Clostridial Gas Gangrene. II. Phospholipase C–Induced Activation of Platelet gpIIbIIIa Mediates Vascular Occlusion and Myonecrosis in Clostridium perfringens Gas Gangrene

Amy E. Bryant,¹ Richard Y. Z. Chen,²,³ Y. Nagata,²
Y. Wang,² C. H. Lee,² Sydney Finegold,²,³
Paul H. Guth,²,³ and Dennis L. Stevens¹,⁴

Clostridium perfringens gas gangrene is a fulminant infection, and radical amputation remains the single best treatment. It has been hypothesized that rapid tissue destruction is related to tissue hypoxia secondary to toxin-induced vascular obstruction, and previous studies demonstrated that phospholipase C (PLC) caused a rapid and irreversible decrease in skeletal muscle blood flow that paralleled the formation of intravascular aggregates of activated platelets, fibrin, and leukocytes. In this study, flow cytometry demonstrated that PLC stimulated platelet/neutrophil aggregation in a gpIIbIIIa-dependent fashion. Pretreatment of animals with heparin or depletion of leukocytes reduced blood-flow deficits, and aggregate formation caused by PLC. It is concluded that fulminant tissue destruction in gas gangrene results from profound attenuation of blood flow caused by PLC-induced, gpIIbIIIa-mediated formation of heterotypic platelet/polymorphonuclear leukocyte aggregates. Therapeutic strategies that target gpIIbIIIa may prevent vascular occlusion, maintain tissue viability, and provide an alternative to radical amputation for patients with this infection.

Materials and Methods

Clostridial toxin preparation. This toxin preparation has been characterized elsewhere [8]. It contained 8120 U of PLC activity/
mL and 3120 hemolytic U of θ-toxin activity/mL, as measured by p-nitrophenylphosphoryleholine hydrolysis assay (NPPC) and hemolysis of sheep red blood cells, respectively [9], and had <1.25 ng/mL endotoxin, as measured by the Limulus amoebocyte lysate assay.

Recombinant PLC (rPLC). rPLC was provided by J. Tso (Protein Design Labs, Palo Alto, CA) [10]. 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 concentration of PLC used in this study (2–12 U) closely correlates with the quantity of PLC produced locally in muscle during active infection [11, 12] and with the amount of PLC required to cause muscle destruction and animal mortality [12].

Toxin-neutralizing antibodies. The toxin-neutralizing antibodies used in this study were in the form of unconcentrated culture supernatant fluid from hybridomas that produce neutralizing monoclonal antibodies against θ-toxin (clone 3H10, IgG1 [13]) and PLC (clone 1C6, IgG1 [14]). Both hybridomas were gifts from Hiroko Sato (National Institutes of Health, Tokyo, Japan) and were cultured in RPMI plus 1-glutamine and 10% fetal calf serum. One microliter of cell-free culture supernatant fluid 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 to rPLC for 15–30 min before use.

Videomicroscopy. Intravital microscopy was performed in 6 groups of rats using methods described in detail elsewhere [8]. Furthermore, for the studies described here, a semiquantitative method for measuring blood flow and intravascular aggregate formation was developed (see below). There were 6 experimental groups: group 1, the control group (n = 6), received saline intravenously (iv) followed by im saline in the abdominal muscle area under observation; group 2, the toxin group (n = 6), received saline iv followed by local im clostridial toxin; group 3, the heparin plus toxin group (n = 5), received 1 U of heparin/g of body weight iv followed by local im clostridial toxin; group 4, the antineutrophil serum plus toxin group (n = 6), received 0.75 mL of antineutrophil serum (Accurate Chemical, Westbury, NY) per 100 g of body weight intraperitoneally 18–24 h before local toxin injection; group 5, the toxin plus anti-PLC antibody group (n = 3), received clostridial toxin that had been incubated for 30 min with a 2-fold excess of the neutralizing anti-PLC antibody, 1C6, before injection into the animal; and group 6, the recombinant PLC group (n = 2), received 8 or 12 U of recombinant PLC. After a 5-min control period, saline or heparin was administered iv. Ten minutes later, using an UltraFine syringe set with a 29-gauge needle (Becton Dickinson, El Paso, TX), clostridial toxin (4 U PLC/0.02 mL) was carefully injected just beneath the fascia in the center of the field under observation. The injection procedure interrupted observation for ~15 s. All in vivo microscopic studies were videotaped for later analysis.

A semiquantitative method for measurement of skeletal muscle blood flow and intravascular aggregate formation was developed for analysis of the videotapes. In brief, 5 areas relative to the site of toxin injection (central, north, south, east, and west) were scored for blood flow and intravascular clot formation by 2 investigators who were blinded to the nature of the material injected and the pretreatment conditions. Vessels within these areas that had diameters of 25–40 μm (as measured by videocaliper with a stage micrometer) were chosen for study. Blood flow was graded independently in arterioles and venules according to the following scoring method: 3, good (rapid flow with little ability to distinguish the movement of cells or aggregates); 2, fair (less rapid flow with ability to distinguish the movement of cells or aggregates); 1, poor (sluggish flow allowing for much clearer observation of cell or aggregate movement); and 0, no blood flow in most of the vessels.

Aggregate formation was similarly quantitated: 0, minimal to no aggregates visible in most vessels; 1, small aggregates in half of the vessels; 2, small aggregates in >half of the vessels; 3, large aggregates in major vessels that still have blood flowing; and 4, large aggregates in major vessels with no blood flow.

The 1st 5 min of videotaping explored all 5 regions just before toxin injection and was designated as time 0. Videotaped observations were repeated 2, 5, 10, 20, and 40 min after toxin injection. Each region was scored independently for each animal. The scores of all 5 regions were then averaged to give a final score at each time point for the individual animal. The blood-flow mean score for all animals in a given group is represented as the percentage of change (± SD) from baseline. Mean aggregate scores were normalized and are reported as the increase above baseline. A repeated-measures analysis of variance (ANOVA) was done to determine the effects of toxins and treatment on changes in normalized blood-flow values and aggregate scores over time. The repeated-measures ANOVA was used to compare the means of the different groups of animals. The Tukey-Fisher least significant difference criterion was used to compute post hoc t tests for pairwise comparisons. P < .05 was considered statistically significant.

Flow cytometry. Whole blood was obtained from healthy human volunteers who had not taken any medications for the previous 10 days and who had platelet counts of 210–265,000/μL. Blood was obtained by venipuncture, using a 19-gauge needle without the aid of a tourniquet, and was collected into heparinized tubes. The first 2 mL of collected blood was discarded. PLC-induced platelet/platelet (homotypic) aggregation was analyzed as previously described [8].

For flow cytometric analysis of activated platelet/neutrophil heterotypic aggregation, whole blood (100 μL) was mixed with 10 μL of fluorescein isothiocyanate (FITC)-conjugated anti-human CD62P (P-selectin, an activated platelet marker) and phycoerythrin-conjugated anti-human CD11b (a granulocyte marker) for 10 min at 37°C. Blood (100 μL) was stimulated with PBS, 5 μM FMLP, clostridial toxins (10 μL of a 1:20 dilution of the shock toxin preparation) or rPLC (1 U). These agonist concentrations were determined to be optimal in preliminary dose-response studies (data not shown). After 5 min at 37°C, samples were immediately prepared for flow cytometric analysis using a commercial formic acid red cell lysis/formalin cell fixation method (Q-prep, Coulter, Hialeah, FL) according to the manufacturer’s instructions. Analysis was performed using an Epic (Coulter) flow cytometer. Gates for granulocytes were drawn on the basis of their characteristic
forward and side scatter profiles. CD11b-positive events within this gate (routinely >95%) were analyzed for percent CD62P positivity and mean fluorescence intensity of the CD62P signal.

In a series of separate experiments investigating the role of mechanisms of PLC-induced platelet/granulocyte adherence, we pretreated whole blood for 5 min with (1) 20 μg/mL each of a neutralizing antibody against platelet gpIIbIIIa (anti-CD41a, clone HIP-8; PharMingen, San Diego) or an isotype-matched IgG control antibody (PharMingen); (2) 600 μM each Arg-Gly-Asp-Ser (RGDS, Sigma, St. Louis, MO), a peptide that represents the principal fibrinogen binding site, or Arg-Gly-Glu-Ser (RGES, Sigma), an analogous but inactive peptide; or (3) the competing fibrinogen-like molecules echistatin (100 nM, Sigma) or fibrinogen fragment 400–411 (600 μM, Sigma). These concentrations were determined in preliminary studies to completely inhibit toxin-induced platelet/platelet aggregation (not shown). To determine the role of P-selectin in platelet/granulocyte adherence, separate samples included fucoidan (40 μg/mL, Sigma), a sulfated glycan that binds to the lectin domain of CD62P and modulates the interaction with its receptor on polymorphonuclear leukocytes (PMNL) [15]. Previous studies had determined that this concentration of fucoidan was sufficient to inhibit 50% of thrombin-induced platelet/granulocyte complex formation (data not shown).

To verify that PLC-induced platelet/granulocyte complex formation was not affected by the inclusion of an antibody directed against P-selectin, a single experiment was performed in which platelets were labeled with monoclonal antibody directed against platelet CD42b (gpIIb, clone HIP1; PharMingen). This glycoprotein is present on all platelets regardless of their activation state. Cytotreated whole blood was incubated for 5 min at 37°C with PBS, PLC (1 U/mL), or thrombin (0.4 U/mL) in the presence or absence of either a rabbit polyclonal antibody that blocks P-selectin (5 μg/mL; PharMingen) or an equal amount of normal rabbit IgG.

Results

Videomicroscopy. Videotapes of blood-flow microscopy were reviewed by 2 blinded observers, and semiquantitative estimates of the blood flow in the arterioles and venules and the number and size of aggregates within these vessels were made, as described in Materials and Methods. Intramuscular injection of sterile saline or uninoculated (sterile) culture medium resulted in a gradual but statistically significant decrease in flow from baseline at all time points. However, venular blood flow in animals receiving the clostridial toxin showed a much sharper, statistically significant decrease in blood flow at each time point until, at 20 and 40 min, flow was only 10% and 7% of baseline, respectively (figure 1A). Blood flow in the arterioles was similarly affected, so that by 40 min, flow was 6% of baseline (figure 1B). ANOVA revealed a significantly greater effect of clostridial toxin on venular blood flow, compared with the flow in the arterioles because of a greater decline in flow at 2, 5, and 20 min in the venular system. These results are comparable with results of our Doppler flow studies [8].

Aggregate formation in the saline treatment/saline challenge control group was minimal and not statistically significant at any time point (figure 2). In contrast, many small aggregates were visible in venules within 2–5 min of clostridial toxin administration (figure 2A). 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 (figure 2B), although their appearance 5–8 min
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.

**Role of PLC in clostridial toxin-induced blood-flow deficits.**

Our previous studies of toxin-induced changes in skeletal muscle microcirculation demonstrated that PLC was responsible for the perfusion deficit induced by im injection of the clostridial toxin preparation [8]. To further confirm this finding, the semiquantitative study described above included 2 animals (experimental group 6) that were injected with rPLC (1 received 8 and the other 12 U). Many small aggregates were seen 2 and 5 min after the injections. The venous aggregate scores were 1.30 (8 U rPLC) and 2.35 (12 U rPLC) at 5 min. Aggregate size and number persisted at this level for 40 min in the animal receiving 8 U (aggregate score: 1.63), and although venular blood flow was initially slowed to 73% of the baseline flow, by 20–40 min it had returned to 93% of baseline. In animals receiving 12 U of rPLC, aggregates in venules and, to a lesser extent, in arterioles progressively increased in size and number from 10 to 40 min: The venule aggregate score was 2.35 at 10 min and 3.50 at 40 min. Venular blood flow in these animals slowed markedly at 2 min (44% of baseline), persisted at that level for 20 min, and then declined further, reaching a nadir (23% of baseline) at 40 min. Arteriolar flow was similarly reduced (44% of baseline at 40 min).

Preincubation of the clostridial toxin preparation with 1C6, a neutralizing monoclonal antibody against PLC, before injection completely prevented both the formation of aggregates and decreases in blood flow in both the venous and arterial circulations (experimental group 5; figures 1 and 2). 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 solely to vasoconstriction; no aggregates were observed in any vascular compartment.

**Role of platelets and granulocytes in toxin-induced perfusion deficits.** We previously demonstrated that the earliest toxin-induced intravascular aggregates consisted of activated platelets [8]. To confirm the role of platelets in toxin-induced perfusion deficits, intravital microscopic analysis of skeletal muscle blood flow and vascular aggregate formation was repeated in animals pretreated with heparin 10 min before toxin injection. In these animals, both venous and arterial blood flow was significantly improved (figure 1), and intravascular aggregate formation was significantly reduced (figure 2) from the 2-min time point through the conclusion of the experiment (40 min), compared with the flow in animals pretreated with saline. Heparin pretreatment did not, however, completely restore blood flow to baseline levels (figure 1).

Because the histologic staining of tissue injected with clo-
tridial toxin demonstrated leukocytes associated with the intravascular aggregates and the vascular endothelium [8], we determined the dynamics of toxin-induced perfusion deficits in animals depleted of circulating neutrophils. Surprisingly, pretreatment of animals for 18–24 h with antineutrophil serum, which reduced the circulating neutrophil count to <200/mm³ (data not shown), completely prevented toxin-mediated aggregate formation (figure 2) and related blood-flow deficits (figures 1) in both the arterial and venous systems.

Analysis of toxin-induced platelet/granulocyte heterotypic aggregation by flow cytometry. Our previous flow cytometry studies of the effects of PLC on platelet aggregation demonstrated the PLC-induced formation of large (i.e., >10 μm) P-selectin–positive aggregates [8]. We have found that this population includes both homotypic and heterotypic aggregations, consisting of platelets alone or platelet/granulocyte complexes, respectively. Therefore, we investigated the dynamics of and mechanisms responsible for PLC-induced formation of platelet/granulocyte aggregates in whole blood. Compared with unstimulated blood, crude clostridial toxin or rPLC stimulated a 30-fold increase in the percentage of granulocytes bearing the platelet activation marker, CD62P (figure 3). Visual inspection of toxin-treated whole blood revealed that platelets bound to granulocytes in a uniform rosette pattern (not shown). The mean fluorescence intensity of granulocyte-associated CD62P induced by the crude clostridial toxin or rPLC was 16.6 ± 4.3 and 13.2 ± 3.5, respectively, compared with 1.7 ± 1.0 for the PBS-stimulated control (mean ± SD of 4 experiments done in duplicate; figure 3). The peptide FMLP (5 μM) was included as a granulocyte activator that does not induce formation of platelet/granulocyte complexes [16].

To examine the molecular mechanism of PLC-induced heterotypic aggregation, we pretreated whole blood with agents that target either gpIIbIIIa or platelet P-selectin. The monoclonal antibody against gpIIbIIIa (anti-CD41a) and peptides that mimic the fibrinogen molecule or its binding epitopes specifically reduced both clostridial toxin– (not shown) and rPLC-stimulated binding of platelets to granulocytes (figure 4). In contrast, a control antibody, a control peptide, and a competitive inhibitor of P-selectin (i.e., fucoidan) had no effect. Furthermore, inclusion of a polyclonal antibody against P-selectin eliminated thrombin-induced complex formation but had no effect on the PLC-mediated response (figure 5).

Discussion

Clinical gas gangrene caused by C. perfringens is remarkable for the speed with which viable tissue is destroyed. The histopathology of gas gangrene is also unique in that few inflammatory cells are found at the site of infection, yet adjacent vasculature contains abundant acute inflammatory cells, largely neutrophils. C. perfringens produces a myriad of extracellular virulence factors that probably all contribute to these unique clinical and histopathologic findings. Yet, using a variety of techniques, we and others have demonstrated that PLC is the major lethal factor (reviewed in [1]). Results presented here and in our companion paper [8] clearly demonstrate a principal and direct role for PLC in the rapid and sustained decline in muscle perfusion, as measured by Doppler flowmetry. In this study, detailed in vivo microscopy analyses of the responses to PLC revealed both the rapid appearance (within minutes) of intravascular platelet aggregates and 4 progressive stages of intravascular events leading to complete cessation of flow in both venules and arterioles. Of interest, histopathologic examination of these poorly perfused tissues in response to PLC demonstrated myonecrosis, an absence of neutrophils in tissue, and accumulation of neutrophils within the vasculature. These data support our hypothesis that PLC-mediated occlusion of the microcirculation contributes heavily to tissue necrosis in gas gangrene.

This study also elucidates a new mechanism of pathogenesis that involves the ability of PLC to activate the platelet fibrinogen receptor gpIIbIIIa. Our previous work demonstrated that injection of PLC into muscle caused the rapid formation of freely mobile intravascular aggregates that consist of activated platelets [8]. This observation suggested that PLC stimulated the conformational change in gpIIbIIIa necessary for platelets in circulation to bind soluble fibrinogen. Indeed, results of this study demonstrated that PLC-induced homotypic aggregation of platelets is mediated by gpIIbIIIa, since aggregation in vitro could be neutralized by antibody against gpIIbIIIa or competitively inhibited by peptides and proteins that mimic the
Figure 4. Mechanism responsible for phospholipase C (PLC)-induced formation of platelet/granulocyte complexes was investigated by flow cytometry. Heparinized whole blood was pretreated with 1 of the following: a neutralizing antibody against gpIIbIIIa (CD41a) or isotype-matched IgG control antibody; Arg-Gly-Asp-Ser (RGDS) or Arg-Gly-Glu-Ser (RGES), an analogous but inactive peptide; competing fibrinogen-like molecules echistatin or fibrinogen fragment 400–411; or P-selectin inhibitor fucoidan. Blood was stimulated with recombinant PLC (rPLC; 1 U/100 µL whole blood) and then processed for flow cytometry. Data represent the mean (± SD) fluorescence intensity of granulocyte-associated CD62P of 3 experiments done in duplicate.

Although P-selectin binding of PMNL glycoproteins has been the paradigm for platelet/PMNL interactions, other investigators have demonstrated that gpIIbIIIa (CD41/CD61) also participates in the adhesion of thrombin-activated platelets to PMNL in vitro [19–21]. These studies have shown that this interaction is fibrinogen-dependent [20, 21], that CD11b/CD18 serves as the PMNL ligand for fibrinogen [22, 23], and that this interaction is further enhanced when the functionally active conformation of CD11b/CD18 is expressed [24]. These findings have been assimilated into a multistep adhesion cascade model in which a platelet P-selectin-dependent recognition step is followed by a gpIIbIIIa/CD18-dependent stabilization step (reviewed in [25]). Although θ-toxin from C. perfringens stimulates the functional up-regulation of CD11b/CD18 [3], our results suggest that PLC bypasses the P-selectin–dependent recognition step and alone is sufficient to produce large, stable heterotypic aggregates. Such cellular activation is probably the consequence of direct diacylglycerol generation and protein kinase C activation induced by PLC [26].

Histologic staining of tissue injected with clostridial toxin demonstrated leukocytes associated with both the intravascular aggregates and the vascular endothelium [8]. Furthermore, flow cytometric analysis of PLC-induced platelet aggregation revealed a population of very large P-selectin–positive cells that fell within the granulocyte gate, which suggested a heterotypic population. Indeed, our results demonstrate that PLC strongly induces the formation of platelet/granulocyte complexes. Because other investigators have shown that adherence of activated platelets to PMNL in response to agonists, such as thrombin, is mediated by the binding of platelet P-selectin to leukocyte glycoproteins (reviewed in [18]) and because PLC induced expression of this adherence molecule in platelets [8], it was expected that P-selectin would mediate PLC-induced heterotypic complex formation. However, agents that markedly inhibited thrombin-induced platelet/granulocyte complexes, including a blocking polyclonal antibody against P-selectin, had no effect on PLC-induced binding of activated platelets to PMNL. In contrast, those anti-gpIIbIIIa strategies found to completely inhibit PLC-induced homotypic platelet aggregation also inhibited heterotypic cellular aggregation induced by PLC.

fibrinogen molecule-binding site. This was expected since activation of gpIIbIIIa is the predominant mechanism responsible for formation of fibrinogen bridges between adjacent platelets (reviewed in [17]). Thus, it is likely this mechanism is also responsible for PLC-induced platelet aggregation in vivo.

That platelet/neutrophil interactions play a critical role in the microvascular dysfunction induced by PLC is supported by the finding that pretreatment of animals with antineutrophil serum abrogated both aggregate formation and blood-flow deficits. In addition, the ability of heparin pretreatment to reduce aggregate formation and partially restore blood flow in tissues injected with PLC suggests a role for thrombin in these events.
These observations may not be unrelated. For instance, platelet/leukocyte complexes could propagate thrombosis, since leukocytes immobilized on a platelet thrombus have enhanced procoagulant activity [27] and release potent soluble platelet activating agents, such as cathepsin G (reviewed in [25]). Furthermore, exposure of platelets to PLC enhances their ability to stimulate prothrombin conversion by coagulation factors Xa and Va because of increased expression of phosphatidylinerine on the platelet surface [28]. Thus, the in vivo effects of heparin can be explained by its ability to modulate thrombin activity leading to muscle destruction in C. perfringens gas gangrene. Therapeutic strategies that target platelet gpIIbIIIa, such as those currently used in treating unstable angina, may prevent vascular occlusion and maintain tissue viability and therefore provide an alternative to radical amputation for patients with this infection. Experimental evaluation of this strategy is currently underway.

Acknowledgments

We thank Hiroko Sato for providing neutralizing monoclonal antibodies against α-toxin and phospholipase C, J. Tso for providing the recombinant phospholipase C, and Nicholas Hadjokas and Cliff Bayer for invaluable assistance with the flow cytometry analyses.

References


