The Role of Clostridial Toxins in the Pathogenesis of Gas Gangrene

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Clostridium perfringens gas gangrene is, without a doubt, the most fulminant necrotizing infection that affects humans. In victims of traumatic injury, the infection can become well established in as little as 6–8 h, and the destruction of adjacent healthy muscle can progress several inches per hour despite appropriate antibiotic coverage. Shock and organ failure are present in 50% of patients and, among these, 40% die. Despite modern medical advances and intensive-care regimens, radical amputation remains the single best life-saving treatment.

Over the past century, much has been learned about the pathogenesis of this disease, and novel therapies are on the horizon for patients with this devastating infection.

THE ORGANISM AND ITS TOXINS

Clostridium perfringens is a gram-positive, spore-forming, nonmotile, rod-shaped organism commonly found in soil and in the intestines of humans and other animals. The species has been divided into 5 distinct types, A–E. Of these subgroups, C. perfringens type A causes the majority of human infections. Although classified as an anaerobe, C. perfringens is somewhat aerotolerant. Under optimal conditions, its generation time can be as little as 8–10 min, and growth is accompanied by abundant gas production.

Gas gangrene caused by C. perfringens is characterized by extensive local destruction of muscle (myonecrosis), rapid destruction of viable tissue, shock, and, ultimately, death. Of the many extracellular toxins produced by this organism, the α (phospholipase C, PLC) and θ (perfringolysin O, PFO) toxins are its major virulence factors. PLC’s role as the major lethal factor in C. perfringens infections is supported by numerous studies that have used a variety of approaches [1]. Both active and passive immunization of animals against PLC or its C-terminal fragment are protective in experimental wild-type infections. Similarly, experimental infections established with genetic mutants of C. perfringens lacking PLC or with strains that produce less PLC were markedly less fulminant, and mortality was significantly reduced [2].

The importance of PFO in the pathogenesis of gas gangrene has been largely controversial, despite the early knowledge of its hemolytic nature and its serological and antigenic relationships to the cholesterol-binding cytolysins from Streptococcus pyogenes, Streptococcus pneumoniae, and Listeria monocytogenes. Recently, the amino acid and nucleotide sequences of pneumolysin, streptolysin O, and PFO have been determined [3–6]. Impressive homology exists among the amino acid sequences of these toxins, particularly in the region that contains the cysteine residue near the amino terminus, where a highly conserved segment of 12 amino acids is identical for all 3 toxins. Some of these toxins, including PFO, have been shown to facilitate the growth of their respective organisms within mammalian phagocytic cells. Furthermore, experimental animal studies have demonstrated the protective efficacy of several antibody preparations against these...
toxins [1]. Thus, these studies support a principal role for thiol-activated cytolysins in the pathogenesis of their respective diseases.

PLC and PFO each contribute to the morbidity and mortality of gas gangrene by uniquely different mechanisms, which are detailed in the following sections. PLC is hemolytic, is cytotoxic to platelets and leukocytes, and increases capillary permeability—effects that are likely related to its ability to cleave sphingomyelin and the phosphoglycerides of choline, ethanolamine, and serine present in eukaryotic cell membranes. PLC requires calcium for optimal activity. Zinc enhances α-toxin production in culture and is essential for its activity in vivo. Histidine residues have been shown to be essential for the binding of zinc ions. Basak et al. [7] have recently crystallized α-toxin and have provided preliminary X-ray diffraction analysis of the protein. Titball et al. [8] have determined that the protein is composed of 2 functional domains: the N-terminal domain possesses the phospholipase C activity and the C-terminal domain binds to eukaryotic cell membranes.

PATHOGENESIS OF SHOCK AND ORGAN FAILURE

Hemodynamic collapse is a common occurrence in patients with gas gangrene caused by C. perfringens. In experimental studies, both PLC and PFO uniquely contribute to the development of shock. For example, a prompt reduction in cardiac index (CI) occurred in rabbits that received either PLC or a crude toxin preparation [9] (figure 1). Although many physiological mechanisms could contribute to such a reduction, we subsequently demonstrated a direct reduction in myocardial contractility ($dF/dt$) in isolated atrial strips bathed with recombinant PLC (rPLC) [9]. As reflected by the increased mortality in the rabbits that received rPLC and crude toxin, a greater reduction in CI was also measured in these groups compared with those that received recombinant PFO (rPFO) or normal saline [9]. Similarly, a marked decline in mean arterial pressure (MAP) was observed in rabbits treated with rPLC and crude toxin, although these effects were delayed until the later stages of the experiment (figure 2) [9]. Thus, rabbits that received PLC-containing toxin preparations maintained MAP in the face of a falling CI by as-yet-uncharacterized compensatory mechanism for a brief period before hypotension ultimately occurred. The physiological mechanisms that maintained MAP did not include significant changes in heart rate or central venous pressure until the terminal stages of the experiments, if at all [9].

In contrast, rabbits treated with rPFO demonstrated changes most characteristic of “warm shock,” with profound falls in peripheral vascular resistance after ~1 h of toxin infusion [9, 10]. Of interest, rabbits that received crude toxin (containing both PLC and PFO activity) demonstrated peripheral vascular

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**Figure 1.** Effects of clostridial exotoxins on cardiac index (CI). Awake rabbits ($n = 6$ per group) were slowly infused (0.33 mL/min) via catheter in the central vein with 50 mL of (1) sterile normal saline, (2) 150 hemolytic units (HU) of recombinant θ toxin, (3) 40–50 units of recombinant phospholipase C (PLC), or (4) a crude clostridial toxin preparation that contained 150 HU θ toxin activity and 50 units PLC activity. CI was measured over a 3-h period by thermodilution. *Values significantly different from baseline. Data adapted from Asmuth et al. [9].
Figure 2. Effects of clostridial exotoxins on mean arterial pressure. Awake rabbits (n = 6 per group) were slowly infused (0.33 mL/min) via catheter in the central vein with 50 mL of (1) sterile normal saline, (2) 150 hemolytic units (HU) of recombinant θ toxin, (3) 40–50 units of recombinant phospholipase C (PLC), or (4) a crude clostridial toxin preparation that contained 150 HU θ toxin activity and 50 units PLC activity. Mean arterial pressure was monitored continuously for 3 h via a catheter placed in the carotid artery. *Values statistically different from baseline. Data adapted from Asmuth et al. [9].

resistance (PVR) values intermediary to the other toxin groups. This latter observation provides insights into possible antagonistic interactions between rPFO and rPLC in terms of PVR. In total, these experiments suggest that PLC initially augments cardiac function, counteracting the vasodilatory effect of rPFO. Later, decreased cardiac output, hypotension, and death occurred. In addition, PLC may contribute indirectly to shock by stimulating production of endogenous mediators such as TNF [11] (figure 3) and platelet-activating factor [12].

PFO likely contributes to septic shock through indirect routes, including the augmented release of TNF, IL-1, and IL-6 [13, 14], platelet-activating factor (PAF), and prostaglandin I₂ [15]. Perhaps PFO-induced synthesis of nitric oxide by host cells such as macrophages or endothelial cells could also play a role in early hypotension. In addition, PLC and PFO may act synergistically in inducing hypotension, hypoxia, and reduced cardiac output. This latter point should be considered when interpreting results from experiments that have used isogenic mutants or single toxins.

Thus, shock associated with gas gangrene may be attributable, in part, to direct and indirect effects of toxins. PLC directly suppresses myocardial contractility [10], thereby contributing to profound hypotension via a sudden reduction in cardiac output [9]. PFO reduces systemic vascular resistance and markedly increases cardiac output [9, 10]. PFO-induced after-load reduction occurs undoubtedly through the induction of endogenous mediators that cause relaxation of blood vessel wall tension [15]. Reduced vascular tone develops rapidly, and, to maintain adequate tissue perfusion, a compensatory host response is required to either increase cardiac output or rapidly expand the intravascular blood volume. In contrast, patients with gram-negative sepsis compensate for hypotension by markedly increasing cardiac output; however, this adaptive mechanism is abrogated in C. perfringens–induced shock because of direct suppression of myocardial contractility by PLC [10].

THE PATHOGENESIS OF TISSUE NECROSIS

C. perfringens gas gangrene is an aggressive infection in which viable tissue is rapidly destroyed despite appropriate antibiotic therapy, leaving the modern physician with few treatment alternatives save the centuries-old practice of radical amputation. Trauma introduces organisms into the deep tissues and produces an anaerobic niche with a sufficiently low redox potential and acid pH for optimal clostridial growth. Histologically, clostridial myonecrosis is remarkable for both the absence of acute inflammatory cells in tissues and the accumulation of leukocytes between fascial planes and within small vessels (leukostasis) [1, 16, 17]. This picture is distinctly different from in-
Figure 3. Phospholipase C (PLC) induces TNF production in human peripheral blood mononuclear cells. TNF-α levels were measured in supernatant fluid from 10^6 human mononuclear cells stimulated with recombinant PLC. Samples were collected at 24 h and assayed in duplicate by commercial enzyme-linked immunosorbent assay. From Stevens and Bryant [11].

Infections caused by bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, or *S. pneumoniae*, which are characterized by minimal tissue destruction and a luxuriant leukocytic response at the site of infection. The rapid progression of infection and tissue necrosis associated with clostridial gas gangrene is related to the absence of an acute tissue inflammatory response, to tissue perfusion deficits resulting from toxin-mediated vascular dysfunction and injury, and to the elaboration of potent cytotoxins and proteases.

**Mechanisms of vascular dysfunction.** We have recently shown that the rapid destruction of muscle involves toxin-mediated impairment of local and regional blood flow [18]. Intramuscular injection of either a clostridial toxin preparation that contains both PFO and PLC activity or of recombinant PLC into rat abdominal musculature caused a rapid, dose-dependent, and irreversible decrease in blood flow (figure 4) that was not due to vasoconstriction but did parallel the formation of freely moving intravascular aggregates initially in venules (<2 min) and, later (8 min), in arterioles [18]. Immunohistochemistry demonstrated that these early aggregates consisted of activated (i.e., P-selectin positive) platelets. Later (20–40 min), aggregates enlarged and consisted of platelets, fibrin, and neutrophils [18]. These heterotypic aggregates became lodged within vessels, completely obstructing local and regional blood flow. Flow cytometry of human whole blood confirmed that PLC induced formation of both activated platelet/platelet (not shown) and platelet/neutrophil aggregates (figure 5) [19]. Neutralization of PLC activity completely abrogated human platelet/neutrophil responses and reduced perfusion deficits in the rat model [18].

Although P-selectin binding of polymorphonuclear leukocyte (PMNL) glycoproteins has been the paradigm for platelet/PMNL interactions, other investigators have demonstrated that the platelet fibrinogen receptor, gpIIbIIa (CD41/CD61), also participates in the adhesion of activated platelets to PMNL in vitro [20–22]. These studies have shown that this interaction is fibrinogen-dependent [21, 22], that CD11b/CD18 serves as the PMNL ligand for fibrinogen [23, 24], and that this interaction is further enhanced when the functionally active conformation of CD11b/CD18 is expressed [25]. The observation that injection of PLC into muscle caused the rapid formation of freely mobile intravascular aggregates consisting of activated platelets suggested that PLC stimulated the conformational change in gpIIbIIa necessary for platelets in circulation to bind soluble fibrinogen. Indeed, PLC-induced platelet/neutrophil aggregation could be neutralized by antibody against gpIIbIIa or competitively inhibited by peptides and proteins that mimic the fibrinogen molecule binding site (figure 6) [19]. In contrast, strategies that targeted P-selectin had no effect (fucoidan, figure 6) [19]. Thus, it is likely that PLC-induced activation of gpIIbIIa is responsible for PLC-induced platelet/platelet and platelet/neutrophil aggregation in vivo.

**Mechanisms of toxin-induced suppression of the acute inflammatory response.** Several plausible mechanisms exist to explain the lack of a tissue inflammatory response in clostridial gas gangrene. First, an absence of bacterial- or host-derived...
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figure 4. phospholipase c (plc) induces a rapid and irreversible decrease in skeletal muscle blood flow. rat abdominal muscles were injected with 0.1 ml of (1) sterile normal saline, (2) 10 μm phenylephrine, or (3) a clostridial toxin preparation that contained 8 units of 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. from bryant et al. [18].

chemoattractants could account for the paucity of leukocytes in the tissues; however, studies have shown that both an extracellular component of bacterial culture and serum incubated with killed bacilli were potent chemoattractants [17]. second, both plc and pfo are cytolytic for leukocytes in high concentrations [26], and the destruction of any infiltrating phagocytes at the site of active bacterial proliferation and toxin elaboration likely contributes to the marked reduction of inflammatory cells in these areas. however, were this the only mechanism responsible for the absence of a tissue inflammatory response, abundant phagocytes should be found in tissues distal to the nidus of infection, but, as the infiltrating phagocytes approached the site of infection, they would be abruptly halted at the point where toxin concentrations reached cytolytic proportions. thus, cytotoxicity alone could account for what is classically observed in both human and experimental cases of gas gangrene—the paucity of inflammatory cells in the tissues—but it does not explain the characteristic leukostasis in adjacent vasculature.

a third possible mechanism involves the effects of sublytic amounts plc and pfo on the function and interaction of leukocytes and endothelial cells. plc and pfo each uniquely affect the normal, physiological mechanisms of leukocyte accumulation, adherence, and extravasation (see below). toxin-induced dysregulation of these events, which orchestrate the pyogenic responses with other infections, could, in part, explain the leukostasis and anti-inflammatory response characteristic of clostridial gas gangrene. furthermore, such dysregulation could lead to local and regional ischemia, thereby extending the region for optimal clostridial proliferation.

effects of clostridial toxins on endothelial cell function. successful transmigration of leukocytes through the vessel and to the site of infection is the culmination of a complex cascade of both leukocyte- and endothelial cell (ec)–dependent adherence and activational events. initially, the circulating, inactivated leukocyte is tethered to the activated ec and rolls along the vessel’s luminal surface—processes mediated by selectins. tethering results in juxta-activation of the leukocyte by paf, functional up-regulation of leukocyte cd11b/ cd18, and firm adhesion to intercellular adhesion molecule–1 (icam-1; cd54) constitutively expressed on the ec [27]. local production of cytokines (e.g., tnf and il-1) augments the inflammatory response by stimulating ec to produce the neutrophil chemoattractant/activator, il-8, to increase icam-1 expression, and to transiently express endothelial leukocyte adhesion molecule–1 (e-selectin). strongly adherent, activated leukocytes move to ec junctions and emigrate between endothelial cells, aided by platelet-endothelial cell adhesion molecule–1.

our work has shown that plc strongly induces the expression of e-selectin and icam-1 on cultured human umbilical vein endothelial cells [28]. the magnitude and duration of these
Platelet/granulocyte complex formation is induced by phospholipase C (PLC). Heparinized whole blood was activated by (1) PBS, (2) n-formyl-methionyl-leucyl-phenylalanine (fMLP), (3) a crude clostridial toxin preparation that contained both PLC and perfringolysin O activity, or (4) recombinant PLC in the presence of fluorescein isothiocyanate–conjugated anti-human CD11b (granulocyte marker) and phycoerythrin-conjugated anti-human CD62P (platelet marker). Dual color-flow cytometry was performed on the CD11b$^+$ events located within the granulocyte gate. Data are the mean ($\pm$ SD) fluorescence intensity of granulocyte-associated CD62P from 4 experiments done in duplicate. fMLP was included as a granulocyte activator that does not induce complex formation. From Bryant et al. [19].

Responses were similar to that reported in other studies that used either TNF or IL-1 or lipopolysaccharide from gram-negative organisms. In addition, PLC also stimulated production of endothelial cell–derived IL-8 [28]. The local production of physiological concentrations of IL-8 in gas gangrene could both amplify the recruitment of leukocytes and prime them for enhanced respiratory burst activity. Alternatively, high concentrations of IL-8 attenuate transmigration of leukocytes through an endothelial cell monolayer in response to chemoattractants—a process termed “heterologous desensitization” [29]. This desensitization results from inhibition of neutrophil F-actin polymerization in response to chemoattractant stimulation [30]. Of interest, we have shown that PFO, but not PLC, at sublytic concentrations also prevents F-actin polymerization in response to chemoattractant stimulation (see below) [17]. Finally, toxin-induced expression of PMNL and endothelial cell adhesion molecules could result in reduced diapedesis [17].

**Effects of clostridial toxins on neutrophil function.** In contrast to PLC, PFO caused a modest, but significant, increase in endothelial ICAM-1, had no effect on E-selectin expression, and did not induce detectable IL-8 synthesis [28]. Yet intramuscular injection of PFO in mice produces marked vascular leukostasis adjacent to the site of toxin injection [17], which suggests that PFO may impair the inflammatory response by primarily affecting neutrophil, rather than endothelial cell, function. Indeed, sublytic concentrations of PFO dose-dependently stimulated random migration of neutrophils but decreased directed migration toward n-formyl-methionyl-leucyl-phenylalanine or a complement-derived chemoattractant [26] and prevented F-actin polymerization by leukocytes in response to chemoattractant factor stimulation [17]. Thus, in vivo, desensitization of neutrophils could contribute to the lack of phagocyte emigration into tissues infected with *C. perfringens*.

**SUMMARY**

These pieces of evidence can be assimilated into a molecular and cellular model of pathogenesis that is initiated by direct toxin effects on venous capillary EC function, leading to the expression of proinflammatory mediators and adhesion molecules and initiation of platelet aggregation. Toxin-induced hyperadhesion of leukocytes with enhanced respiratory burst activity due to toxins directly or to toxin-induced IL-8 or PAF synthesis by host cells and toxin-induced chemotaxis deficits could result in neutrophil-mediated vascular injury. Direct toxin-induced cytopathic effects on EC may also contribute to vascular abnormalities associated with gas gangrene. Over prolonged incubation periods, PLC at sublytic concentrations causes EC to undergo profound shape changes similar to those described after prolonged TNF or IFN-γ exposure. In vivo, conversion of EC to this fibroblastoid morphology could con-
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Figure 6. Phospholipase C (PLC)–induced platelet/granulocyte complex formation is mediated by gplIbIIa. Heparinized whole blood was pretreated with a neutralizing antibody against gplIbIIa (anti-CD41a) or isotype-matched control IgG or peptides or proteins that mimic fibrinogen, including (1) Arg-Gly-Asp-Ser (RGDS) or Arg-Gly-Glu-Ser (RGES; an analogous but inactive peptide), (2) echistatin, an RGD-containing polypeptide and potent anticoagulant from the venom of the viper Echis carinatus, (3) fibrinogen fragment 400–411, or (4) the P-selectin inhibitor, fucoidan. Blood was stimulated with recombinant PLC and processed for dual color-flow cytometry. Data represent the mean (±SD) fluorescence intensity of granulocyte-associated CD62P expression of 3 experiments done in duplicate. From Bryant et al. [19].

tribute to the localized vascular leakage and massive swelling observed clinically with this infection. Similarly, the direct cytotoxicity of PFO could disrupt endothelial integrity and contribute to progressive edema both locally and systemically.

Thus, via the mechanisms outlined above, both PLC and PFO may cause local, regional, and systemic vascular dysfunction. For instance, the local absorption of exotoxins within the capillary beds could affect the physiological function of the endothelium lining the postcapillary venules, resulting in impairment of phagocyte delivery at the site of infection. Toxin-induced endothelial dysfunction and microvascular injury could also cause loss of albumin, electrolytes, and water into the interstitial space, resulting in marked localized edema. These events, combined with intravascular platelet aggregation and leukostasis, would increase venous pressures and favor further loss of fluid and protein in the distal capillary bed. Ultimately, a reduced arterial flow would impair oxygen delivery, thereby attenuating phagocyte oxidative killing and facilitating anaerobic glycolysis of muscle tissue. The resultant drop in tissue pH, together with reduced oxygen tension, might further decrease the redox potential of viable tissues to a point suitable for growth of this anaerobic bacillus. As infection progresses and additional toxin is absorbed, larger venous channels would become affected, causing regional vascular compromise, increased compartment pressures, and rapid anoxic necrosis of large muscle groups. When toxins, or the cytokines they induce, reach arterial circulation, systemic shock and multiorgan failure rapidly ensue, and death is common.

References