Clostridial Gas Gangrene. I. Cellular and Molecular Mechanisms of Microvascular Dysfunction Induced by Exotoxins of *Clostridium perfringens*

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Mechanisms responsible for the rapid tissue destruction in gas gangrene are not well understood. To examine the early effects of *Clostridium perfringens* exotoxins on tissue perfusion, a rat model of muscle blood flow was developed. Intramuscular injection of a clostridial toxin preparation containing both phospholipase C (PLC) and θ-toxin caused a rapid (1–2 min) and irreversible decrease in blood flow that paralleled formation of activated platelet aggregates in venules and arterioles. Later (20–40 min), aggregates contained fibrin and leukocytes, and neutrophils accumulated along vascular walls. Flow cytometry confirmed that these clostridial toxins or recombinant PLC induced formation of P-selectin–positive platelet aggregates. Neutralization of PLC activity in the clostridial toxin preparation completely abrogated human platelet responses and reduced perfusion deficits. It is concluded that tissue destruction in gas gangrene is related to profound attenuation of blood flow initiated by activation of platelet responses by PLC.

Clostridium perfringens type A is the most common organism isolated from patients with trauma-induced gas gangrene [1]. Although initial growth of the organism occurs within the devitalized anaerobic milieu, acute invasion and destruction of healthy, living tissue rapidly ensues, with margins of tissue destruction often advancing several centimeters per hour despite appropriate antibiotic therapy [1, 2]. Even with modern medical advances and intensive care regimens, the centuries-old practice of radical amputation is often the best life-saving treatment alternative for patients with this infection.

Although several theories have been proposed to explain these pathologies, the consensus is that the local and systemic manifestations of gas gangrene are related to the elaboration of potent extracellular protein toxins, especially the α-toxin, a phospholipase C (PLC), and θ-toxin, a thiol-activated cytolysin [1, 3, 4]. Recent elegant genetic-based studies have elucidated some structure/function relationships of both PLC and θ-toxin (reviewed in [5]). Yet, despite this formidable body of sophisticated knowledge, the pathogenic mechanisms underlying the rapid destruction of viable tissue have not been elucidated. However, the classic signs and symptoms of human disease provide some clues.

First, an early manifestation of gas gangrene is the sudden onset of severe pain in the infected region. MacLennan [1] remarked that such pain is "sometimes so sudden as to suggest a vascular catastrophe" [1]. Indeed, such pain is also a prominent feature in other clinical conditions that involve occlusion of the arterial blood supply, such as acute arterial embolism or myocardial infarction. Second, the speed (centimeters/hour) with which skin, subcutaneous tissue, fascia, and muscle are destroyed in gas gangrene is similar to the rate of tissue death following acute arterial thrombosis. Third, the host response to soft-tissue infection with other organisms, such as *Staphylococcus aureus, Haemophilus influenzae,* or *Streptococcus pneumoniae,* usually involves dilation of blood vessels and increased blood flow, as evidenced by local *calor, rubor,* and *tumor*. In contrast, tissues that are being rapidly destroyed in the progression of gas gangrene do not bleed. This age-old observation has become dictum in surgery such that it is recommended to remove necrotic tissue until tissue that bleeds is encountered. Indeed, nearly a century ago, McNee and Dunn [2], who after evaluating serial histopathologic sections of muscles taken from freshly amputated limbs of soldiers with gas gangrene, remarked that "[t]he muscle fibres at the margin [between healthy

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For studies involving human subjects, 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 of the University of Washington, Seattle. Experiments involving animals were approved by the Animals Subjects Committee, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles.

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and necrotic tissues] are paler and duller than the normal, [which] might be accounted for by total absence of blood in the part involved." Taken together, these observations suggest that destruction of tissue in clostridial gas gangrene is initially a local ischemic process that, in time, expands regionally until an entire limb is destroyed.

Thus, we hypothesized that an early, toxin-mediated microvascular event constitutes the primary vascular "catastrophe" that initiates the rapid progression of tissue necrosis. The present study sought to establish an in vivo model to study the early effects of clostridial toxins on local blood flow in muscle and to investigate the cellular and molecular mechanisms of the host response.

Materials and Methods

Clostridial toxins. A crude clostridial toxin preparation was generated by ammonium sulfate fractionation (35%–55%) of culture filtrate from log-phase *C. perfringens* (ATCC 13124) grown in basal proteose-peptone medium (Difco, Detroit) supplemented with amino acids, salts, and vitamins to provide optimal conditions for toxin production, as described elsewhere [6]. The pelleted proteins were resuspended in and dialyzed exhaustively against pyrogen-free deionized water and then were aliquotted and frozen at −70°C until use. An ammonium sulfate fraction of uninoculated (sterile) culture medium was prepared in identical fashion for use as a negative control. The clostridial toxin preparation contained 8120 U of PLC activity/mL and 3120 hemolytic U of θ-toxin activity/mL, as measured by *p*-nitrophenylphosphorylcholine hydrolysis assay (NPPC) and hemolysis of sheep red blood cells, respectively [7], and had <1.25 ng/mL endotoxin, as measured by the *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA).

Analysis of the clostridial toxin preparation by SDS-PAGE with silver staining revealed 5 protein bands. Two proteins had molecular weights of 43,000 and 55,000, which migrated in identical fashion with recombinant α- and θ-toxins, respectively. The third prominent band had a molecular weight of 109,900. Two minor bands had estimated molecular weights of 11,700 and 18,800; these bands were also found in the ammonium sulfate fraction of uninoculated sterile culture medium and probably represent small proteins from the basal medium. Dilutions of the clostridial toxin preparation were made in sterile, pyrogen-free normal saline for administration to experimental animals or in pyrogen-free Dulbecco's PBS with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ (DPBSi) for whole-blood flow cytometry studies.

Recombinant PLC (rPLC). rPLC was provided by J. Tso (Protein Design Labs, Palo Alto, CA) [8]. Phospholipase activity (determined by NPPC assay) of stock PLC was 284 U/mL (specific activity 0.024 PLC U/μg protein). This toxin preparation yielded a single band on SDS-PAGE with silver staining and contained 22.7 ng LPS/U of PLC, as measured by the *Limulus* amoebocyte lysate assay. The concentrations of PLC used in this study (i.e., 2–12 U) closely correlate with the quantity of PLC produced locally in muscle during active infection [9, 10] and with the amount of PLC required to cause muscle destruction and animal mortality [10].

Neutralizing antibodies. Toxin-neutralizing antibodies used in this study were in the form of unconcentrated culture supernatants from hybridomas that produce neutralizing monoclonal antibodies (MAbs) against θ-toxin (clone 3H10, IgG1 [11]) and α-toxin (clone 1C6, IgG1 [12]). Both hybridomas were gifts from Hiroko Sato (National Institutes of Health, Tokyo) and were cultured in RPMI plus L-glutamine and 10% fetal calf serum. One microliter of cell-free culture supernatant of 1C6 neutralized 7.3 U of PLC activity, and 1 μL of 3H10 neutralized 6.4 hemolytic U, as measured by inhibition of NPPC hydrolysis and red cell hemolysis, respectively. Hybridoma YN1 (anti–human CD54, IgG1; American Type Culture Collection, Rockville, MD) was similarly prepared for use as a negative, isotype-matched, control. In both the in vitro and in vivo studies, a 2-fold excess of each neutralizing antibody or an equal volume of control antibody was added to diluted toxin or rPLC for 15–30 min before use.

Blood-flow measurements. Male Sprague-Dawley rats (225–250 g) were anesthetized with urethane (1.25 g/kg intraperitoneally). A carotid artery was cannulated to monitor blood pressure, and a tracheostomy was performed to maintain a patent airway. The skeletal muscle blood flow on the abdominal wall was measured by a Doppler laser blood perfusion monitor (LASERFLO BFM; Vasamedics, St. Paul, MN). An incision was made on the skin, and a flow probe was placed directly on the abdominal skeletal muscle. A period of 20–30 min was allowed for the baseline blood flow to stabilize. Normal saline (100 μL), phenylephrine (10 μM) or the clostridial toxin preparation diluted to contain 8, 4, or 2 U of PLC activity was then injected into the muscle immediately below the center of the probe, where the blood flow was measured for an additional 40 min.

The Doppler laser blood-flow readings were expressed as percentage of baseline blood flow for each rat (i.e., the posttreatment blood-flow measurement at each time point was divided by the pretreatment value and then multiplied by 100). The repeated-measure analysis of variance (SAS Procedure MIXED; SAS, Cary, NC) 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 *t* tests for pairwise comparisons. $P < .05$ was considered statistically significant. In separate groups of animals, the experiment was repeated, and tissue from the injection site was removed at various times for routine histopathology studies.

Videomicroscopy. The progression of clot formation and vascular occlusion was visualized and quantitated in separate groups of animals by videomicroscopy. Specifically, while animals were under urethane anesthesia, midline, high, and low transverse abdominal incisions were made through the abdominal skin, and the skin was retracted to expose the underlying muscle. The muscle was transilluminated by inserting the light rod through a small incision and by placing it just beneath the exposed muscle area to be studied. A gold-plated copper plate with a shallow well (~0.5-mL capacity) with a 5-mm diameter center hole was placed over the exposed abdominal muscle and sealed fluid-tight with a silicone elastic adherent (Silly Putty; Binny and Smith, Easton, PA). The copper plate was secured to a 3-dimensional manipulator. The temperature of the copper plate was kept constant at 36–37°C in the vicinity of the shallow well by an attached warm-water chamber.

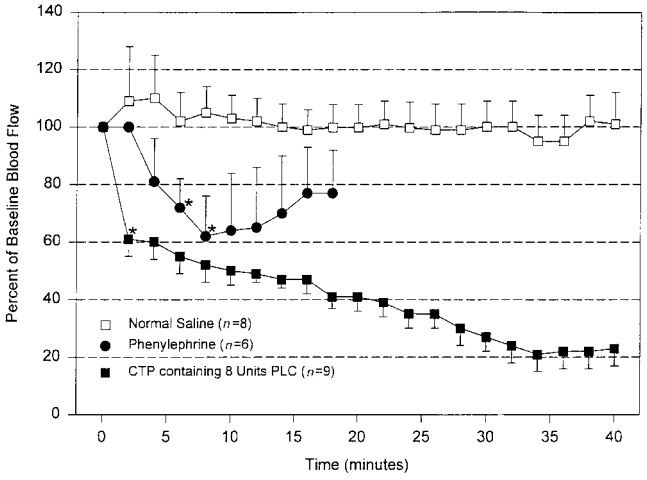

Figure 1. Blood flow in rat abdominal muscles that were injected with 0.1 mL of normal sterile saline, 10 μM phenylephrine, or a clostridial toxin preparation (CTP) diluted to contain 8 U of phospholipase C (PLC) activity. Blood flow was measured for 40 min by laser Doppler blood perfusion monitor and is expressed as the mean percentage (±SE) of baseline perfusion. Repeated-measure analysis of variance was used to compare mean results for the different groups of rats. Tukey-Fisher least significant difference criterion was used to compute post hoc *t* tests for pairwise comparisons. Level of significance was set at $P < .05$. *Statistically different from respective baseline value.

The exposed muscle was superfused periodically with prewarmed normal saline solution at 35–37°C, to maintain constant temperature and hydration of the exposed muscle. After a 10-min control period, 0.02 mL of clostridial toxin (containing the same amount of PLC activity as in the Doppler flow studies above) or rPLC (8 or 12 U) was injected into the abdominal muscle just beneath the fascia in the center of the field of observations by using an ultrafine syringe set with a 29-gauge 12 × 75–mm needle. The observation continued for another 40 min. In 2 animals, the toxin preparation was incubated with a 2-fold excess of neutralizing MAb against PLC for 30 min before injection into the animal. All in vivo microscopic studies were videotaped for later analysis. Vessel diameters were measured by videocaliper with a stage micrometer, and those with diameters of 25–40 μm were chosen for study.

Histopathology. At 2, 5, 10, 20, and 40 min after injection of either normal saline or clostridial toxin into rat abdominal musculature, we excised the tissue, fixed it in neutral buffered formalin, embedded it in paraffin, and processed it for routine hematoxylin-eosin staining. In some specimens, sections from the paraffin blocks were stained by the Ledrum Acid Picro-Mallory method [13] for the detection of intravascular fibrin accumulation. For demonstration of P-selectin, sections of formalin-fixed, paraffin-embedded tissues from clostridial toxin–injected rat abdominal muscle were deparaffinized and rehydrated, and the P-selectin antigen was recovered by microwaving the sections in 10 mM citrate buffer, pH 6.0. P-selectin expression was visualized by commercial avidin-biotin conjugate immunohistochemistry (Zymed, San Francisco) using a polyclonal antibody against human P-selectin, which cross-reacts with rat P-selectin (PharMingen, San Diego).

Flow cytometry. Whole blood was obtained by venipuncture from healthy human volunteers and collected into heparinized tubes. The volunteers had not taken any medications for the previous 10 days and had platelet counts of 210–265,000/μL. Blood was obtained with a 19-gauge needle without the aid of a tourniquet. The first 2 mL of collected blood was discarded. Blood (100 μL) was placed into 12 × 75 tubes containing 10 μL of an isotype control or phycoerythrin-conjugated monoclonal anti–human CD42b (a platelet-specific marker, clone HIP1, mouse IgG1, PharMingen) alone or in combination with fluorescein isothiocyanate (FITC)–conjugated monoclonal anti-human CD62P (P-selectin, a platelet-activation marker; clone AK-4, mouse IgG1, PharMingen). Samples were gently mixed and placed at 37°C for 5–10 min. Next, 10 μL of one of the following was added, and incubation continued at 37°C for another 5 min: PBS, a 1:10 dilution of clostridial toxin preparation, rPLC (0.124 U/10 μL), or 200 μM ADP (Chronolog, Havertown, PA).

Samples were then immediately prepared for flow cytometric analysis using a commercial formic acid red cell lysis/formalin cell fixation procedure (Q-prep; Coulter, Hialeah, FL), according to the manufacturer's recommendations. In a separate experiment, whole blood was stimulated for 5 min with PBS, clostridial toxin, or rPLC that had been pretreated for 15 min with 2-fold excess of neutralizing MAb against PLC (clone 1C6, [12]), θ-toxin (clone 3H10, [11]), or an equal volume of irrelevant isotype-matched antibody.

Flow cytometry analysis was done using a Epics (Coulter) flow cytometer. Platelets and platelet aggregates were distinguished from

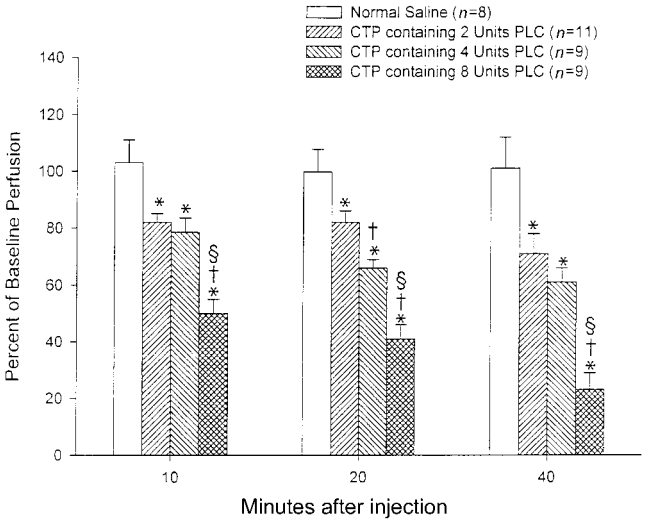

Figure 2. Blood flow in rat abdominal muscles that were injected with 0.1 mL of normal saline or a clostridial toxin preparation (CTP) diluted to contain 2, 4, or 8 U of phospholipase C (PLC) activity. Blood flow was measured for 40 min by the laser Doppler blood perfusion monitor and is expressed as the mean percentage (±SE) of baseline perfusion. Repeated-measure analysis of variance was used to compare mean results for the different groups of rats. Tukey-Fisher least significant difference criterion was used to compute post hoc *t* tests for pairwise comparisons. Level of significance was set at $P <$ .05. *Significantly different from respective normal saline group; ‡significantly different from respective 2 U of PLC group; §significantly different from respective 4 U of PLC group.

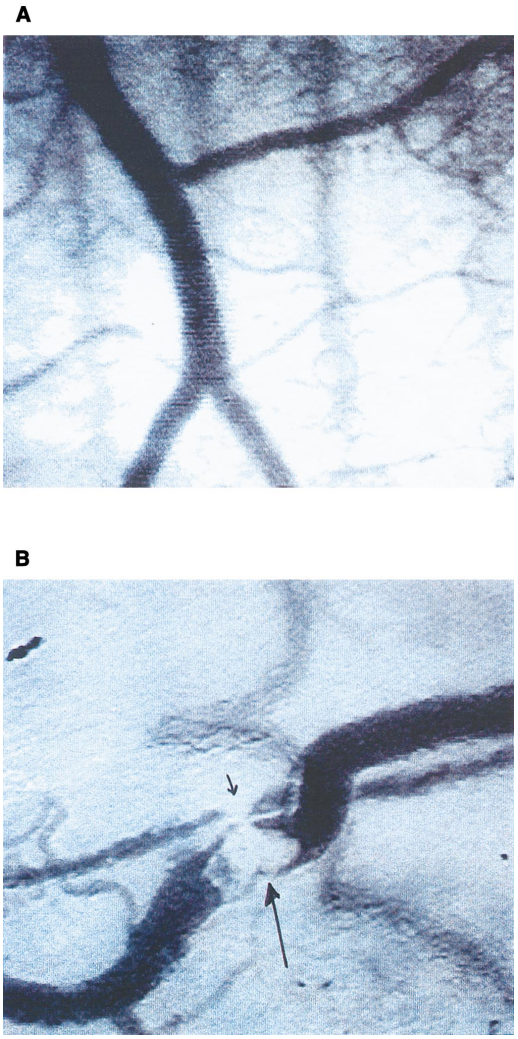

Figure 3. Still image of intravascular aggregates in rat vessels. Images are from representative videotapes following intramuscular injection of a clostridial toxin preparation. See text for complete methodology. *A,* Venule under baseline conditions (i.e., before toxin injection; original magnification, ×210). *B,* Venule *(large arrow)* and arteriole *(small arrow)* at 11 min after toxin injection. Venule is occluded by a large stationary aggregate, and arteriole contains small, flowing aggregates (original magnification, ×267).

nonplatelet events (i.e., residual erythrocytes and leukocytes) by their fluorescence due to phycoerythrin-conjugated anti-CD42b bound to the particle surface. Thirty thousand $CD42b^+$ events were collected per sample, and further analyses were done only on this population. Aggregated and nonaggregated platelets were distinguished from each other and from platelet microparticles by their forward scatter profiles, and gates were drawn accordingly. Internal cytometry bead standards of 3.6 and 10 μm were included for size reference. A threshold was defined for increased P-selectin expression as the point below which 95% of all platelet events from unactivated control samples stained with FITC-labeled P-selectin were located. These experiments were done with duplicate blood from 3 different donors.

Results

Blood-flow studies in skeletal muscle. Intramuscular injection of the clostridial toxin preparation containing 8 U of PLC activity caused an initial 40% decrease in blood flow in 2 min, which continued to decline over the 40-min observation period (figure 1). Phenylephrine also caused a 40% reduction in blood flow, but this reduction was slower in onset and transient, compared with that induced by injection of clostridial toxins (figure 1). Intravital microscopy confirmed that the phenylephrine-induced but not the clostridial toxin–induced reduction in blood flow was due to vasoconstriction (see next section). The perfusion deficits induced by clostridial toxins were time- (figure 1) and dose-dependent (figure 2) and were not spontaneously reversible.

Videomicroscopy of blood flow. Intramuscular injection of sterile saline or uninoculated (sterile) culture medium resulted in no discernable alteration in vessel blood flow within any vascular compartment (data not shown). In contrast, small aggregates were visible in venules within 1 min of clostridial toxin administration. These aggregates, when smaller than the vessel diameter, were freely moving or only transiently arrested on the vessel wall. Aggregates also formed in the arterial circulation, although their appearance (at 8 min after toxin administration) was later than those in venous channels. As time progressed, the growing aggregates became irreversibly immobilized on the endothelium, and eventually blood flow was markedly attenuated as vessels became completely obstructed (figure 3).

Videotapes of blood-flow microscopy of 10 rats (5 control rats and 5 rats given clostridial toxin containing 4 U of PLC activity) were reviewed by 2 blinded observers, and semiquantitative estimates of the number and size of aggregates were

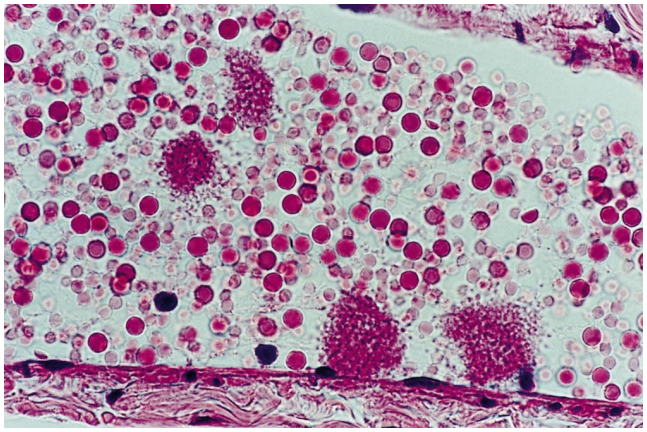

Figure 4. Routine hematoxylin-eosin stain (original magnification, ×100) of rat abdominal muscles that were injected with a clostridial toxin preparation diluted to contain 4 U of phospholipase C activity. Small aggregates, such as those illustrated here, were apparent in numerous large and small vessels as early as 2 min after injection.

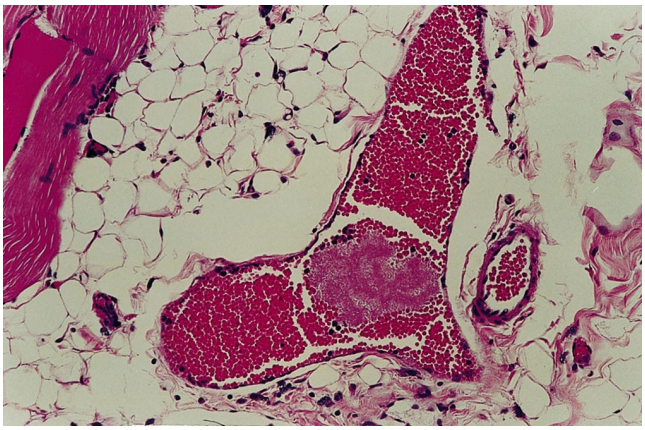

Figure 5. Routine hematoxylin-eosin stain (original magnification, ×40) of rat abdominal muscles that were injected with a clostridial toxin preparation diluted to contain 8 U of phospholipase C activity. Intravascular aggregates became larger and more widespread after 40–60 min.

made. In animals injected with saline, aggregates in both the venules and arterioles ranged from none to a few small aggregates during the 40-min observation period. In contrast, in rats receiving toxin, many small aggregates appeared in the venules at 2–5 min after toxin injection and grew progressively in size and number to many large aggregates at 10, 20, and 40 min, so that the blood flow was completely and persistently occluded in some venules. In arterioles, aggregates also increased in number and size but at a slower rate and to a lesser extent than in venules with occasional complete but transient arteriolar occlusion.

To obtain evidence that PLC was responsible for the perfusion deficit induced by crude toxin, we repeated the semiquantitative study described above using rPLC (8 or 12 U) or saline solution in 3 rats. In the control rat (saline), only a few small aggregates that were freely flowing in arterioles and venules were seen during the 40-min observation period. Blood flow in both arterioles and venules remained at baseline levels during this time. In contrast, many small aggregates were seen at 2 and 5 min after injection of 8 or 12 U of rPLC. Aggregate size and number persisted at this level for 40 min in the animal receiving 8 U of rPLC, and although venular blood flow was initially slowed, by 20–40 min it had returned to near-baseline levels. In animals receiving 12 U of rPLC, aggregates in both venules and, to a lesser extent, in arterioles progressively increased in size and number from 10–40 min. Venular blood flow in these animals slowed markedly at 2 min, persisted at that level for 20 min, and then declined further from 20–40 min. Arteriolar flow was also reduced in a similar dose-dependent manner.

At no time was vasoconstriction observed after injection of either crude clostridial toxin or rPLC. In stark contrast, attenuation of blood flow in response to phenylephrine administration was related only to vasoconstriction; no aggregates were observed in any vascular compartment. Preincubation of the clostridial toxin preparation or rPLC with a neutralizing MAb against PLC before injection completely prevented both the formation of aggregates and any decrease in blood flow (see companion manuscript in this issue [15]).

The changes described above occurred in the immediate area surrounding the site of toxin injection, and all vessels in the field of view were affected. As the microscope stage was moved to view fields away from that area, the intensity of the changes diminished. At more distant sites (e.g., the contralateral side of the abdominal wall), microcirculatory flow showed no disturbances whatsoever.

Histopathology and immunohistochemistry. As early as 2 min after injection, routine histopathology of muscles injected with clostridial exotoxins revealed formation of untethered intravascular aggregates (figure 4), which became larger and more widespread after 20–40 min (figure 5). These aggregates stained positively for P-selectin (figure 6), which suggests that they consisted of tightly-adherent, activated platelets. Endothelial cells adjacent to platelet aggregates were also positive for P-selectin at 2–10 min (figure 6), which suggests that this

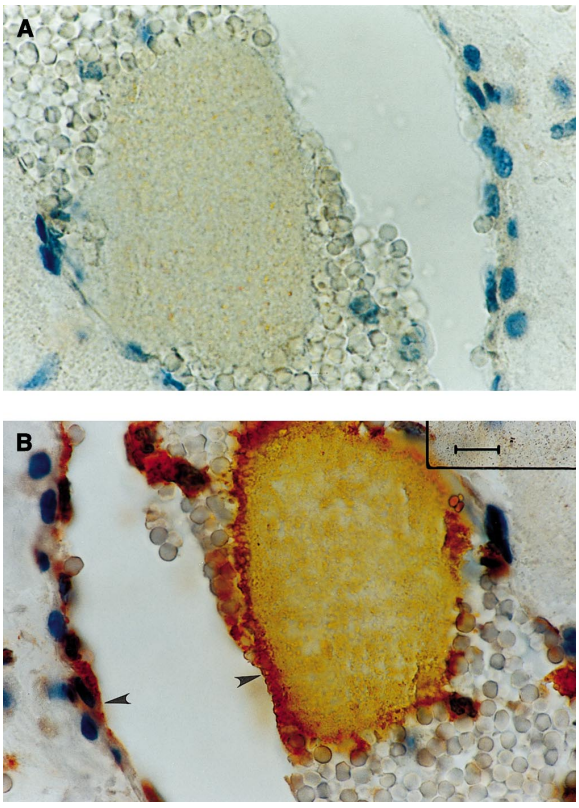

Figure 6. Immunohistochemistry rat abdominal muscle 2 min after injection of a clostridial toxin containing 4 U of phospholipase C activity. *A,* Negative, isotype-control. *B,* Intravascular aggregates and the adjacent vessel walls *(arrows),* which were positive for P-selectin.

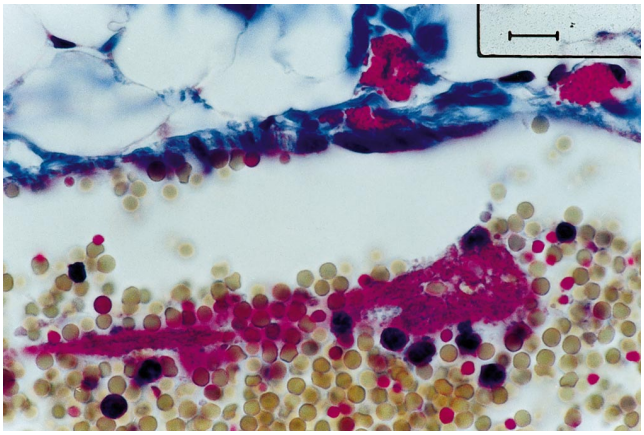

Figure 7. Staining of rat abdominal muscles that were injected with a clostridial toxin preparation diluted to contain 4 U of phospholipase C activity. Aggregates that formed at 2 min were negative for fibrin (not shown) but were positive by 20 min (original magnification, ×100; Ledrum Acid Picro-Mallory method for specific staining of fibrin in formaldehyde-fixed, paraffin-embedded tissue: red, fibrin; orange-brown, erythrocytes; blue, collagen; blue-black, nuclei).

adherence molecule was expressed by endothelial cells, that P-selectin–positive platelets were bound to the endothelium, or both. The small platelet aggregates formed at 2 min were negative for fibrin, whereas the larger aggregates formed at 20–40 min had a demonstrable fibrin component and were complexed with leukocytes (figure 7). Intravascular leukocyte accumulation was not apparent at the early time points (<10 min; not shown), but at 40 min, margination of polymorphonuclear leukocytes (PMNL) along the vascular endothelium was striking (figure 8).

Flow cytometry. Preliminary experiments that used platelet-rich plasma established 3 distinct platelet populations on the basis of size: resting, nonaggregated platelets were <5 μm, whereas ADP-activated platelets formed aggregates of 5–10 μm and >10 μm in size. Similarly, when whole blood was used as the source of platelets, 94.9% ± 1.3% of resting platelets fell within the <5-μm gate, whereas stimulation with ADP for 5 min caused 24% ± 2% of platelets to move into the 5–10-μm gate. These results are in agreement with the ADP-activated platelet flow cytometry studies of Jy et al. [14]. Activation of whole blood for 5 min with either the clostridial toxin preparation or rPLC also caused an increase in the forward scatter of the platelet population (indicative of aggregation), so that a significant percentage of the platelet events moved into the 5–10-μm and >10-μm gates (figure 9). Pretreatment of the clostridial toxin preparation and rPLC with neutralizing MAb against PLC completely abrogated both toxin-induced activation (not shown) and aggregation (figure 10), whereas MAb against clostridial θ-toxin (perfringolysin O) or an irrelevant, isotype-matched control antibody had no effect.

Only 4.1% ± 1.3% of unstimulated platelets were positive for P-selectin expression (mean fluorescence intensity [MFI], 1.6; figure 11*A*). Activation of whole blood with ADP caused 26.9% of nonaggregated platelets to express P-selectin at a low level (MFI, 2.2; figure 11*A*). In contrast, exposure of whole blood to either the clostridial toxin preparation or rPLC strongly induced 91.5% ± 0.8% and 86.8% ± 4.6% of the platelets <5 μm in size to express P-selectin, respectively ($MFI_{clostridial\ toxin}$, 7.7 [not shown]; MFI_{rPLC}, 7.1; figure 11*A*). Furthermore, platelet aggregates in the 5–10-μm gate that had been stimulated with clostridial toxins or rPLC (and, to a lesser extent, with ADP) were strongly positive for P-selectin ($MFI_{clostridial\ toxin}$, 41.1 [not shown]; MFI_{rPLC}, 41.6; MFI_{ADP}, 3.1; figure 11*B*).

In a single flow cytometry experiment, we examined the effects of rPLC on the activation and aggregation of rat platelets. Stimulation of heparinized whole blood (obtained from a 210-g male Sprague Dawley rat by cardiac puncture) with rPLC caused a 7.3-fold increase in the number of 5–10-μm platelet aggregates, compared with a 3.1-fold increase in PLC-stimulated human whole blood (data not shown). In addition, 95% of all rat platelets in the 5–10-μm gate were positive for P-selectin expression (not shown). This data, together with previous in vitro aggregation studies that used rabbit platelets and purified PLC [16], suggest that *C. perfringens* PLC uniformly activates platelets from a variety of mammalian species.

Discussion

We previously hypothesized that the rapid progression of tissue destruction in clostridial gas gangrene is related, in part, to regional tissue hypoxia secondary to toxin-induced vascular

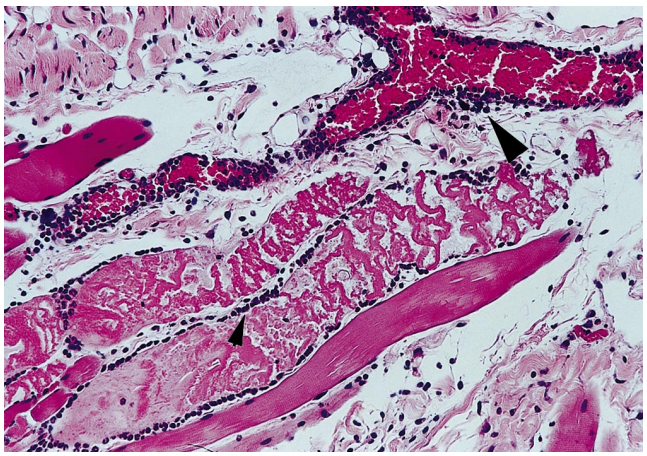

Figure 8. Routine hematoxylin-eosin stain of rat abdominal muscles that were injected with a clostridial toxin preparation diluted to contain 4 U of phospholipase C activity (original magnification, ×40). Margination of leukocytes in vessels *(large arrowhead)* and endomysial channels *(small arrowhead)* was striking at 40 min.

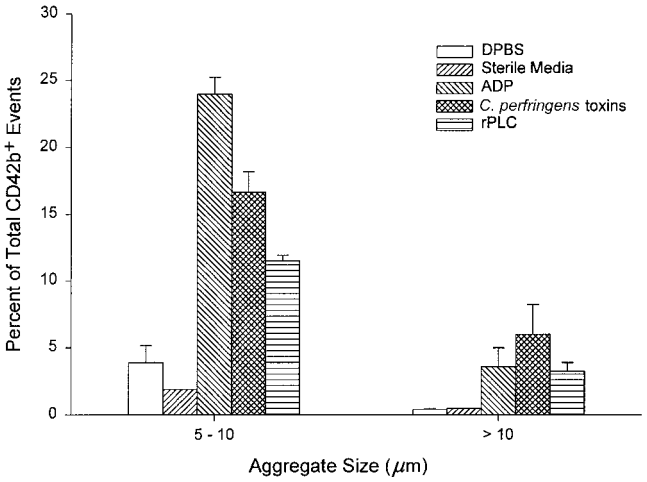

Figure 9. Flow cytometry analysis of platelet events in heparinized whole blood (100 μL) that was stimulated with 10 μL of PBS (DPBS), a 1:10 dilution of uninoculated (sterile) culture medium preparation, 200 μM ADP, a 1:10 dilution of a stock clostridial toxin preparation, or recombinant phospholipase C (rPLC; 0.142 U/10 μL) for 5 min. Flow cytometry was done on 30,000 $CD42b^+$ platelet events, with gates drawn for platelets <5 μm (not shown), 5–10 μm, and >10 μm in size on the basis of internal cytometry bead standards. Data are mean ± SE from 3 experiments using 3 blood donors.

obstruction or endothelial cell injury (or both) [17]. We developed a model of skeletal muscle blood flow to investigate the early events of toxin-induced microvascular dysfunction.

In this model, intramuscular injection of clostridial toxin resulted in a rapid and sustained decline in local blood flow that was initiated by the formation of intravascular aggregates of activated platelets. Incubation of the clostridial toxin preparation with a neutralizing MAb against PLC abrogated the toxin-induced perfusion deficits, which suggests that PLC was the active principle. This finding is in agreement with earlier studies that demonstrated rapid aggregation of platelets after administration of clostridial toxin preparations containing PLC [18, 19]. Although aggregation of platelets is a well-known biologic activity of PLC, the mechanism has not been elucidated and its relevance to pathogenesis has not, heretofore, been determined.

Our results demonstrate that PLC circumvents the physiologic series of events that occur in a normal hemostatic response and irreversibly shifts the balance to a pathologic, prothrombotic state. Support for this conclusion is provided by careful inspection of the dynamics of PLC-induced blood-flow changes. These events can be temporally and conceptually divided into 4 phases. First, formation of freely flowing intravascular aggregates of activated platelets occurred rapidly in venules (1–2 min) and arterioles (4–8 min; phase I), which caused a precipitous decline in local blood flow as smaller vessels became occluded. These mobile aggregates quickly (5–10 min) increased in size and were composed of platelets, fibrin, and leukocytes (phase II). Only at the later stages (20–40 min) did these large heterotypic aggregates appear fixed to the vessel lining (phase III). At this time, most larger vessels were occluded. Thereafter, muscle necrosis ensued with only minimal inflammatory cell infiltration into adjacent tissues (phase IV). It is important to note that the histologic features of phase IV resemble what is classically observed in necrotic tissue from human cases of gas gangrene [1, 17]. In addition, progressive intravascular aggregate formation is characteristic of experimental wild-type *C. perfringens* myonecrosis but is notably absent from vessels in tissues infected with a PLC-negative isogenic mutant strain (unpublished observations).

This sequence of PLC-induced thrombotic events is markedly different from that occurring in response to traumatic vessel injury, such as a laceration. These differences provide insight into the molecular mechanisms that control the responses to PLC. First, in phase I, PLC-induced platelet aggregates are seen freely moving within the vasculature. In contrast, following vessel injury, platelets are specifically immobilized at the site of injury. This localization is mediated by the sequential binding of platelets gpIb and gpIIbIIIa to the von Willebrand factor (in the case of vessels with high shear forces) or by platelet gpIIbIIIa binding to immobilized fibrinogen (in areas of low shear stress; reviewed in [20]). To restore vessel integrity and maintain vessel patency, local thrombus formation then pro-

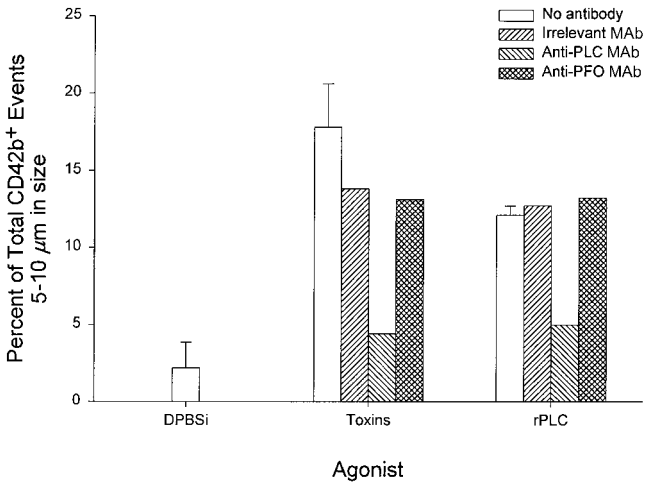

Figure 10. Flow cytometry analysis of platelet aggregation in whole blood that was stimulated for 5 min with PBS (DPBSi), a clostridial toxin preparation (toxins), or recombinant phospholipase C (rPLC) alone or in the presence of 2-fold excess of neutralizing monoclonal antibody (MAb) against PLC (clone 1C6), clostridial θ-toxin (clone 3H10, perfringolysin O [PFO]), or an equal volume of isotype-matched irrelevant antibody. Analysis was performed on 30,000 $CD42b^+$ platelet events. Platelet aggregates were gated by size on the basis of internal cytometry bead standards (Material and Methods). Data are the mean ± SE of 3 experiments using 3 blood donors.

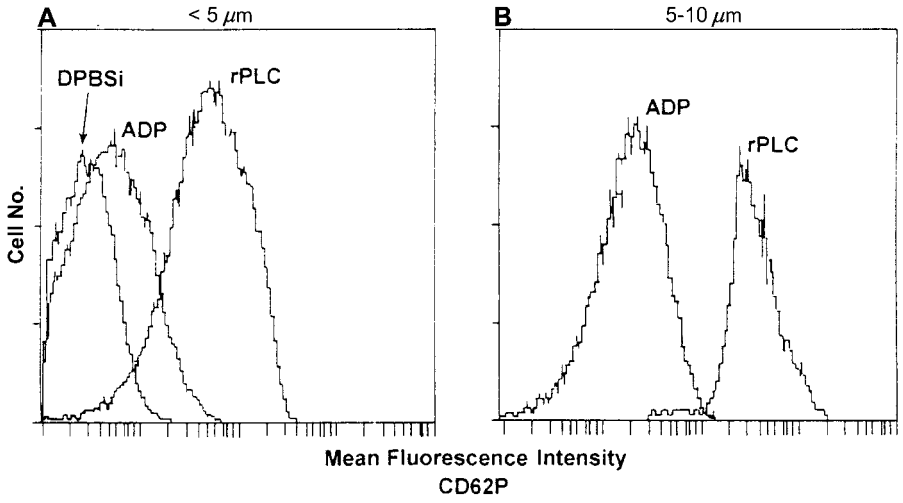

Figure 11. Mean fluorescence intensity of P-selectin expression from a representative experiment for platelet events <5 μm *(A)* and 5–10 μm *(B)* in size. Whole blood was stimulated with PBS, ADP, or recombinant phospholipase C (rPLC) for 5 min. Dual-color flow cytometry analysis was done on 30,000 $CD42b^+$ platelet events.

ceeds only at the injured site through the formation of fibrinogen (or the von Willebrand factor) bridges between activated gpIIbIIIa molecules of adjacent platelets. In contrast, the appearance of nonadherent, freely mobile platelet aggregates suggests that PLC causes the conformational change in platelet gpIIbIIIa that is necessary for binding soluble fibrinogen (reviewed in [20]). Data presented in the companion manuscript elsewhere in this issue [15] support this concept.

In phase II, PLC-induced intravascular platelet aggregates enlarge and become associated with leukocytes. The PLC-induced process again differs from the normal hemostatic response to vessel injury in 2 additional aspects. First, the continued propagation of platelet thrombi suggests that endogenous antithrombotic mechanisms are incapable of inhibiting intravascular aggregate formation. Second, the participation of leukocytes in a growing, mobile platelet aggregate is not observed in simple vessel injury and has important pathologic significance. Such heterotypic interaction is known to initiate cellular crosstalk, which stimulates the functions of both cell types (reviewed in [21]). For example, PMNL become hyperadhesive and primed for enhanced respiratory burst activity and superoxide anion release after their interaction with activated platelets [22, 23]. A triggering of this response under conditions of PLC-induced vascular leukostasis would result in destruction of the vessel and an irreversible interruption of the blood supply. Thus, in response to PLC, these physiologic interactions could contribute to the propagation of thrombosis and vascular injury. The fact that leukocytes are active participants in this process in our model is supported by the observations that (1) leukocytes are adherent to and not simply buried or trapped within the growing thrombus (this study), (2) PLC stimulates PMNL to bind platelets in a uniform rosette pattern (see companion manuscript in this issue [15]), and (3) depletion of circulating PMNL in experimental animals eliminated aggregate formation and blood-flow deficits caused by PLC.

Most studies of platelet-leukocyte interaction have shown that the adherence of activated platelets to PMNL is mediated in part by the binding of platelet P-selectin to leukocyte glycoproteins (reviewed in [20]). However, other investigators have demonstrated that gpIIbIIIa (CD41/CD61) also participates in the adhesion of activated platelets to PMNL in vitro [24–26]. Although the current study demonstrates that PLC strongly induces expression of platelet P-selectin, we have shown that PLC also activates platelet gpIIbIIIa. Furthermore, we have shown that PLC-induced platelet/PMNL heterotypic aggregation is mediated solely by this ligand (see companion manuscript [15]).

Last, in phase III, large, heterotypic aggregates irreversibly occlude blood flow. There are 2 possible explanations for this observation. First, these aggregates simply may be larger than the vessel diameter and thus physically block blood flow. Second, PLC may induce up-regulation of specific adherence mechanisms among platelets, leukocytes, and endothelial cells. Our previous work showed that PLC stimulates proinflammatory changes in endothelial cells [27], including de novo synthesis and expression of the leukocyte adherence molecules E-selectin and intercellular adhesion molecule 1 [28]. These observations suggest that the endothelium actively immobilizes the heterotypic aggregates at this stage of the process.

In summary, this study demonstrates that rapid tissue necrosis in gas gangrene is the result of PLC-induced, platelet/leukocyte-mediated vascular injury. Absorption of toxin saturates first the postcapillary venous system and then affects

arterioles. Aggregates of activated platelets and platelet/PMNL complexes sequentially occlude small, medium, and large vessels, which reduces local perfusion and contributes to endothelial cell damage. The resultant decreases in tissue pH and oxygen tension provide for optimal growth of the anaerobe. As infection progresses, major blood vessels become affected, which causes regional vascular compromise and increased compartment pressures, leading to anoxia and necrosis of large muscle groups. Left unchecked, systemic shock, multiorgan failure, and death rapidly ensue.

Further elucidation of the cellular and molecular events that contribute to microvascular dysfunction in this setting may provide rationale for novel therapeutic interventions to enhance perfusion and to maintain tissue viability.

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