M-type 1 strain rapidly and significantly reduced local blood flow after an im injection and that this dose-dependent process was irreversible at the highest amount of toxin tested. Furthermore, the toxin-induced reduction in perfusion occurred within minutes and was associated with the formation of occlusive intravascular cellular aggregates. These dynamics suggest either a direct toxin effect on circulating cells themselves or an indirect toxin-induced release of cell stimulatory molecules, such as platelet-activating factor [15]. Vasocostriction was definitely not a factor. Although we cannot completely rule out the contribution of rapidly expressed endogenous mediators, we have clearly demonstrated a direct effect of streptococcal toxins on the formation of heterotypic platelet/PMNL complexes. That SLO is responsible for these effects is suggested by the following: (1) the active, complex-inducing moiety in the toxin preparation was inhibited by both MAb against SLO and by cholesterol; (2) toxins from an SLO-deficient strain of GAS did not elicit complex formation; and (3) recombinant SLO itself stimulated platelet/PMNL complexes. Taken together, these findings support the concept that toxin-induced ischemia results in necrosis.

A role of platelet/leukocyte complexes in the pathogenesis of
Various diseases has heretofore been made only by association. Compared with those in healthy control subjects, increased numbers of circulating platelet/leukocyte complexes are found in patients with diseases characterized by thrombosis and inflammation, such as meningococcemia [16] and septic shock [17–19]. In patients with sepsis, an inverse correlation was found between the severity of illness and the extent of platelet/neutrophil adhesion complexes [18, 19]. From these observations, the authors of those studies surmised that the reduced number of complexes was due to sequestration of these aggregates in the microvasculature of the major organs. Such sequestration, they reasoned, contributed to multiple organ dysfunction. However, histopathologic studies confirming this hypothesis have not been conducted, and therapeutic strategies designed to limit complex formation in these settings have not, to our knowledge, been tested.

In contrast, the present study has clearly demonstrated a role of platelet/PMNL complexes in GAS myonecrosis. In addition, we have recently shown that the toxin-induced formation of occlusive intravascular aggregates of platelets and PMNL contributes to the destruction of viable tissue in C. perfringens gas gangrene [9, 10]. Furthermore, rendering the experimental animals neutropenic or pretreating them with heparin prevented both intravascular aggregate formation and the associated perfusion deficits [10]. Thus, a role of platelet/PMNL complexes in the pathogenesis of necrotizing soft-tissue infections has been established.

The direct binding of platelet P-selectin to PMNL glycoproteins has been the paradigm for platelet/PMNL interactions induced by most physiological agonists, such as thrombin [20]. In addition, other researchers have shown that the platelet fibrinogen receptor gpIIbIIIa (CD41/CD61) also participates [21–23], using fibrinogen as a bridging molecule between gpIIbIIIa and PMNL CD11b/CD18 [24, 25]. High-affinity binding of fibrinogen requires a conformational change in platelet gpIIbIIIa [26] after platelet activation. Fibrinogen-dependent platelet/PMNL complex formation is further enhanced when the functionally active conformation of PMNL CD11b/CD18 is expressed [27]. These findings have been assimilated into a multistep adhesion cascade model in which the formation of platelet/PMNL complexes is initiated by a platelet P-selectin-dependent recognition step and is followed by a gpIIbIIIa/CD18-dependent stabilization step [20].

How well does this model describe events stimulated by nonphysiological agonists such as bacterial toxins? In the case of C. perfringens, the formation of large platelet/PMNL complexes was principally mediated by the phospholipase C–induced activation of gpIIbIIIa [10, 28]. This activation produced large platelet/ platelet aggregates that bound PMNL in vitro via both gpIIbIIIa [10] and, to a lesser extent, P-selectin (data not shown). In the present work with toxins from Streptococcus pyogenes, the ligand hierarchy was reversed. Specifically, anti–P-selectin antibodies reduced the number of SLO-induced platelet/PMNL complexes to background levels, whereas strategies targeting gpIIbIIIa were less effective. In further contrast to phospholipase C, GAS toxins did not induce significant numbers of large platelet/platelet aggregates, which suggests that exotoxins from GAS do not directly activate gpIIbIIIa—a necessary step for nonadherent platelets to bind other platelets [26]. Instead, it is likely that gpIIbIIIa becomes activated and mediates adherence only after the initial binding via P-selectin has occurred. Furthermore, such secondary gpIIbIIIa-mediated binding is likely enhanced by SLO-induced functional up-regulation of CD11b/CD18 [12]. In this sense, the stimulation of platelet/PMNL complexes by SLO is analogous to that induced by thrombin.

Taken together, our findings demonstrate that the rapid tissue destruction characteristic of both streptococcal and clostridial myonecrosis results from toxin-induced, platelet/PMNL aggregate–mediated vascular occlusion. As infection progresses and greater quantities of toxins are produced and absorbed, this local ischemic process likely expands regionally until an entire limb is destroyed. Systemically, microvasculature occlusion may also contribute to the shock and organ failure associated with these infections. Although the end result of platelet/PMNL complex formation is the same in both GAS and clostridial necrotizing infections (i.e., microvascular thrombosis and ischemic necrosis of tissue), the principal mechanisms are different. Thus, immunotherapeutic strategies designed to block cellular adherence molecules, limit microvascular thrombosis, and maintain perfusion may need to be tailored to the etiologic agent. Testing of such strategies in experimental GAS myonecrosis and clostridial gas gangrene is currently under way in our laboratory.

References