Investigation of the role of the toxins perfringolysin O (PFO) and sialidase in *Clostridium perfringens* gas gangrene infections

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ABSTRACT

Clostridium perfringens is the causative agent of gas gangrene. A lethal infection in mice requires a large inoculum suggesting that the immune system is involved in inhibiting disease. Human monocytic cells and neutrophils killed *C. perfringens* in vitro when complement was present. Macrophages and neutrophils co-localized with *C. perfringens* in vivo when bacterial numbers were low. Depletion of neutrophils and monocytes in mice revealed that monocytic cells play a role in inhibiting *C. perfringens* gas gangrene in mice infected with an intermediate dose.

C. perfringens can persist in the tissues and this could be mediated by persistence within macrophages. To examine if the toxin perfringolysin O (PFO) could mediate this, less active variants of PFO were used to examine what occurs between phagosomal escape and cell lysis. The mutant forms of PFO did mediate phagosomal escape in macrophages and were found within macrophages at higher numbers than wild-type *C. perfringens*. Our data were preliminary but may indicate that less active PFO mediates intracellular persistence.

To investigate the role of sialidase in *C. perfringens* gas gangrene we made *nanI*-, *nanJ*-, and *nanI*-/*nanJ*- mutants. We observed that NanI is responsible for the majority of sialidase activity of *C. perfringens* strain 13, that NanJ is an extracellular sialidase, and that these genes are transcriptionally regulated by sialic acid. Murine infection trials revealed that these sialidases may be protective for mice during infection.

In conclusion, murine monocytes inhibit disease onset and *C. perfringens* sialidase enhances mouse survival. However, the toxin PFO if less active promotes the survival of *C. perfringens* with macrophages.

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I. General Overview

A. *Clostridium perfringens*

Clostridium perfringens is an anaerobic, gram-positive, spore-forming rod shaped bacterium. This organism is a soil microbe and part of the normal intestinal flora of animals and humans (111). *C. perfringens* is also the most frequently encountered bacterium in gas gangrene infections (myonecrosis) (79, 129). In humans, *Clostridium perfringens* can also be the causative agent of food poisoning and necrotic enteritis. In addition, the bacterium causes a variety of gastrointestinal tract diseases in animals, such as food poisoning, necrotic enteritis, dysentery, and enterotoxaemia (111). *C. perfringens* produces 13 different toxins, which ascribe to it these pathogenic characteristics. The known toxins that *C. perfringens* produces are: α, θ, β, ε, δ, ι, κ, λ, μ, ν, enterotoxin (CPE), sialidase, and urease (Table 1-1). There are five types were classified by the specific combination of the above mentioned toxins that they produced (Table 1-2); however, the enterotoxin, α, θ, β, and ε, toxins are the primary virulence factors in *Clostridium perfringens* diseases (111).

B. Gas gangrene

C. perfringens type A, the leading pathogen in gas gangrene infections, produces α-toxin, phospholipase C (PLC), and θ-toxin, perfringolysin O (PFO), as its chief virulence factors (75). Gas gangrene is the bacterial invasion of healthy living tissues, which were previously undamaged by trauma or ischemia (75, 132). The infection, on average, develops within 24 hours after introduction of bacteria to the tissue through a deep, penetrating injury that compromises blood supply to the tissues creating a less oxygenated environment where *C. perfringens* can proliferate (129). In 1990, the incidence of gas gangrene was reported to be between 1,000 and 3,000 cases in the United States each year and the mortality rate for advanced cases was between 28–50%. Gas gangrene can also occur post-operatively or spontaneously. These cases have the highest rate of mortality, 24% and 38% respectively, due to the rapid spread of the disease and difficulty in diagnosis (100).

Once *C. perfringens* is introduced to the tissues, it quickly multiplies, dividing as rapidly as every 12 minutes, if the redox potential is sufficiently low (less than 0.074 mV (90)), and necrosis can advance ten centimeters per hour (75, 108). Obvious signs of infection can develop as quickly as three hours after infection or can be delayed as long as six weeks (79, 100).

Table 1-1. Toxins produced by *Clostridium perfringens*.[a]

Toxin	Activity
α (PLC)	Phospholipase C, sphingomyelinase
θ (PFO)	Cholesterol specific hemolysin, cytolysin
β	Forms cationic selective channels in cell membranes
ε	Alteration of cell membrane permeability
δ	GM2 specific hemolysin, cytolysin
ι	Forms pores, ADP-ribosyltransferase
κ	Collagenase
λ	Protease
μ	Hyaluronidase
ν	DNase
Enterotoxin (CPE)	Pore forming (changes cell membrane permeability)
Sialidase	Sialidase
Urease	Urease

[a] Based on a review by Rood and Cole (111).

Table 1-2. The five types of *C. perfringens*, the toxins they produce and the diseases they cause.[a]

	α	θ	β	ε	δ	ι	κ	λ	μ	ν	CPE	Neuraminidase	Urease	Diseases
A	x	x	-	-	-	-	x	-	x	x	x	x	x	Gas gangrene, food poisoning, necrotic enteritis in human infants and poultry
B	x	x	x	x	x	-	x	x	x	x	-	x	x	Dysentery in lambs, food poisoning in sheep, foals, goats
C	x	x	x	-	x	-	x	-	x	x	x	x	x	Necrotic enteritis in animals and humans, food poisoning in sheep
D	x	x	-	x	-	-	x	x	x	x	x	x	x	Food poisoning in sheep, pulpy kidney disease
E	x	x	-	-	-	x	x	x	-	x	-	x	x	Necrotic enteritis in rabbits

[a] Based on a review by Rood and Cole (111).

Symptoms include swelling, gas in the tissues, darkening of the skin, severe pain, fever, and rapid heart rate (75, 100, 160). Infected muscle tissue may appear superficially pale, non-contractile, and does not bleed. Infected muscle has a foul odor and is black and brittle at the end stages of disease (79, 100). Upon microscopic examination, the advancing edge of infection contains few bacteria and a moderate amount of leukocytes. There is edema, degradation of connective tissue fibers, occluded vessels, and red blood cell hemolysis. At the locus of infection, there are large numbers of bacteria, widespread fragmentation of muscle fibers and edema. Surrounding vessels are occluded, nuclear karyolysis, connective tissues fibers are destroyed, and no inflammatory cells are present. However, leukocytes can be seen within the fascia and vessels distal to the site (79, 103). Gas gangrene causes severe muscle damage and progresses rapidly, requiring aggressive treatment with large doses of antibiotics, debridement, and amputation (100, 135). In conjunction with these treatments, hyperbaric oxygen has been shown to be an effective therapy, but it is not a common or readily available treatment option (57).

C. Phospholipase C

Phospholipase C (PLC), or α-toxin, is the major lethal toxin in a *C. perfringens* gas gangrene infection. Evidence for the necessity of PLC in infection was shown by mouse infections with a *Bacillus subtilis* strain expressing *C. perfringens* PLC that caused severe gangrene symptoms and death in mice (86). Also, mice infected with a *plc*- strain of *C. perfringens* did not develop gangrene (5, 88). Vaccination with the C-terminal domain of PLC protected mice from severe gangrene symptoms and death following infection with wild-type *C. perfringens* (138, 156).

PLC causes many tissue changes within the host. Purified PLC injected intramuscularly into mice causes no influx of polymorphonuclear leukocytes to the site of infection, myonecrosis, thrombosis, the formation of intravascular aggregates, and death of the host (16, 17). In mice infected with the *plc*- strain, PMNs were found to move into the site of infection and myonecrosis was less than observed in wild-type infections (140). Also, mice injected with a *plc*- strain had a reduced number of intravascular aggregates and thrombi at the site of infection. The tissues of mice injected with a *plc*-/*pfoA*- (*pfoA* is the gene for the toxin

perfringolysin O) mutant of *C. perfringens* and complemented with *plc* displayed rapid and extensive spread of myonecrosis, intravascular thrombi, and moderate leukostasis, the inability of leukocytes to move out of the vasculature (6). In summary, PLC is important for a lethal infection, necrosis, thrombosis, leukostasis, and intravascular aggregate formation.

PLC is a phosphohydrolase that preferentially cleaves phosphatidylcholine and sphingomyelin (115). It is composed of 370 amino acids and has two domains (42). The N-terminal domain is the active domain and the C-terminal domain is the binding domain. The C-terminal domain is believed to bind three calcium ions to allow for interaction with phospholipid aggregates and lipid micelles (50, 83). It is postulated that the binding of the membrane induces a conformational change that allows the N-terminal domain to interact with the membrane, resulting in cleavage of the phospho-head groups (116). The N-terminal domain contains the active site and has an active and inactive form. The inactive from has two tightly bound zinc ions, while the loose binding of a third zinc ion results in the active form (38). Site-directed mutagenesis of the residues involved in binding the third zinc ion within the active site resulted in a nearly complete loss of lecthinase, sphingomyelinase, hemolytic activities, platelet aggregation, myotoxicity, and toxicity showing, that binding of rthe third zinc ion is required for PLC activity (1, 51).

PLC acts on host, i.e. eukaryotic cell membranes, where it degrades phospholipids, lecithin, and sphingomyelin, causing cell leakiness and death (73). PLC directly affects cell surfaces and the immune system. The effects of PLC on the immune system include leukostasis, the inability of leukocytes to leave the vasculature, by the upregulation of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and interleukin-8 (IL-8) on human umbilical vein endothelial cells (HUVECs) (19). Leukocyte-endothelial binding of these molecules results in weak adhesion of PMNs to the vasculature; however, PLC also causes β2 and β3 integrin binding events allowing stronger adhesion to the endothelium (40, 91). PLC also induces the formation of intravascular aggregates, which decreases blood supply to the tissues and interferes with diapedesis. Most vessels near the site of injection are at least partially occluded by these aggregates twenty to forty minutes after PLC toxin injection. PLC also increases production of platelet activating factor (PAF) and P-selectin on HUVECs,

resulting in increased neutrophil adhesion and intravascular aggregate accumulation (21). The intravascular aggregates began as free flowing platelet aggregates that grew in size due to the trapping or binding of leukocytes and fibrin (16). The receptor gpIIbIIIa mediates the binding of platelets to platelets and platelets to neutrophils within these aggregates (17). The depletion of neutrophils or blocking gpIIbIIIa results in decreased aggregate formation and increased blood flow to PLC-treated tissues. PLC also activates neutrophils and stimulates them to produce superoxide (91). Neutrophil activation, while neutrophils are trapped within the vasculature, could result in damage to the endothelium and possibly the subendothelium (19, 91, 137).

PLC also has profound effects on the cardiovascular system that may result in shock, which ultimately kills the host (139). It causes a large reduction in arterial pressure and heart rate by decreasing myocardial contractility (4). Decreased cardiac output combined with accumulation of intravascular aggregates and decreased oxygen pressure in the blood leads to a less oxygenated environment in which *C. perfringens* can proliferate (4).

In summary, PLC triggers an inflammatory response of the host endothelium and dysfunctional chemotaxis of host neutrophils causing the tissue changes observed in infection. PLC causes necrosis by cleavage of phosphotydalcholine and sphingomyelin in host cell membranes. The host further degrades the lipid tails to lecithin and diacylglycerol. This may activate the phospholipases and the arachidonic acid cascade of the host, resulting in the indirect activation of endothelial cells by PLC to release inflammatory mediators. These changes lead to a conformational change of gpIIbIIIa, which causes platelets to bind to fibrinogen decreasing blood flow due to platelet aggregates and platelet-leukocyte aggregates within the vasculature. PLC also induces leukocytes to remain attached to the endothelium and to have enhanced respiratory burst activities, thereby injuring the endothelium. These vascular injuries and the systemic diffusion of toxins could result in myocardial dysfunction culminating in the shock and death of the host. Also, the restriction of blood flow due to platelet-leukocyte intravascular aggregates reduces oxygen delivery and phagocytic killing allowing *C. perfringens* to flourish.

D. Perfringolysin O

Perfringolysin O (PFO), or θ-toxin, is a 53 kDa protein that belongs to the cholesterol-dependent cytolysin (CDC) superfamily of β-pore forming toxins (147, 158) and has been

implicated as an important toxin to the pathogenesis of gas gangrene (88, 140). PFO is a 494-residue preprotein requiring the removal of a 28-residue signal peptide for activation (111). Defining characteristics of a CDC, also known as thiol-activated cytolysin (73), include: inhibition by free cholesterol, activity on cholesterol-containing membranes, having a unique cysteine, and inactivity when oxidized (98). A region of homology characteristic of all CDCs is the undecapeptide near the C-terminus of the protein (ECTGLAWEWWR) (92, 148). The undecapeptide contains the single cysteine residue that gives CDCs oxygen-sensitivity. CDCs can only bind membranes with at least 40 mole percent cholesterol, or a closely related sterol, making them highly active on mammalian cells (146). The mature CDC protein has four domains; domain three and four are conformationally and functionally coupled even though they are not directly attached to each other (54). Conformational changes in domain three or four are communicated to the opposite domain through conformational changes in domain two.

CDCs form unusually large, aqueous pores that can range from 25 to 30 nm in diameter and are composed of 40 to 50 monomers (15, 31, 112) allowing for the efflux of large particles such as hemoglobin and IgG (56). These pores are formed when the water-soluble monomers recognize and bind the membrane via domain four (56, 62). Binding induces a conformational change in domain four that is communicated to domain 3 through domain 2. The monomers oligomerize on the surface of the membrane prior to insertion (124). Before insertion, the six α-helices of domain three change conformation to form two amphipathic β-hairpins (32, 122, 123). Stalling insertion until the completion of oligomerization allows for the alignment of the β-hairpins and the formation of hydrogen-bonds between the β-hairpins of adjacent monomers (122, 123). Hotze, et al., suggest that oligomerization is required prior to insertion to decrease the transitional energy barrier, i.e. it is more energetically favorable for the monomers to insert simultaneously (59). The β-hairpins are not exposed to the membrane prior to pore formation (55). The β-barrel inserts into the membrane causing the oligomer to undergo a 40 Å vertical collapse (31) due to structural changes in domain two. The β-hairpins of domain three span the membrane, whereas domain four is embedded in the outer leaflet of the membrane (Fig. 1-1), and domains one and two are not associated with the membrane (54, 95, 101). It is unclear how these cytolysins lyse phagosomal membranes and allow for bacterial escape from the

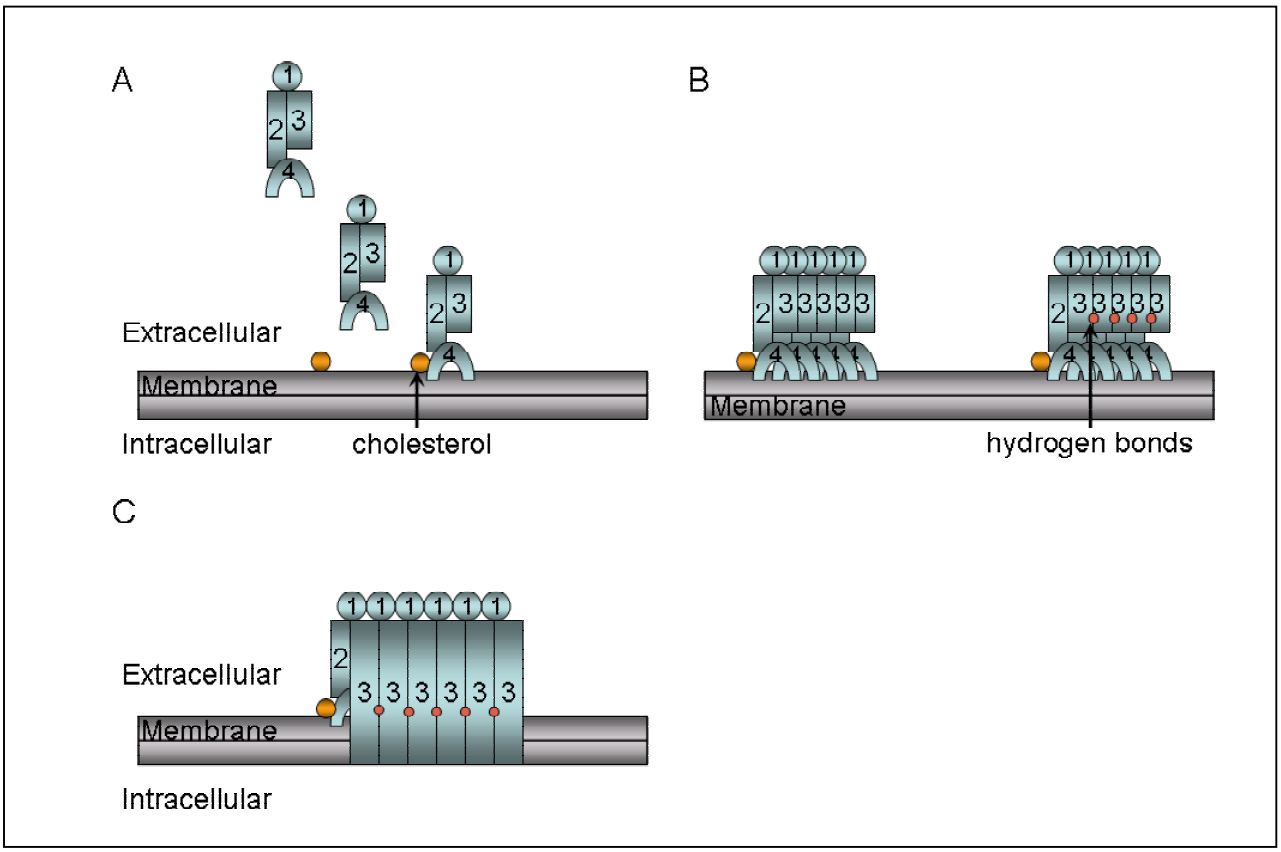

Figure 1-1. The mechanism of cholesterol-dependent cytolysin pore formation Cholesterol-dependent cytolysins bind cholesterol containing membranes and lyse these cells, presumably by forming pores in the plasma membrane. These proteins have four domains; domain 3 and 4 are the only domains that interact with the membrane. First, soluble monomers attach to the cell membrane in proximity to cholesterol and oligomerize on its surface (**A**). A ring or arch-like structure is created by the oligomerization of the monomers on the surface of the membrane. Hydrogen bonds form between the domain 3 β-hairpins of the different monomers (**B**). The oligomers form a pore by simultaneously inserting into the membrane (**C**). Reviewed in (54, 112, 124, 146).

phagosome, but it has been shown that they lyse erythrocytes by colloid osmotic lysis (52, 98, 146).

The role of cholesterol in binding and insertion of CDCs to membranes is unclear. Initially cholesterol was thought to be the receptor for the CDC membrane binding (37, 61, 63), but recent work has shown that its role may be associated with insertion into the membrane (47). Depletion of cholesterol on erythrocytes resulted in the oligomerization of three different CDCs (including PFO) on the membrane, but they failed to insert into the membrane and lyse the cell (47). Rossjohn et al, created a model for insertion using crystallographic data of PFO (112) suggesting that cholesterol binds in the tryptophan pocket of domain four and induces conformational changes that result in insertion of the oligomer. Their model also presents a role for cholesterol in stabilizing insertion by its association with the β-hairpins of the monomers (112).

PFO causes necrosis and hemolysis in disease as a direct result of its activity on cell membranes, but it also affects immunological signaling and the inflammatory response (134, 140). PFO is implicated as a main pathogenicity factor in inducing leukostasis in gas gangrene (15). PFO has been shown to upregulate the expression of CD11b/CD18 on PMNs and ICAM-1 on endothelial cells (15, 19). Also, PFO is cytotoxic to leukocytes at high concentrations and at low concentrations with prolonged exposure. PFO activates oxidative metabolism and leukotriene release by PMNs that are exposed to sublethal doses (14, 15, 19). Other CDCs such as streptolysin O and alveolysin were also shown to trigger leukotriene release by PMNs (13, 14). Granulocyte leukotriene release increases vascular permeability of local vessels and induces granulocyte chemotaxis (13). The activation of leukocytes while adhered to the vascular endothelium could result in severe vascular damage and increased edema (15, 19).

A *C. perfringens* mutant lacking the main virulence factor, PLC, *plc*- mutant, and having intact PFO (encoded the gene *pfoA*) was found to produce less myonecrosis than wild-type in a mouse, produced little to no vascular thrombi, was non-lethal, and attenuated PMN chemotaxis for 12 hours after infection (40, 140). Mice injected intramuscularly with killed washed *C. perfringens* and purified PFO exhibited similar susceptibility and pathology supporting the results of the genomic mutants (15, 140). These findings indicate that PFO is not required for

virulence (5), but that it does contribute to myonecrosis and is required to cause leukostasis (6) in a *C. perfringens* gas gangrene infection.

PFO also indirectly affects the cardiovascular system of the infected host. Intravenous injection (i.v.) of purified PFO causes a decrease in cardiac output and an increase in heart rate and central venous pressure (4, 139). PFO has no effect on myocardial contractility ex vivo, but i.v. administration of PFO resulted in hypotension and bradycardia of the host (4, 139). PFO may have a role in inducing shock in the patient, a common fatal complication of gas gangrene, by decreasing cardiac output through an unknown mechanism or vascular mediator (4, 139).

The *pfoA* gene has a major and minor promoter. The major promoter is regulated by the two-component virulence factor regulators the sensor kinase, VirS, and the response regulator, VirR (8). In the absence of VirR, *pfoA* is not transcribed (8). VirR binds upstream of *pfoA* at two imperfect direct repeats (CCCAGTTNTNCAC) named VirR boxes (25). Both VirR boxes and the exact spatial organization of these boxes are required for transcriptional activation by VirR and RNA polymerase. The VirR boxes must be eight base-pairs apart and the second VirR box must be six base-pairs from the -35 region (24).

E. Listeriolysin O

Listeriolysin O (LLO) is a 60 kDa cholesterol dependent cytolysin (CDC) secreted by *Listeria monocytogenes*, an intracellular pathogen (46). LLO like other CDCs is inhibited by low amounts of free cholesterol, activated by reducing agents, and has the consensus undecapeptide (81). LLO is required for growth and survival of *L. monocytogenes* within host cells. *B. subtilis* expressing LLO was found to escape macrophage phagosomes and live within the cytosol (97). LLO is active after internalization of the bacterium within host cells and acts on the phagosomal membrane to allow for escape and intracellular growth within the cytosol (98). The drop in pH that occurs as the phagosome is acidified is necessary for activation of LLO (30). LLO has maximal activity a pH 5.5 and little to no activity at pH 7, giving LLO pH specificity such that it can form pores on the phagosomal membrane, allow for escape from the acidic phagosome, but then is deactivated upon exposure to the neutral cytosol preventing damage to the host cell membrane (64). Increasing the pH of the phagosome by adding bases exogenously resulted in the inability of LLO to perforate the vacuole and grow intracellularly (10). *L.*

monocytogenes expressing a more active CDC, PFO, active at neutral and acidic pH, allowed the bacterium to escape the phagosome; however, it could not replicate intracellularly due to its lysis of the host cell (64). LLO has a leucine at residue 461 that is unique among the CDCs. Mutation of L461 to threonine, as in PFO, increased its activity at neutral pH and decreased virulence indicating the importance of this residue in the pH specificity of LLO (48).

LLO is rapidly degraded in the host cytosol, which may be due to the PEST-like motif at its N-terminus. This motif has been associated with promoting the cytosolic degradation of proteins (72). A form of LLO lacking the PEST-like motif accumulated in the host cell and permeabilized the membrane suggesting that this sequence is required for degradation of LLO in the cytosol (34).

F. Sialidase

Sialidase is one of the thirteen different toxins produced by *C. perfringens*. Sialidases are glycohydrolases that hydrolyze α-glycosidic bonds between sialic acid (Table 1-3) and the glycoconjugate (70). Seven species of clostridia make sialidase *C. perfringens*, *C. sordelli*, *C. septicum*, *C. chauvei*, *C. tertium*, some *C. butyricum* strains, and one *C. nexile* strain (108). Bacterial sialidases and all animal sialidases are characterized by two conserved sequences: (1) an Asp-box (Ser-X-Asp-X-Gly-X-Thr-Trp) which repeats four to five times within the protein sequence and (2) the (F)RIP-region ((Phe)-Arg-Ile-Pro) located upstream of the first Asp-box and has a possible role in substrate binding (106). There are two groups of clostridial sialidases, (1) relatively large proteins with a broad substrate range and an optimum pH of 5 to 5.5 and (2) smaller proteins with a specific substrate (α(2,3)- bound sialic acid) and an optimum pH of 6 (108). Bacterial sialidases are induced by free sialic acid, N-acetylmannosamine, glycoconjugates (e.g. fetuin, mucin), polysaccharides (e.g. colominic acid, α(2,3/6)-sialyllactose), and some media (e.g. brain heart infusion agar, blood agar) (29).

C. perfringens has two characterized sialidases, a small sialidase, NanH, and a large sialidase, NanI. NanH is a 43 kDa sialidase with an optimum temperature of 37 °C, an optimum pH of 6.1, which removes α(2,3)- linked sialic acid (105, 107). NanI is a 73 kDa sialidase with an optimum temperature of 55 ºC and an optimum pH of 5. NanI has a broad substrate range, its highest activity is on detergent treated gangliosides, and has a high affinity for polyvalent

Table 1-3. Functions of sialic acid in vivo.[b]

Functional characteristic	Examples of effects
Negative charge	Mutual repulsion of circulating blood cells
	Viscoelasticity of mucins
	Directs intramolecular interactions influencing molecular shape and form
	Binding/transport of positively charged compounds
	Aggregation and disaggregation of cells
Dictates biological function	Involved in blood clotting
	Involved in complement activation
	Regulation of molecular and cellular recognition
Antirecognition	Effects half-life of RBCs, thrombocytes, lymphocytes, and sialoglycoconjugates in circulation
	Exposure of T-antigen
Receptor functions	Macrophage- lymphocyte interactions
	Neutrophil migration by selectin binding
	Attachment of microorganisms
	Attachment of bacterial fimbri to mucosal cells
	Essential components of receptors
Conformation of cell surface glycoproteins	Maintain activity of glycoprotein enzymes
	Resistance to proteases

[b]Based on reviews (29, 119).

substrates (105, 143, 145). NanI and a newly annotated *C. perfringens* sialidase, NanJ, are highly homologous modular enzymes containing what appears to be an N-terminal binding domain and a C-terminal catalytic domain (85).

Sialidases can be beneficial to bacteria in two ways: nutrition and pathogenesis. Some bacterial strains can metabolize sialic acid or use its sialidase to cleave off sialic acid and thereby gain access to metabolize the glycoconjugate (29). *C. perfringens* uses its extracellular sialidase, NanI, to cleave off sialic acid, transport sialic acid into the cell through NanT, a permease, and cleave sialic acid to N-acetylmannosamine and pyruvate by NanA, a lyase (84, 154). NanE, N-acetylmannosamine-6-phosphate epimerase, converts N-acetylmannosamine-6-phosphate to N-acetylglucosamine-6-phophate which can be used further in anabolic or catabolic pathways (93). NanH is an intracellular sialidase that removes sialic acid from small substrates that are transported into the cell (105). NanA, N-acetylneuraminate lyase, and NanE like NanI and NanH are induced by sialic acid (84, 154).

Sialidases can also be beneficial in pathogenesis. The sialidase of *Vibrio cholera* enhances the binding of cholera toxin, a key virulence factor in cholera (45). In pneumococcal hemolytic uremia the sialidase increases the susceptibility of host cells to hemolysis, agglutination, and phagocytosis (121). The *C. septicum* sialidase has been shown to be a swarming factor for *C. septicum*, which could play a role in infections of the gut (74). In *Streptococcus pyogenes* glomerulonephritis, the sialidase desialyates IgG and promotes the formation of IgG and IgM complexes (78). *C. perfringens*, *C. sordelli*, and *C. septicum* cause life threatening diseases in humans and produce vast amounts of sialidase, which may correlate to its having a role in pathogenesis (109). Many sialidases have a role in disease but none have been shown to be required for virulence (29).

G. Innate immune system

The innate immune system works by nonspecific action to clear infectious agents. Phagocytosis is a primary line of defense. Monocytes, macrophages, and PMNs are phagocytes that are part of the initial response to infectious agents (35, 82, 153). In humans, monocytes are 3-7% of leukocytes in the blood and PMNs are 50 to 70%. Macrophages differentiate within the tissues and can exist there for two to three months (82). PMNs respond to inflammation within

thirty to sixty minutes whereas macrophages are thought to move into the site of inflammation by five or six hours post-infection. PMNs take-up and digest microbes and are killed during this process (58). Monocytes and macrophages constitutively express receptors for bacteria and attempt to phagocytose them by these generalized receptors (71, 89). During phagocytosis monocytes and macrophages activate the inflammatory response by releasing cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tissue necrosis factor-α (TNF-α) (102). Inflammation is a series of vascular events that include clotting, increased blood flow, increase vascular permeability, and an influx of phagocytic cells.

Phagocytosis is achieved by encircling the pathogen with membrane extensions called pseudopodia. The pathogen is brought into the cytosol within a phagosome. ATPase pumps in the membrane of the phagosome move protons into the phagosome decreasing the phagosomal pH to 5 (35). Early and late endosomes fuse with the phagosome and then lysosomes fuse with the vacuole (67). The lysosome releases lysozyme, proteases, and defensins into the phagosome (152). The NADPH oxidase complex forms on the membrane and produces superoxide. Hydrogen peroxide, hypochloric acid, and nitric oxide can be formed from the reaction of superoxide with other substances in the phagosome (76). These oxygen metabolites are potent bactericidal substances (35, 127).

Another key component of the innate immune system is complement. Complement proteins, C1- C9, are produced by the liver and cleaved for activation. Complement can opsonize microbes for phagocytosis or form the membrane attack complex and punch holes in the cell wall of the invading organism (149). The complement cascade can be activated by three pathways: (1) collectins binding mannose residues on the bacterial surface, mannose-binding pathway, (2) antibodies binding the antigenic epitopes on the bacterial surface, classical pathway, (3) complement binding of lipid or carbohydrate complexes at the bacterial surface, alternative pathway (36, 68). All of these pathways converge at the complement component, C3, which is cleaved to C3a and C3b. C3a and C5a stimulate mast cells which results in vasodilation. C5a and cytokines signal phagocytes to transmigrate, and a gradient of C5a guides them to the foci of infection (128). C5b can recruit C6, C7, C8, and C9 to bind the microbe and form the membrane attack complex (MAC) to perforate the microbial cell wall (94).

The movement of leukocytes from the vasculature across the vascular endothelium and into the tissues is called transmigration or diapedesis (Fig. 1-2). Initially, endothelial cells are stimulated to express TNF-α, IL-1, and interferon-γ (INF- γ) (153). PMNs loosely bind to selectins and become detached unless an additional signal, IL-8, stimulates PMNs to produce integrins (Table 1-4). Integrins bind ICAM-1 on the endothelial cells resulting in margination, the flattening of PMNs against the endothelium. PMNs move between the endothelial cells by interactions with PECAM-1 (142). Transmigration is enchanced by mast cell production of vasodilators such as leukotrienes and prostaglandin. TNF-α, IL-1, IL-8, and PAF are required for PMN recruitment, monocyte recruitment and maturation, and the activation of tissue macrophages (58, 82).

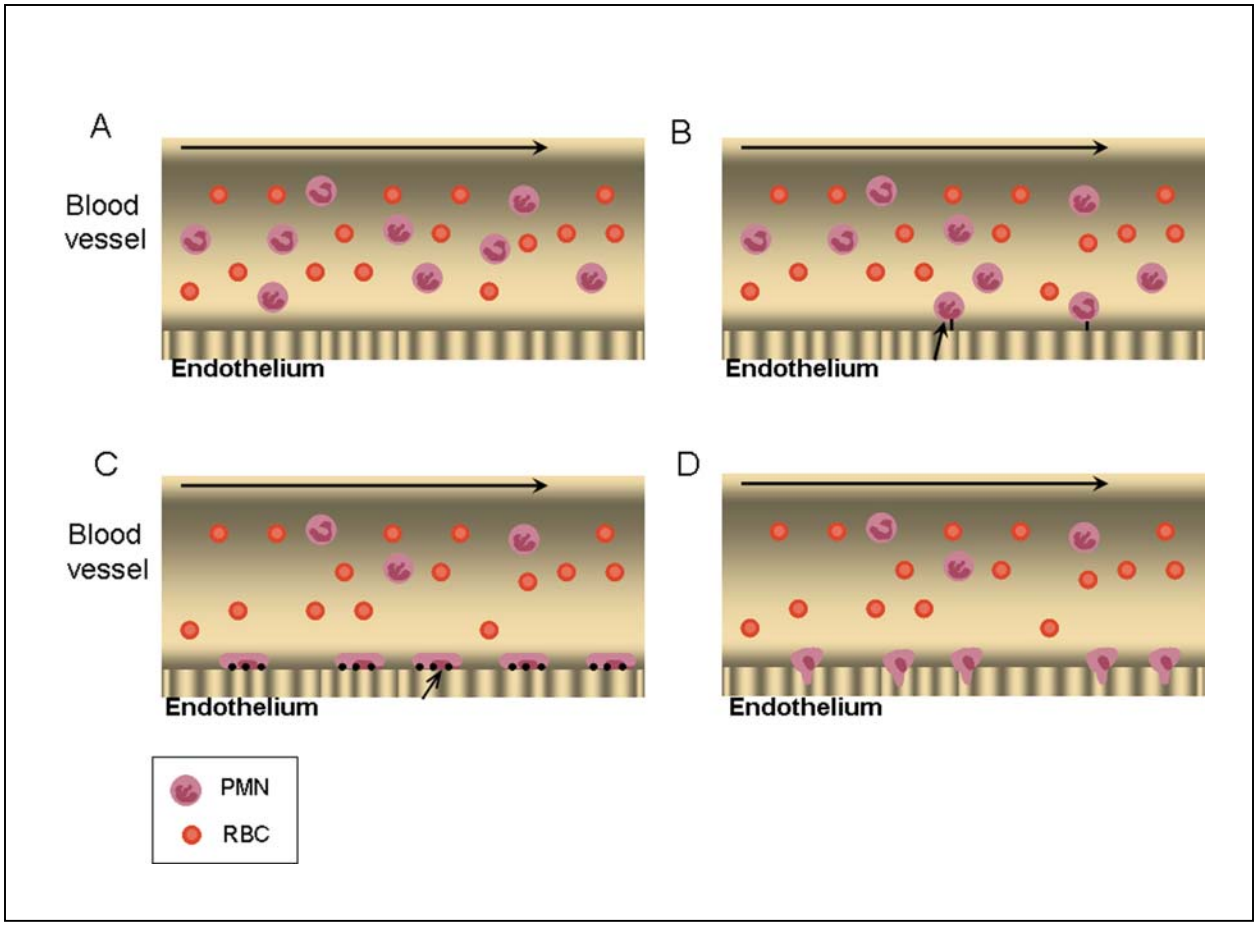

Figure 1-2. Polymorphonuclear phagocytes leave the circulation through a process called transmigration. PMNs are initially stimulated to bind the endothelium loosely by molecules that are called selectins (B, arrow). Additional inflammatory stimulus can trigger the expression of integrins and ICAM-1 on PMNs and endothelial cells, respectively (C, arrow). Firm adhesion via these molecules results in margination (C) of the PMNs and they move across the endothelium via PECAM-1 (D). Reviewed in (153)

Table 1-4. The activities of specific molecules in inflammation and diapedesis.[c]

Molecule	Cell	Activity of interest	Net result
Adhesins			
L-selectin	Neutrophil	Binds sulfated sialomucin on the endothelium	Tethering to endothelium
ELAM-1 (E-selectin)	Endothelial cell	Binds E-selectin ligand-1 on neutrophils	Tethering to endothelium
P-selectin	Endothelial cell	Binds P-selectin glycoprotein ligand-1 on neutrophils	Initiates capture
β_2 integrins (CD11/CD18)	Leukocytes	Binds ICAM-1 on the endothelium	Firm adhesion to the endothelium
ICAM-1	Endothelial cell	Binds β_2 integrins on leukocytes	Firm adhesion to leukocyte
PECAM-1	Neutrophil & endothelial cell	Binds PECAM-1 on opposing cells	Transmigration (diapedesis)
Cytokine			
TNF-α	Endothelial cell	Induces production of P-selectin, ICAM-1, IL-8	Neutrophil is bound by the enothelial cell
	Neutrophil	Induces shedding of L-selectin and CD18 expression	Inhibits neutrophil chemotaxis
Chemoattractants			
PAF	Neutrophil/ endothelial cell	Induces adhesin expression	Binding of neutrophil and the endothelium
	Neutrophil	Attractant	Neutrophil chemotaxis
Leukotriene	Neutrophil/ endothelial cell	Induces adhesin expression	Binding of neutrophil and the endothelium
	Neutrophil	Attractant	Neutrophil chemotaxis
Chemokine			
IL-8	Neutrophil	Attractant	Neutrophil chemotaxis
		Induces shedding of L-selectin and CD18 expression	Inhibits neutrophil chemotaxis

[c] Based on a review by Wagner and Roth (153).

II. The role of monocytes and neutrophils (polymorphonuclear cells) in initiation of *Clostridium perfringens* gas gangrene infections

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Abstract

Clostridium perfringens is the most common cause of a highly lethal disease, gas gangrene. Established gangrene infections are notable for the rapid spread of the disease and an absence of leukocytes at the site of infection. Yet in the murine animal model, in which bacteria are injected into the hind leg muscles of mice, a large inoculum ($1 \times 10^8 - 1 \times 10^9$) of bacteria is needed to initiate an infection. This suggests that leukocytes may play a role in inhibiting the initiation of a gangrene infection. To test this hypothesis we used in vitro and in vivo assays to examine the role that monocytic cells and PMNs play in the early stages of a gangrene infection, when bacterial numbers and toxin levels are low. Human PMNs and monocytes effectively killed *C. perfringens* if complement was present and if the phagocytes and bacteria were actively mixed, allowing for toxin dispersal. In vivo, PMN and tissue macrophages moved into the site of infection when toxin levels were low and the dose was sublethal (1×10^6), but not with a lethal (1×10^9) dose. Also, mice were depleted of PMNs or circulating monocytes before infecting them with increasing inoculums of *C. perfringens*. In these infections monocytes appeared to play a role in inhibiting a gas gangrene when an intermediate inoculum was used, but PMNs did not enhance survival at any inoculum. Together, these experiments indicate that, in tissues, *C. perfringens* can be killed by phagocytes when toxin levels are low and that monocytic cells appear to play a more important role in inhibiting a gangrene infection than do PMNs.

Acknowledgements

I would like to acknowledge David K. O'Brien and his work on the mouse studies, performing the PMN depletion study, and the mouse infections. Michael Woodman worked as an undergraduate research student and isolated human PMNs and monocytes and performed in vitro survival assays with *Clostridium perfringens*. I would like to thank Stephen B. Melville for his guidance in planning and executing experiments and for assistance with results interpretation.

Introduction

Clostridium perfringens is a gram-positive spore-forming bacterium that is the causative agent of gas gangrene. Bacteria are introduced to the tissues through a deep, penetrating wound such as a gunshot or knife wound, crush injury, or intramuscular injection (75). Gas gangrene can occur post-operatively especially following bowel or billiary surgery or improperly performed abortion (75, 129). Spontaneous occurrences of gangrene are most often due to tumors in the gastrointestinal tract (22). It is estimated that there are 1,000 to 3,000 cases of gangrene in the US each year and 80% are due to *C. perfringens* (75, 100). Fifty percent of patients with the disease develop shock and 40% of these patients die (53). Mortality rates are the highest for cases of spontaneously occurring gangrene, 38%, because of difficulty in diagnosis (100). Post-operative cases of gangrene have a 24% mortality rate and gangrene due to traumatic injury has a 10% fatality rate. Death can occur as quickly as 12 hours if the infection is sudden and severe and if treatment is not immediate (100).

On average, the infection develops within one to three days and ranges between six hours and several days (130, 131). Gangrene is characterized by the sudden onset of severe pain at the site of infection, gas in the tissues, and changes in skin color (131). Inspection of the tissues reveals muscle that does not bleed or contract and connective tissue that is damaged or destroyed. (79, 100). Microscopic examination of the tissue demonstrates rod shaped bacteria, deteriorating tissues, and an absence of inflammatory cells (18, 79, 140). The disease rapidly advances infecting several centimeters of tissue per hour (75). If bacteremia or intravascular hemolysis occurs, the patient will most likely die due to shock (53).

Treatment of gas gangrene must be aggressive and immediate. Infected tissues are removed by debridement or amputation (100, 130, 131) and broad-spectrum antibiotics are administered in high doses for several days. Hyperbaric oxygen has also been shown to be an effective additional therapy and may improve the survival of patients with non-extremity gas gangrene (100, 130).

Gangrene is a highly lethal, rapidly advancing infection in humans. To create this type of infection in mice, a large inoculum is required. The most commonly used mouse model of *C. perfringens* gas gangrene uses an intramuscular injection of 10^8 or 10^9 bacteria (5, 6, 15, 40, 88,

140). This infection in mice is lethal, causes severe tissue damage and leukostasis, and the infection advances quickly killing mice within the first 6 to 24 hours of infection. The major virulence factor in gas gangrene, phospholipase C (PLC), has been shown to be necessary for lethality in mice (5, 88). In vivo experiments have also demonstrated that the pore-forming toxin perfringolysin O (PFO), along with PLC, was needed to cause characteristic pathology such as leukostasis and wild-type levels of necrosis in mice (5, 6, 40).

The necessity of a large inoculum to induce a lethal infection in mice may indicate that the immune system is inhibiting the initiation of disease. To test this hypothesis, we examined the ability of polymorphonuclear leukocytes (PMNs) and monocytes to kill *C. perfringens* in vitro and their ability to move into the site of infection in vivo. We also depleted PMNs and monocytes prior to infection to identify their role in enhancing mouse survival. PMNs and monocytes were able to kill *C. perfringens* in vitro and to co-localize with *C. perfringens* in vivo when toxin levels were low. Infection experiments indicated a role for monocytes in inhibiting gas gangrene and enhancing mouse survival when an intermediate inoculum was used, but PMNs did not significantly enhance survival.

Methods

Bacterial strains and growth conditions

C. perfringens strain 13 (obtained from C. Duncan) was used for all studies. *C. perfringens* was grown in PGY media (30 g proteose peptone, 20 g of glucose, 10 g of yeast extract, and 1g of sodium thioglycolate per liter) in a Coy anaerobic chamber (Coy Laboratory Products) (162).

Isolation of human peripheral blood neutrophils, monocytes, and serum

Blood obtained from healthy human volunteers was layered on a Histopaque gradient (Sigma-Aldrich). The gradient was centrifuged at 700 *x g* for 30 minutes at room temperature. The layers containing neutrophils or monocytes were collected and washed twice in Dulbecco's Modified Eagle's Medium (DMEM). Sterile distilled water was used for hypotonic lysis of the red blood cells. The viability of the cells was determined to be > 98% using the trypan blue staining. To obtain serum, blood taken from human volunteers was allowed to clot at 37 °C. Serum was collected by centrifugation of the clotted blood at 700 *x g* for 10 minutes and removal of the supernatant. For heat-treated serum, serum was incubated at 56 °C for 30 min. Human volunteers were recruited for blood donation in accordance with procedures approved by the Institutional Review Board of Virginia Tech.

Bacterial killing assays using human peripheral blood monocytes and neutrophils

C. perfringens cells were grown to mid-log phase in PGY medium and washed 3 times in phosphate buffered saline (PBS). Neutrophils or monocytes were incubated at a one-to-one ratio in DMEM with washed *C. perfringens*. For the assays, 1 x 10^6 neutrophils or monocytes were added to two-ml screw-cap plastic tubes. DMEM and serum were added to bring the volume to 400 µl, as noted, and two µl of bacteria, an inoculum of 1 x 10^6 bacteria, was added to each tube. Tubes were rotated for two hours at 37 °C. The percent survival was determined by serial dilution in PBS and plating on PGY. For anaerobic assays, the plastic tubes were placed in a Coy anaerobic chamber before the screw-caps were tightened, allowing the oxygen to diffuse out of the tube. When assays were performed using 24-well tissue culture plates, the phagocytes and bacteria were added to each well in the same fashion as stated above for the rotating tube assay.

The kinetics of leukocyte recruitment in the early stages of an infection

C. perfringens was grown to mid-log phase in PGY media and washed twice with PBS. Samples were resuspended in PBS to a final concentration of ~1 x 10^{10} colony forming units per ml (cfu/ml) or diluted to a final concentration of ~1 x 10^{7} cfu/ml. Twelve BALB/c mice were anesthetized with Isoflurane and inoculated in the right hind leg with 0.1 ml of the 10^{10} cfu/ml or 10^{7} cfu/ml bacterial suspensions (i.e., 10^{9} or 10^{6} cfu). Three mice were euthanized at ten minutes and one, two, or three hours post-infection. The hind leg muscle was rapidly excised, fixed in neutral-buffered formalin, and stored in 70% ethanol. The muscles were then embedded in paraffin and three μm thick sections were made at two millimeter intervals and the sections were fixed to glass slides. Sections were stained with hematoxylin and eosin and examined for the co-localization of leukocytes (mononuclear and polynuclear) and bacteria. A Leitz Dialux 20 phase-contrast microscope with a Moticam 1300 camera was used to take 30 to 90 random images of bacteria within the tissues for each mouse muscle sample (Table 2-1). The percent of fields with PMNs, monocytic cells, or lymphocytes in the presence of bacteria was enumerated. A field measured 100 μm in diameter at 1000x magnification. The presence of one or more leukocytes co-localizing with one or more bacterial cells within 45 μm was counted as positive.

Preparation of liposomes

Liposomes were prepared as described by Van Rooijen and Sanders (150). Briefly, 33 μg/ml of 1,2 Distearoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)] (DSPG from Avanti), 33 μg/ml of cholesterol (Sigma), and 100 μg/ml of 1,2 Distearoyl-*sn*-Glycero-3-Phosphocholine (DSPC from Avanti) were dissolved in 10 ml chloroform. Chloroform was removed by reverse-phase evaporation and the remaining lipid film was dissolved in 10 ml 0.6 M clodronate (Sigma) in water or 10 ml PBS. The resulting solution was evacuated with nitrogen gas and stored at room temperature for two hours. The solution was then put on ice, sonicated for three minutes, and incubated under nitrogen gas at 4 °C overnight.

Before use, the liposomes were washed in PBS three times, resuspended in a final volume of four milliliters of PBS, and kept under nitrogen gas until used.

Depletion of peripheral monocytes

Monocytes were depleted by intravenous tail-vein injection of 100 μl clodronate-filled

Table 2-1. Number of fields examined for co-localization of bacteria and leukocytes.

Inoculum	10 minutes	60 minutes	120 minutes	180 minutes
10^6	238	66	94	0
10^9	204	195	189	204

liposomes (or 100 µl PBS-filled liposomes for control mice). Following monocytic cell phagocytosis and degradation of the liposomes, clodronate is released intracellularly and kills the cell (150). To determine the level of monocyte depletion, 200 µl blood samples were taken from the tail-vein of each mouse before and 24 hours after liposome injection. Each blood sample was stained with F4/80 monoclonal antibody (60) labeled with Alexa Fluor 488 (Molecular Probes) and then treated with Pharm Lyse (Becton Dickenson) to remove red blood cells from the sample. The total leukocyte cell number in each blood sample was determined on a hemacytometer and the number of monocytes labeled with the fluorescently tagged monoclonal antibody specific for the F4/80 surface antigen on monocytes was counted. For each sample the number of monocytes was quantified using fluorescence at 488 nm. Four mice were tested for depletion with each type of liposome.

Depletion of polymorphonuclear leukocytes

Six BALB/c mice received an intraperitoneal (i.p.) injection of 100 µg of the monoclonal antibody RB6-8C5 (Cedarlane), which is specific for the Gr-1 surface antigen present on granulocytes of mice but not on monocytes or lymphocytes (104). RB6-8C5 has been shown to bind and lyse PMNs (113). To verify that PMNs were depleted, blood smears were taken from mice at one, three, and five days post-antibody-injection, and stained using Hema 3 Stain Set (Fisher). The total number of PMNs was enumerated by microscopy (Fig. 2-1). Control mice received similar i.p. injections of PBS instead of the antibody.

***C. perfringens* infections of depleted mice**

Female BALB/c mice were used for all animal experiments. Preceding infection with *C. perfringens*, mice were depleted of either their PMNs or peripheral monocytes as described above. *C. perfringens* was grown to mid-log phase and injected into the right hind leg at inoculums of ~1 x 10^9, 10^8, 10^7, or 10^6 cfu. Once the onset of gangrene was observed, the mice were euthanized to eliminate any unnecessary suffering. Any mouse that did not show signs of gangrene was euthanized 36 hours after the start of the experiment. Six mice were used for each inoculum.

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Tech.

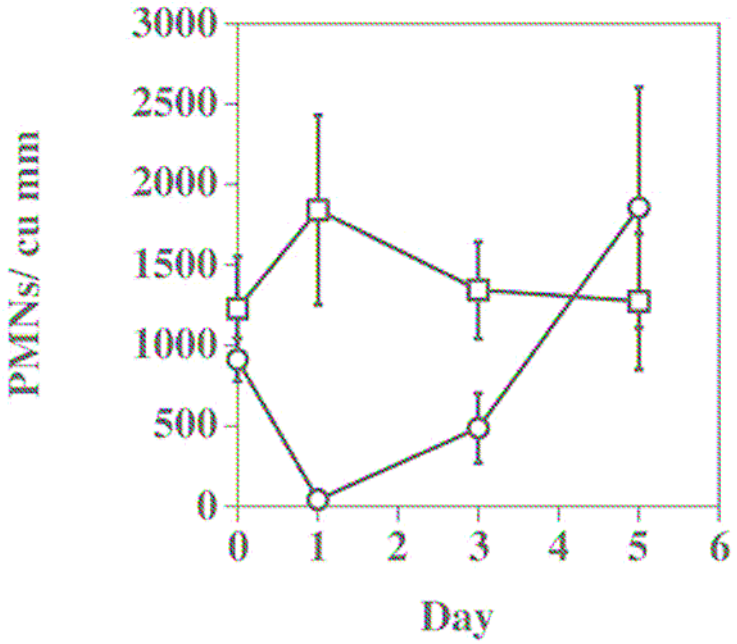

Figure 2-1. Time course showing depletion of neutrophils using the monoclonal antibody RB6-8C5 The number of neutrophils/ cu mm of peripheral blood from BALB/c mice was determined after injection with the monoclonal antibody (circles) or PBS alone (squares). Depletion performed by David K. O'Brien.

Results

PMNs and monocytes can kill *C. perfringens* in vitro.

Human PMNs and peripheral blood monocytes infected with *C. perfringens* at a one-to-one ratio were tested for their ability to kill *C. perfringens*. Infections were performed under aerobic or anaerobic conditions in a rotating tube or under aerobic conditions in a stationary 24-well plate. PMNs and monocytes were unable to kill *C. perfringens* efficiently when incubated alone with bacteria. PMNs and monocytes incubated with serum resulted in a 1 to 2.5 log decrease in *C. perfringens* survival as compared to bacteria incubated with phagocytes alone (Fig. 2-2A & 2-2B). Infections performed with heat-treated serum (inactive complement) resulted in similar survival levels to those observed for infections lacking serum.

The in vitro killing assay was also performed in rotating tubes under anaerobic conditions. A similar trend was seen when *C. perfringens* cells incubated with PMNs or monocytes and serum were effectively killed by these phagocytes (Fig. 2-2D). Under these conditions, survival was decreased by 2.5 to 3.5 logs when incubated with PMNs and 1 to 2 logs when incubated with monocytes. However, when these assays were performed in a stationary 24-well plate using the same parameters as the aerobic rotating tube assays, *C. perfringens* survival did not significantly decrease regardless of the presence of serum in the media (Fig. 2-2C).

Tissue macrophages and PMNs co-localize with *C. perfringens* in vivo at a sublethal dose.

Mice were injected in the hind leg with 10^9 bacteria, a lethal inoculum, or 10^6 bacteria, a sublethal inoculum, and infiltration of leukocytes was scored at ten minutes and one, two, and three hours post-infection. Myonecrosis, edema, fascitis, and microvascular occlusions were observed at one and two (Fig. 2-3) hours post-infection. We could not detect bacteria within the tissues by three hours post-infection with the 10^6 inoculum, but tissue sections from the three hour time point from mice injected with 10^9 bacteria had abundant necrosis and bacteria at the site of infection. The ability of all leukocytes to move into the site of infection (i.e. localize to the vicinity where the bacteria were) was severely limited within the first three hours of infection with a 10^9 inoculum (Fig. 2-4). However, when bacterial numbers were lower, 10^6 cfu, the influx of PMNs present in the observed fields increased from 17% to 71% over the first two

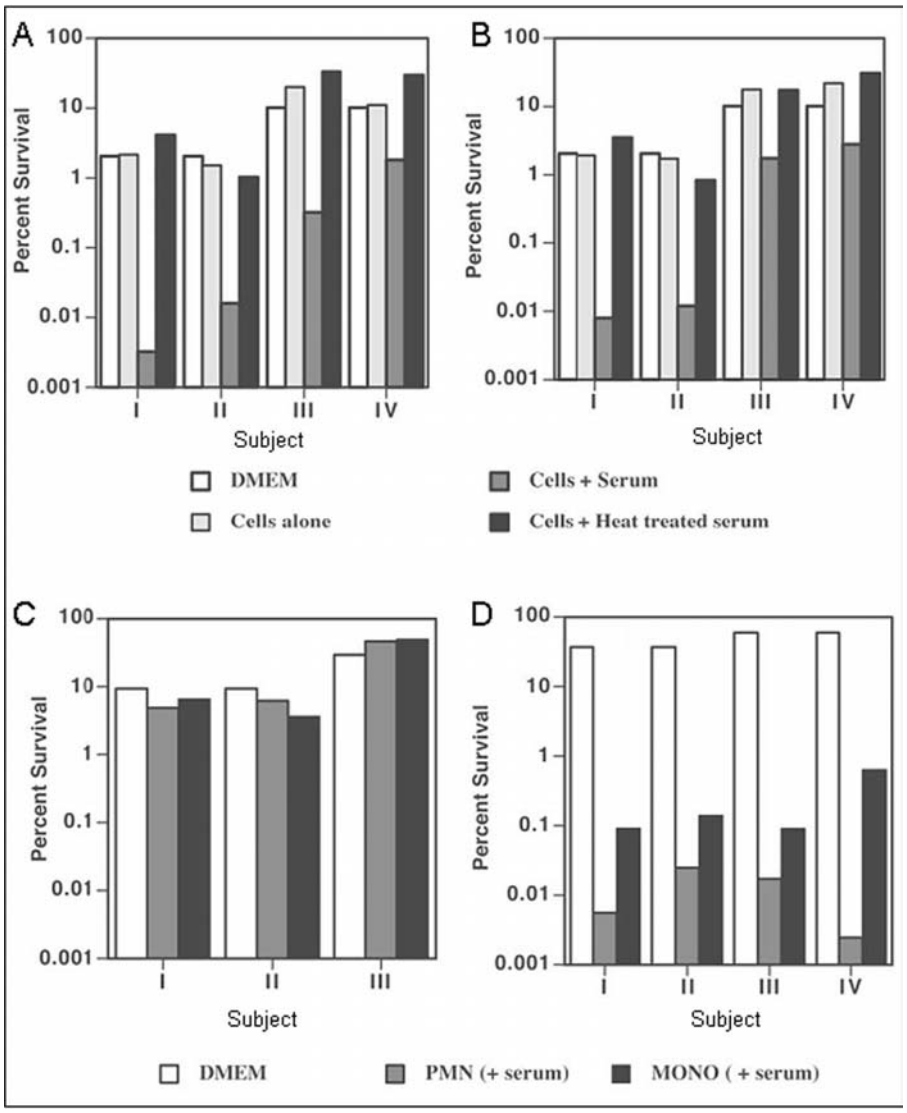

Figure 2-2. Killing of *C. perfringens* by human neutrophils and monocytes. Neutrophils (**A**) and monocytes (**B**) isolated from different human volunteers (subjects) were incubated with *C. perfringens* at an MOI of 1:1 under aerobic conditions in rolling tube assays or under anaerobic conditions in rolling tube assays (**D**). (**C**) Human neutrophils and monocytes incubated with *C. perfringens* in stationary conditions (i.e., in a 24-well plate, without shaking). DMEM: Dulbecco's modified Eagles medium. Assays were performed by Michael Woodman.

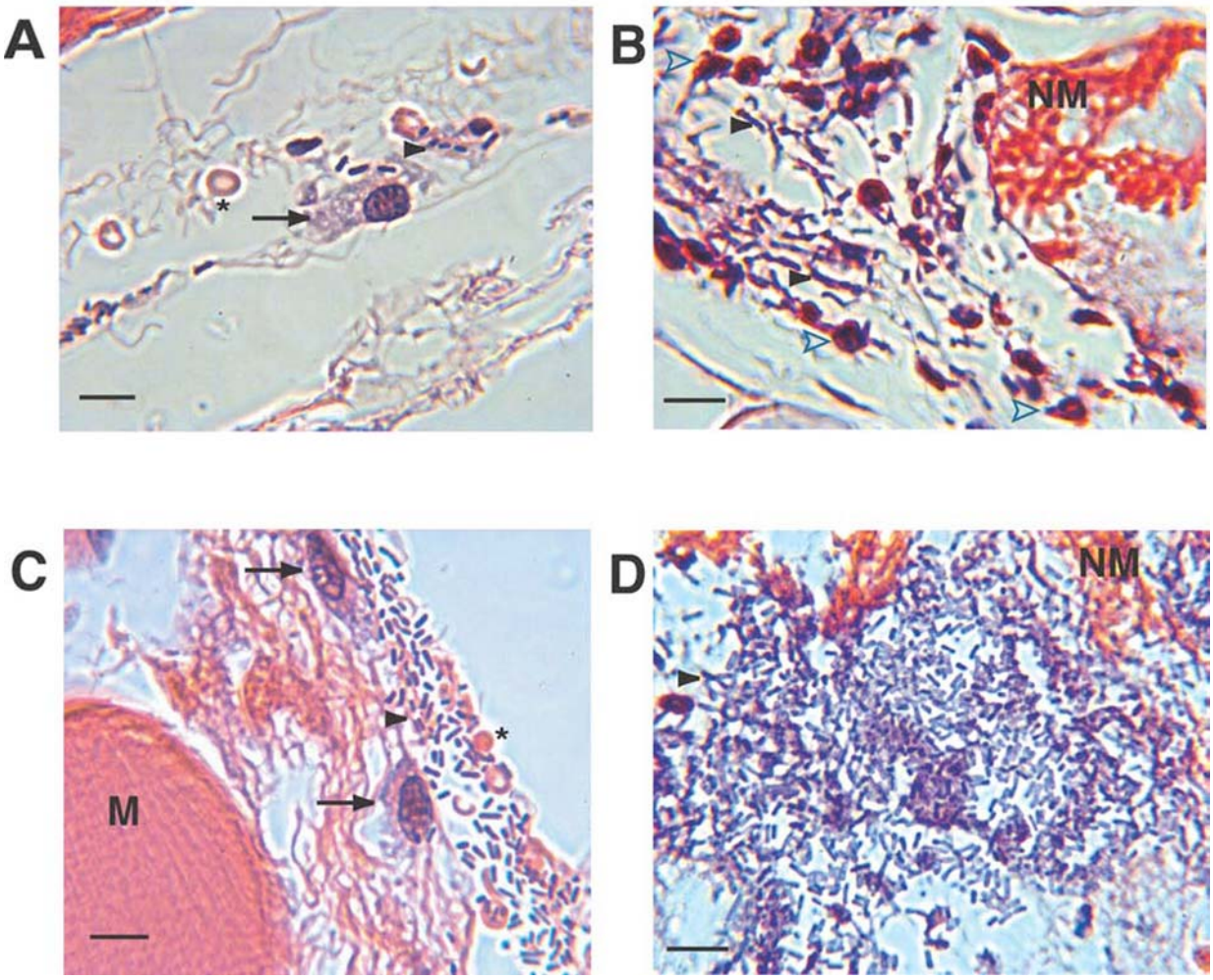

Figure 2-3. Images showing co-localization of leukocytes and bacteria at the site of infection. Tissue sections from mice infected in the hind leg muscle at 10 min. or 2 hr. with 10^6 (A and B) or 10^9 (C and D) *C. perfringens*. Sections were made from paraffin-embedded tissue and stained with hematoxylin and eosin. Arrows point to macrophages, arrowheads to bacteria, open arrowheads to neutrophils, asterisks indicate red blood cells. M, Muscle fibers; NM, necrotic muscle fibers. Panels A and C show representative images in which bacteria were present, panels B and D show regions of high levels of bacteria. Bars = 10 μm. Images were taken using a phase-contrast microscopy.

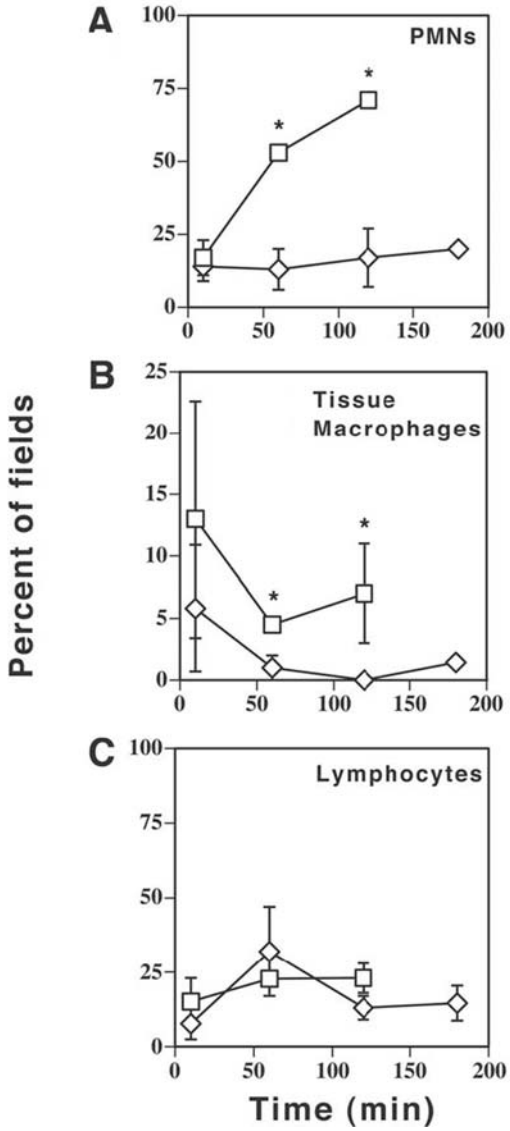

Figure 2-4. Kinetics of infiltration of leukocytes into the site of infection. Hematoxylin and eosin stained slides of the mouse muscle tissue were examined for the co-localization of bacteria and leukocytes. The movement of neutrophils into the site of infection (A) was significantly higher at one and two hours for the 10^6 inoculum (squares) than the 10^9 (diamonds) inoculum (P = 0.0005 and P = 0.0290, respectively). The infiltration of monocytes (B) was significantly higher with the 10^6 inoculum than the 10^9 inoculum at one and two hours (P = 0.0037 and P = 0.0257, respectively). The presence of lymphocytes was variable at the site of infection, and no significant difference between the inoculums (C).

hours of infection**.** (Fig. 2-3B & 2-4A). PMNs were present at statistically higher levels at one (P= 0.0005) and two (P= 0.0290) hours post-infection in mice infected with 10^6 bacteria as compared to mice infected with 10^9 bacteria.

Tissue macrophages were observed in the presence of bacteria by ten minutes post-infection in mice infected with 10^6 and 10^9 bacteria (Fig. 2-3A & 2-3C). The presence of tissue macrophages decreased during the interval between ten minutes and one hour in both sets of mice (10^9 and 10^6 inoculum). At one hour post-infection, the presence of tissue macrophages was at a statistically higher level in mice infected with 10^6 bacteria (P = 0.0037, Fig. 2-4B). The occurrence of macrophages with bacteria was 7% higher at two hours with the 10^6 inoculum, statistically higher than the 10^9 inoculum (P = 0.0257, Fig. 2-4B). The number of lymphocytes associated with bacteria varied over the course of the experiment, but did not increase with either dose of *C. perfringens* (Fig. 2-4C).

PMNs do not significantly affect the initiation of a *C. perfringens* gangrene infection.

To determine the effectiveness of RB6-8C5-mediated PMN depletion, five mice injected with the monoclonal antibody RB6-8C5 or PBS were measured for their PMN levels one, three, and five days post-injection. The maximal level of PMN depletion was observed 24 hours post-injection as compared to the control mice (PBS injected, Fig. 2-1). Therefore, all differential blood samples and in vivo infections were performed 24 hours after RB6-8C5 injection.

PMN depleted mice were infected intramuscularly with 10^9, 10^8, 10^7, or 10^6 bacteria, and the time to the appearance of gangrene was recorded. Mice depleted of their PMNs with the monoclonal antibody RB6-8C5 showed a decreased survival rate within the first six hours after infection with 10^9 bacteria (Fig. 2-5A). This was found to be a statistically significant decrease in survival by a Fisher's exact test (P = 0.0303). However, the presence of PMNs did not affect the ability of mice to survive—all mice died by 24 hours post-infection. The 10^8, 10^7, and 10^6 inoculums showed no significant difference in survival between the control and neutropenic mice.

Monocytic cells appear to have a role in inhibiting the initiation of a *C. perfringens* gas gangrene infection.

To deplete circulating monocytes mice received intravenous injections of clodronate-filled liposomes, and control mice were injected with PBS liposomes. Mice injected with clodronate-filled liposomes showed a 4.5-fold decrease in their overall level of monocytes 24 hours post-injection; however, control mice showed no significant only a one-fold decrease in monocytic numbers (Table 2-2). Mice depleted of their peripheral monocytes were infected with 10^9, 10^8, 10^7, or 10^6 bacteria and the time to death was recorded. Mice infected with 10^9 bacteria and depleted of their peripheral monocytes displayed no significant difference in survival. Mice infected with 10^8 bacteria and depleted of their peripheral monocytes began to show a decreased rate of survival at 12 hours post-infection (Fig. 2-5B). By 24 hours post-infection, only 33% of mice survived in comparison to 100% of the PBS treated mice. This was found to be statistically significant by a Fisher's exact test ($P = 0.0303$). No difference was found in the survival of mice infected with 10^6 or 10^7 bacteria.

Table 2-2. Percent average fluorescence of F4/80 labeled cells in murine blood samples before and after liposome treatment.

Average % Fluorescence	Before Treatment (Range)	After Treatment (Range)
Clodronate Liposomes	1.8% (0.5 - 2.6)	0.4% (0.0 – 1.1)
PBS Liposomes	0.9% (0.71 - 1.1)	0.75% (0.6 – 1.0)

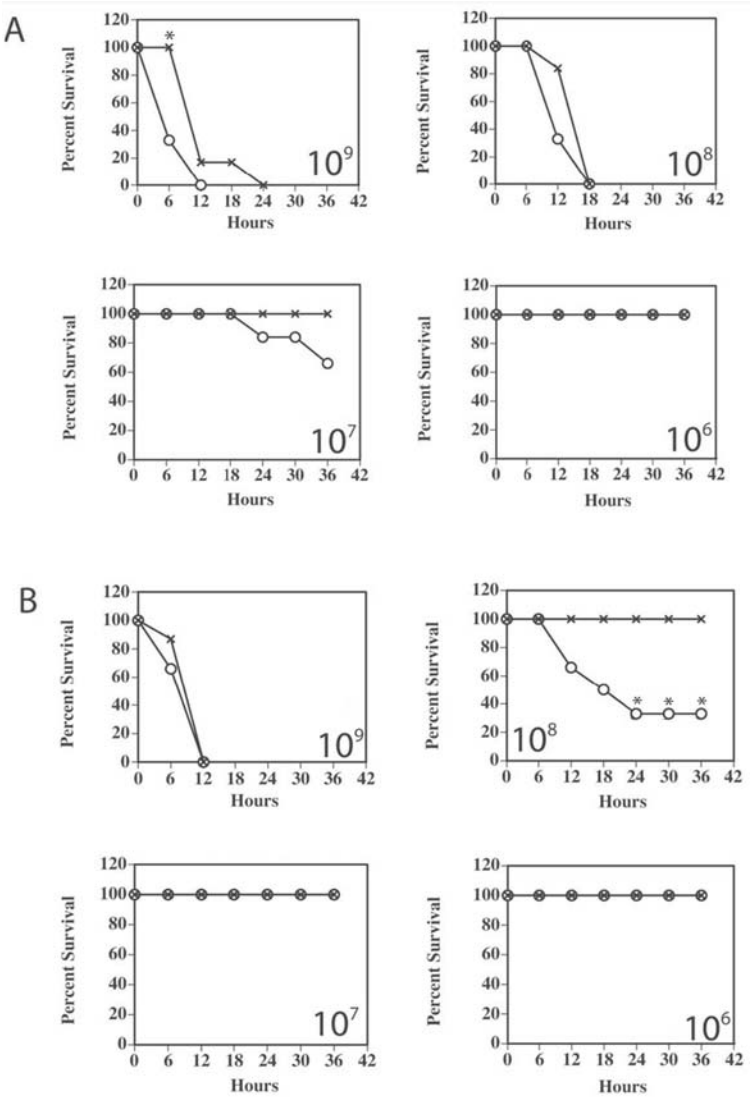

Figure 2-5. Mouse infections following PMN or monocyte depletion. A) Survival rate of RB6-8C5 treated mice (circles) and PBS treated mice (cross hairs) infected with *C. perfringens*. Mice treated with RB6-8C5 showed no significant difference in overall survival as compared to the PBS treated mice. **B)** The survival rate of clodronate liposome treated mice (circles) and PBS liposome treated mice (cross hairs) infected with *C. perfringens*. Mice treated with clodronate liposomes showed a significant decrease in survival as compared to mice injected with PBS liposomes at the 10^8 inoculum. The starred values were determined to be statistically significant by a Fisher's exact test, $P < 0.05$. Infections were performed by David K. O'Brien.

Discussion

Gas gangrene is a rapidly advancing, lethal disease that requires immediate, aggressive treatment. *C. perfringens* is the most commonly encountered bacterium in gas gangrene infections. Lethal *C. perfringens* gangrene infections in mice are only created by injection of 10^9 or 10^8 bacteria, suggesting that the host immune system may inhibit initiation of infection. We found that human PMNs and peripheral monocytes can kill *C. perfringens* in vitro when complement was present and a mechanical stimulus was applied. Cells alone and cells with heat-treated serum (inactivated complement) were unable to kill *C. perfringens* in vitro. These results indicate that killing was most likely mediated by phagocytosis following opsonization by complement. A previous study with J774 macrophage-like cells showed that phagocytosis of *C. perfringens* was inhibited by cytochalasin D (87) indicating that phagocytosis is actin-mediated. In vitro, phagocytes were found to be able to kill *C. perfringens* in a rotating-tube assay but not in a stationary assay. We believe that the tumbling of the rotating tube dispersed toxins throughout the media and increased the frequency of *C. perfringens*-phagocyte interactions allowing for better killing as compared to the stationary assay.

PMNs and monocytes were scored for their ability to co-localize with *C. perfringens* in vivo when mice were infected with 10^9 or 10^6 bacteria. We observed that if PMNs and tissue macrophages can move into the site of infection. Tissue macrophages are initially depleted but after one hour, they gained the ability to move into the site. The presence of lymphocytes is variable. The 10^9 bacterial inoculum prevented the infiltration of phagocytes indicating that at high bacterial levels toxins prevented extravasation and leukostasis occurred. The 10^6 bacterial inoculum produced local pathology characteristic of gangrene, by two hours post-infection--muscle bundle fragmentation and connective tissue degradation was evident (Fig. 2-3B). Tissue macrophages and PMNs co-localized with *C. perfringens* at a higher frequency with the 10^6 bacterial inoculum than with the 10^9 bacterial inoculum, indicating enhanced co-localization when toxin levels are lower. The amount of co-localization between tissue macrophages and bacteria increased between one and two hours post-infection possibly indicating that macrophages were moving into the site of infection from surrounding tissues. The pattern of

lymphocyte co-localization (Fig. 2-4C) may indicate that these cells were present only due to vascular leakage (21, 39) at the site, and not due to a directed immune response.

We were unable to score co-localization at three hours post-infection with 10^6 bacteria and this may be due to (1) the dispersal of bacteria throughout the tissue and inability to locate single cells or (2) the bacteria were within phagocytes and could not be distinguished using H & E staining. The same infection done previously in our lab used plate counts to demonstrate the presence of viable bacteria within the tissues three hours after intramuscular injection of 10^6 bacteria leading us to believe that bacteria were present but not detected (88).

To determine what role PMNs and monocytes played in effecting a *C. perfringens* gas gangrene infection, we depleted these cells in mice prior to infection. Neutropenic mice infected with *C. perfringens* showed a significant decrease in survival at six hours post-infection when given a 10^9 inoculum, but the overall survival of the depleted mice was not significantly lower than non-depleted mice. These results suggest that PMNs may briefly attenuate the advancement of gangrene within the first few hours of infection with a high bacterial inoculum, but play no role in enhancing host survival as seen by all mice dying by 24 hours post-infection.

Mice depleted of their peripheral monocytes and infected with 10^8 *C. perfringens* did have an overall decreased level of survival. Our results showed a statistically significant increase in survival at 24 hours post-infection for mice with their peripheral monocyte population intact and infected with 10^8 bacteria. A statistically significant decrease in the ability of depleted mice to survive following infection with 10^9 bacteria was not observed most likely due to the fact that such a large number of bacteria would overwhelm the host immune system and cause death of all mice regardless of the health of the host immune system. Also, no significant difference in survival was seen in mice infected with 10^6 bacteria because the small size of this inoculum may not sufficiently disrupt the blood supply to allow for the growth of *C. perfringens*, an anaerobe.

Clodronate-filled liposomes, injected intravenously, deplete only peripheral monocytes and tissue macrophages in organs such as the liver and spleen but, because the liposomes cannot escape the vasculature they are unable to deplete tissue macrophages in the muscle (20, 150). We believe that these results present a role for peripheral monocytes moving into the site of

infection, differentiating into mature macrophages, and reducing bacterial numbers to prevent gangrene in a mouse. We conclude that monocytic cells can kill *C. perfringens* when toxin concentrations are low and that these cells play a role in inhibiting gangrene in mice that are infected with an intermediate dose of *C. perfringens*.

III. Perfringolysin O mutants allow for phagosomal escape of *Clostridium perfringens*

Blair H. Therit, David K. O'Brien, and Stephen B. Melville

Abstract

Clostridium perfringens is the causative agent of gas gangrene in humans and animals. The two major virulence factors in a *C. perfringens* gas gangrene infection are phospholipase C (PLC) and perfringolysin O (PFO). PFO is a pore-forming toxin and a cholesterol-dependent cytolysin (CDC). Listeriolysin O (LLO) a CDC produced by *Listeria monocytogenes*, it has a narrow pH optimum, acidic pH, and a short half-life, which allows it to mediate phagosomal escape and intracellular persistence. PFO also mediates phagosomal escape, but it is highly cytotoxic due to its activity at acidic and neutral pH, acting on the phagosomal membrane and the cell membrane, and it has a longer half-life. To determine if PFO can mediate intracellular persistence in macrophages, two less active variants of PFO were used. The variants were different from wild-type PFO in one of two respects: decreased optimum pH or decreased intracellular half-life. The decreased pH mutant had very low hemolytic activity in comparison to wild-type PFO, but was able to mediate phagosomal escape in J774 macrophage-like cells. The decreased half-life mutant had 53% to 78% hemolytic activity of wild-type PFO. Both of the PFO variants escaped the phagosome at rates comparable to wild-type after the first hour of infection, but these rates declined to about 50% by two hours post-infection. Thirty random images were taken of macrophages infected with each strain, and the number of intracellular bacteria two hours post-infection was enumerated. On average, more bacteria were found within macrophages infected with the strains expressing variant forms of PFO than those infected with wild-type PFO expressing strains. Although this data is preliminary it could indicate that a less cytotoxic form of PFO can mediate intracellular persistence.

Acknowledgements

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Introduction

Clostridium perfringens is an anaerobic, gram-positive, spore-forming rod shaped bacterium. *C. perfringens* type A, the leading pathogen in gas gangrene infections (79, 129), produces α-toxin, phospholipase C (PLC), and θ-toxin, perfringolysin O (PFO) as its chief virulence factors (5, 18, 75, 88, 133, 140). Gas gangrene is the bacterial invasion of healthy living tissues, which were previously undamaged by trauma or ischemia (75, 132). The infection, on average, develops within 24 hours after introduction of the bacterium to the tissue through a deep, penetrating, or traumatic injury that compromises blood supply to the tissues creating a less oxygenated environment where *C. perfringens* can proliferate (129).

Phosoholipase C (PLC) has been shown to be the required virulence factor for *C. perfringens* gas gangrene (88). Previous research in our lab using a *pfoA*- (the gene for PFO) mutant showed that PLC can also mediate phagosomal escape from macrophages and contributes minimally to they cytotoxicity of *C. perfringens* (88). However, we did demonstrate that PLC along with PFO is required for *C. perfringens* persistence with primary macrophages in vitro and survival in vivo at sublethal (10^6) concentrations.

PFO has been implicated as an important pathogenicity factor in *C. perfringens* gas gangrene. PFO was shown to be required for leukostasis and wild-type levels of necrosis in a murine infection model (137, 140). PFO is a potent cytotoxic protein, which aids in the progression of a gas gangrene infection by damaging and lysing host cells, disregulating endothelial cell and PMN interactions, and priming PMNs for burst activity (15, 19, 134). These effects lead to lowered blood pressure, endothelial cell injury, and leukostasis (40, 139). Purified PFO intravenously administered to rabbits caused decreases in peripheral vascular resistance, hypotension, and bradycardia (4, 16, 139), suggesting a role for PFO in causing shock in the host, a commonly fatal complication of *C. perfringens* gas gangrene infections.

Perfringolysin O (PFO) is an oxygen-sensitive, pore-forming cytolysin (120, 147) and a member of the family of cholesterol-dependent cytolysins (CDC) (41, 112). CDCs are inhibited by free cholesterol, active only on cholesterol containing membranes, and they have a single unique cystine, which causes the protein to be inactive when it is oxidized (98, 146). PFO shares these characteristic properties with CDCs from other gram-positive bacteria, such as: *Listeria*

monocytogenes, *Streptococcus pyogenes*, and *Streptococcus pneumoniae,* which produce listeriolysin O (LLO), streptolysin O (SLO), and pneumolysin, respectively (64, 98). The CDC mechanism of action (Fig. 1-1) is first initiated by the binding of toxin monomers to cholesterol in the membrane, followed by their oligomerization on the membrane (112, 122, 124). These toxins oligomerize into ring or arch-like structures inducing the insertion of the β-barrel and forming a pore resulting in cell lysis due to the rapid influx and efflux of solutes (55, 59).

Listeriolysin O (LLO) is useful for our purposes because it shares many properties with PFO (64, 98). LLO is a cholesterol-dependent cytolysin that facilitates escape from the phagosome and permits bacterial survival and spread in the host (12, 97). LLO is less cytotoxic to host cells than PFO because LLO has a lower optimum pH range making it only active within the phagosome (48, 97). Once *L. monocytogenes* has escaped the phagosome, pH 5.5, and enters the cytosol, pH 7, its activity dramatically decreases (48, 98). PFO has also been shown to mediate phagosomal escape under aerobic and anaerobic conditions in vitro (88); however, due to its activity at acidic and neutral pH, it lyses the macrophage soon after escape (98). LLO also has less activity than PFO because it has a shorter half-life (65) within the host cell, therefore, decreasing the amount of time in which it is active (34, 72).

Jones et al., have created two PFO mutants by ethyl-methane sulphonate mutagenesis (65). Both forms of mutated PFO are secreted in amounts equal to that of wild-type PFO (65). PFO mutant DP-L2795 with a leucine 462 to phenylalanine mutation gave a lower optimum pH, more active at pH 5.6 and less active at pH 7.4, similar to LLO. The second PFO mutant, histidine 438 to tyrosine mutation, DP-L2817, has a decreased half-life of 30 minutes, which is similar to LLO, while wild-type PFO has a half-life of 60 minutes. The PFO mutants are useful because they reduce the cytotoxicity of PFO allowing us to separate its ability to mediate phagosomal escape from its cytotoxicity.

Previous research in our lab demonstrated that *C. perfringens* can persist in tissues for up to 72 hours following infection with a sublethal inoculum (10^6) (88). Also cases of recurrent gas gangrene due to *C. perfringens* have been reported in sites of previous infection where additional injury was minor indicating that the infection was most likely due to latent bacteria or spores that persisted in the tissues (75, 136). Also, we have found that *C. perfringens* survival is

significantly enhanced under aerobic conditions in vitro when macrophages were present (87). This evidence suggests to us that *C. perfringens* can persist in tissues even though it is an anaerobe and that its persistence could be mediated by persistence or growth within macrophages.

The purpose of this research was to investigate the importance of PFO mediated phagosomal escape to the survival of *C. perfringens* within macrophages. Plasmids carrying variant forms of the *pfoA* gene (DP-L2795 and DP-L2817), which encodes PFO, or the *hlyA* gene, which encodes listeriolysin O (LLO), were used to complement DOB4, a non-virulent *plc-/pfoA-* mutant of *Clostridium perfringens* strain 13. We wanted to eliminate the activity of PLC from our experiments because PLC also mediates phagosomal escape (88). Large amount of research has been done to examine the role of PLC in infection but only a few studies have looked at the role of PFO in infection. For this reason we are using less active variants of PFO to examine their ability to mediate phagosomal escape and the role PFO plays in intracellular survival of *C. perfringens*. The PFO mutants are less cytotoxic but were found to mediate escape from the phagosome and were found within the cytosol at higher concentrations compared to wild-type *C. perfringens* and DOB4. These findings may indicate that *C. perfringens* could persist within the cytosol of macrophages if the cytotoxic activity of PFO was limited.

Methods

Bacteria and growth conditions

All *C. perfringens* strains were grown in PGY medium (80) at 37 °C in a Coy anaerobic chamber. *Listeria monocytogenes* was grown overnight in brain heart infusion broth (BHI) at 30 °C without shaking (64). *E. coli* was grown aerobically at 37 °C in luria bertani (LB) media. Following electroporation (2), *C. perfringens* transformants were plated on BHI and incubated in the anaerobic chamber at 37 °C. When needed, antibiotics were added to the medium at the following concentrations: 20 μg/ml of chloramphenicol, 30 μg/ml of erythromycin, and 5 μg/ml or 10 μg/ml of tetracycline for *C. perfringens* and *E.*coli, respectively.

The macrophage-like cell line, J774.6, was grown without shaking in Dulbecco's Modified Eagle's Medium (DMEM) with glucose and L-glutamine, 10% fetal bovine serum, and 5 mM sodium pyruvate. The cells were grown at 37 °C with 5% CO_2.

Hemolysis assay

DOB4(pBT102), DOB4(pBT103), strain 13, *plc*-, and DOB4 were grown to stationary phase and subcultured. Supernatants were collected from log phase cultures by centrifugation and filtered through a 0.22 μm filter. The supernatants were incubated anaerobically for 30 minutes with an equal volume of buffer (2 mM EDTA, 20 mM DDT, 64 mM KH_2PO_4, 156 mM NaCl, 6 mM cysteine, 50% washed red blood cells) at pH 5.6 or pH 7.4. After incubation, the tubes were centrifuged at 200*x g* for 5 minutes. Spectrophotometer readings were taken at an absorbance of 550 nm to detect the release of hemoglobin. A hemolytic unit was defined as the difference between the absorbance at 550 nm between the sample and the blank per microgram of protein. The protein concentrations were measured with a Biorad Protein Assay kit, following manufacturer's instructions.

Macrophage infections for transmission electron microscopy

J774.6 cells were grown to confluency in Dulbeco's Modified Eagle's Medium (DMEM) in 50-ml tissue culture flasks. *C. perfringens* was grown to mid-log phase. The cells were pelleted and washed three times in phosphate buffered saline (PBS). J774.6 cells were infected with *C. perfringens* at a multiplicity of infection (MOI) of one-to-one at 37 °C for one and two hours under aerobic conditions. An MOI of one-to-one was used to ensure that the number of

wild-type *C. perfringens* was not sufficient to lyse the majority of the macrophages prior to analyzing them. Infected macrophages were washed in PBS and fixed in 2.5% glutaraldehyde in PBS for five minutes. The cells were pelleted at 20*x g* for one minute and incubated in glutaraldehyde for one to two hours at room temperature. The infected cells were further processed for viewing with a transmission electron microscope. At least 50 images of bacteria were taken randomly from each sample and were determined to be within the phagosome or within the cytosol. Each bacterium that was considered had to be completely visible in the section being analyzed, surrounded by the macrophage, and electron dense.

The same grids were used to determine the total number of bacteria within a macrophage. Images of 30 macrophages were taken randomly for each sample. The entire section of the macrophage had to be visible (not blocked by grid bars) and had to have one or more bacteria. Each bacterium that was counted had to be surrounded by the macrophage and electron dense. The number of intracellular bacteria was quantified for each macrophage and recorded.

Table 3-1. Strains and plasmids used in mutant PFO tests

Strains/Plasmid	Relevant characteristics	Source or reference
E. coli		
DH10B	F- *mrcA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*dlacZ*ΔM15 Δ*lacX74 deoR recA1 araΔ139* Δ(*ara*, *leu*)7697 *galU galK* λ- *rpsL endA1 nupG*	(49)
C. perfringens		
13	PFO+ PLC+	C. Duncan
PLC-	PFO+ PLC-	(66)
DOB4	PFO- PLC-	(88)
Plasmids		
pJIR751	*C. perfringens*- *E. coli* shuttle vector, *ermBP*+	(9)
pJV5	*C. perfringens*- *E. coli* shuttle vector, *catP*+ *kanR*+	This study
pDOB28	pJIR751/ *pfoA* from strain 13	This study
pBT106	*C. perfringens*- *E. coli* shuttle vector, *tetM*+	This study
pBT102	pBT106/ *pfoA* from DP-L2795 (decreased optimum pH mutant)	This study
pBT103	pBT106/ *pfoA* from DP-L2817 (decreased half-life mutant)	This study
pBT109	pBT106/ *hlyA*	This study
pBT110	pDOB28/ *ermBP*- *tetM*+	This study

Results

PFO mutants are hemolytic when expressed by *C. perfringens*.

Hemolysis assays were performed using defibrinated sheep blood and culture supernatants. Activities were compared to wild-type *C. perfringens*, because the wild-type *pfoA* shuttle vector construct was not expressed in DOB4 as shown by a lack of hemolysis on sheep's blood agar plates. The activity of DOB4 (pBT103) showed a similar pattern of activity as was observed by Jones et al. (65). Its hemolytic activity was 78% of wild-type at pH 7.4 and 53% of wild-type at pH 5.6 (Fig. 3-1A). DOB4 (pBT102) activity was much lower than expected, 2.5% and 8% of wild-type at pH 7.4 and pH 5.6, respectively. However, DOB4 (pBT102) had a definite induction of activity at pH 5.6. The ratio of its activity at pH 5.6 and pH 7.4 was the highest induction of activity of all five strains (Fig. 3-1B). Listeriolysin O was poorly expressed in DOB4 having only 6% activity of wild-type *C. perfringens* at pH 5.6 and pH 7.4 and showing no induction of activity at lower pH. The *plc*- strain of *C. perfringens*, with an intact *pfoA* gene, had lower levels of hemolysis than wild-type, 63% at pH 7.4 and 39% at pH 5.6. The double mutant DOB4 had less than 1% activity of wild-type *C. perfringens* (Fig. 3-1A). The activity of the wild-type PFO (pBT110) construct was not measured due to its lack of hemolysis on blood plates when expressed by DOB4.

Transmission electron micrographs show that the PFO mutants can mediate phagosomal escape in *C. perfringens*.

Transmission electron micrographs (TEMs) were taken of J774.6 macrophages infected with wild-type *C. perfringens*, DOB4, *plc*-, DOB4 (pBT102), and DOB4 (pBT103) for one and two hours. 70% of wild-type *C. perfringens* escaped macrophage phagosomes at one and two hours post-infection. The PFO mutants escaped at rates comparable to wild-type at one hour, but at two hours decreased to 49% and 51% for DOB4 (pBT102) and DOB4 (pBT103), respectively (Fig. 3-2B). DOB4 escaped at an average of two and five times less than the other strains at one and two hours post-infection, respectively.

Macrophages containing DOB4 (pBT102) and DOB4 (pBT103) qualitatively seemed to contain more bacteria than macrophages infected with the other strains. When this observation was scored at two hours post-infection, DOB4 (pBT102) and DOB4 (pBT103) averaged 3.06

and 2.73 bacteria per macrophage, respectively, and ranged between one and eight bacteria per macrophage (Fig. 3-3). In comparison, wild-type averaged 1.2 bacteria per macrophage and ranged between one and three bacteria per macrophage after two hours.

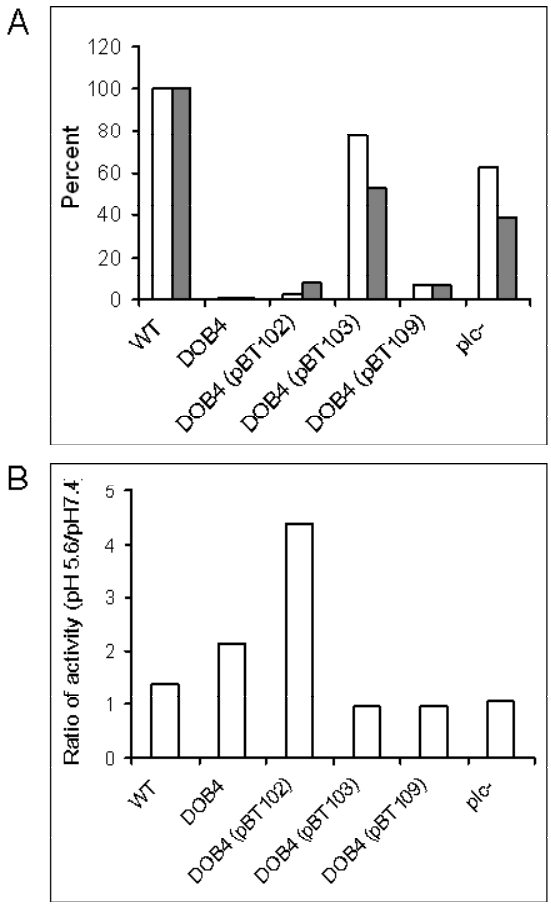

Figure 3-1. Hemolytic activity of DOB4 expressing the mutant forms of PFO. Washed, defibrinated sheep blood was incubated with culture supernatants anaerobically for thirty minutes. Spectrophotometer readings of the samples were taken at 550 nm to detect the release of hemoglobin. A hemolytic unit was calculated as the difference between the blank and the sample at 550 nm per milligram of protein. (A) The average percent activity in comparison to wild-type. White bars indicate samples tested at pH 7.4 and gray bars indicate tests done at pH 5.6. (B) The ratio of activity for each sample at pH 5.6 and pH 7.4.

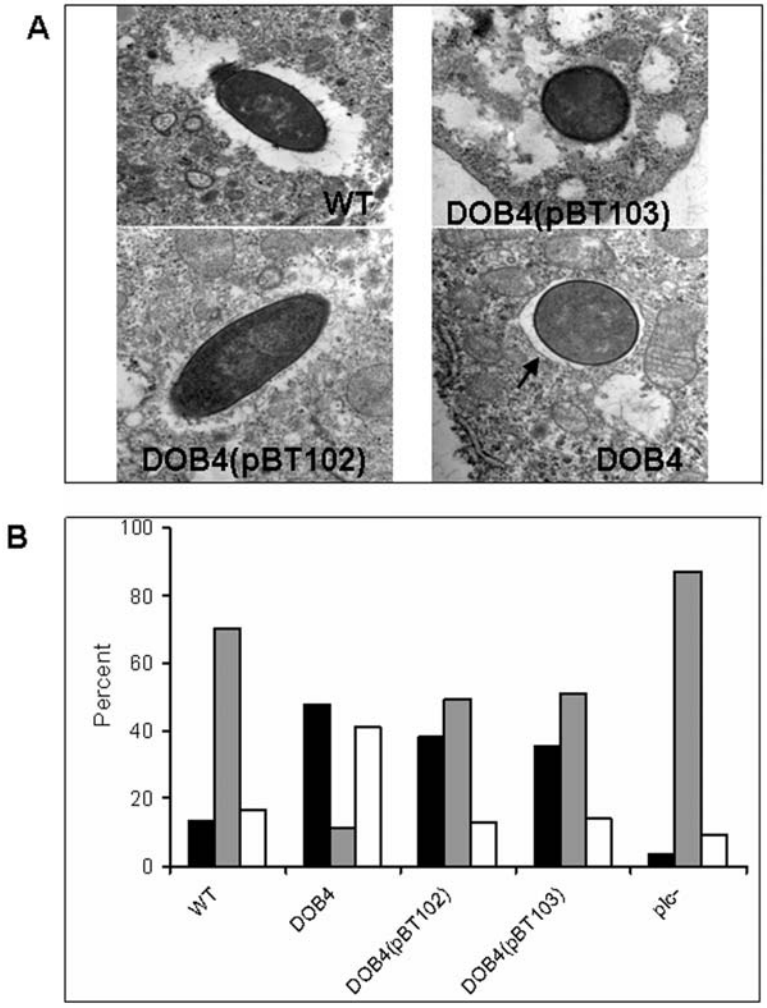

Figure 3-2. The variant forms of PFO mediate phagosomal escape. Representative images of WT, DOB4(pBT102), and DOB4pBT(103) within the cytoplasm and of DOB4 within the phagosome are shown in **A**. The arrow indicates the phagosomal membrane. To determine the percent of bacteria that escaped the phagosome 50 bacteria were analyzed for each strain (**B**). Bacteria were judged to have escaped if only one-third of the bacterium was not surrounded by the phagosomal membrane or was completely in the cytoplasm (gray bars). Bacteria that were at least two-thirds surrounded by the phagosomal membrane were graded as being in the phagosome (black bars). Bacteria that were scored as not determined did not have a clearly defined or visible phagosomal membrane (white bars).

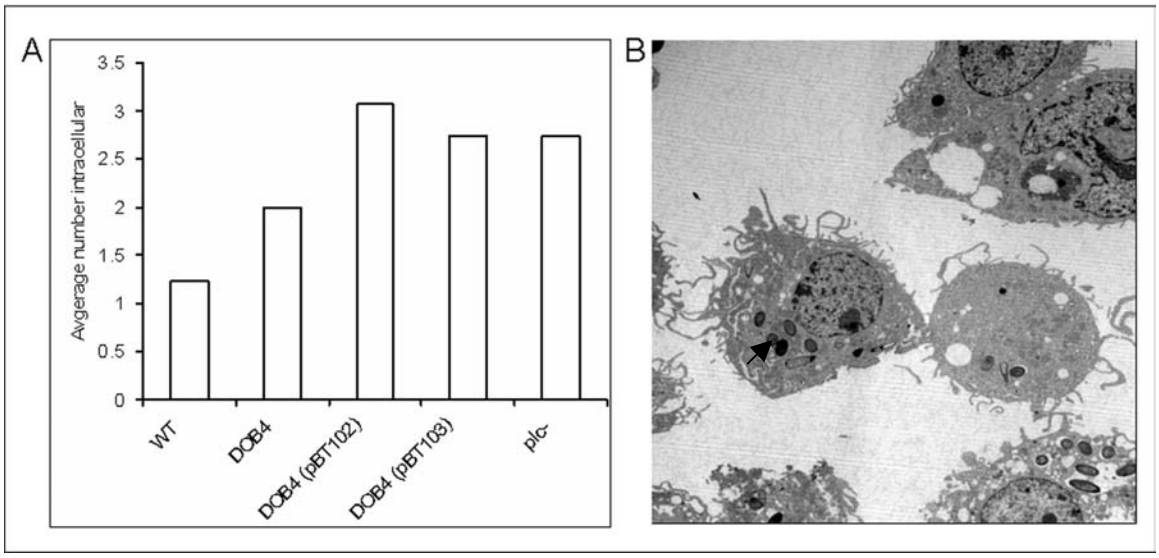

Figure 3-3. Average number of intracellular bacteria found for each strain in J774.6 infections (A). Macrophages were infected for two hours, aerobically at a MOI of one to one. Images of entire macrophages were taken to determine the number of intracellular bacteria. Thirty macrophages infected with each strain were examined to determine the number of intracellular bacteria. Bacteria were counted as intracellular if they were surrounded by the macrophage and if they were electron dense. A representative image of bacteria (arrow) within macrophages is given in **B**.

Discussion

PFO is a potent cytotoxin in *C. perfringens* gas gangrene infections. It has been shown to mediate phagosomal escape and induce vascular leukostasis (40, 88). LLO is a cytotoxin produced by *L. monocytogenes* and is required for phagosomal escape and virulence in listeriosis (96). LLO and PFO are both cholesterol-dependent cytolysins, but LLO has the unique characteristic of being pH specific (146). Its inactivity in the neutral cytosol allows *L. monocytogenes* to persist and grow in the cytosol of host cells (48). Mutant forms of PFO created by Jones, et al., have properties similar to LLO, lower optiumum pH and shorter half-life, and were shown to mediate phagosomal escape and intracellular growth for *L. monocytogenes* (65). Using these mutant forms of PFO we analyzed their ability to mediate phagosomal escape for *C. perfringens* strain DOB4.

DOB4 (pBT102), decreased optimum pH mutant, had very low hemolytic activity as compared to wild-type *C. perfringens* (Fig. 3-1A), even so, it did mediate phagosomal escape at levels close to wild-type one hour post-infection. DOB4 (pBT103), decreased half-life mutant, also escaped the phagosome at a rate comparable to wild-type at one hour post-infection. DOB4 (pBT102) and DOB4 (pBT103) rate of escape decreased by two hours post-infection, which may be due to the activation of the macrophages (127). The ability of wild-type *C. perfringens* to escape did not significantly decrease between one and two hours post-infection. Perhaps, this is due to wild-type activity levels of PFO and PLC in wild-type *C. perfringens*. It was previously shown that wild-type *C. perfringens* is not efficiently killed by activated macrophages in a stationary assay; in fact, these macrophages are more sensitive to its cytotoxic activities (87), so that if the macrophages were more activated by two hours post-infection this would not affect the ability of *C. perfringens* to escape or be cytotoxic to the macrophages.

DOB4 (pBT102) and DOB4 (pBT103) were found at higher numbers within macrophages in comparison to wild-type *C. perfringens*. Macrophages had as many as eight intracellular DOB4 (pBT102) and DOB4 (pBT103) bacteria, but these macrophages only had a maximum of three intracellular wild-type *C. perfringens*. This could be an indication of intracellular persistence or growth within macrophages when cytotoxicity is limited. The higher intracellular numbers of the strains expressing mutant forms of PFO were most likely not due to

their being more susceptible to phagocytosis since we observed more bacteria within macrophages infected with strains producing the mutant forms of PFO than DOB4, which presumably would be the strain most susceptible to phagocytosis. The data, however, is preliminary and indirect; therefore, future work will be done to test the ability of the less active variants of PFO to mediate intracellular persistence using in vitro invasion assays and video microscopy studies. If it is found that the modified forms of PFO do allow for intracellular persistence or growth of *C. perfringens*, then this would also indicate the oxygen level in the macrophage cytosol is equal to if not below the level required for the anaerobe *C. perfringens* to grow.

The wild-type *pfoA* and *hlyA* constructs did not produce functional proteins in DOB4. Restriction digests and gel-electrophoresis indicated that the fragments used to make the constructs were the correct size and sequencing did not show any mutations in the genes or their promoter regions. The inactivity of these constructs may be due to post-translational modification or regulation of the proteins causing them to be non-functional.

C. perfringens has been found in the muscle of mice up to 72-hours post-infection with a sublethal dose (88). The ability of *C. perfringens* to persist in an aerobic wound could be mediated by its persistence or growth within tissue macrophages. *C. perfringens* has been shown to increase significantly in its ability to survive under aerobic conditions in the presence of macrophages in vitro (87). Also, intracellular *C. perfringens* has been shown to prevent the co-localization of the LAMP-1 phagosome-lysosome marker with *C. perfringens* containing phagosomes (87). Averting the bactericidal components of the macrophage by preventing maturation of the phagosome and escaping the phagosome would permit *C. perfringens* to remain alive within the macrophage (87, 88). The ability to persist within tissue macrophages, at least transiently before the cell is lysed, would provide an environment of reduced oxygen where the anaerobic bacterium could reside. Then if the tissues become hypoxic due to toxin mediated vascular occlusions (16, 17) or additional injury compromising blood supply, *C. perfringens* could multiply rapidly and cause gangrene.

IV. Characterization of *Clostridium perfringens* strain 13 sialidases

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Manuscript in preparation

Abstract

Clostridium perfringens produces at least thirteen different toxins including sialidase. *C. perfringens* has two sialidases NanH and NanI that were previously observed to be induced when cultures were grown with sialic acid. We examined eleven different *C. perfringens* strains for their ability to produce NanI and observed that strains isolated from a food poisoning outbreak in Great Britain in 1950 did not produce NanI. A novel sialidase for *C. perfringens* annotated as NanJ was found to be highly homologous to the sialidase of *Clostridium septicum*. Primer extension identified three putative start sites for *nanI* at 66-bp, 163-bp, and 308-bp upstream of the start codon. Primer extension also showed that *nanI* is transcriptionally induced when sialic acid is present. For *nanJ*, two putative transcription start sites were identified at 86-bp and 146-bp upstream of the start codon. *nanI*-, *nanJ*-, and *nanI*-/*nanJ*- mutants were created by homologous recombination in strain 13 and tested for sialidase activity. We demonstrated for the first time that NanJ is an extracellular sialidase. NanJ is more active at 37 °C than at 50 °C and is repressed by the presence of sialic acid in the growth media. We identified a 17-bp conserved motif among genes with sialic acid-related regulation, which may be the binding site for a transcriptoinal regulator. The *nanI*-/*nanJ*- double mutant was used in murine gas gangrene infection, and *C. perfringens* sialidase appeared be protective for infected mice.

Acknowledgements

I would like to acknowledge David K. O'Brien for performing the Southern blot and sialidase assay to determine the presence of the *nanI* gene in and production of NanI by different strains of *Clostridium perfringens*. David O'Brien also performed the β-glucuronidase assay using the *nanI-gusA* promoter fusion. Veronica L. Stirewalt worked as a technician in the lab and performed the primer extension for *nanI*. I would like to thank Stephen B. Melville for guidance with experimental design and results interpretation.

Introduction

Clostridium perfringens is an anaerobic, gram-positive, rod shaped bacterium, and the causative agent of gas gangrene (79, 129). *C. perfringens* produces 13 different toxins including phospholipase C (PLC) and perfringolysin O (PFO), which are the major virulence factors of *C. perfringens* gas gangrene infections (5, 88, 111). Of these 13 toxins that *C. perfringens* produces, several are probable pathogenicity factors, for example, urease, collagenase, and sialidase. *C. perfringens* has two sialidases, a large extracellular sialidase, NanI, and a small intracellular sialidase, NanH (44, 105, 145). Genome sequencing results of the genetically tractable, gangrene causing strain, strain 13, revealed that it has the *nanI* gene, does not have the *nanH* gene, and was recently annotated to have *nanJ*, a novel sialidase for *C. perfringens* (126). NanJ is predicted to have 1173 amino acids and a molecular weight of 129 kDa making it the largest known clostridial sialidase (85).

Sialidases are glycohydrolases that cleave the terminal sialic acid from sialoglycoconjugates (29). Sialidases have two conserved regions: two to five "Asp-box" (Ser-X-Asp-X-Gly-X-Thr-Trp) repeats and a (F)RIP ((Phe)-Arg-Ile-Pro) sequence located upstream of the first Asp-box (145). The Asp-boxes are thought to be involved in catalysis, and the (F)RIP sequence seems to be involved in substrate binding (69, 110).

Sialidases can be metabolically useful by supplying substrates for metabolism (29, 108). In *C. perfringens*, the permease, NanT, imports sialic acid for degradation by N-acetylneuraminate lyase (NanA), which cleaves sialic acid to pyruvic acid and N-acetylmannosamine (154). These components can be used for energy or as building blocks for cellular components. Sialidases can also be pathogenicity factors. In *Psuedomonas aeruginosa*, the sialidase allows for improves attachment of pili to cells within the infected host (33, 157). The sialidase of *Vibrio cholera* enhances binding of cholera toxin to host cells (45).

There is some preliminary evidence of a role for sialidases in the pathogenesis of gas gangrene. Cells with a ganglioside (sialylated galactose-containing lipid compound) deficiency were more susceptible to PLC induced membrane damage, and in vivo results of injected toxin indicate that NanI enhances PLC activity (43). Some evidence indicates that membrane-bound gangliosides disrupt PLC activity on membranes without affecting the ability of PLC to bind to

membranes (11). Also, sialylated gangliosides on canine kidney cells were shown to inhibit the binding of the epsilon toxin from *C. perfringens* types B and D indicating that the sialidase may enhance epsilon toxin binding (125).

C. perfringens strain 13, *C. septicum*, and *C. sordellii* produce sialidases in large amounts and these three species also cause diseases in animals suggesting that the sialidases produced by these organisms may enhance their pathogenicity (108). Sialic acid is present on the surface of all mammalian cells and is essential for intercellular and intracellular signaling; suggesting that cleavage of sialic acid by pathogens can be an advantage in pathogenesis. The aim of this study was to characterize some aspects of *C. perfringens* strain 13 sialidases. We investigated the regulation and of *nanI* and *nanJ* and generated a *nanI*-/*nanJ*- double mutant to test for an in vivo role for sialidase in virulence. Sialic acid was found to induce transcription of *nanI* and repress transcription of *nanJ*. Our results demonstrate that sialidases may be protective for mice with a *C. perfringens* gas gangrene infection.

Methods

Bacterial strains, plasmids and growth media.

Bacterial strains and plasmids used in this report are listed in Table 4-1. Luria broth (LB) (10 g tryptone, 5 g NaCl, 5 g yeast extract) was used to grow *E. coli*. To grow *C. perfringens*, anaerobic PGY medium (30 g proteose peptone, 20 g glucose, 10 g yeast extract, and 1 g sodium thioglycollate per liter) or PY medium (PGY lacking glucose) was prepared and stored in a Coy anaerobic chamber (Coy Laboratory Products, Inc.), as previously described (162).

Plasmid constructs and DNA manipulations.

To determine if the region upstream of *nanI* can function as a promoter, a PCR product containing 664 bp of the intergenic region between *nanI* and the upstream gene (145) was placed upstream of the *gusA* gene in plasmid pSM218 (154), to create plasmid pSM230 (Table 4-1). Plasmid pSM218 is an *E. coli-C. perfringens* shuttle vector containing a promoterless *cpe-gusA* gene fusion, which retains the ribosomal binding site and first 13 amino acids of the *cpe* gene, preceded by a polylinker region and four tandem terminators (154). The *nanI-gusA* fusion contained the entire promoter region and the first three codons of the *nanI* structural gene. The resulting plasmid was then transformed by electroporation into *C. perfringens* SM101, as previously described (80).

To create the *nanI*- mutant, BT1, a *C. perfringens* suicide vector containing an internal fragment of the *nanI* structural gene was constructed. An internal fragment of the *nanI* gene was amplified with oligonucleotides ODOB 11 (5'-CCTTAATGGTACAATCATAAAAGAAGTTAAAGATA-3') and ODOB 12 (5'-ATAAGGTTCTAGAATTTATTATTTTTCCATTTTC-3') and ligated to pSM300, a *C . perfringens* suicide plasmid (151), to form pDOB18 (Table 4-1). The suicide vector pSM300 encodes the *ermBP* gene, conferring erythromycin resistance.

A *C. perfringens* suicide vector containing an internal region of the *nanJ* structural gene was constructed and used to make the *nanJ*- mutant, BT2 (Table 4-1). An internal fragment of the *nanJ* gene was amplified by PCR with oligonucleotides OBT21 (5'-GGGGAAACTAAGGCGCCTGCAGAGG-3'), located 900 bases downstream of the putative

Table 4-1. Strains and plasmids used in the characterization of sialidases.

Strains/Plasmid	Relevant characteristics	Source or reference
E. coli		
DH10B	F- *mrcA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*dlacZ*ΔM15 Δ*lacX74 deoR recA1 araΔ139* Δ(*ara*, *leu*)7697 *galU galK* λ- *rpsL endA1 nupG*	(49)
JM107	*endA' gyrA96 thi hsdR17 supE44 relA1* λ- Δ(*lac*$^-$ *proAB*) F' *traD36 proAB lac19 lacZ* ΔM13	(159)
C. perfringens		
NCTC 8239	Cpe+	R. Labbe
NCTC 8679	Cpe+	R. Labbe
NCTC 8798	Cpe+	R. Labbe
NCTC 10240	Cpe+	R. Labbe
ATCC 3624	Cpe+	R. Labbe
T-65	Cpe-	R. Labbe
3663	Cpe+	R. Labbe
4246	Cpe+	R. Labbe
FD1	Cpe-	R. Labbe
13	Cpe-	R. Labbe
6234 LI	type C	J. G. Songer
SM101	High frequency electroporation derivative of NCTC 8798	(162)
BT1	NanI- NanJ+	This study
BT2	NanI+ NanJ-	This study
BT3	NanI- NanJ-	This study

Table 4-1. (Cont.)

Strains/Plasmid	Relevant characteristics	Source or reference
Plasmids		
pSM218	Transcription vector, *catP*+	(162)
pSM300	*C. perfringens* suicide vector, *ermBP*+	(88)
pJIR750	*E. coli*-*C. perfringens* shuttle vector, *catP*+	(9)
pJV50	*C. perfringens* suicide vector, *catP*+	This study
pSM230	pSM218/ *nanI* promoter region from strain 13	This study
pSM240	pSM218 with polylinker of pBluescript SK+ (Stratagene corp.)	This study
pDOB18	pSM300/ internal fragment of *nanI* from strain 13	This study
pDOB1	pJIR750/ *nanI* from strain 13	This study
pBT5	pJV50/ internal fragment of *nanJ* from strain 13	This study

nanJ start codon, and OBT22 (5'-GCCTGCCCAAGCTTCCTCG-3'), located 1-kb upstream of the *nanJ* stop codon. The PCR product was ligated to pGEM-T Easy vector (PROMEGA) per manufacturer's instructions. The insert was then digested with HindIII and PstI, giving a 1.3-kb fragment, which was ligated to pJV50, to form pBT5 (Table 4-1). pJV50 is a suicide vector that confers chloramphenicol resistance.

pDOB18 and pBT5 were first electroporated into JM107 *E. coli*. This cell line is *rec*+ and produces multimeric forms of the plasmid by recombination (159). This allows for greater insertion frequency into the chromosome of *C. perfringens*, presumably because more copies of the sequence of interest are present. Following passage through the JM107 cell line, the plasmids were purified by cesium gradient. The resulting highly pure plasmid was used to transform overnight cultures of *C. perfringens* strain 13 by electroporation with 4 mm-gap cuvettes and 60 μg of plasmid DNA. The resistant colonies were selected for on BHI plates with the appropriate antibiotic in the following concentrations: 30 μg/ml erythromycin, 20 μg/ml chloramphenicol.

To create the *nanI*-/*nanJ*- double mutant, BT3, BT1 was electroporated with the suicide vector pBT5 and selected for chromosomal insertion on BHI plates with 30 μg/ml of erythromycin and 20 μg/ml of chloramphenicol.

Extraction of chromosomal DNA

Chromosomal DNA was extracted according to the method of Pospiech and Neumann (99) as follows. Five mls of overnight culture were concentrated, washed with one ml of STE (75 mM NaCl, 25 mM EDTA, 200 mM Tris pH 7.5), and resuspended in 500 μl of STE with 10 μg/ml of RNase and one mg of lysozyme. The samples were incubated at 37 ºC for two hours. Then 50 μl of 10% SDS and 10 μl of proteinase K were added and the samples were incubated at 55 °C for two hours with frequent mixing. At the end of two hours 165 μl of 5 M NaCl and 600 μl of chloroform was added to each sample. Each sample was incubated at room temperature for 30 minutes with frequent inversions and burping. The samples were centrifuged at 5000 rpm for 10 minutes and the top clear layer was transferred to a new tube. One volume of isopropanol was added, the sample was mixed, and the chromosomal DNA was spooled out. The DNA was washed with 70% ethanol and 95% ethanol and dried before resuspension in 100 μl of TE.

Southern hybridization analysis

For Southern blot analyses to detect the presence of *nanI* (Fig. 4-1), 5 μg of chromosomal DNA was digested to completion with Hind III, and following agarose gel electrophoresis the DNA was transferred to a nylon membrane, done as previously described (117). To detect hybridization to the probes, the Phototope Star detection system was used (New England Biolabs) according to manufacturer's instructions. For *nanI*, the probe was a 1.5 kb EcoRI-Hind III fragment from a PCR product containing the *nanI* gene from strain 4246 (unpublished results).

Southern hybridization analysis was done on BT1, BT2, and BT3. Chromosomal DNA from the wild-type and mutant strains was isolated and digested with NdeI to check for an insertion in *nanI* or ScaI to check for the *nanJ* insertion. Digests were run on 0.8% agarose gel and transferred to a nylon membrane (118). Membranes were probed with the internal *nanI* or *nanJ* fragment that was amplified with ODOB11 and ODOB12 or OBT21 and OBT22, respectively. Probes were labeled and hybridized according to the protocol of the NEBlot Phototope kit (New England BioLabs). After digestion, the *nanI* gene probe was predicted to hybridize to a 5.1-kb fragment (i.e., a 1.5-kb chromosomal region plus 3.6-kb for pSM300) and the *nanJ* gene probe was predicted to hybridize to a 6.1-kb fragment (3.3-kb chromosomal region and 2.6-kb for pJV50). To detect hybridization to the probes, the Phototope Star detection system was used (New England Biolabs) according to manufacturer's instructions.

Sialidase enzyme assay

For sialidase assays, *C. perfringens* cultures were grown overnight in an anaerobic chamber in two or five mls of PY media, in either the presence or absence of one mg/ml sialic acid (N-acetylneuraminic acid). When sialic acid was not added, water was used to replace the volume taken up by the sialic acid solution. For extracellular sialidase activity, the overnight cultures were pelleted and the supernatants were removed. For intracellular sialidase activity, the cell pellets were washed three times with one ml of PBS. Pellets were frozen on dry ice and resuspended in one ml TE containing 10 mg/ml of lysozyme and incubated at 37 °C for 20 minutes. The bacterial cells were lysed by adding one ml of water and then centrifuged to remove cell debris. For the cell wall fraction, the cell debris pellet was washed three times in

one ml PBS. All samples were resuspended, if needed, in a final volume of five mls and were kept on ice until use.

The measurement of sialidase activity was performed as previously described (105). The data shown in Figure 4-1 represent the means of at least three independent measurements of NanI activity, where the standard deviation was no greater than 30% of the mean values. The data shown in Figure 4-6A and 4-6B are the means of triplicate experiments and the error bars indicate the standard deviation from the mean.

Primer extension experiments

To determine transcription for *nanI* and *nanJ*, *C. perfringens* strain 13 was grown to mid-log phase on either PY or PY plus one mg/ml sialic acid and total RNA was extracted from the cells using the TRIZOL® reagent (Invitrogen), as previously described (80). Twenty μg of RNA were used as a template for primer extension reactions for *nanI*, using the Promega Primer Extension System kit according to the manufacture's instructions. The primer, OSM91, was designed to anneal to the first 10 codons and 6-bp upstream of the *nanI* structural gene.

Primer extension for *nanJ* was done using one μg of RNA with the primer OBT 34 (5'- CAAGTGTAGCTATAATTTTTTTACTTTTCACTAGCTC -3'), which includes the first 10 codons of the *nanJ* structural gene, and was tagged at the 5'-end with the fluorescent tag 6 FAM.

β-glucuronidase assay

β-glucuronidase assays in *C. perfringens* were performed as previously described (80). The constructs were tested in strain SM101 because it does not have a *gusA* gene. Briefly, one ml of overnight culture, grown with or without sialic acid, was snap frozen and lysed with toluene. Supernatants were mixed with the substrate ρ-nitrophenly β-D-glucuronidate and incubated at 37 °C until yellow color developed. The reaction was stopped with bicarbonate buffer and absorbance readings were taken at 405 nm. Specific activity was calculated as the absorbance at 405 nm multiplied by 1000 and divided by the product of the OD_{600}, time, and culture volume. The means of duplicate samples of a representative experiment for the promoter region of *nanI* are shown in Fig. 4-5.

Animal studies

Six to eight week old BALB/c mice were injected in the hind leg muscle with mid-log phase, washed *C. perfringens* strain 13 or BT3, the *nanI-/nanJ-* double mutant. The *C. perfringens* cultures were pelleted, washed three times in 1x PBS, and diluted. Ten mice were injected with concentrations of either ~ 1 x 10^9, 1 x 10^8, or 1 x 10^7 bacteria for wild-type *C. perfringens* and BT3. As a control, mice were injected with 1x PBS.

In accordance with Virginia Polytechnic Institute and State University animal care policy all mice were euthanized by CO_2 asphyxiation when gangrene symptoms became apparent or 36 hours after injection. The time to appearance of gangrene was recorded for each mouse.

Results

Some strains of *C. perfringens* lack the *nanI* gene.

In order to detect the *nanI* gene in different strains of *C. perfringens*, we used PCR techniques to amplify an internal segment of the *nanI* gene to be used as a molecular probe for Southern blots. Degenerate primers designed to anneal to regions of the *nanI* coding sequence (145) amplified a fragment from strain 13, but failed to give a PCR product using NCTC 8798 chromosomal DNA (unpublished results). To determine if strain NCTC 8798 carried a copy of the *nanI* gene, a Southern blot analysis was done using the *nanI* fragment from strain 13 as a probe, but a *nanI* gene of sufficient similarity to hybridize to our probe using chromosomal DNA from NCTC 8798 was not detected. Eight other strains (Table 4-1) available in our laboratory were also tested and found to display an interesting pattern: four strains with the enterotoxin (CPE) designated as Hobbs serotypes 3, 6, 9, and 13, isolated from food poisoning outbreaks in Great Britain during the 1950s (141), lack the *nanI* gene (Fig. 4-1, top). Each strain lacking the *nanI* gene also lacked NanI enzymatic activity (Fig. 4-1, bottom). This apparent lack of the *nanI* gene in strains isolated from a distinct geographical location and time period (i.e., Great Britain in the 1950s) suggests a common origin for these strains. The fact that the strains lack *nanI* and are CPE+ (they cause of food poisoning in humans) does not appear to be relevant since strains 4246 and 3663 are also CPE+ and yet they carry the *nanI* gene (Fig. 4-1).

Some of the strains containing *nanI* showed a 1.5 to 3-fold induction of activity for this gene, when grown in the presence of sialic acid (Fig. 4-1), while others, including strain 13, showed small induction of activity between the different growth conditions. We can not explain this but later experiments (Fig. 4-6) did show a larger induction of activity for *nanI* strain 13 when grown with sialic acid.

nanJ annotation and sequence alignment

Genome sequencing of strain 13 revealed that this strain did have the gene for the "large" sialidase, *nanI*, but it did not have the gene for the "small" sialidase, *nanH*. Additionally a new gene was annotated as an exo-alpha-sialidase, *nanJ* (126). NCBI BLASTP (3) of the NanJ sequence yielded a hit of highest homology with the sialidase of *C. septicum*, e-value of zero,

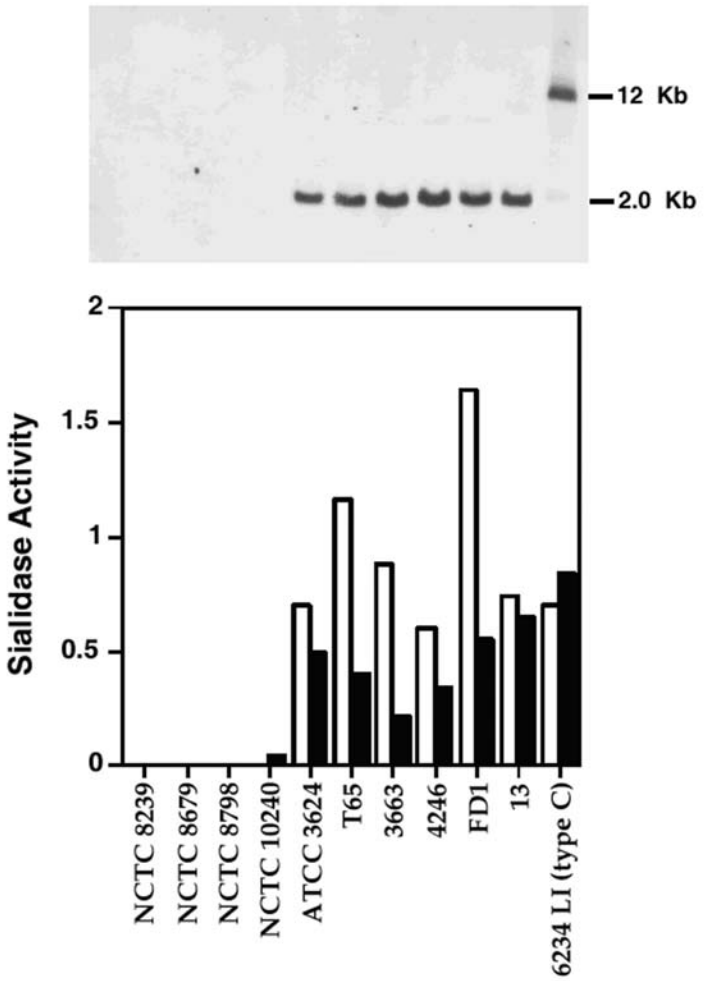

Figure 4-1. Detection of *nanI* in various strains of *C. perfringens*. Southern blot (**Top**) of the strains listed at the bottom of the figure was performed by digestion of the DNA with HindIII and probing with a *nanI*-specific DNA fragment (see Methods). The numbers on the right show the sizes of two of the hybridizing bands, as estimated using lambda phage DNA digested with EcoRI and HindIII. Sialidase enzyme activity for these strains was tested (μmoles 4-methylumbelliferyl-α-D-N-acetylneuraminic acid hydrolyzed/min/mg protein) using the NanI-specific enzyme assay (**Bottom**). Open bars represent bacteria grown in PY plus 1 mg/ml sialic acid, solid bars were grown in PY only. Experiments performed by David O'Brien and Veronica Stirewalt.

49% identity, and 66% positive. These two proteins are most dissimilar at the C-terminus. Alignment of NanJ, NanI and the *C. septicum* sialidase on NCBI showed that these sialidases share several conserved domains (Fig. 4-2). All three sialidases have an N-terminal domain characteristic of sialidases and the sialidase domain containing the Asp boxes, characteristic motifs of sialidases (106). NanJ and *C. septicum* sialidase both have a FA58C domain at the N-terminus. FA58C domain is a cell surface carbohydrate binding domain present on eukaryotic cells. At its C-terminus, NanJ has a FN3 domain, which is a domain present within fibronectin and in eukaryotic proteins.

Identification of sialic acid-inducible transcription start sites for *nanI* and *nanJ* by primer extension experiments.

Nees and Schauer (84) reported that sialidase activity was inducible by the addition of sialic acid to the growth medium. To determine whether this induction occurs at the level of transcription and to identify the promoters of *nanI* and *nanJ*, primer extension analysis was done with RNA extracted from strain 13 growing with or without sialic acid as a carbon source. We identified three 5' ends that may represent promoters in the region upstream of the *nanI* gene (Fig. 4-3A). Three 5'-ends located 66, 163, and 308 bp upstream of the start of the *nanI* coding sequence were identified (Fig. 4-3B). In addition, all of these potential transcription start sites were induced by the presence of sialic acid in the growth medium.

Detection of transcripts labeled with the 6 FAM tag yielded two peaks indicating transcripts of 116 or 117-bp and 176 or 177-bp in length (Fig. 4-4A). These two products indicate that *nanJ* may have two promoters with transcription start sites located ~ 86-bp upstream and ~ 146-bp upstream of the *nanJ* start codon (Fig. 4-4B). The peaks have different areas indicating the relative concentration of that transcript (Table 4-2). Peak areas are larger for reactions using more RNA, and in the case of *nanJ*, are larger for cultures grown in the absence of sialic acid. The samples tested for cultures grown without sialic acid had 1.3 to 3.8 times more fluorescence than cultures grown in the presence of sialic acid. The peak areas also indicate that the first promoter (P1) is weaker than the second (P2) with activity that is an average of 20% of the activity of the second promoter; however it does demonstrate a larger repression of activity when sialic is present (Table 4-2, Fig. 4-4).

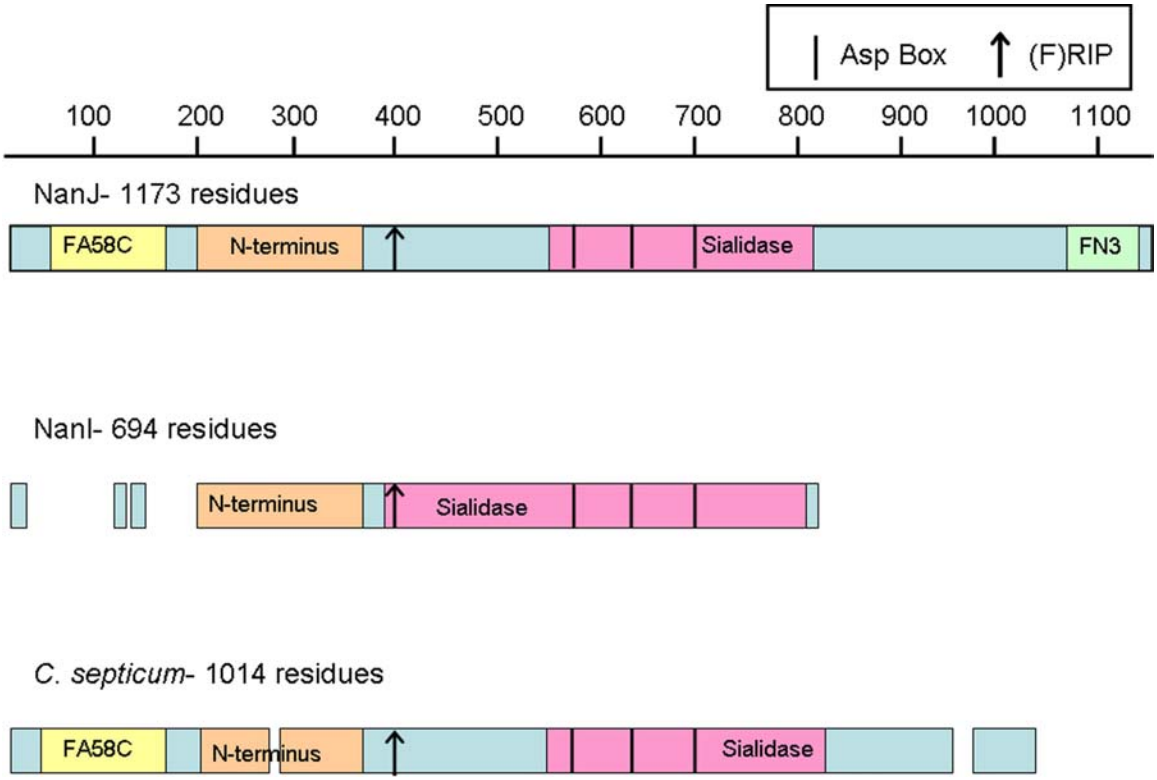

Figure 4-2. Schematic of *nanI, nanJ*, and the sialidase of *C. septicum* indicates that these proteins share several conserved domains.

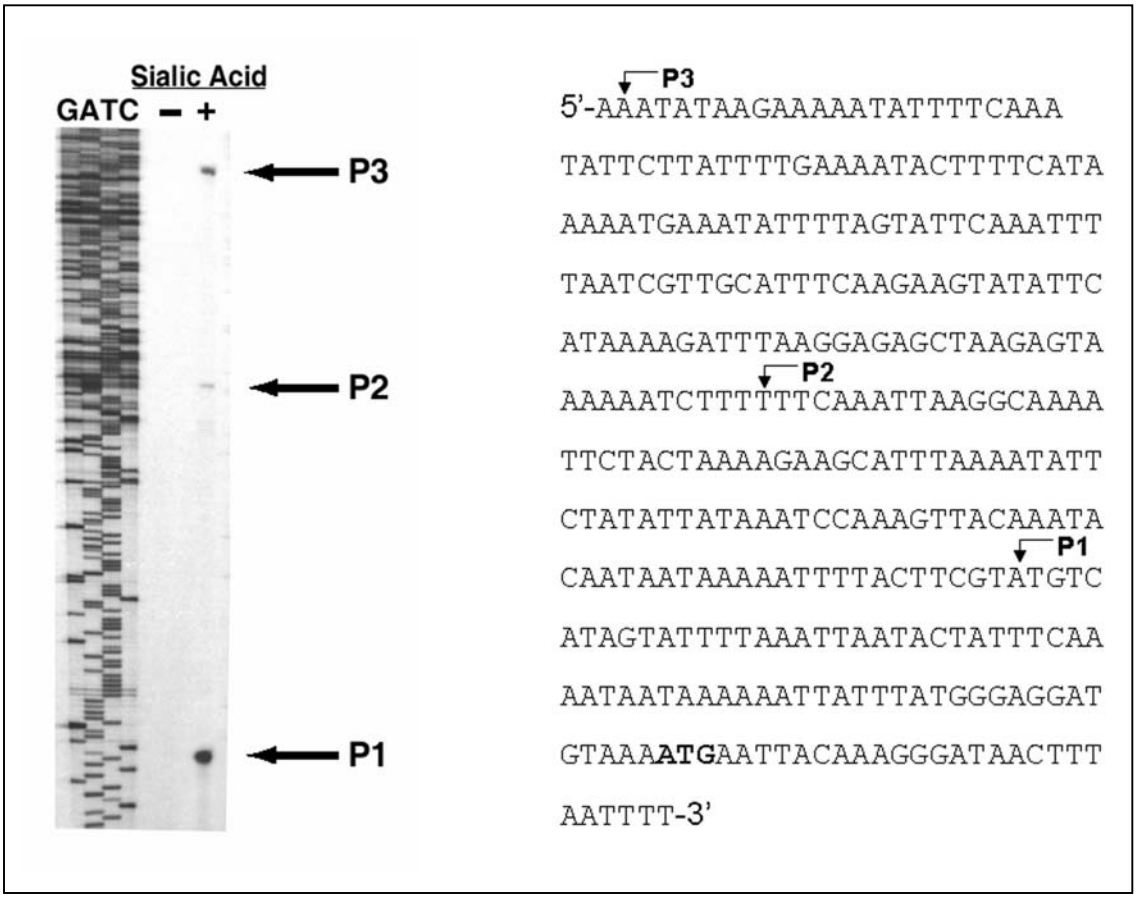

Figure 4-3. Primer extension results on RNA isolated from *C. perfringens* strain 13 using a primer specific for *nanI* mRNA. The cells were grown on PY (- sialic acid) or PY plus 1 mg/ml sialic acid (+ sialic acid). The GATC lanes show the results of sequencing using the same primer used for the primer extension experiments (**A**). The arrows labeled P1, P2, and P3 on the sequence (**B**) indicate the position of the putative start sites. Primer extension performed by Veronica Stirewalt.

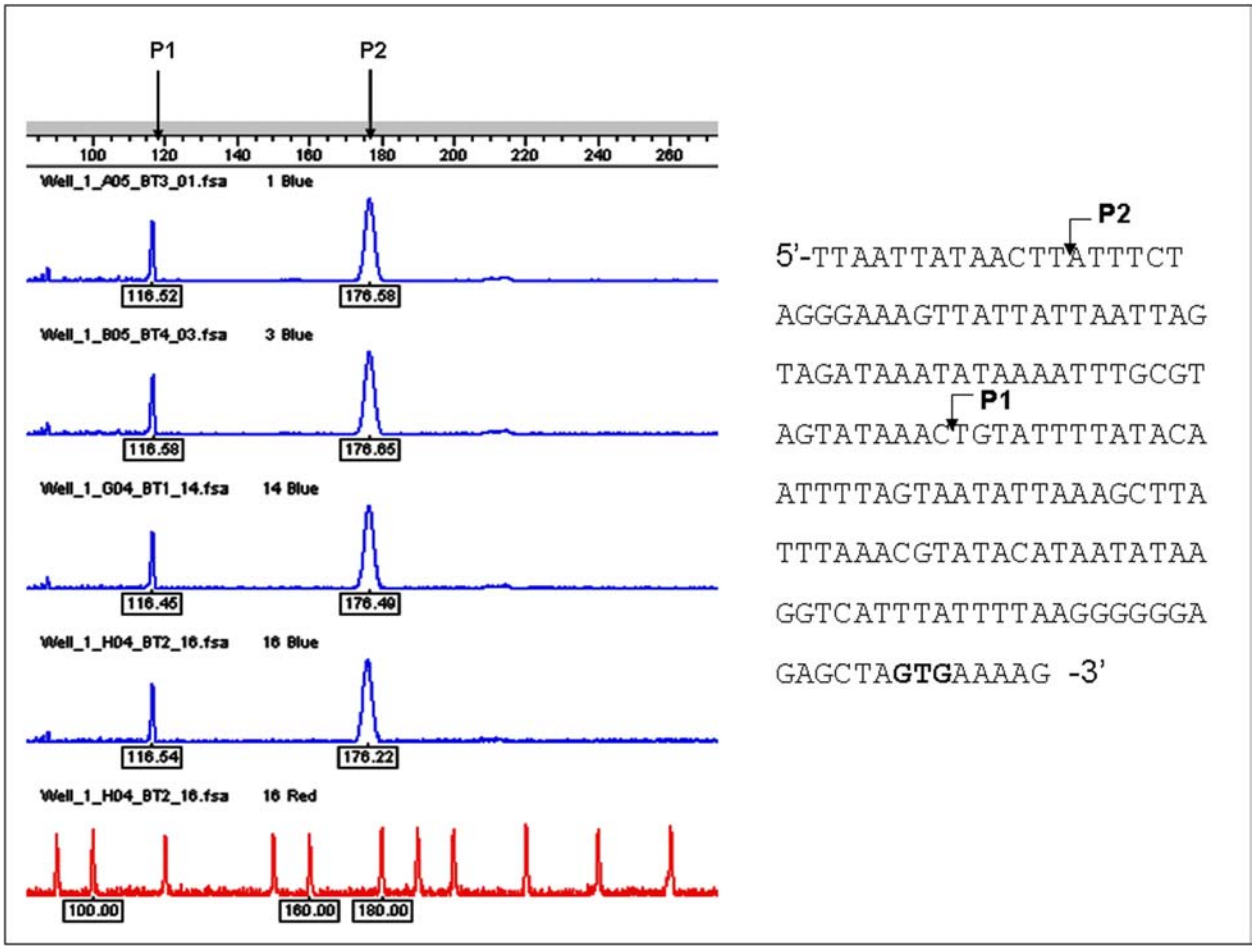

Figure 4-4. Primer extension results for *nanJ* from *C. perfringens* strain 13. The graph shows peak fluorescence of the 5'- 6 FAM labeled RT-PCR product for *nanJ* (**A**). Numbers below each peak indicate the length of the product. The area of each peak is recorded in Table 4- 2. The putative transcription start sites are indicated on the sequence by arrows labeled P1 and P2 (**B**).

Table 4-2. Peak areas from *nanJ* primer extension results

Conditions	Length (bp)	Peak area
1 μg RNA (no SA)	116.52	10,933
	176.58	46,589
1 μg RNA (SA)	116.45	3,352
	176.49	36,170

The *nanI* promoter region can drive transcription of an indicator gene (*gusA*) in *C. perfringens* strain SM101.

To confirm that the 5' ends we detected in the *nanI* primer extension experiment were actual promoters, we fused a fragment containing 664 bp of the intergenic region between *nanI* and the upstream ORF 1 and the first three codons of *nanI* to the promoterless indicator gene, *gusA*. The use of *gusA* fusions allow β-glucuronidase activity to be measured and used as an indicator of promoter activity on the multi-copy plasmid pSM218 (154). The *nanI* promoter region did show a significant level of transcriptional activity, but this activity was induced only 2-fold by sialic acid (Fig. 4-5). This may be due to plasmid copy number effects, where regulatory proteins could have been titrated out by the multicopy plasmid, resulting in the loss of regulation. A similar effect was seen with another sialic acid-inducible promoter upstream of the *nanE/A* operon in *C. perfringens* (154).

A region 315-bp in length located 35-bp upstream of the *nanJ* start codon and containing the two putative *nanJ* promoters was fused to the promoterless *gusA* gene in pSM240. Only very low levels of β-glucuronidase activity were detected for this construct in SM101, which may be due to the lack of transcriptional activation of the *nanJ* promoter (which was obtained from strain 13) in SM101.

***nanI* and *nanJ* are both responsible for extracellular sialidase activity in *C. perfringens* strain 13.**

We amplified the internal fragment of the *nanI* gene by PCR, cloned this fragment into an erythromycin suicide vector for *C. perfringens*, and transformed strain 13 with the plasmid. We were able to verify that the plasmid carrying the internal fragment had inserted into the chromosome and disrupted the *nanI* gene by Southern blot and sialidase enzyme assays. On the Southern blot the putative *nanI* mutant (BT1) lacked the band for wild-type *nanI* and had a band which indicated a multimeric insertion of the plasmid. Sialidase activity of BT1 was only 9% of wild-type, indicating that this mutant lost 91% of its inducible sialidase activity (Fig. 4-6A). When the culture was uninduced BT1 lacked 81% sialidase activity and had a residual of 19% activity. Complementation of BT1 restored its activity to 74% of wild-type when uninduced and 24% activity when induced (Fig. 4-6A). We created the *nanJ* mutant by homologous

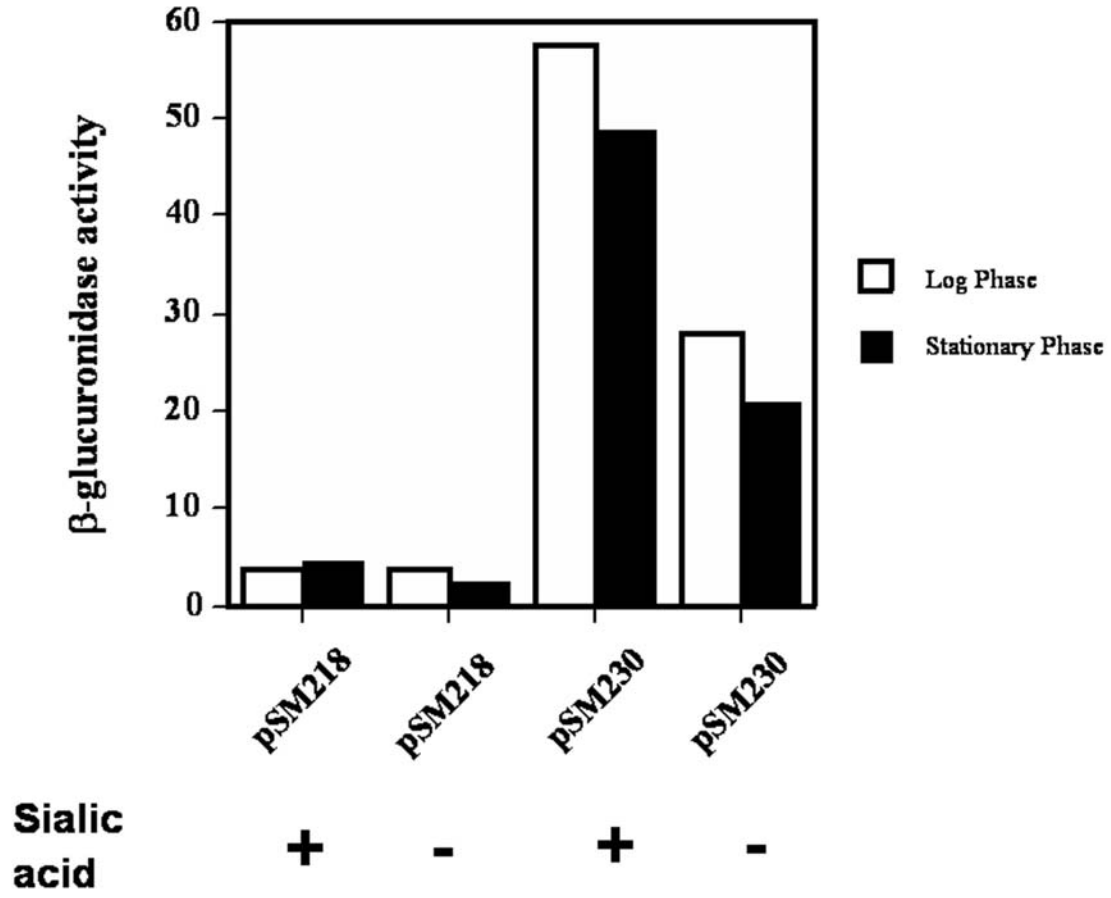

Figure 4-5. **β-glucuronidase activity of *C. perfringens* strain SM101 expressing a *nanI-gusA* promoter fusion.** Cells were grown in PY with or without 1 mg/ml sialic acid, as indicated. pSM218 is our transcriptional vector control with no insert and pSM230 is pSM218 with the *nanI* promoter from strain 13. β-glucuronidase activity was calculated as the product of the absorbance at 405nm and 1000 divided by the product of the OD_{600}, culture volume, and time to appearance of the yellow color in each sample. Assays performed by David O'Brien.

recombination with an internal fragment of *nanJ* (as with *nanI*). Southern blot showed similar results as those observed in the Southern blot for BT1, the putative *nanJ* mutant (BT2) lacked the wild-type band and had a band indicating multimeric insertion of the plasmid. Sialidase assays showed a 53% reduction in activity at 50 °C (Fig. 4-6A) and 64% at 37 °C (Fig. 4-6B) when the culture was uninduced as compared to wild-type, these are statistically significant differences with *P* values equal to 0.0004 and 0.0022, respectively. NanJ seems to be responsible for a limited amount of *C. perfringens* strain 13 sialidase activity as observed under these assay conditions (Fig. 4-6A). Complementation of BT2 was not successful due to the apparent inability of *E. coli* to maintain the plasmid.

BT3 was created by transforming the BT1 with pBT5. By Southern blot the double mutant was found to lack the wild-type *nanJ* band and have a band indicating multimeric insertion of the plasmid. Checking BT3 sialidase activity we found that it lacked sialidase activity under induced and uninduced conditions (Fig. 4-6A).

Identification of a conserved 17 bp sequence motif in sialic acid-associated genes from *C. perfringens*

Since we have identified two sialic acid related promoters that are inducible by sialic acid (i.e., *nanI* and *nanE/A* (154)) or repressed by sialic acid (*nanJ*) we examined the sequences of the promoter regions to determine if there were any conserved sequence elements. We also included the putative promoter region of *nanH*, the small intracellular *C. perfringens* sialidase that has been shown to be inducible by sialic acid (26, 84), in our analysis. Interestingly, we discovered a conserved 17-bp element that was present in the promoter regions of *nanI*, *nanJ*, *nanE*, and *nanH* (Fig. 4-7). There are identical copies present in the *nanE* and *nanI* promoters. These conserved elements in the *nanI*, *nanJ*, and *nanE* promoter are in the regions known to be the start sites of transcription for these genes (Fig. 4-3, 4-4, and (154)), suggesting that they may function as a recognition sequence for a transcriptional regulator.

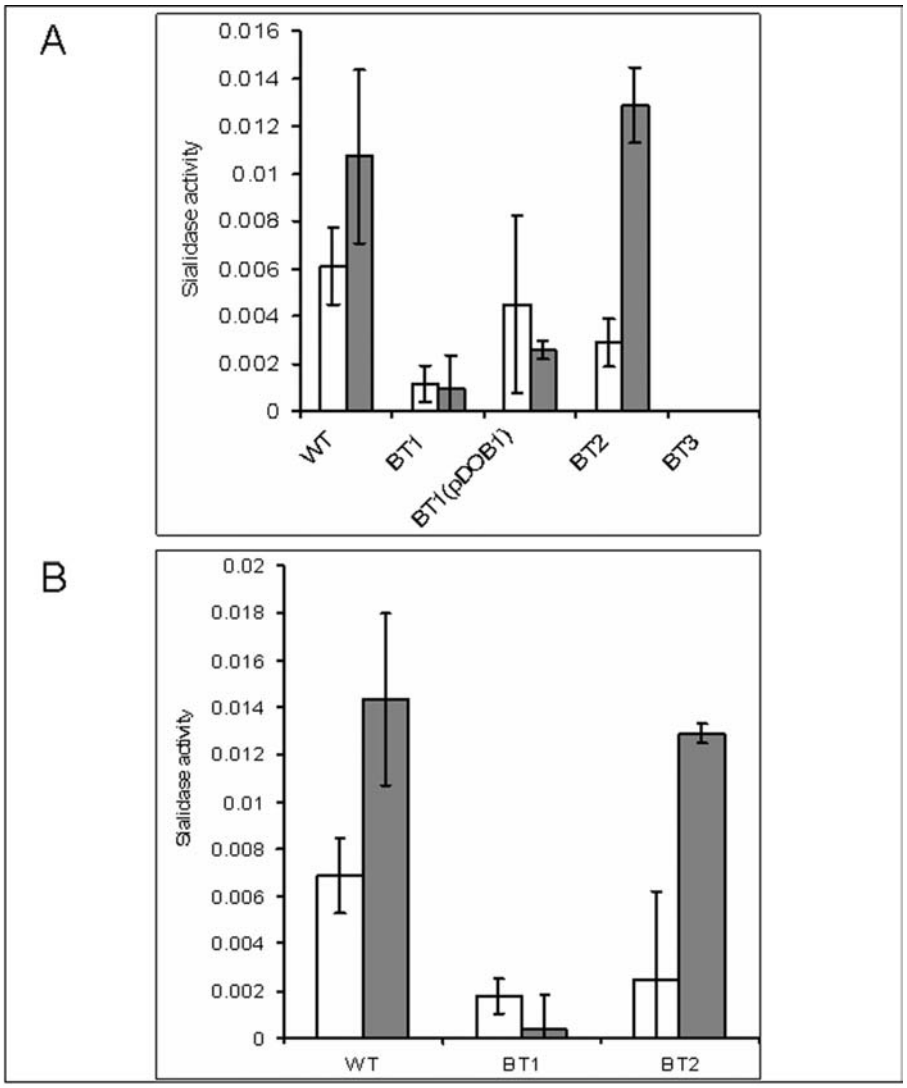

Figure 4-6. Extracellular sialidase activity of wild-type *C. perfringens*, BT1, BT2, and BT3. Cells were grown overnight in 5 mls of PY with (closed bars) or without (open bars) 1 mg/ml sialic acid as indicated. Sialidase activity was tested at 50 ºC (**A**) and 37 ºC (**B**) using the substrate 4-methylumbelliferyl-α-D-N-acetylneuraminic. Sialidase activity was defined as μmoles of 4-methylumbelliferyl-α-D-N-acetylneuraminic acid hydrolyzed per minute per milligram of protein.

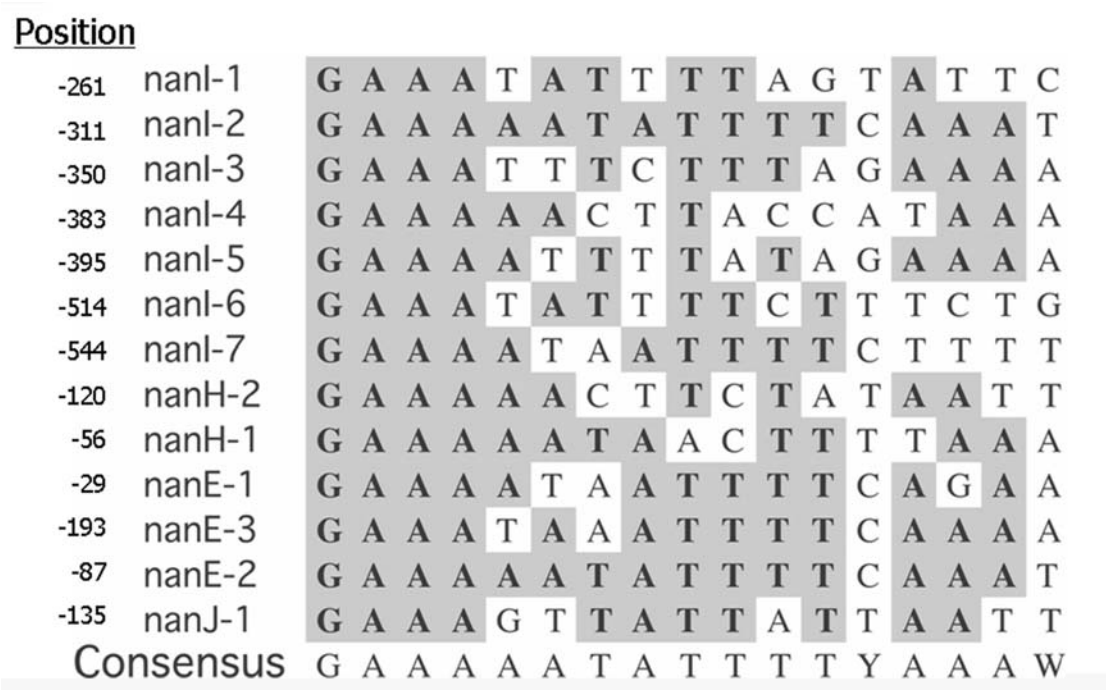

Figure 4-7. Alignment of conserved 17-bp elements located in the promoter regions of sialic acid-related genes. The position refers to the distance from the adenosine in the initiator methionine residue for each gene shown to the left. Shaded bases show bases that are conserved between the upstream region and the consensus sequence, shown at the bottom. The numbers following each gene indicate the proximity of the sequence to the start codon with the sequences closest to the start represented by the lowest number.

Animal studies

Mice infected with ~ 1 x $10^7$and 1 x 10^9 wild-type *C. perfringens* and BT3 showed no statistically significant difference in survival at any inoculum tested (Fig. 4-8). At the 10^8 inoculum the BT3 infected mice succumbed to infection at a faster rate than did mice infected with wild-type *C. perfringens*. This difference was statistically significant at 18 hours post-infection as determined by a Fisher's exact test (P = 0.0349). All mice infected with the 10^7 inoculum survived and by 18 hours post-infection all mice infected with the 10^9 inoculum had been sacrificed when gangrene symptoms became apparent. A few mice at the 10^9 and 10^8 inoculum died spontaneously from symptoms that were not characteristic of gangrene. These mice were scruffy and lethargic, but they did not have advanced swelling of the limb or blackening of the foot. Mice injected with PBS had no symptoms of disease and survived for the length of the experiment (data not shown).

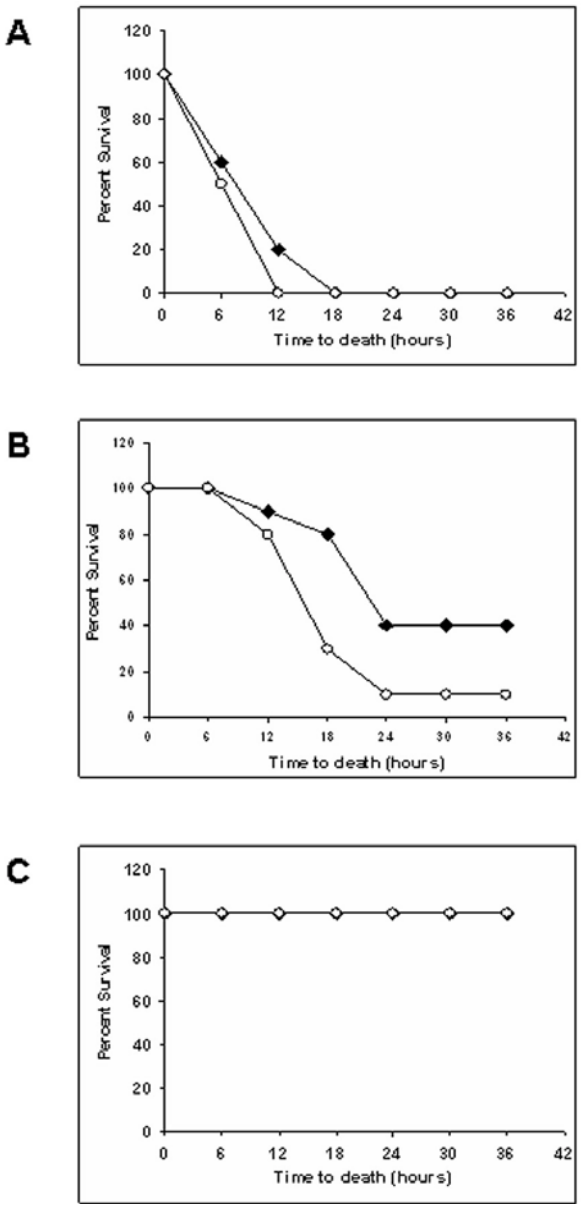

Figure 4-8. Time to the appearance of gangrene symptoms for mice infected with wild-type *C. perfringens* and BT3. Mice were injected in the hind leg muscle with each strain and euthanized when symptoms of severe gangrene became apparent. Ten mice were used for each inoculum, 10^9 (**A**), 10^8 (**B**), 10^7 (**C**). No statistical difference in survival was seen for either strain at these inoculums. Solid diamonds represent wild-type *C. perfringens* and open circles represent BT3.

Discussion

We found that NanI is positively regulated by the presence of sialic acid in the media. The induction of its activity occurred at the transcriptional level and accounted for the majority of sialidase activity produced by *C. perfringens*. The fact that NanI is regulated by positive feedback is advantageous for *C. perfringens*. It may allow for the complete desialyation of any sialic acid-conjugates in the immediate environment, which could result in a nutritional advantage over other bacteria. *C. perfringens* does not only have the ability to take up and use sialic acid, but desiaylation could also provide better access to the conjugated molecule leading to further degradation by or attachment for its numerous other toxins.

Shimizu's group in 2002 identified a previously unidentified sialidase in *C. perfringens* strain 13, NanJ (126). Analysis of the putative amino acid sequence for NanJ with BLASTP on NCBI yielded a hit of high homology with the sialidase of *Clostridium septicum*. The sialidase of *C. septicum* is a secreted toxin that is most active at pH 5 and 37 °C and assists in pathogenicity by cleaving off the sialic acid in mucin aiding in colonization of the bowel (74, 161). We found NanJ to not only have high amino acid homology to the sialidase of *C. septicum* but to be secreted and to be most active at pH 5 and at 37 °C as is the sialidase of *C. septicum*.

We made sialidase mutants BT1, BT2, and BT3 by homologous recombination. Interestingly, BT1 had some residual activity in the supernatant when sialic acid was not present. Sialidase assays and primer extension results indicated that this was the condition under which NanJ was the most active. We also found by use of BT1 that the majority of the sialidase activity of *C. perfringens* strain 13 is due to NanI. We had expected this result due to the fact that NanI had previously been isolated and characterized (105, 145). As a result of making a *nanJ*- mutant and a *nanI*-/*nanJ*- double mutant we found that the residual activity was in fact due to NanJ proving that NanJ does have sialidase activity even though it had not been previously characterized. We determined that NanJ was secreted by testing the extracellular, cell wall, and intracellular extracts for sialidase activity. We found low levels of activity in the cell wall and no detectable activity in the intracellular extracts (unpublished data).

NanJ sialidase activity was present in media without sialic acid and was nearly absent in media with sialic acid. This suggests an interesting mode of regulation. NanJ is not induced by

sialic acid but instead appears to be repressed by it. The difference in *nanI* and *nanJ* activity may be the lack of a proper substrate for NanJ under our assay conditions. NanJ may have higher levels of activity with a different substrate. Methylumbelliferyl-linked sialic acid was found to be inhibitory to the *C. septicum* sialidase (161).

Conversely, NanJ may not be a true sialidase, but instead NanJ may be a trans-sialidase. Trans-sialidases have sialidase activity but also have the ability to attach the sialic acid residues to the exterior of the organism (28, 77). This type of activity has traditionally been thought to permit a microbe to mask itself and to hide from the host immune system (28). With trans-sialidase activity it would be more understandable for the sialidase activity of NanJ to be repressed by free sialic acid in the growth media, because repressing its sialidase activity would prevent the cleavage of sialic acid residues from the exterior of the bacterium and allow it to remain hidden from the host immune system.

Sialidases do play a role in virulence for *P. aeruginosa* and *V. cholera*. Sialidases have been shown to enhance bacterial binding to host surfaces, toxin binding, and colonization (45, 144, 155, 157). In the *C. perfringens* gas gangrene mouse model we found no direct role for sialidase in virulence, but our results suggest a role for sialidase in protecting the mouse when infected with an intermediate inoculum. Some research has been done to show that sialidases can activate murine cytotoxic T-cells. The alteration of surface glycosylation of cytotoxic T-cells with *C. perfringens* sialidase resulted in increased production of interleukin-2, interferon gamma, and granzyme B (114). Although, T-cells may not be the cells involved in enhancing mouse survival in a gas gangrene infection when sialidase is present, this evidence should be considered as a possible explanation for the results that we observed. Further experimentation should be done to determine if *C. perfringens* sialidases can activate immune cells such as mononuclear and polynuclear leukocytes, which have been shown to interact with *C. perfringens* in the initial stages of infection.

V. Concluding Remarks

Clostridium perfringens is found in 80% of gas gangrene infections and each year 1,000 to 3,000 people contract gas gangrene (75, 100). The disease spreads rapidly and can quickly cause death if treatment is not immediate (75, 100, 130). Treatment of gas gangrene almost always involves the aggressive removal of large amounts of tissue (27, 131). A large inoculum of 10^8 to 10^9 bacteria is required to create this kind of infection in mice (5, 15, 88, 135). The reason for this size inoculum may be, as our results indicate, that monocytic cells are involved in inhibiting the initiation of gas gangrene in mice infected with a moderate dose of *C. perfringens*. The difference in mouse survival was observed 24 hours after infection. This observation along with previous evidence that clodronate-filled liposomes only deplete peripheral monocytes (150) possibly indicates that tissue macrophages are inhibiting the infection.

Previous research in our lab demonstrated that *C. perfringens* survives better under aerobic conditions, in vitro, when macrophages are present (87) and when mice were infected with a sublethal dose of *C. perfringens* the bacteria persisted in the tissues for 72 hours (88). Cases of recurrent *C. perfringens* gas gangrene have also been documented where the injury was non-traumatic and in some cases the skin was not perforated suggesting that either spores or latent cells from the previous infection were activated and caused disease (129, 136). We hypothesized that the persistence of *C. perfringens* in the tissues could be mediated by persistence within macrophages. To examine if the toxin perfringolysin O (PFO) could mediate this, as its homolog listeriolysin O (LLO) does, we used mutant forms of PFO with properties similar to LLO. These variant forms of PFO were less cytotoxic allowing us to examine what occurs between phagosomal escape and cell lysis. PFO lyses cells so quickly that it is difficult to examine what role phagosomal escape and transient presence within the cytosol plays. We found that these mutant forms of PFO did mediate phagosomal escape in macrophages and were found within macrophages at higher numbers than wild-type *C. perfringens*. Our data was preliminary, but these results may indicate that the mutant forms of PFO do allow for intracellular persistence. Further experimentation should be done to measure intracellular persistence directly. Video microscopy of infected macrophages and invasion assays could be used to elucidate if a less cytotoxic PFO could mediate escape and allow for intracellular persistence.

We created *nanI*-, *nanJ*-, and *nanI*-/*nanJ*- sialidase mutants in *C. perfringens* strain 13 and determined that the majority of its sialidase activity with the substrate 4-methylumbelliferyl-α-D-N-acetylneuraminic acid is due to NanI and that the remaining activity is due to NanJ. This is the first experimental evidence to indicate that NanJ is an extracellular sialidase. Primer extension experiments also revealed putative transcription start sites for NanJ and NanI and indicated that NanI transcription is induced when sialic acid is present and NanJ is repressed when exposed to free sialic acid. Further experimentation should be done to determine the optimal temperature and pH for NanJ as well as its optimal substrate.

In a mouse model of infection with an intermediate inoculum of the *nanI*-/*nanJ*- mutant and wild- type *C. perfringens* we found that mice infected with the double mutant had decreased survival in comparison to mice infected with wild-type. This was an unexpected result. Future experimentation needs to be done to identify how sialidase could be protective for mice with *C. perfringens* gas gangrene. In vitro tests need to be done with primary mononuclear and polynuclear leukocytes to determine if sialidase can activate these immune cells.

C. perfringens type A is an opportunistic pathogen that can cause lethal gas gangrene infections in humans. It produces at least thirteen different toxins that aid in its pathogenesis and virulence. PLC is required for lethality in a gas gangrene infection and has been shown to be required for leukostasis and the formation of vascular occlusions in vivo. PFO is also considered to be an important virulence factor because necrosis and vascular leukostasis is enhanced when it is present along with PLC in vivo. PLC and PFO are required for phagosomal escape from primary macrophages in vitro and PFO has been shown to be highly cytotoxic to macrophages in vitro. Other toxins produced by gangrene causing strains of *C. perfringens* include hyaluronidase, collagenase, and nuclease (111). Hyaluronidase and collagenase are possible pathogenicity factors that may aid in the progression of the disease by degrading connective tissue (7, 23). These toxins are not all required for virulence but they all may prove to play a role in promoting the survival of *C. perfringens* in vivo.

VI. **References**

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Curriculum Vitae

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Education:

2003-present: Degree to be earned: Masters of Science in Biological Sciences (GPA: 3.92/4.0)
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Thesis title: " Investigation of the role of the toxins perfringolysin O (PFO) and sialidase in *Clostridium perfringens* gas gangrene infections"

2000-2003: Bachelor of Science earned in Biology (GPA: 3.88/4.0)
Minor earned in Chemistry
Meredith College- Raleigh, North Carolina

Experience:

Spring 2005: Mentor for an undergraduate research student- advisor for experimental design and results interpretation

2003-2004: Graduate Teaching Assistant, General Microbiology Lab,
Virginia Tech

2002-2003: Undergraduate Research, Meredith College- bacterial transformations, GFP expression vector cloning, PAGE

Submitted manuscripts:

"The role of monocytes and neutrophils a *Clostridium perfringens* gas gangrene infection" (Submitted to *Microbes and Infection* on August 18, 2006)

Manuscripts in preparation:

"Characterization of *Clostridium perfringens* strain 13 sialidases: NanI and a novel *C. perfringens* sialidase NanJ"

Presentations:

February 11, 2006: Poster presentation for Biology Research Day, "Characterization of sialidases from *Clostridium perfringens*", Delivered at Virginia Tech

Presentations (Cont.):

June 8, 2005: Poster presentation for American Society for Microbiology Meeting, "A role for monocytes in a *Clostridium perfringens* gas gangrene infection", Delivered at Georgia World Conference Center in Atlanta, Georgia

February 6, 2005: Poster presentation for Mid-Atlantic Microbial Pathogenesis Meeting, "The role of phagocytes in *Clostridium perfringens* gas gangrene infections", Delivered at Wintergreen Conference Center in Virginia

October 6, 2004: Seminar presentation, "*Listeria monocytogenes* molecular mechanisms of virulence", Delivered at Virginia Tech

Awards and Honors:

Spring 2005: Inducted into Phi Kappa Phi Honor Society
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Fall 2004: Awarded Graduate Research Development Project grant, From Virginia Tech