Supplementary Appendix

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Supplementary Appendix

Supplement to: Stevens DL and Bryant AE. Necrotizing Soft Tissue Infections

Contents

Pathogenesis of Group A Streptococcal and Clostridial Necrotizing Soft Tissue Infections

The classical clinical and histologic features of necrotizing group A streptococcal and clostridial infections are mediated by potent bacterial exotoxins and by mediators of the host response (for detailed review, see [1]).

Pain and Rapid Tissue Destruction: In high concentrations, some exotoxins are cytotoxic – contributing directly to tissue destruction and organ dysfunction, whereas lower concentrations can hyper-augment cellular responses, including cytokine production, cell-cell interactions and leukocyte degranulation. For instance, acute onset of severe pain and rapid destruction of healthy tissues have been attributed to tissue hypoxia from toxin-mediated microvascular thrombosis caused by occlusive platelet/leukocyte complexes (reviewed in [2]) (Supplemental Figure 1). 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 which are being rapidly destroyed in the progression of gas gangrene and group A streptococcal necrotizing infection do not bleed. This age-old observation has become dictum for surgeons who routinely remove necrotic tissue until bleeding is encountered. Indeed, histologic examination of necrotic tissues obtained from patients with StrepTSS at biopsy or amputation [3, 4] or experimental animals challenged with group A streptococcus or *C. perfringens* [5, 6] reveals thrombi and fibrin clots in capillaries, post-capillary venules and arterioles of the affected musculature and tissues.

Pain may also be attributed, in part, to the ability of pore-forming toxins to directly activate peripheral pain-sensing neurons as has been described for the alpha hemolysin from *S. aureus* [7]. Lastly, toxin-mediated cleavage of endogenous granulocyte chemoattractants (e.g.,

C5a, IL-8) by group A streptococcal exotoxins may also contribute to the reduced tissue inflammatory response [8, 9]

Perfusion Deficits. Though surgeons have, for centuries, relied on the ability of tissue to bleed to guide debridement, human studies have not provided any insights into the mechanisms responsible for the perfusion deficits associated with necrotizing, toxin-mediated infections. Our experimental studies of monomicrobial infections caused by group A streptococcus or *C. perfringens* have provided some clues and have identified possible therapeutic targets. For example, in a rat abdominal musculature model, vascular perfusion was rapidly (<2 min) and irreversibly (>40 min) decreased by streptolysin O (SLO) from group A streptococcus [10] and by alpha toxin from *C. perfringens* [6, 11] (Supplemental Figure 1a). In contrast, perfusion deficits caused by the vasoconstrictor, phenylephrine, were only transient over 5-10 min. Time-lapse videomicroscopy with subsequent immunohistochemistry showed that neither toxin caused vasoconstriction; instead reduced tissue perfusion was due to time-dependent occlusion of vessels by intravascular aggregates of platelets, fibrinogen and leukocytes that lodged first in the capillaries and microvessels (Supplemental Figure 1b), then proceeded to occlude venules and arterioles, ultimately resulting in ischemic necrosis of tissue. Neutrophil depletion (by anti-neutrophil serum) or inactivation of platelets (by aspirin or heparin treatment) prevented the toxin-mediated blood flow deficits [6, 11]. That this process occurs in humans is suggested by the arteriogram of a patient with group A streptococcus NF of the right leg (Supplemental Figure 1c).

Absence of a Tissue Inflammatory Response. In most classical descriptions of necrotizing streptococcal and clostridial infections, neutrophils are notably absent from the site of infection, yet such cells are abundant in the adjacent vasculature. This sequestration is associated, in part, with paralysis of diapedesis mechanisms since neutrophils burdened with large numbers of adherent platelets are unable to cross an endothelial cell barrier [12]. With *C.*

perfringens infection, dysfunctional diapedesis was mediated largely by alpha toxin-induced activation of the platelet fibrinogen receptor, glycoprotein IIb/IIIa [13]. With group A streptococcus, the predominant molecular mechanism of platelet/neutrophil interaction was streptolysin O-induced upregulation of platelet P-selectin binding to its cognate ligands on the neutrophil [10]. In addition, *in vivo* migration of neutrophils to sites of infection was hindered by the ability of cholesterol-dependent cytolysins such as PFO and SLO to functionally dysregulate CD11b-mediated binding of neutrophils to the vascular endothelium [14], to impair neutrophil chemotaxis [15-17] and to stimulate production of platelet activating factor by endothelial cells [18]. At high concentrations, such as those at the nidus of infection, these exotoxins are cytolytic which further explains the absence of leukocytes in the tissues. Lastly, these toxins directly impair neutrophil and macrophage phagocytic activities [15 19, 20].

Cardiomyopathy and Hemodynamic Collapse. Cytokines clearly mediate shock and organ failure in bacterial infections including those due to group A streptococcus. Experimental evidence suggests that TNFα is central to this process. Specifically, high levels of TNFα were observed in a baboon model of *S. pyogenes* bacteremia when profound hypotension was manifest [21]; administration of a neutralizing anti-TNFα antibody restored normal blood pressure and reduced mortality by 50% [21]. Diffuse capillary leak also contributes to hypotension in StrepTSS and is likely attributable to cytokines and other mediators, though may also be related to circulating M protein–fibrinogen complexes [22].

Multiple *S. pyogenes* exotoxins (e.g., streptococcal pyrogenic exotoxins [Spe] A, B and C, MF, SSA [23]) and potentially M protein fragments [24] act as superantigens to cause watershed induction of both monocyte- and lymphocyte-derived cytokines (tumor necrosis factor [TNF] -α, interleukin [IL]-1β, IL-6 and TNF-β, IL-2, interferon-γ, respectively) [23-29]. Superantigens also drive the clonal proliferation of specific Vβ T-cells, in part through induction of IL-2. Thus it would be expected to find expansion of superantigen-specific Vβ T-cell clones in an infected

individual. Yet studies in patients with StrepTSS demonstrate depletion, rather than expansion, of such subsets [30]. This enigma remains to be reconciled.

Among the 4 alleles of SpeA, alleles 2 and 3 are most common and have the highest affinity for MHC class II on antigen presenting cells [31]. Some clinical studies have suggested that variation in human leukocyte antigen (HLA) haplotype may predispose to worse outcomes in some patients with StrepTSS [29]. Finally, the lack of anti-SpeA antibodies is a predisposing factor for development of StrepTSS [32].

Other streptococcal virulence factors can also induce mononuclear cell pro-inflammatory cytokine production. Specifically, SpeB releases active IL-1β from preformed intracellular pools [33]. SLO also stimulates mononuclear cells to produce TNF-α and IL-1β and, in the presence of SpeA, has synergistic effects on IL-1β production [34]. Heat-killed *S. pyogenes* as well as isolated peptidoglycan and lipoteichoic acid are also potent inducers of TNF-α and IL-1β [35, 36]. In total, toxin-mediated, leukocyte-derived cytokines in circulation likely contribute to hypotension and organ dysfunction.

Recent evidence suggests that cardiomyocyte-derived cytokines are produced following direct *S. pyogenes* stimulation and after exposure to *S. pyogenes*-activated inflammatory cells [37]. In addition, viable *S. pyogenes* induced production of cardiomyocyte-derived stimulator/s that boosts macrophage production of matrix metalloproteinase-9, pro-inflammatory cytokines (IL-1β, IL-6) and cardio-depressant factors (iNOS) [38]. These locally produced, cardiomyocyte-derived cytokines (termed “cardiokines”) may mediate cardiac contractile dysfunction observed in some patients with StrepTSS who develop a unique and reversible form of cardiomyopathy characterized by global hypokinesia and reduced cardiac index [39].

Other non-cytokine-mediated mechanisms of shock may also play a role. For example, SpeB has been shown to release bradykinin from a high-molecular-weight kininogen [40]. Bradykinin is a potent vasodilator of systemic and pulmonary vasculature and could be

responsible, at least in part, for the early hypotension observed in StrepTSS [21]. In addition, recent studies demonstrated that SLO, via its ability to form membrane pores, is causes early, direct cardiomyocyte contractile dysfunction [41]. Within minutes of exposure, SLO disrupted the normal contraction response of isolated murine cardiac cells to electrical pacing. Later, SLO induced spontaneous, non-paced contractions characterized by hyper-augmented contractile force. These effects were mediated by an influx of calcium through SLO-induced membrane pores. Upon removal of SLO, normal electrical pacing resumed, suggesting that membrane lesions were repaired and normal intracellular calcium levels were restored. These observations are consistent with the clinical observation that cardiomyopathy is a reversible condition in patients who survive StrepTSS [39].

Cryptogenic Necrotizing Group A Streptococcal Infections. Mechanisms that underlay development of cryptogenic group A streptococcal infection following non-penetrating injury have been recently proposed. Using a murine model of this infection in which repeated eccentric contraction exercise created a moderate muscle strain injury, Bryant and colleagues have shown that circulating group A streptococcus specifically traffics to the injured site [42]. Trafficking correlated with peak expression of vimentin [42] – a key group A streptococcal ligand [43]. Because this intermediate filament protein is highly expressed on activated and proliferating skeletal muscle precursor cells (myoblasts) but not on mature healthy myofibers [44], these findings provide the first molecular mechanism to explain the development of severe GAS soft tissue infections precisely at sites of prior minor muscle trauma.

Figure S1

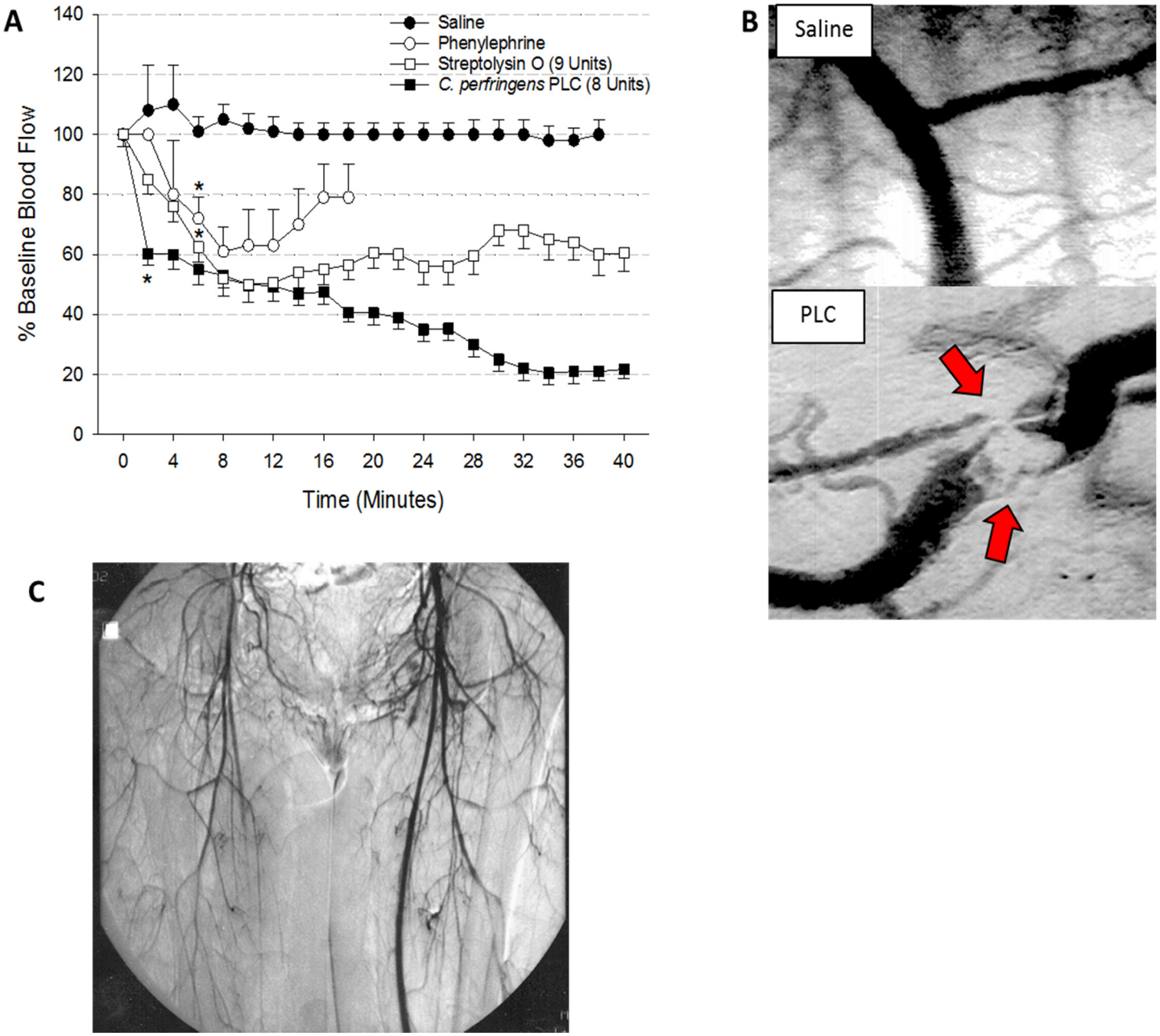

Figure S1. Perfusion Deficits in Necrotizing Infections. ***A,B)*** Rat abdominal muscles were injected with either sterile normal saline, phenylephrine, *C. perfringens* phospholipase C (PLC), or *S. pyogenes* streptolysin O (SLO). ***A)*** Blood flow was measured by laser Doppler blood perfusion monitor and is expressed as the percent of baseline perfusion ± standard error.

Values less than or equal to those marked with asterisks (*) are statistically different from respective baseline value. ***B)*** Top: Normal blood flow following saline injection; Bottom: SLO- (not shown) and PLC-induced regional reduction in blood flow was associated with the formation of occlusive intravascular aggregates in adjacent venules and arterioles (arrows) and later in medium- to large-sized vessels (not shown). The muscle supplied by these vessels ultimately became blackened and necrotic, suggesting that destruction of tissue was due to ischemia; adapted from [6, 10]. ***C)*** Arteriogram of patient with group A streptococcal necrotizing fasciitis/myonecrosis of the leg showing a marked reduction of blood flow in the affected tissue.

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