THE INTERACTIONS OF *CLOSTRIDIUM PERFRINGENS* WITH PHAGOCYTIC CELLS

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
Doctorate of Philosophy
in
Biology

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April 18, 2003
Blacksburg, VA

Keywords: *Clostridium perfringens*, anaerobe, macrophages, polymorphonuclear leukocytes, phagocytosis, phagosome, lysosome, LAMP-1, macrophage receptors, theta-toxin, alpha-toxin

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(ABSTRACT)

Clostridium perfringens is the most common cause of gas gangrene (clostridial myonecrosis), a disease that begins when ischemic tissues become contaminated with *C. perfringens*. *C. perfringens* quickly multiplies in ischemic tissues and spreads to healthy areas, leading to high levels of morbidity and mortality. As a species, the bacterium can synthesize thirteen different toxins. The alpha toxin (PLC) and perfringolysin O (PFO) are thought to be important virulence factors in gangrene. We wished to understand how *C. perfringens* is capable of avoiding killing by the host immune system, and determine if PLC and PFO play a role in this avoidance. We found *C. perfringens* was not killed by J774-33 cells or mouse peritoneal macrophages under aerobic or anaerobic conditions. Using electron microscopy, we showed that *C. perfringens* could escape the phagosome of J774-33 and mouse peritoneal macrophages. We believe the ability of *C. perfringens* to survive in the presence of macrophages is due to its ability to escape the phagosome. Using a variety of inhibitors of specific receptors, we identified those used by J774-33 cells to phagocytose *C. perfringens*. The scavenger receptor, mannose receptor(s), and complement receptor (CR3) were involved in the phagocytosis of *C. perfringens*. To determine if PFO or PLC were involved in the ability of *C. perfringens* to survive in the presence of macrophages, we constructed *C. perfringens* strains lacking these toxins.

The ability of *C. perfringens* to survive in the presence of J774-33 cells is dependent on PFO, while survival in mouse peritoneal macrophages is dependent on PFO and PLC. The ability of *C. perfringens* to escape the phagosome of J774-33 cells and mouse peritoneal macrophages is mediated by either PFO or PLC. Using a mouse model, we found that PFO and PLC were necessary for *C. perfringens* to survive *in vivo* using infectious doses 1000 times lower than those required to initiate a gangrene infection. We propose that PFO and PLC play a critical role in the survival of *C. perfringens* during the early stages of gangrene infections, when phagocytic cells are present and bacterial numbers are low.

DEDICATION

This dissertation is dedicated to my parents, and my fiancé Marie Faini for their love and support always.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Stephen Melville, and the other members of my committee, Dr. David Popham, Dr. Klaus Elgert, Dr. Ann Stevens, and Dr. Rich Walker for their guidance, comments, and assistance. I would like to thank my friends at the University of Tennessee, especially Sheri Klas, Chuck Dorsy, and Chris Guglielmo for their support, encouragement, and stress relief. I would also like to thank my friends at Virginia Tech especially Derrell McPherson, John Varga, Mike Woodman, Wes Black and Marie Faini for their support, encouragement, and stress relief.

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CHAPTER ONE

General Overview

A. Introduction to *Clostridium perfringens*

All of the topics to be discussed are involved or can be involved in the pathogenesis of gas gangrene. Therefore, my goals were to understand what role PLC and PFO played in a gangrene infection. Research to date has focused primarily on what is happening once the onset of gangrene has been detected. Researchers are mostly involved in finding ways to cure the disease rather than trying to find a way to prevent the disease from progressing. Very little research has been done concerning the early stages of an infection. With that in mind, my research dealt with the early stages of *C. perfringens* infections in hopes of finding ways to prevent the disease from occurring and progressing into gas gangrene. More specifically, I focused my work on the early stages of a gangrene infection with special attention to the interactions between *C. perfringens* and host phagocytic cells. My work with the interactions between *C. perfringens* and host immune cells is still in the early stages, but I believe we are taking the right approach to understanding the role this pathogen plays in disease.

Therefore, understanding *C. perfringens* interactions with phagocytic cells, will grant us a better understanding as to how host tissue is damaged and how the bacteria survive during the early stages of infection. Greater understanding of how *C. perfringens* initially interacts with host phagocytic cells could possibly provide new methods of preventing infections from progressing to clinical cases of gangrene.

Clostridium perfringens is a Gram-positive, spore-forming, non-motile, rod shaped anaerobic pathogen. It produces 13 different toxins (197), which cause a variety

of diseases in humans including: gas gangrene (clostridial myonecrosis), enteritis necroticans (Pigbel) (80), and food poisoning (93). *C. perfringens* is also a possible cause in sudden infant death syndrome (SIDS) (114). *C. perfringens* is divided into five biotypes A-E, depending on which of the 13 different toxins they produce (173) (Table 1-1). The wide variety of diseases caused by *C. perfringens* and its associated biotypes are listed in Table 2-2. Of these five types, type A and C are the only two types that cause disease in humans.

In the United States, *C. perfringens* induced gas gangrene affects over 3000 people annually, with a mortality rate of 25%, even when aggressive medical treatment is administered (78, 158). *C. perfringens* is been implicated either alone or in combination with other organisms in 50-100% of all gas gangrene infections (6) and *C. perfringens* type A is the most common organism isolated from patients with trauma-induced gas gangrene (118).

Gas gangrene caused by *C. perfringens* type A, most often occurs when the bacteria enter the host through a deep penetrating wound. If this wound is anaerobic, *C. perfringens* will grow and the clinical signs of gangrene will appear as soon as 6 hours after infection (204). The disease can progress through healthy living tissues at a rate of several inches per hour (118, 204). Although much is unknown about the initial stages of a gangrene infection, many researchers believe that the local and systemic stages of a gangrene infection are related to the production of potent extracellular toxins (117, 208). If left untreated, gangrene is always fatal because of the severe shock that follows the release of toxins into the bloodstream (10, 208). Of the thirteen different toxins produced

Table 1-1. *C. perfringens* biotypes and related toxins.

Biotype	Toxins Produced												
	α[1]	β[2]	β-2[3]	ε[4]	ι[5]	δ[6]	θ[7]	κ[8]	λ[9]	μ[10]	ν[11]	Sial.[12]	Ent.[13]
A	+	-	-	-	-	-	+	+	-	+	+	+	+
B	+	+	+	+	-	+	+	+	+	+	+	+	+
C	+	+	-	-	-	+	+	+	-	+	+	+	+
D	+	-	-	+	-	-	+	+	+	+	+	+	+
E	+	-	-	-	+	-	+	+	+	-	+	+	+

1 – phospholipase C
2 – necrotizing heat and trypsin sensitive protein
3 – related to horse intestinal disorders
4 – responsible for tissue necrosis
5 – responsible for tissue necrosis
6 – hemolysin
7 – perfringolysin O
8 – collagenase
9 – caseinase
10–hyaluronidase
11–probable endonuclease
12–Sialidase
13–Enterotoxin

Based on Rood and Cole 1991 (173).

Table 1-2. *C. perfringens* biotypes and associated diseases.

Biotype	Associated Diseases
A	Gas gangrene, food poisoning, necrotic enteritis of infants and poultry, porcine necrotic enterocolitis, equine colitis, and canine hemorrhagic gastroenteritis
B	Lamb dysentery and enterotoxemia of sheep, ovine hemorrhagic enterotoxemia, equine and bovine hemorrhagic enteritis
C	Enteritis necroticans (Pigbel), enterotoxemia of sheep (struck), necrotic enteritis in animals
D	Enterotoxemia of sheep and calves, caprine enterocolitis, bovine enterotoxemia
E	Enteritis of rabbits, and bovine and ovine enterotoxemia

Based on Rood and Cole 1991 (173).

by *C. perfringens*, the α, β, ε, and θ toxins are considered the major lethal toxins. The two main toxins in gas gangrene infections are PLC (alpha toxin, α), which is a phospholipase C and a sphingomyelinase (220), and PFO (theta toxin, θ), a thiol-activated cytolysin (228).

1. <u>Pathogenesis of Gas Gangrene</u>

Shortly after infection, the anaerobic environment of the wound, and the presence of amino acids and peptides needed for growth (198), allow the bacteria to become metabolically active and produce toxins. At the same time, immune cells are recruited to the site of infection. However, once gas gangrene has developed, immune cells are not present at the site of an infection. Another hallmark of gangrene, is that polymorphonuclear leukovytes (PMNs) get stuck in blood vessels and accumulate there, a phenomenon known as leukostasis. It is believed that the alpha and theta toxins are responsible for this activity. One explanation for the lack of immune cells could be the lethality of the theta toxin to leukocytes at high concentrations (207). It is proposed that the lethality of these toxins would then destroy any cells migrating to the site of infection. A second hypothesis is that these toxins interfere with the migration of leukocytes by upregulating the expression of intercellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1) on endothelial cells, a function normally assigned to interleukin-8 (IL-8). Bryant and Stevens (30) supported this theory by demonstrating that alpha toxin induced the expression of ICAM-1 and ELAM-1, as well as the secretion of IL-8 in human umbilical vein endothelial cells (HUVEC). Theta toxin only increased the expression of ICAM-1 but not ELAM-1 or IL-8. Stevens et al.

(207) provided additional support to this theory by demonstrating that PFO altered the chemotactic migration and morphology of polymorphonuclear leukocytes. These effects are illustrated in Figure 1-1.

The production of alpha and theta toxin may also interfere with endothelial cell integrity leading to swelling and edema associated with gas gangrene. Edema, along with the production of hydrogen gas and carbon dioxide from the anaerobic fermentation process of the bacteria, produces pressure that constricts minor blood vessels supplying blood to the site of infection further decreasing the oxygen concentration, making conditions favorable for *C. perfringens* growth (147). *C. perfringens* requires an oxidation-reduction potential lower than +0.074 to grow in human tissues (147). The increasingly anaerobic environment favors the growth of the bacterium, as well as the increased production of toxins and virulence factors that destroy surrounding tissues.

As the disease spreads, the toxins enter the bloodstream causing regional vascular compromise and increased compartment pressure (29). These reactions to the toxins are thought to lead to shock, multiple organ failure and ultimately death (10). Evidence for this comes from studies done by injecting rabbits with either alpha or theta toxin. The alpha toxin decreased the cardiac index of rabbits while the theta toxin decreased peripheral vascular resistance (10). This decrease in cardiac output along with venous dilation causes a shortage of blood flow to the organs, resulting in their failure.

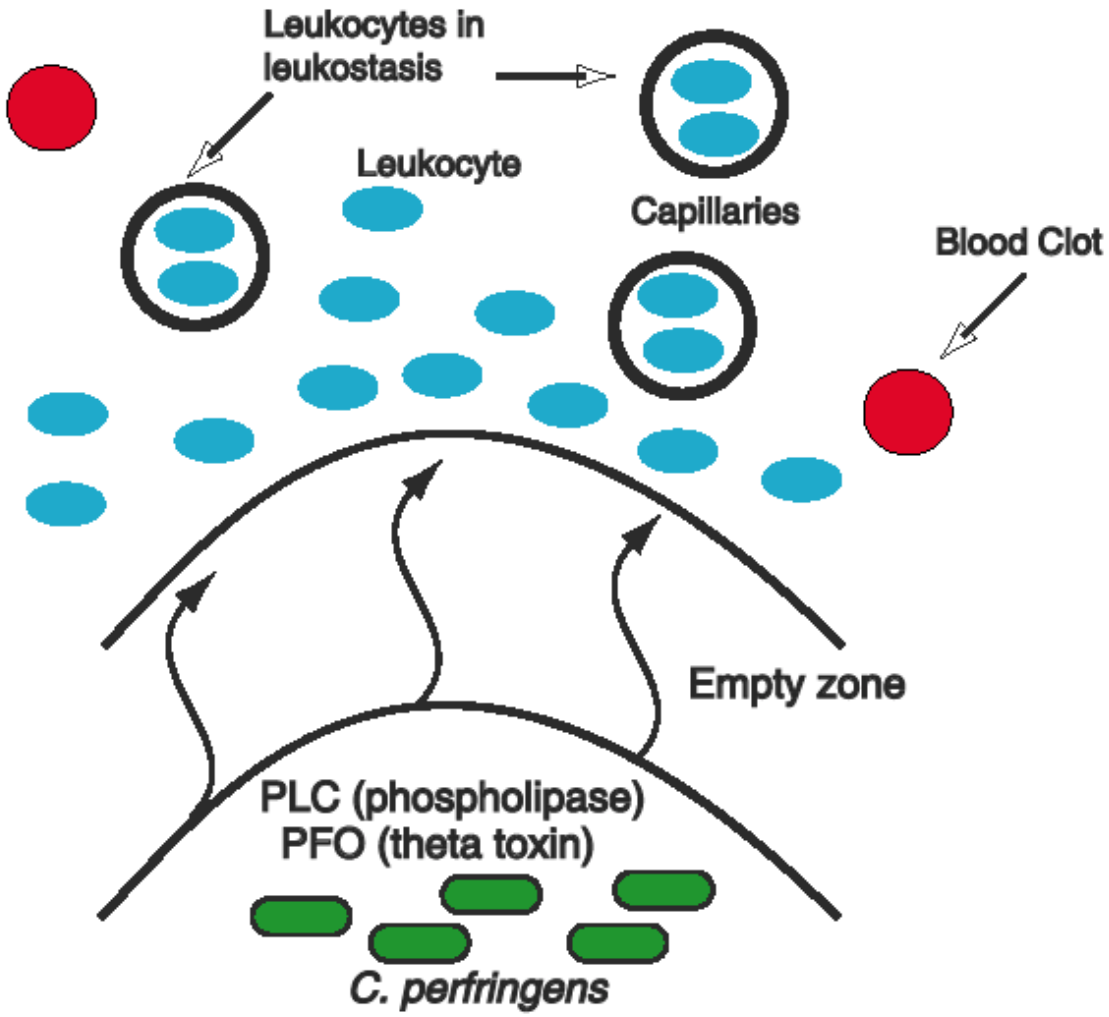

Figure 1-1. Model explaining the lack of an inflammatory response to gangrene. This represents the late stages of a gangrene infection where the bacteria (green rods) are located at one site of the infection while the phagocytic cells (blue ovals) are located in another. This occurs when ICAMs and ELAMs are upregulated on endothelial cells by the diffusion of PFO and PLC through the tissue. These toxins also create an empty zone where no phagocytic cells are present.

Based on Stevens et al. 1997 (209).

2. Properties of the Alpha Toxin (PLC) and Theta Toxin (PFO)

a. PLC

The alpha toxin (PLC) or phospholipase C serves two functions in *C. perfringens*. One function is to degrade lecithin found in the membrane of eukaryotic cells (225). The other function is to degrade sphingomyelin that is also present in eukaryotic cell membranes (226). These functions can act together to degrade cell membranes. PLC is a monomeric protein (109, 134), comprised of an N-terminal domain and a C-terminal domain (16). Titball et al (222) showed that a fragment of the N-terminal domain made up of amino acids 1-249 retained PLC activity, but did not have hemolytic or lethal activities and showed a reduced sphingomyelinase activity. A C-terminal domain made up of amino acids 247-370 possessed no enzymatic or lethal activity. However, when the two fragments were mixed together, hemolytic activity was partially restored, suggesting that the C-terminus is responsible for conferring hemolytic and, perhaps, lethal activities to the toxin (221).

b. PFO

PFO, encoded by the *pfoA* gene, is commonly referred to as a thiol-activated or oxygen-labile toxin. Thiol-activated toxins are found in Gram-positive bacteria such as *Clostridium*, *Streptococcus*, and *Listeria*. PFO is a cholesterol-dependent cytolysin and is a highly water-soluble monomeric protein, which oligomerizes to form large pores upon contact with membranes containing cholesterol (192). Up to 50 of these individual monomers can partake in forming these pore complexes (150). Recently, it was shown that a prepore complex forms on the membrane surface before these pore complexes are

inserted into the membrane (89, 193). The pores produced by these complexes are approximately 20-30 nm in diameter (82). The resulting pores are capable of causing leukocytes and red blood cells to lyse (209).

c. Lethal Effects of PLC and PFO

Alpha and theta toxins are the most widely studied extracellular toxins produced by *C. perfringens*. Evidence for the lethality of these toxins comes from experiments using purified toxins, as well as strains of *C. perfringens* that have been genetically altered by inactivating these toxin genes. Purified alpha and theta toxins are lethal when injected into mice (208). Purified theta toxin is also cytotoxic to macrophages *in vitro* while alpha toxin is not. When mice were infected with strains of *C. perfringens* carrying an inactive *plc* gene there is a marked decrease in virulence (11). However, when mice were infected with a *pfoA* mutant strain of *C. perfringens*, no significant decrease in virulence occurs (11). Recently, Rood *et al*. (12) created a *pfoA/plc* mutant and found most of the clinical characteristics of a gangrene infection, which include limping, malaise, swelling, and blackening of the thigh and footpad, were absent when injected into mice. When this mutant was complemented with *plc* or both *pfoA* and *plc*, the clinical characteristics of a gangrene infection reappeared. Complementing the mutant with *pfoA* showed increased virulence characteristics, but they were different from that observed from a typical gangrene infection. There was essentially no blackening or swelling of the footpad, and reduced levels of malaise and limping.

B. The Role of Immune Cells in Disease

Professional phagocytes play a crucial role in protecting the host from infections. These cells include polymorphonuclear cells such as neutrophilic granulocytes, eosinophils, basophils as well as monocytes/macrophages. The main function of these cells is to ingest foreign particles and eliminate them, and macrophages are also responsible for antigen presentation. Phagocytosis can be broken down into three steps: the binding of the particle to the phagocyte, internalization, and maturation of the phagosome. The phagocytosis process begins when phagocytes bind to ligands present on the surface of foreign particles. This receptor engagement leads to the internalization of the particle into a phagosome by a complex sequence of events (234). Shortly after ingestion, the phagosome undergoes numerous changes including fusion with endosomes and lysosomes, leading to a phagolysosome (154). The phagolysosome possesses many degradative properties including low pH, hydrolytic enzymes, defensins, and their ability to generate toxic oxidative compounds that ultimately destroy the bacteria (75).

1. Polymorphonuclear Leukocytes (PMNs)

Polymorphonuclear leukocytes (PMNs) comprise the majority of leukocytes circulating in human blood, about 50-60%, and only 18% in mice. PMNs eliminate pathogens by phagocytosing them via Fc and complement receptors (48). PMNs develop in the bone marrow where for two weeks they undergo proliferation and differentiation. Six stages are present in this process beginning with a myeloblast and ending with a

segmented neutrophil. Segmented neutrophils are fully functional and active cells. Their cytoplasm contains granules and a lobed chromatin-dense nucleus with no nucleolus. Approximately 1 x 10^{11} neutrophils are produced in humans each day. Once released from the bone marrow, they have a half-life of only 4 to 10 hours in the blood before they enter into the tissue where they can survive for one to two days (92). Their major role is to phagocytose and destroy infectious agents, but they also inhibit the growth of some microbes. They are the first line of defense and are the first cells to be recruited to the site of infection or injury. This innate defense mechanism is dependent upon the ability of PMNs to ingest and subsequently eliminate pathogens by oxidative and nonoxidative processes (141).

2. Macrophages

Macrophages are a population of ubiquitously distributed mononuclear phagocytes responsible for a number of homeostatic, immunological and inflammatory processes. Their development takes place in the bone marrow where they undergo seven maturation steps starting with a stem cell and maturing into a tissue macrophage. This process is called hematopoiesis and is regulated by at least 11 growth factors. Prior to maturation, a macrophage is called a monocyte. This transition from monocyte to macrophage occurs very quickly, between one to three days. During monocyte differentiation, granules form in the monocytes that are very similar to those found in PMNs (92).

Macrophages can be divided into two classes, normal and inflammatory. Inflammatory macrophages are recruited to inflammation sites and are mostly found in

various exudates. Normal macrophages include macrophages found in connective tissue (histiocytes), liver (Kupffer's cells), lung (alveolar macrophages), lymph nodes and spleen (free and fixed macrophages), bone marrow (fixed macrophages) as well as other tissues. The normal life span of macrophages ranges days to months depending on what type of tissue they are present in (92).

A primary role of macrophages is to protect the host from the invasion of foreign particles such as bacteria. After the foreign particles have been phagocytosed, they can prevent the intracellular replication of these organisms in three ways. The first is due to the low pH and lack of nutrients in the phagolysosome. Secondly, macrophages may become activated allowing for the production of reactive oxygen intermediates. Finally, macrophages also produce microbiostatic effector molecules that can maintain the bacteria in a non-replicative state.

C. Receptors Involved in the Phagocytosis of Bacteria

Upon contact with bacteria, receptors on host macrophages induce cytoskeletal rearrangements that lead to the internalization of bacteria into a phagosome (2). These actin rearrangements are very complex, due to the numerous factors involved in the cytoskeletal rearrangements. In humans, the receptors involved in the uptake of bacteria can be classified into two groups. The first group consists of mannose and β-glucan receptors that recognize surface components of bacteria (47, 59). Other receptors in this group include CD14, which recognizes surface components like lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, and scavenger

receptor A. The second group recognizes humoral components of the host immune system that coat or opsonize the bacteria. The most important opsonins are immunoglobulin G (IgG) and the complement fragments C3b and C3bi. These receptors work by first binding the bacteria, and then clustering around the bacteria leading to localized actin polymerization at the site of particle ingestion. The progressive recruitment of receptors at the site of particle binding is required for the actin-driven engulfment of the particle (72). Examples of phagocytic cells, their receptors and ligands are shown in Table 1-3.

1. <u>Mannose Receptor</u>

The mannose receptor of macrophages recognizes mannose and fucose residues on the surface of bacteria (202) allowing it to have broad pathogen specificity. The mannose receptor is a single chain receptor with a short cytoplasmic tail and an extracellular domain that includes eight lectin-like carbohydrate-binding domains. The cytoplasmic tail is essential to endocytic and phagocytic functions, however, little is known about the signals that lead to phagocytosis (59, 202). During phagocytosis the actin cytoskeleton is assembled around the phagosome and proteins such as F-actin, talin, and Myosin I are recruited (4). Uptake by the mannose receptor also induces the release of many pro-inflammatory signals such as IL-1β, GM-CSF, TNF-α, and IL-12 (65, 194, 203, 245). The role of the mannose receptor in phagocytosis has mostly been deduced with competitors of the receptor including monosaccharides and mannan (115). COS-1 cells transfected with the mannose receptor show their use in the phagocytosis of *Candida albicans*, *Pneumocytis carinii*, and *Klebsiella pneumoniae* (59, 60, 96). Prior to

Table 1-3. Examples of phagocytic receptors in mammalian cells.

Cell Type	Receptor	Target	Ligand
Leukocytes	FcγRs	Pentaxin-opsonized zymosan	Serum amyloid P, C-reactive protein
PMN, Mono, Macs	CR1 (CD35)	Complement opsonized bacteria and fungi	C3b, C4b, mannan-binding lectin
PMN, Mono, Macs	CR3 (CD11b-CD18; Mac-1)	Complement opsonized bacteria and fungi	C3bi, C3d
Mast Cells	CD48	Enterobacteria	FimH
Macs	Mannose receptor	*Pneumocystis carinii* , *Candida albicans*	Mannosyl/fucosyl residues
Macs	Scavenger receptor AI/AII	Gram-positive cocci	Leipoteichoic acid
Macs	MARCO	*Escherichia coli* , *Staphalococcus aureus*	?
Macs	CD36	Apoptotic PMN	PS/thrombospondin
Macs	CD14	*Pseudomonas aeruginosa*	LPS
Macs	αvβ3	*Apoptotic cells*	Thrombospondin
Epithelial	E-cadherin	*Listeria*	InlA

Based on Greenberg and Grinstein 2002 (72).

recent work by Lee et al. (112), no one had looked at the role of the mannose receptor in innate immunity due to the lack of a mannose receptor deficient mouse. Lee et al. (112) demonstrated that mannose receptor deficient mice exhibited no difference in their susceptibility to *Candida albicans* as compared to wild-type mice.

2. <u>Complement Receptors</u>

Complement receptors recognize complement proteins present in the serum that have opsonized bacteria. The complement receptors CR1, CR3, and CR4 are expressed on macrophages and recognize these opsonins (36, 191). CR1 is a single chain transmembrane protein consisting of a large extracellular lectin-like complement binding domain and a short cytosolic domain. CR1 binds C3b, C4b, and C3bi and it is thought to be involved primarily in particle binding (24). The CR3 and CR4 receptors are integrins made up of heterodimers of different α-chains (α_M for CR3 and α_X for CR4) and a shared β-chain (β_2) (191). These receptors bind to C3bi and are involved in the internalization of the particle. In the absence of phagocytic cells, complement can form pores in bacteria leading to their lysis and death (Figure 1-2). Complement receptors of peritoneal macrophages bind, but do not internalize, particles in the absence of other stimuli such as PKC, TNF-α, GM-CSF, or by attachment to laminin- or fibronectin-coated surfaces (156, 242, 243). Particles phagocytosed by the complement receptor appear to sink into the macrophage and have point-like contacts between the membrane and the bacteria (4). Uptake by complement receptors induces a non-inflammatory response that can be somewhat advantageous for the bacteria. Experiments done with CR3-deficient mice

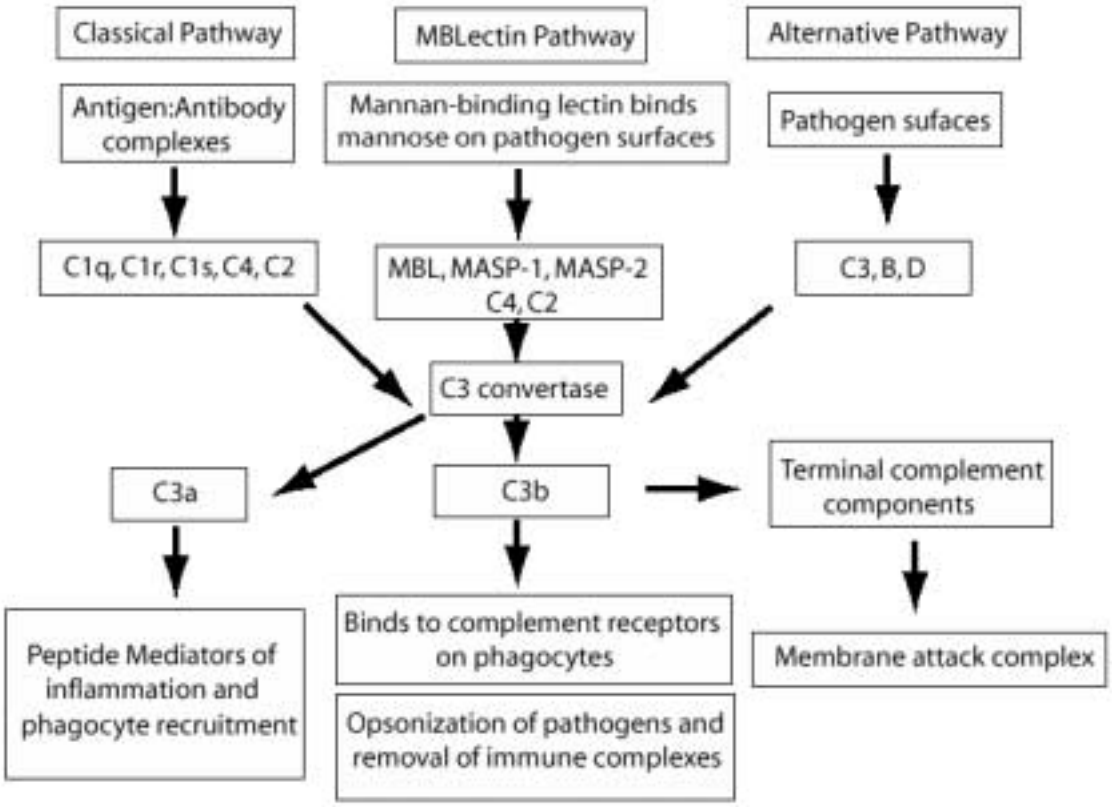

Figure 1-2. The complement cascade.

Based on Janeway 1999 (92).

show that these mice are defective in adhesion and phagocytosis (4, 116). These mice are also defective in bacterial clearance in an acute septic peritonitis model (175).

3. Fc Receptor

There are two types of Fc receptors; one type activates effector functions while the other type inhibits these functions (165). The receptors used for phagocytosis in humans are part of the activation class and include FcγRI, FcγRIIA, and FcγRIIIA (164), whereas murine macrophages lack the FcγRIIA receptor. FcγRI and FcγRIIIA have extracellular Fc binding domains, but lack cytoplasmic tails. As a result they need to bind dimers of γ-subunits needed for signal transduction (7, 216). During Fc receptor mediated phagocytosis, veils of membranes develop above the cell surface and surround the bacteria before it is internalized (4, 99). The uptake of bacteria by the Fc receptors leads to the production and secretion of proinflammatory molecules like reactive oxygen intermediates and arachidonic acid metabolites (3, 244). Mice lacking FcγRII expression showed a significant increase in their survival when infected with *Staphylococcus aureus in vivo* (66). The authors present multiple possible reasons for the increase in survival of FcγRII-deficient mice but they say the primary reason is due to the increased production of IL-10 and increased phagocytic ability of granulocytes and monocytes in FcγRII-deficient mice.

4. Scavenger Receptors

Scavenger receptors are cell surface glycoproteins and are defined by their ability to bind chemically modified low density lipoprotein (25, 108). Most scavenger receptor ligands are polyanionic in nature, but not all polyanions are ligands, suggesting that

structure also plays a role in the binding of ligands by the scavenger receptor (68). Another defining property of scavenger receptors is the ability to bind a wide range of ligands (107). Other cell types such as endothelial cells and smooth muscle cells express scavenger receptors (14, 153). There are six classes of scavenger receptors, A-F, and all are muti-domained transmembrane proteins, but there is no single domain common to all scavenger receptors (68). The class A and C scavenger receptors can bind whole pathogens. The class C scavenger receptors are only found on *Drosophila* haemocytes/macrophages during embryogenesis (68).

The class A scavenger receptor is further divided into three groups SR-AI, SR-AII, and SR-AIII (69, 106). The class A receptors can bind to lipopolysaccharide of Gram-negative bacteria as well as lipoteichoic acids of Gram-positive bacteria (71, 76). Using SR-A deficient mice, a role for the class A scavenger receptor in host defense has been elucidated. These mice are more susceptible to infection by *Listeria monocytogenes* as well as herpes simplex type-1 (214).

Using receptor deficient mice, macrophage receptors can be shown to be either beneficial or harmful to the bacteria. The lack of the complement receptor CR3 is beneficial to the multiple bacteria present in an acute septic peritonitis infection. The lack of the CR3 receptor allowed the bacteria to persist, multiply and cause disease more readily. Lacking the scavenger receptor SR-A also had a negative effect on the host in much the same way. The lack of the mannose receptor led to no difference in host susceptibility to *Candida albicans,* suggesting this receptor is not the major receptor utilized to clear a *Candida* infection. Mice lacking the FcγRII receptor were more

resistant to *Staphylococcus aureus* infections. These experiments show the importance and multiple roles that macrophage receptors can play during bacterial infections.

5. Phagosome Maturation

Once bacteria are phagocytosed by receptors they enter into a compartment known as a phagosome. In order for a phagosome to become a bactericidal phagolysosome, it must fuse with a variety of organelles ranging from early endosomes to lysosomes in a pathway called the endocytic pathway (Figure 1-3) (50). The first vesicles to fuse phagosomes are sorting endosomes that function to re-route the phagocytosed particles. Sorting endosomes are poor in proteases and have a pH of approximately 6.0. They can be identified by the presence of Rab5 or early endosome antigen 1 (EEA1) (15). If the particle is not going to be degraded, the phagosome is directed towards recycling endosomes, which are less acidic with a pH of approximately 6.5, and can be detected by the presence of Rab11 (113). Particles that are to be degraded such as bacteria are sorted towards late endosomes. Late endosomes are more acidic than sorting endosomes with a pH of approximately 5.5. They also contain hydrolytic enzymes that can begin to break down the bacteria. Late endosomes can be identified by the presence of Rab7, Rab9, lysobisphosphatidic acid, mannose-6-phosphate and lysosomal-associated membrane proteins (LAMPS) (136, 200). The last step in phagosomal maturation is the fusion with lysosomes. Lysosomes contain most of the degradative enzymes like active proteases and lipases, and are very acidic with pH less than 5.5 (217).

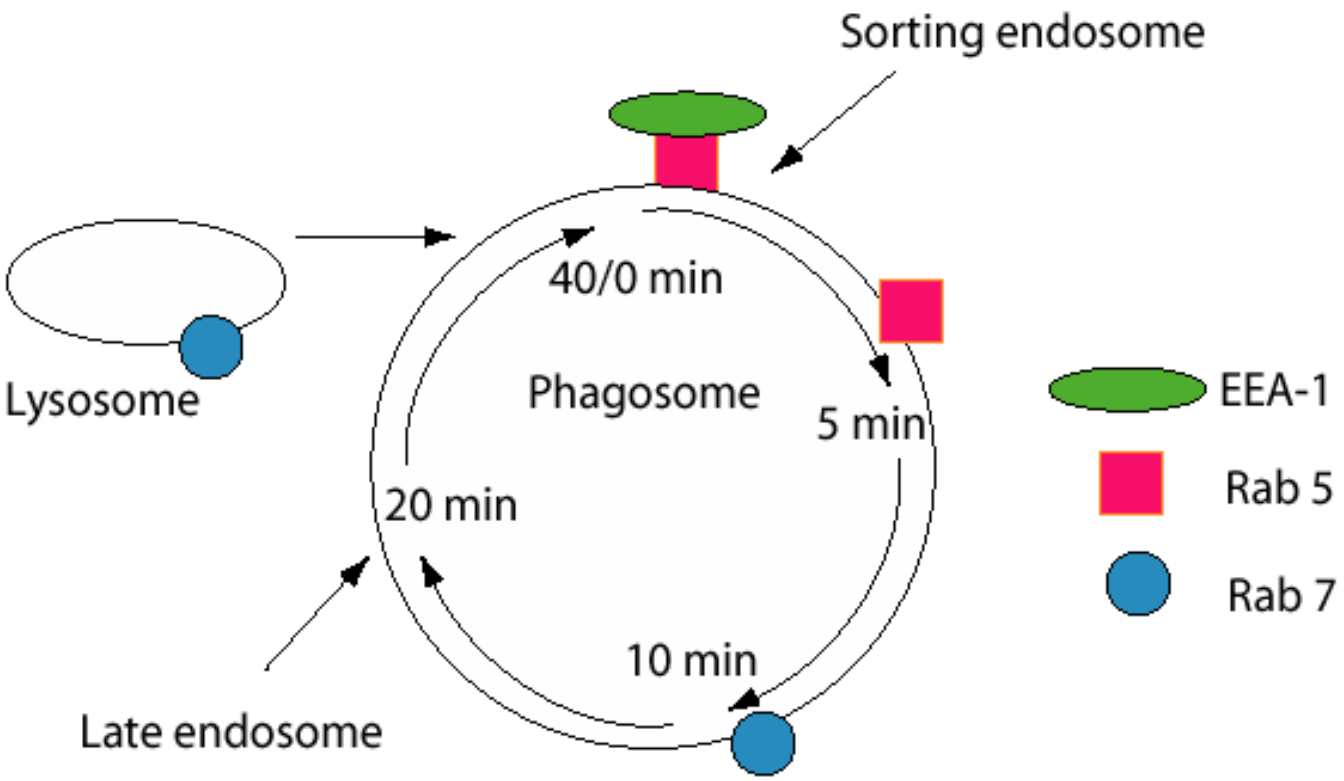

Figure 1-3. A schematic illustrating the approximate time course of the maturation of the phagosome.

Based on Vieira et al. 2002 (234).

D. Escape Mechanisms of Bacteria

In order for pathogens to survive, they must be capable of entering a host, finding a unique niche, somehow avoiding or subverting the host's normal defenses, and multiplying in that setting (Table 1-4) (63). Infectious agents such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Salmonella typhimurium* enter macrophages and modify vacuolar maturation to ensure their survival (2).

Mycobacterium tuberculosis binds to multiple receptors on the surface of macrophages including the complement receptors CR1 and CR3, the mannose receptors and scavenger receptors (58, 190). Once internalized *M. tuberculosis* resides in a membrane bound vacuole that resists lysosomal fusion and fails to acidify below pH 6.5 (79, 212). The vacuole does however fuse with early endosomes (43, 182).

Legionella pneumophila is phagocytosed due to binding by the complement receptor CR3. An outer membrane protein, called MOMP, on the surface of *L. pneumophila* is capable of fixing the complement component C3 to the surface of the bacterium allowing phagocytosis to occur via the complement receptor CR3 (19). After binding has occurred, the bacterium is taken up in a structure called a "coiling phagosome" (86). The resulting phagosome does not fuse with endosomes or lysosomes and does not acidify below a pH of 6.1 (85, 87). However, the phagosome does fuse with smooth vesicles ultimately forming a ribosome-studded vacuole composed of endoplasmic reticulum-derived membranes (88, 215). The combination of these traits allow the bacterium to replicate and lyse the host cell.

Table 1-4. Intracellular pathogens and their non-phagocytic host cells and methods used by these pathogens to ensure their survival.

Intracellular pathogens	Non-phagocyte	Evasion mechanism
Rickettsia	Endothelial cells, hepatocytes	Escape into the cytosol
Orientia	Endothelial cells, cardiac myocytes	Escape into the cytosol
Listeria	Intestinal and amniotic epithelial cells, hepatocytes	Escape into the cytosol
Shigella	Intestinal epithelial cells	Escape into the cytosol
Salmonella	Intestinal epithelial cells, hepatocytes	Prevent Phago/lyso fusion
Mycobacterium leprae	Schwann cells, endothelial cells	Hide from macs in schwann cells
Campylobacter jejuni	Intestinal epithelial cells	Possesses SOD and catalse
Chlamydia spp.	Intestinal, opthalmic, and genital epithelial cells	Grows inside non-acidified vacuoles
Malaria sporozoites, Plasmodium spp.	Hepatocytes, red blood cells	Clonal antigenic variation

Based on Ismail et al. 2002 (247).

The receptors involved in the uptake of *Salmonella typhimurium* have not been identified, although the bacterium is taken up by an actin-dependent mechanism. After binding to the macrophage, the bacterium induces membrane ruffling that results in its internalization into a vacuole called a "spacious phagosome" (5). It is thought that the spacious vacuole remains immature and does not fuse with lysosomes, allowing the bacteria to replicate and spread (163).

However, not all intracellular bacteria remain within a vacuole. For instance, *Shigella*, *Rickettsia*, and *Listeria* rapidly gain access to the cytosol where they replicate (62). Once phagocytosed, *Shigella* escapes the phagosome into the cytoplasm where it can replicate and spread to adjacent cells. *S. flexneri* escapes the phagosome by secreting proteins into the host cell using a type III secretion apparatus (83, 185). The effector proteins secreted by this type III secretion apparatus that are responsible for the escape from the phagosome are the invasion plasmid antigens, IpaB, IpaC and IpaD (128).

The *Rickettsia* species is another group of pathogens that, once inside of a host cell, can escape the phagosome. *Rickettsia* first mediates its entry into host cells using a phospholipase A2 (195). Once inside the cell, it is proposed that this phospholipase is involved in escape from the phagosomal membrane (239).

L. monocytogenes possesses two different phospholipase C's necessary for its escape and survival, a phospholipase C (PC-PLC), which cleaves most phospholipids and a phosphatidyl inosotol specific phospholipase (PI-PLC). Making mutations in either PLC or both PLC's, Smith *et al*. (196) showed that a PI-PLC mutant affected escape from the primary vacuole but did not affect cell-to-cell spread (196). Conversely, a mutation

in the PC-PLC affected cell-to-cell spread but no affect on the ability to escape the primary vacuole.

Other researchers report that when the non-pathogenic *Bacillus subtilis* was transformed with the Listeriolysin O (LLO) gene, *hly*, or the *pfoA* gene of *C. perfringens*, they allowed *B. subtilis* to lyse the phagocytic vacuole and grow in the cytoplasm (22, 157). When the chromosomal *hly* gene of *Listeria monocytogenes* was replaced by the *pfoA* gene of *C. perfringens*, *L. monocytogenes* is capable of escaping from the host cell vacuole and can replicate in the cytoplasm (94).

E. Activation of Macrophages

Professional phagocytes (neutrophils, eosinophils, monocytes, and macrophages) are critical components of the human host defense system when dealing with the elimination of bacterial pathogens.

When macrophages phagocytose bacteria they become activated, leading to the secretion of several cytokines (230). Macrophages require two signals for activation. One is provided by IFN-γ, and the other, which is needed to sensitize the macrophage so that it will respond to IFN-γ, can be provided by a variety of means including lipopolysaccharide (LPS) (92).

Activated macrophages are more efficient killers of bacteria than unactivated macrophages because they can fuse their lysosomes more efficiently to phagosomes, exposing recently phagocytosed bacteria to degradative lysosomal enzymes (92).

Following activation, these cells are able to produce potent reactive intermediates of oxygen metabolism (42). For example, phagocytic cells make reactive oxygen intermediates (ROI) as well as reactive nitrogen intermediates (RNI) to assist in killing the intracellular pathogen (135).

1. <u>Reactive Oxygen Intermediates (ROI)</u>

When phagocytic cells phagocytose bacteria, they undergo a burst of oxygen consumption that is caused by the assembly of an NADPH oxidase complex in the membrane (Figure 1-4). The superoxide anion ($O_2^{\cdot -}$) is the initial product of the respiratory burst (13), and is formed by the monoelectronic reduction of O_2 with NADPH as the specific electron donor. The respiratory burst also generates hydrogen peroxide (H_2O_2), a dismutation product of $O_2^{\cdot -}$. In neutrophils, myeloperoxidase released from granules during degranulation catalyzes the reaction of hydrogen peroxide with the abundant chloride present in the cells, to generate hypochlorous acid (HOCl), a potent antibacterial compound (135). Another reaction involving hypochlorous acid is the N-chlorination of nitrogen-containing compounds, yielding chloroamines (135). Two other by-products are also produced to aid in the elimination of pathogens: the hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$). The formation of these intermediates is shown in Figure 1-5. The ability of phagocytes to produce ROI and their ability to use them for killing intracellular pathogens, was determined in studies done with phagocytic cells from people who have chronic granulomatous disease (CGD), a genetic disease in which the

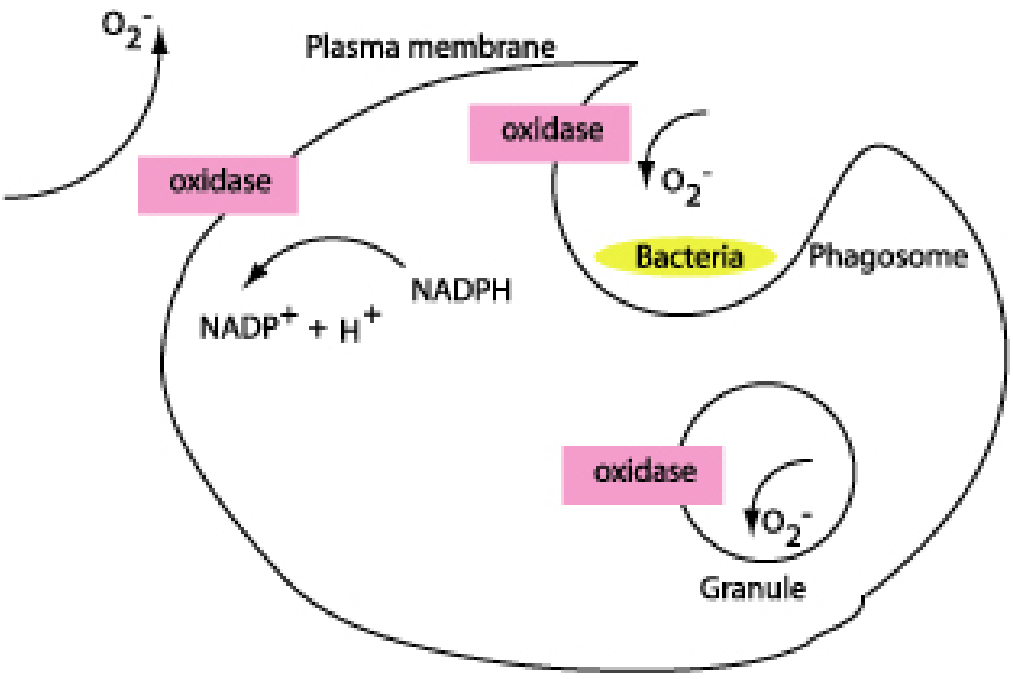

Figure 1-4. Possible sites for the NADPH-oxidase assembly and activity in immune cells.

Based on Dahlgren and Karlsson 1999 (100).

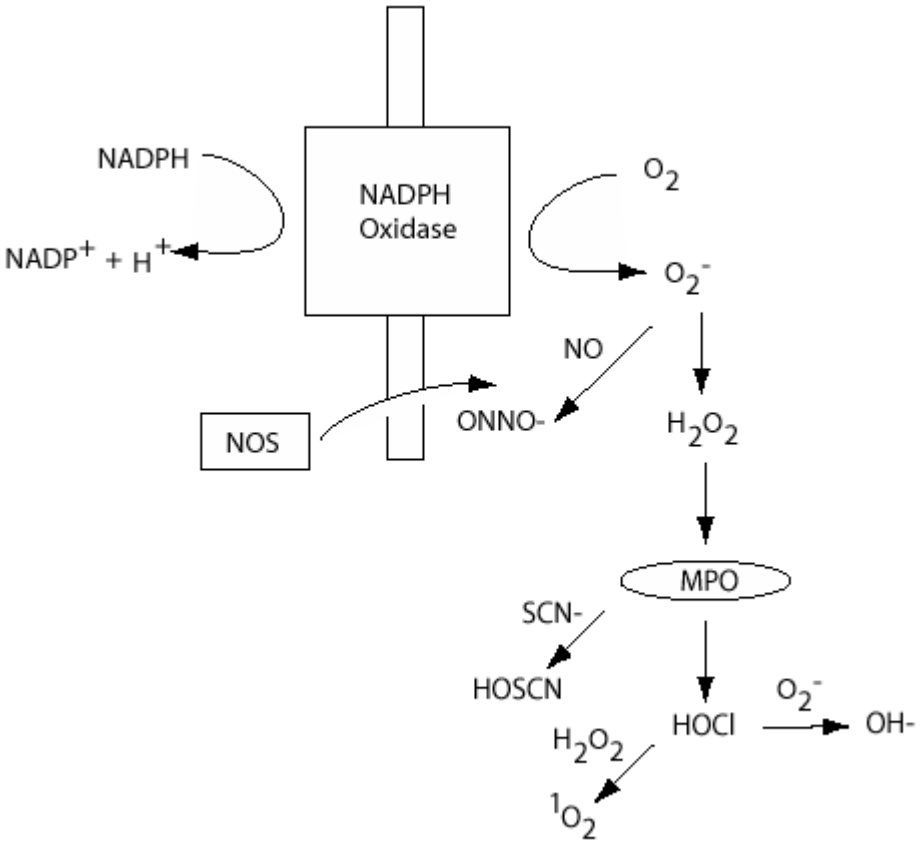

Figure 1-5. Possible intermediates made by stimulated neutrophils.
NOS, nitric oxide synthase; MPO, myeloperoxidase; NO, nitric oxide; OH, hydroxyl radical; O_2, Superoxide anion; HOCL, hypochlorous acid; ONNO-, peroxinitrate; HOSCN, chloroamine.

Based on Hampton et al. 1998 (75).

NADPH oxidase is inactive (199). These individuals are very susceptible to bacteria and fungal infections because their phagocytic cells cannot undergo a respiratory burst.

2. <u>Reactive Nitrogen Intermediates (RNI)</u>

RNI are also produced when phagocytic cells are activated. Nitric oxide ($NO^{\cdot}$) is made from L-arginine, oxygen, and NADPH. The reaction is catalyzed by nitric oxide synthase (NOS) (121) (Figure 1-5). The role of $NO^{\cdot}$ as a primary macrophage antimicrobial effector molecule has been addressed *in vitro,* where its actions can be specifically blocked using the inhibitor NG-monomethyl-L-arginine (L-NMMA). Using this system, macrophages activated with IFN-γ can inhibit the intracellular replication of a variety of intracellular bacteria in a NO-dependent manner (186).

F. The Role of Type IV Pili in the Pathogenesis of Gangrene

Type IV pili are usually found on the surface of Gram-negative bacteria (124, 211). Upon examination of the sequenced genome of *C. perfringens*, we found the presence of two *pil* genes, *pilC* and *pilT*. Because these genes are present in a Gram-positive bacteria, and the fact that *C. perfringens* has no twitching motility, the role of these gene products in gangrene needs to be elucidated. Type IV pili are distinguished from other pili by their polar location, sequence conservation of their pilins and pilus assembly proteins, and their role in bacterial motility (151, 211). Type IV pili are typically 5-7 nm in diameter and can extend to several μm in length. They are composed

primarily of a single small subunit called pilin (PilA), which is arranged in a helical conformation (123). Type IV pili can bind to a variety of surfaces including inert surfaces, other bacterial cells, and eukaryotic cells, where they can mediate both colonization and attachment through pili retraction (129, 213). These genes are also involved in the uptake of DNA by *Pseudomonas aeruginosa* among others (159, 181). A sensory role is proposed for the type IV pili. It is hypothesized that a signal could pass from the tip of a fiber to its base by the propagation of a helix dislocation or a mechanical force such as tension, compression or flexion (119). A similar model is used by *Vibrio haemolyticus* where they can activate genes in response to pressure on its polar flagellum (104).

Type IV pili are involved in secretion due to their homology with ten type II secretion proteins (159, 181). Prepilin, a precursor to pilin, in secreted into the cytoplasmic membrane in sec-independent manner (103), suggesting that the type IV pili could act as secretory channels. Other evidence comes from psuedopilins, proteins homologous to type IV pilins, that are used in type II secretion and in the related DNA uptake systems (40). It has been hypothesized that these pseudopilins form a structure similar to type IV pili between the inner and outer membrane and are involved in macromolecular transport (123, 159).

1. PilT

The main functions for PilT seem to be competency for natural transformation and twitching motility. Mutants that lacked PilT were deficient in both these activities (240). Pujol et al. (160) showed that although *Neisseria meningitides* lacking PilT still

adhered to T84 cells, they lacked intimate attachment suggesting that pilus retraction may be involved in this process. The finding that *pilT* mutants express pili but lack motility has led to another theory that PilT may function as a motor (227). *pilT* may also be associated with the pathogenesis of some organisms. Schoolnick et al. (21) show that *bfpF* (homologue of pilT in Enteropathogenic *E. coli* (EPEC)) mutants are avirulent even though they can still make pili, as well as bind and colonize tissue monolayers.

2. PilC

The main function of PilC is in adherence. Purified recombinant PilC imparts human epithelial cell binding activity on latex beads and blocks the binding of piliated organisms to epithelial cells (179). Also, piliated mutants lacking PilC fail to adhere to these cells (180, 241). The binding of *Neisseria gonorrhoeae* and *Neisseria meningitides* to epithelial and endothelial cells requires the gene product of *pilC* (189, 241). Most strains of *N. gonorrhoeae* have two copies of *pilC*, where the expression of at least one is required for the production of extracellular pili (178, 183). A loss of these genes can be complemented by a compensation mutation in *pilT* (241).

G. The Role of Clostridial Sialidases in Disease

Sialidases have been identified in numerous bacteria, especially in bacteria that are in close contact with animals or commensals (46, 187). Clostridial sialidases or neuraminidases are glycohydrolases that cleave terminal sialic acid residues from sugar chains of glycoproteins, glycolipids, oligosaccharides and sialoglycoconjugates. Along

with a lyase that *C. perfringens* produces, these enzymes are thought to be beneficial to the bacteria because they can cleave the sialic acid residues and then process them into pyruvic acid and N-acetylmannosamine, which *C. perfringens* can use as a nutritional source (Figure 1-6). This release of sialic acid residues exposes underlying structures that can be degraded by other hydrolytic enzymes and used as additional carbon sources. Therefore, the sialidases are thought to be mainly for nutritional purposes (46). It is also possible they may play a role in virulence. However, little is known about their role in virulence, but they might facilitate the degradation of tissues to allow for more efficient cell-to-cell spread of the bacteria (233). Another theory is that they may suppress the biological processes mediated by sialic acids in the host. Bacteria could also use sialidases for adherence by cleaving off the sialic acids, allowing for their adhesion to underlying galactose residues. Sialidases are also implicated in preventing the uptake of lymphocytes by high endothelial venules present in lymph nodes, which may prevent the migration of leukocytes to the site of infection (174). Sialidases also have a minor connection with gas gangrene. Patients with *C. perfringens* contaminated wounds, have sialidases present in their serum and wounds (188). Antibodies have also been used to confirm the presence of silaidases (168, 170, 188). *C. perfringens* has two sialidases, a large sialidase, NanI, and a small sialidase, NanH, which greatly differ in their properties.

1. <u>Nan I</u>

The large sialidase or NanI is a 71 kDa monomeric protein that is secreted (224). The protein functions optimally at a pH of 5.0 and a temperature of 55ºC (169). NanI can act on a large number of substrates including large and small sialoglycoconjugates (169).

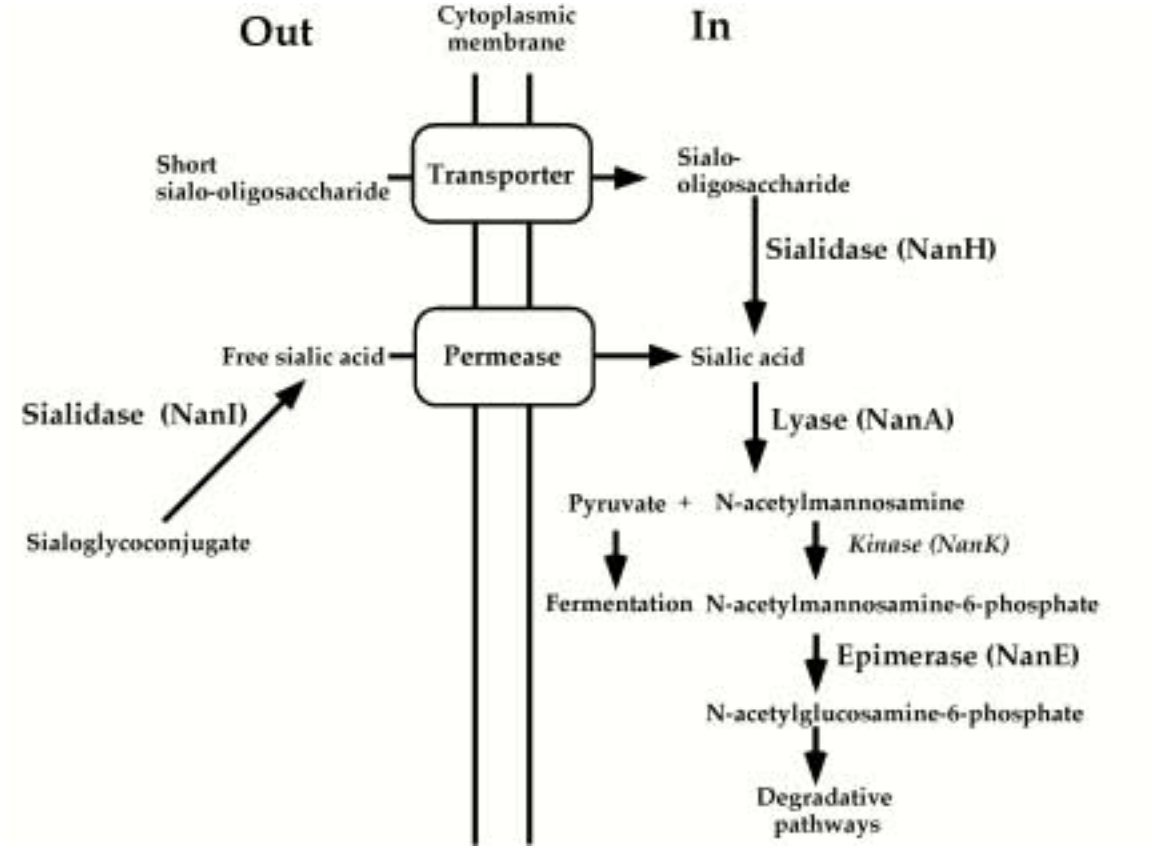

Figure 1-6. Schematic illustrating a hypothesized pathway of sialic acid metabolism in *C. perfringens.*

Based on Walters et al. 1999 (236).

2. Nan H

The small sialidase or NanH is a 43kDa monomeric protein that is not secreted because it lacks a signal sequence (224). The protein functions optimally at a pH of 6.1 and a temperature of 37ºC (169). The preferred substrates for NanH are oligosaccharides although complex sialoglycoconjugates can be degraded at very low rates (169). Upon completion of the sequenced genome of *C. perfringens*, it was found that it has a third sialidase, NanJ. However, no work has been done on NanJ to identify a role for it.

CHAPTER TWO

The anaerobic pathogen *Clostridium perfringens* can escape the phagosome of macrophages under aerobic conditions

This paper has been published in *Cellular Microbiology*

David K. O'Brien and Stephen B. Melville. (2000) The anaerobic pathogen *Clostridium perfringens* can escape the phagosome of macrophages under aerobic conditions. *Cellular Microbiology* **2**(6), 505-519.

ABSTRACT

Clostridium perfringens is the most common cause of gas gangrene (clostridial myonecrosis), a disease that begins when ischemic tissues become contaminated with *C. perfringens* vegetative cells or spores. An aerotolerant anaerobe, *C. perfringens* quickly multiplies in ischemic tissues and spreads to healthy areas, leading to a high level of morbidity and mortality. As a species, the bacterium can synthesize thirteen different toxins, and these are thought to be the major virulence factors of the disease. However, we present evidence here that *C. perfringens* can also persist inside macrophages, under aerobic conditions, by escaping the phagosome into the cytoplasm. *C. perfringens* was not killed by the cells of a clone (J774-33) of the macrophage-like murine cell line J774A.1, under aerobic or anaerobic conditions, while the non-pathogenic bacterium, *Bacillus subtilis* was killed by J774-33 cells in both conditions. Electron microscopy images showed that *C. perfringens* cells were intact and resided mostly in the cytoplasm of J774-33 cells, while *B. subtilis* was in the phagosome. Immunofluorescence microscopy showed that intracellular *C. perfringens* bacteria failed to co-localize with the late endosome-lysosomal marker glycoprotein LAMP-1, while *B. subtilis* did co-localize with LAMP-1. *C. perfringens* also appeared to escape the phagosome of both activated and unactivated mouse peritoneal macrophages, but not as efficiently as was seen with the J774-33 cell line. In addition, cytochalasin D was used to show that phagocytosis of *C. perfringens* was dependent on actin polymerization and that the bacteria attach to J774-33 cells at distinct areas of the cell membrane. We propose that the ability to escape

the phagosome and persist inside macrophages is an important factor in the early stages of a gangrene infection, when bacterial numbers are low and phagocytic cells are present.

INTRODUCTION

Clostridium perfringens is a gram-positive anaerobic pathogen that causes a wide spectrum of diseases in humans and animals. In humans, *C. perfringens* causes gas gangrene (clostridial myonecrosis), necrotic enteritis in infants, and enteritis necroticans (Pigbel). Traumatic gas gangrene occurs in deep, penetrating wounds that restrict the blood supply and result in ischemia and tissue damage (204). Reduced arterial blood flow leads to hypoxia and lowered E_h levels. Because *C. perfringens* is an aerotolerant anaerobe and can grow at the relatively high E_h level of +166 mV (77), it can initiate infections as rapidly as 6-8 hours after the injury (204).

After the induction of ischemia, *C. perfringens* infections follow three steps: (1) Contamination of the wound. (2) Rapid growth of *C. perfringens* in the ischemic tissue (3) Spread of the bacterium to healthy tissue. In the late stages, *C. perfringens* can enter the bloodstream and cause a systemic infection. If left untreated, the disease is always fatal due to the severe shock that follows the release of toxins by the bacterium into the bloodstream (10, 208). Despite some success with vaccines in animal experiments (237), there is no effective vaccine against *C. perfringens* infections.

Five different biotypes (A-E) of *C. perfringens* produce an array of thirteen different toxins (197). The two most important toxins produced during a gangrenous infection are thought to be PLC (α-toxin), which is both a phospholipase C and a sphingomyelinase (220), and PFO (θ-toxin or perfringolysin), a thiol-activated cytolysin (228). PLC has hemolytic properties, and is cytotoxic to macrophages and polymorphonuclear leukocytes (PMN) in high concentrations (205, 207, 209), but the

precise biochemical nature of the toxic effect is still unknown (220). The importance of PLC as a virulence factor was demonstrated when a strain of *C. perfringens* had a mutation introduced into the *plc* gene, which encodes PLC. The *plc*- strain showed reduced virulence in a mouse gas gangrene animal model (11, 55, 209). Another indication of the role of PLC in virulence was demonstrated when the *C. perfringens plc* gene was transformed into the non-pathogenic bacterium *Bacillus subtilis*. The *plc*+ *B. subtilis* strain showed a 100-fold decrease in LD_{50} when injected into the peritoneum of mice (145).

PFO is a member of a large family of thiol-activated cytolysins that includes listeriolysin (LLO), streptolysin (SLO), and pneumolysin (228). These toxins bind to cholesterol in eukaryotic membranes, aggregate, and form a pore in the membrane(228). PFO is hemolytic and is leukocidal at high concentrations *in vitro* (207). A mutation was introduced into the gene encoding PFO, *pfoA*, but the resulting mutant failed to show a significant reduction in virulence in the mouse gas gangrene model (11). However, later studies demonstrated that PFO plays a role in the vascular leukostasis peculiar to *C. perfringens* infections (55, 209).

In addition to its cytotoxic effects, a potential role for PFO in escaping the phagocytic compartment was demonstrated by Jones and Portnoy (94). They used a strain of the intracellular pathogen *Listeria monocytogenes* carrying a *hly* (LLO-) mutation that could not escape the phagosome and was avirulent in mice. They showed that the cloned *pfoA* gene from *C. perfringens* could restore the ability of the *hly* mutant to escape into the cytoplasm (94). However, the recombinant strain carrying the *pfoA*

gene exhibited an increased level of cytotoxicity and was still avirulent in mice (94). The authors suggested the increased cytoxicity was because PFO is active over a wide pH range, while LLO is active only under acidic conditions; i.e., LLO would only be active in the acidic compartment of the phagosome while PFO would be active in the cytoplasm as well, leading to cell leakiness and death. Jones et al. (95) used a mutagenesis strategy on the *C. perfringens pfoA* gene to isolate mutants that would allow an *L. monocytogenes hly* mutant to escape into the cytoplasm and still support intracellular growth. They identified one class of mutants in which the PFO activity was reduced at neutral pH, but still active under acidic conditions, providing support for their model that the pH-dependent activity of these toxins was important in their intracellular roles. Portnoy et al. (157) also demonstrated that transforming *B. subtilis* with the *C. perfringens pfoA* gene allowed the normally non-pathogenic *B. subtilis* to grow in the mouse macrophage-like cell line, J774, but not in primary bone marrow cells. These results led the authors to speculate that PFO may play a role for *C. perfringens* in killing professional phagocytes after they phagocytose the bacterium (157).

An importance aspect of pathogenesis of *C. perfringens*-induced gas gangrene infections involves the role of the immune system in ischemic tissues. In response to ischemia, connective tissue cells and resident macrophages release monocyte chemoattractant protein-1 (MCP-1) (23, 70, 90). This chemokine leads to the recruitment of monocytes to the ischemic area. In response to anoxia, macrophages have also been shown to release the proinflammatory cytokines macrophage inflammatory protein-1 alpha (MIP-1-α) and tumor necrosis factor-alpha (TNF-α) (232). These proinflammatory

cytokines lead to further recruitment of macrophages and polymorphonuclear leukocytes (PMNs) to the ischemic tissue. In addition, macrophages have a remarkable capacity for anaerobic survival. Alveolar macrophages isolated from guinea pigs were reported to be viable for 96 hours in the absence of oxygen (32). In response to anaerobiosis, macrophages have been shown to induce the enzymes involved in glycolysis, and ATP is obtained from this pathway (37). However, despite the strong recruitment of actively phagocytic cells to ischemic tissues, small amounts of contaminating *C. perfringens* are able to grow rapidly and initiate a gangrene infection.

We describe in this report the bactericidal activities of J774 macrophage-like cells against *C. perfringens* under aerobic and anaerobic conditions. Surprisingly, it was discovered that J774 cells not only fail to kill *C. perfringens* under anaerobic conditions, they also fail to kill *C. perfringens* under *aerobic* conditions. We propose that the ability of *C. perfringens* to escape the phagosome plays an important role in its survival inside macrophages.

RESULTS

Isolation of a J774A.1 cell line clone exhibiting high levels of attachment and phagocytosis of *C. perfringens*.

The mouse macrophage-like cell line J774A.1 (162) was chosen as a model system for studying *C. perfringens* interactions with macrophages. After receiving the cell line from American Type Culture Collection, it was determined that, under aerobic conditions, only ~10% of the macrophages had at least one cell-associated *C. perfringens* after infection (see Experimental Procedures). Fifty-five single clones were isolated using a cell sorter, propagated, and assayed for *C. perfringens* attachment and/or phagocytosis (i.e., cell-associated bacteria). One clone, J774-33, exhibited a frequency of cell-associated bacteria of ~90% under aerobic conditions. Therefore, this clone was chosen for further experiments with *C. perfringens*. J774-33 cells also phagocytosed other gram-positive bacteria, *Staphylococcuc aureus* and *B. subtilis* (unpublished data), indicating the clone was not specific in its phagocytic properties to *C. perfringens*. Interestingly, the large majority of the clones tested showed ~0% phagocytosis, suggesting the cell line we received was a mixed population with different phenotypes.

Because the phagocytosis capacity of the J774-33 clone was to be tested under both aerobic and anaerobic conditions, it was necessary to determine if the cell line could survive under anaerobic conditions. This clone was tested for viability under anaerobic conditions by measuring the amount of lactate dehydrogenase (LDH) that was released by lysis of dead cells. Total cell numbers were estimated by determining the total amount of LDH in the culture (Fig. 2-1A). After 48 h under anaerobic conditions, the total

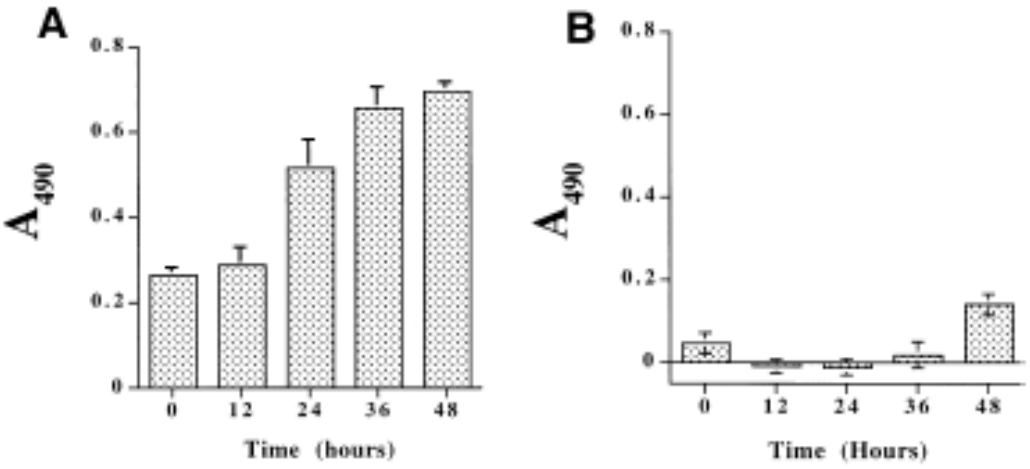

Figure 2-1. Survival of clone J774-33 under anaerobic conditions.
A. Changes in the cell population were estimated using the total amount of lactate dehydrogenase (LDH) at increasing times. The total LDH concentration was determined by complete lysis of all the macrophages using Triton X-100, before carrying out the LDH assay (see Experimental Procedures). The means +/- SD of quadruplicate samples are shown at each time point.
B. Spontaneous lysis of J774-33 cells was estimated by measuring the amount of released LDH in the supernatants of wells containing the J774-33 cells. The means +/- SD of quadruplicate samples are shown at each time point.

amount of LDH increased two-fold (Fig. 2-1A); however, no additional growth was seen when the cells were examined using a light microscope (data not shown); suggesting that the cells increased the synthesis of LDH in response to the shift to anaerobic conditions (32, 37). In Fig. 2-1B, spontaneous lysis of the macrophages was measured as released LDH activity. The cells showed only a small amount of lysis over the course of 48 h, which was confirmed by light microscopy (not shown). Therefore, J774-33 cells were not sensitive to anaerobic conditions and could be used as a model cell line for *C. perfringens* infections.

***C. perfringens* is not killed by J774-33 cells under aerobic and anaerobic conditions.**

The survival of *C. perfringens* strain 13 incubated with J774-33 cells was examined under aerobic conditions at a multiplicity of infection (MOI) of 1:1 (Fig. 2-2A). Over the first 6 hours the levels of surviving bacteria were similar whether macrophages were present or not. However, the bacteria incubated with J774-33 cells actually increased from 9-24 h, while the bacteria alone exhibited an exponential decline in numbers over the same period (Fig. 2-2A). Similar results were seen when the MOI was increased to 5:1 or 10:1 (data not shown).

As a control, the identical experiment was carried out, but *B. subtilis* was substituted for *C. perfringens*. *B. subtilis* is a motile, primarily aerobic, non-pathogenic, gram-positive, rod shaped bacterium. In Fig. 2-2B it can be seen that, in the absence of J774-33 cells, *B. subtilis* grew rapidly in DMEM, but its growth in the presence of J774-33 cells was greatly curtailed. The *B. subtilis* population may not drop to very low levels since its motility prevented some bacteria from interacting with the macrophages (we did

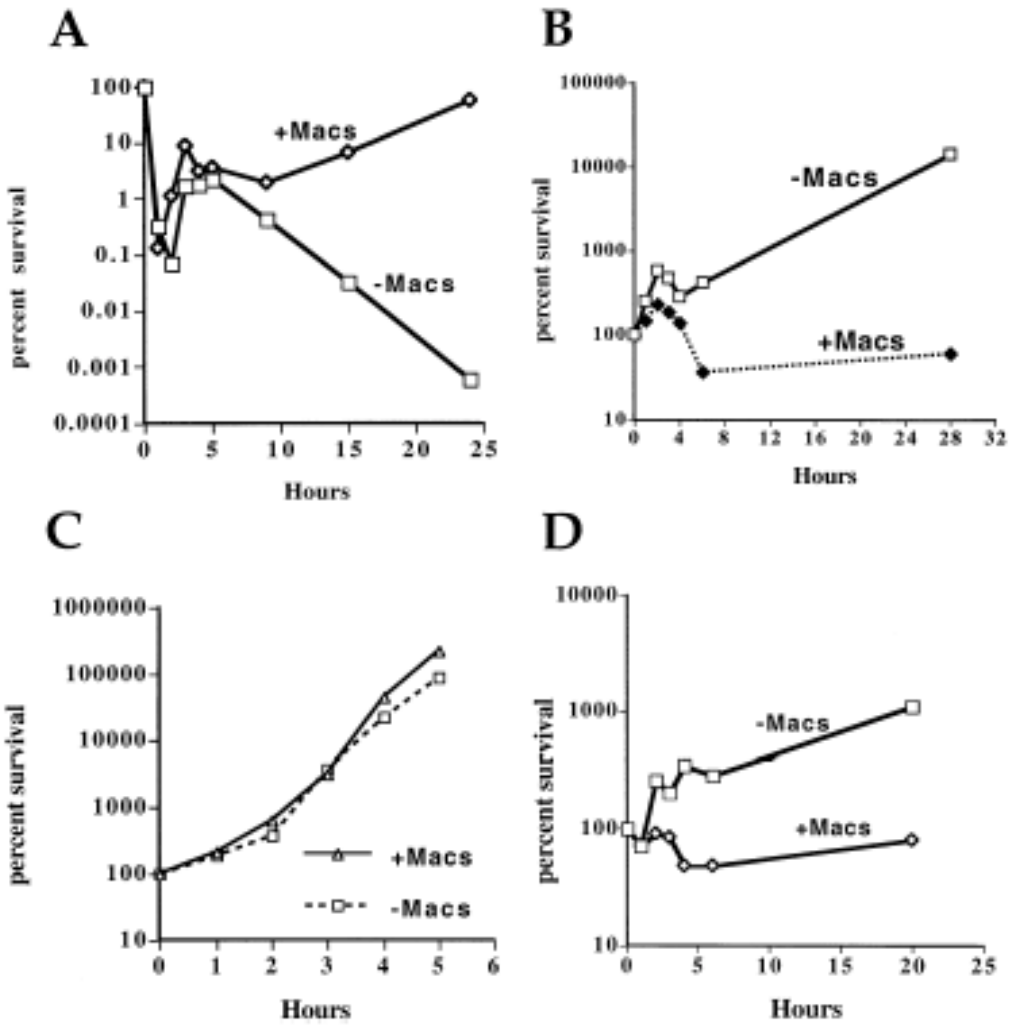

Figure 2-2. Survival of *C. perfringens* and *B. subtilis* incubated with J774-33 cells under aerobic and anaerobic conditions.

A. *C. perfringens* incubated +/- J774-33 cells under aerobic conditions.
B. *B. subtilis* incubated +/- J774-33 cells under aerobic conditions.
C. *C. perfringens* cells incubated +/- J774-33 cells under anaerobic conditions.
D. *B. subtilis* cells incubated +/- J774-33 cells under anaerobic conditions.

not centrifuge the bacteria onto the macrophages, in order to be consistent with the *C. perfringens* experiments).

Similar bacterial survival experiments were also performed under anaerobic conditions. Macrophages were given fresh medium and then placed in the anaerobic chamber for two hours, before the addition of bacteria, to reach an anaerobic state in the tissue culture medium. Under anaerobic conditions, with or without macrophages, *C. perfringens* cells grew at nearly an exponential rate during the first 5 h (Fig. 2-2C). In contrast, *B. subtilis*, while increasing 10-fold in the absence of macrophages, showed a slight decline when J744-33 cells were present (Fig. 2-2D). Though often considered an obligate aerobe, *B. subtilis* does have the capacity to grow in the absence of oxygen on certain substrates (139), Fig. 2-2D). These results were interpreted by us as showing J774-33 macrophages can kill *B. subtilis* but cannot kill *C. perfringens* under both aerobic and anaerobic conditions.

C. perfringens does not co-localize with the late-endosome-lysosomal marker protein LAMP-1 under aerobic conditions.

The fusion of lysosomes to the phagosome is the primary bactericidal mechanism of macrophages (18). To determine if phagosome-lysosome fusion was occurring in the J774-33 cells, we used rat monoclonal antibody 1D4B, which is directed against the mouse late endosome-lysosomal marker glycoprotein LAMP-1, as a marker for fusion. Polyclonal rabbit anti-sera directed against whole cells of *C. perfringens* was used to localize both *C. perfringens* and *B. subtilis* (see Experimental Procedures). J774-33 cells were infected with either *C. perfringens* or *B. subtilis* under aerobic conditions and

examined in a Zeiss LSM 510 laser confocal microscope (Fig. 2-3). Due to the large size of the bacteria, two features of infected cells could be determined using the three-dimensional images produced by the laser confocal microscope: Whether the cell-associated bacteria were intra or extracellular and whether the intracellular bacteria co-localized with the LAMP-1 marker protein. For *C. perfringens* and *B. subtilis*, 66% and 89%, respectively, of the cell-associated bacteria were intracellular (Table 2-1). However, only 17% of intracellular *C. perfringens* co-localized with LAMP-1, while 78% of intracellular *B. subtilis* were found to co-localize with LAMP-1 (Table 2-1). Therefore, the majority of cell-associated bacteria were intracellular and the ability to prevent lysosomal association appeared to play a major role in the survival of *C. perfringens* inside J774-33 cells.

The LAMP-1 staining, described above, defined only the lysosomes and late endosomes of the cell and not the entire cytoplasmic volume. As an additional control, J77-33 cells infected with *C. perfringens*, were stained with the eukaryotic cytoplasmic-staining marker Cell Tracker Green CMFDA (green fluorescence) (Molecular Probes, Inc.), while the bacteria were stained with propidium iodide (red fluorescence). These infected cells were examined in the laser confocal microscope and it was found that 85% of the bacteria were intracellular (data not shown). Because the LAMP-1 staining actually underestimated by ~19% the amount of intracellular bacteria, we were confident that each of the bacteria determined to be intracellular with LAMP-1 staining was intracellular and that a co-localization determination with LAMP-1 was appropriate for that bacterium.

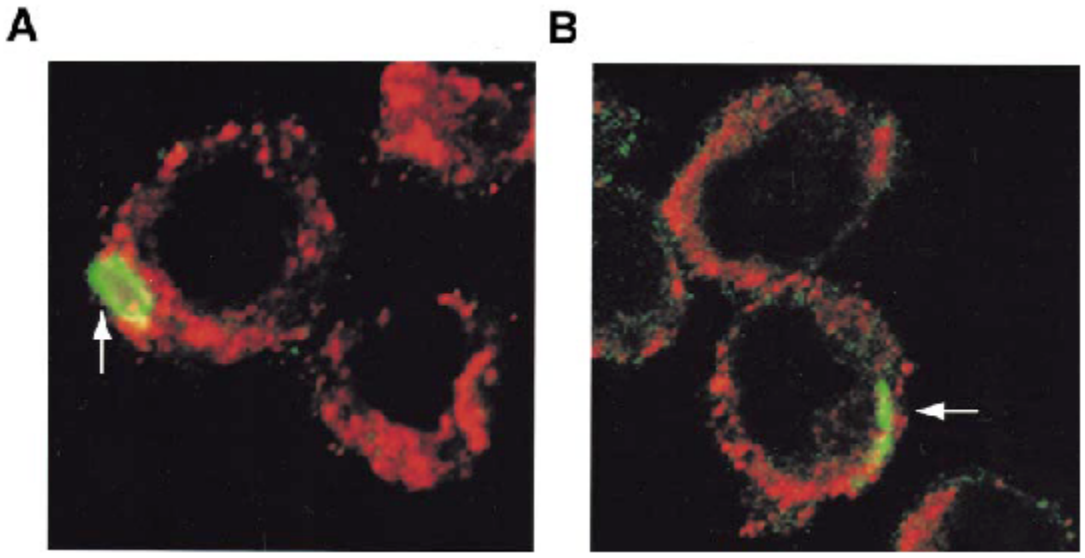

Figure 2-3. Co-localization of LAMP-1 with *C. perfringens* and *B. subtilis*.
Two-dimensional laser confocal microscopy images of immunofluorescently labeled LAMP-1 (red) and bacteria (green)
A. *C. perfringens* in J774-33 cells under aerobic conditions. Arrow points to a single bacterium.
B. *B. subtilis* in J774-33 cells under aerobic conditions. Arrow points to two bacteria.

Table 2-1. Summary of results obtained from laser confocal microscopy experiments.

Bacteria	% Bacteria intracellular	% Intracellular bacteria co-localized with LAMP-1	% Bacteria intracellular (+ cytochalasin D)
C. perfringens	**66 (n = 170)**	**17 (n = 112)**	**4 (n = 103)**
B. subtilis	**89 (n = 123)**	**78 (n = 109)**	**9 (n = 44)**

***C. perfringens* can escape the phagosomal compartment in aerobic J774-33 cells.**

Intracellular bacteria can prevent association with lysosomes by preventing phagosome-lysosome fusion or by escaping the phagosome into the cytoplasm. To determine if either of these mechanisms was used by *C. perfringens*, J774-33 cells were infected with *C. perfringens* for 90 min under aerobic conditions and then processed for transmission electron microscopy (TEM). Representative TEM figures are shown in Fig. 2-4. It was observed that the cytoplasmic membrane was intact in most J774-33 cells (Fig. 2-4). For *C. perfringens*, the cell wall is intact and the cytoplasm is electron dense, indicating the bacteria were not degraded at the time of fixation. Most importantly, we were unable to detect an intact phagosomal membrane around the majority (>70%) of intracellular *C. perfringens* cells. As a control to insure that we can visualize a phagosomal membrane, we infected J774-33 cells with *B. subtilis* bacteria for 60 min under aerobic conditions and examined them using the electron microscope. Representative images, shown in Fig. 2-5, clearly show an intact phagosomal membrane around the intracellular *B. subtilis* cells. These images provide a mechanism to explain how *C. perfringens* can survive inside J774-33 cells, while *B. subtilis* is killed: *C. perfringens* can escape the phagosome while *B. subtilis* cannot, and is subsequently killed by the normal bactericidal activities of the lysosomal contents.

Electron microscopy was also done on J774-33 cells infected by *C. perfringens* for 90 min under anaerobic conditions. The bacteria were highly cytotoxic to the J774-33 cells, leading to rapid lysis and cell death (Fig. 2-6). This supports our observation that *C. perfringens* can grow rapidly in the presence of J774-33 cells under anaerobic

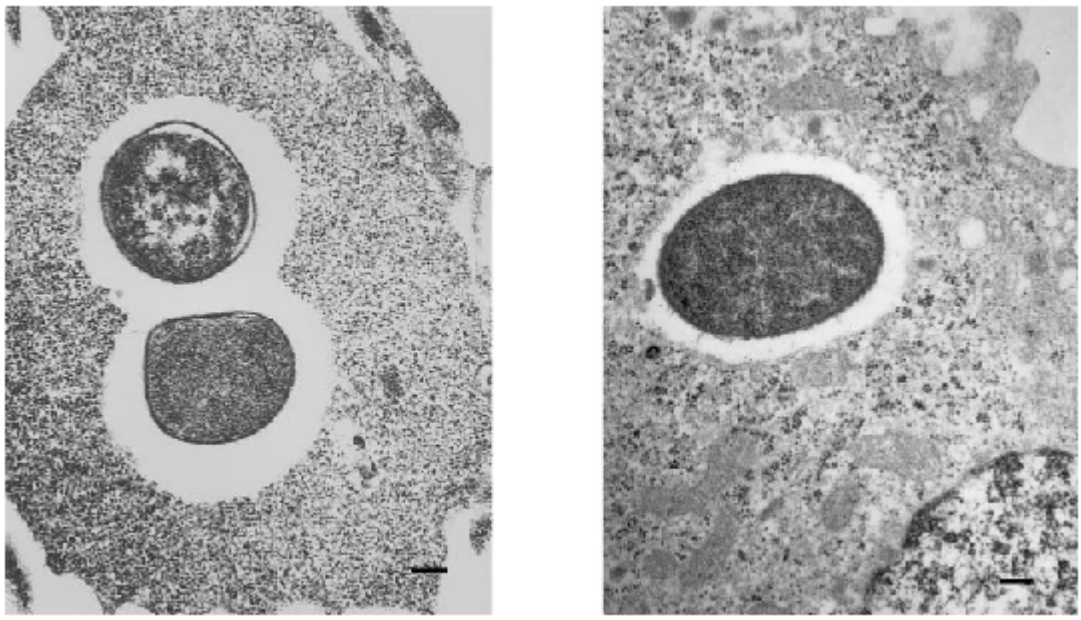

Figure 2-4. *C. perfringens* appears to escape the phagosome under aerobic conditions.
Transmission electron micrographs (TEM) of J774-33 cells infected with *C. perfringens* under aerobic conditions. Note the absence of phagosomal membranes around the bacteria. Bars: 200 nm.

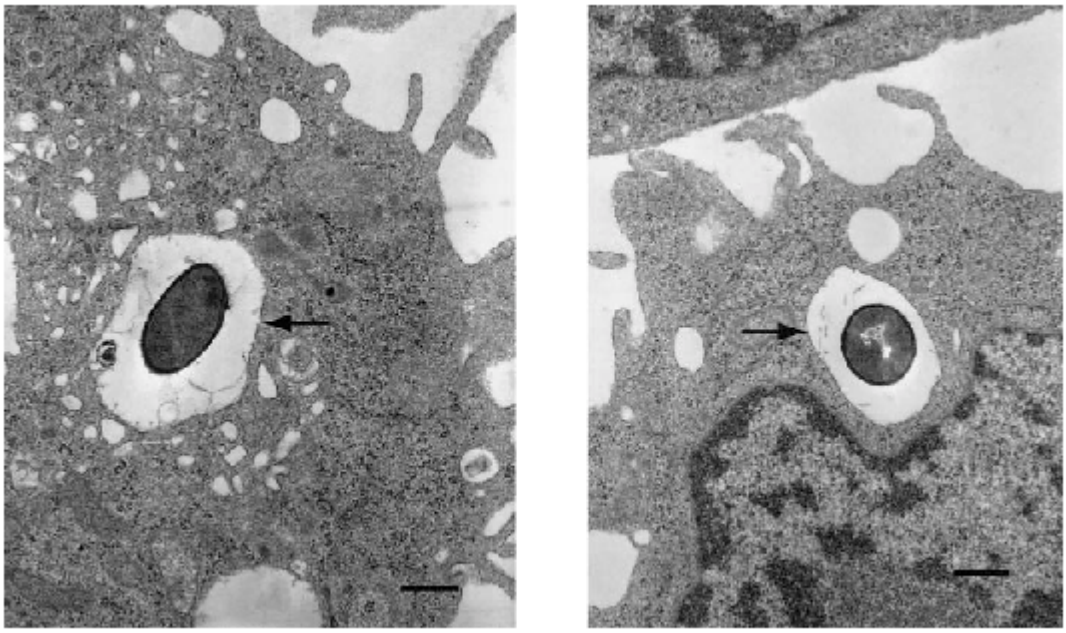

Figure 2-5. *B. subtilis* remains in the phagosome under aerobic conditions.
TEM of J774-33 cells infected with *B. subtilis* under aerobic conditions. Arrows point to a clearly visible phagosomal membrane. Bars: 500 nm.

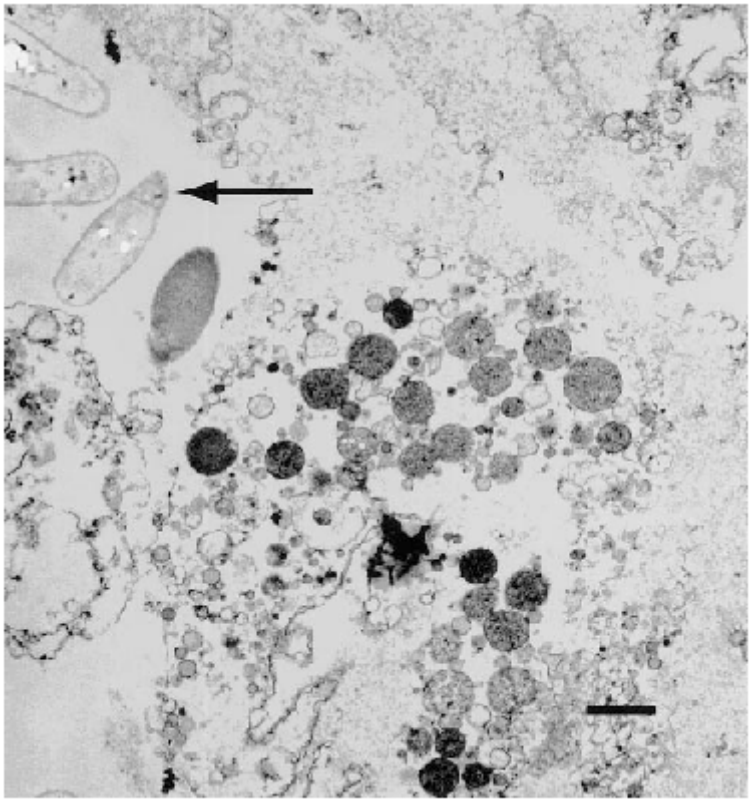

Figure 2-6. TEM of J774-33 cells infected with *C. perfringens* under anaerobic conditions.
Arrow points to bacteria. Note the lysed, degraded appearance of the J774-33 cells. Bar: 1 μm.

conditions (Fig. 2-2C). We also infected J774-33 cells with *B. subtilis* for 60 min under anaerobic conditions and examined the cells in the light microscope. The J774-33 cells appeared intact and contained numerous intracellular bacteria, as determined using differential interference contrast (DIC) microscopy (data not shown).

The timing of *C. perfringens* escape from the phagosome.

In order to determine if the point in the phagocytosis process in which *C. perfringens* escaped from the phagosome could be detected, additional electron microscopy was done with J774-33 cells infected with *C. perfringens* under aerobic conditions. A time course analysis of *C. perfringens* infections of J774-33 cells indicated that the highest numbers of cell-associated bacteria were seen at 60 min (data not shown), therefore, we used this time to look at the uptake of the bacteria. Using an electron microscope, large numbers of intracellular bacteria could be seen (data not shown) and also bacteria that appeared to be in the process of being taken up by J774-33 cells (Fig. 2-7A and 2-7B). Note the degraded appearance of the phagosomal membrane around the bacterium in Fig. 2-7A, despite the visual evidence that this bacterium has not yet been completely phagocytosed. In Fig. 2-7B, a bacterium, apparently in the early stages of phagocytosis, already shows a discontinuity in the developing phagosome (inset). These images suggest *C. perfringens* may be able to escape the phagosome very rapidly, perhaps even before phagocytosis is complete. Another type of process often seen in these cells is shown in Fig. 2-7C and 2-7D. In these images, the bacteria appear to be breaking out of the cell itself, perhaps by localized lysis of the cytoplasmic membrane. In Fig. 2-7C, the boxed bacterium is in the cytoplasm and cell material can be seen

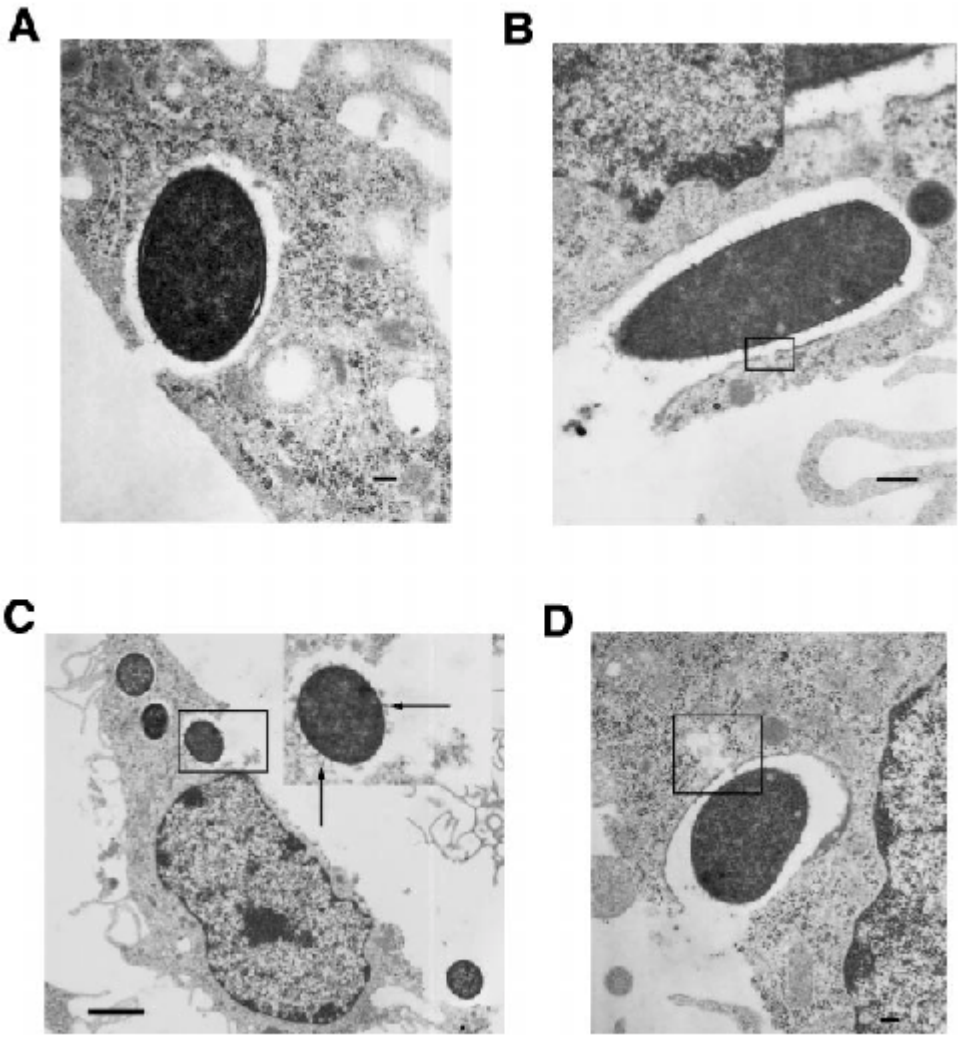

Figure 2-7. Timing of *C. perfringens* escape from the phagosome.
TEM images of J774-33 cells infected with *C. perfringens* under aerobic conditions.
A. The bacterium is nearly completely engulfed in a phagosome, but the membrane surrounding the bacterium shows pronounced signs of degradation. Bar: 200 nm.
B. Earlier stage of engulfment, with a discontinuity in the phagosomal membrane (box and inset). Bar: 500 nm.
C. A bacterium (boxed) apparently escaping from the J774-33 cell. Note the patchy cellular material attached to the bacterium (arrows in inset). Bar: 2 μm.
D. Another bacterium apparently escaping from the phagosome. Note the large disruption in the phagosomal membrane and extracellular material diffusing into the cell (box). Bar: 200 nm.

adhering to the bacterium in patches (inset). Some of the intracellular bacteria still appear to be in a phagosome (Fig. 2-7C). Unfortunately, with a static assay like electron microscopy, it cannot be determined if all of the intracellular bacteria will eventually escape the phagosome. In figure 2-7D, the bacterium has broken a hole in the phagosomal membrane and also appears to be breaking out of the cell. However, for Fig. 2-7C and 2-7D, another possible interpretation is that the bacteria were in the process of being phagocytosed, but were able to break down the phagosomal membrane before it could completely engulf the bacterium. Images of the types shown in Fig. 2-7 were not seen when *B. subtilis*-infected cells were examined (data not shown).

The actin cytoskeleton is required for phagocytosis of *C. perfringens*.

The actin polymerization inhibitor, cytochalasin D, was used to determine if phagocytosis of *C. perfringens* was actin dependent. J774-33 cells were infected for 60 min in the presence of 1 μg/ml cytochalasin D. One μm sections were stained with toluidine blue, to identify cell-associated bacteria. In the absence of cytochalasin D, the majority of bacteria were intracellular (Fig. 2-8, top), while in the presence of cytochalasin D, the large majority of bacteria, while still cell-associated, were extracellular (Fig. 2-8, bottom). We also used cytochalasin D for laser confocal microscopy experiments, where the bacteria and LAMP-1 were labeled with fluorochrome-conjugated antibodies, as described above. The presence of cytochalasin D lowered the intracellular percentage of

C. perfringens from 66% to 4% (Table 2-1). These results demonstrate that actin polymerization is essential for phagocytosis of *C. perfringens*.

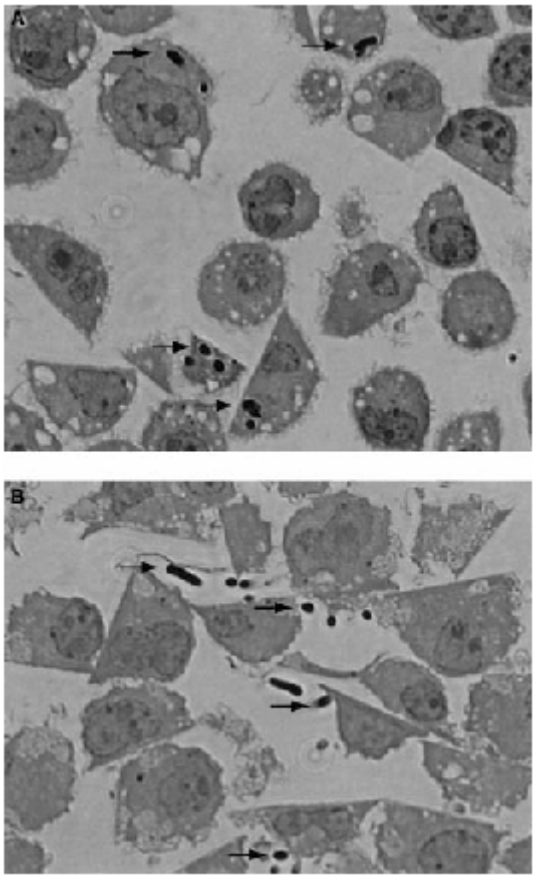

Figure 2-8. Cytochalasin D inhibits phagocytosis of *C. perfringens* by J774-33 cells.
Phase contrast light micrographs.
Top: J774-33 cells infected with *C. perfringens* under aerobic conditions were fixed, embedded in resin, and sectioned. Sections were stained with toluidine blue. Note the majority of bacteria (small dark circles) are intracellular (arrows).
Bottom: Same as the top panel, but the J774-33 cells were treated with 1 μg/ml cytochalasin D. The majority of bacteria are extracellular (arrows).

Since *C. perfringens* was still attached to cytochalasin D-treated J774-33 cells, TEM was also performed on cytochalasin D-treated J774-33 cells that were infected with *C. perfringens*, to examine the point of attachment. Interestingly, we saw that nearly all of the attached bacteria were associated with a J774-33 cell at discrete "patches", usually seen at the end of protrusions from the surface of the J774-33 cytoplasmic membrane (Fig. 2-9).

***C. perfringens* invasion assay.**

Although the bacterial survival curves indicated *C. perfringens* can survive in the presence of J774-33 cells, they did not address whether the bacterium can grow in the intracellular environment. To answer this question, a method for selectively killing extracellular bacteria, i.e., an "invasion assay" (57), was developed. A typical invasion assay uses gentamycin, which does not cross the cytoplasmic membrane of macrophages, to kill extracellular bacteria (57). However, *C. perfringens* is not sensitive to gentamycin. Therefore, an invasion assay using penicillin G to kill extracellular *C. perfringens* was developed. Penicillin does not penetrate mammalian cells (238) and has been used for invasion assays with other gram-positive bacteria (110, 177). As a positive control, *Salmonella enterica* subsp. Typhimurium (*S. typhimurium*), which is known to survive inside J774 cells (229), was used. The invasion assay was carried out under aerobic and anaerobic conditions, as described in Experimental Protocols, and the results are shown in Fig. 2-10A and 2-10B. Penicillin G was effective at killing extracellular *S. typhimurium*, because the addition of the phagocytosis inhibitor cytochalasin D led to a 10-fold increase in cell killing (Fig. 2-10A). Penicillin G was also effective at killing

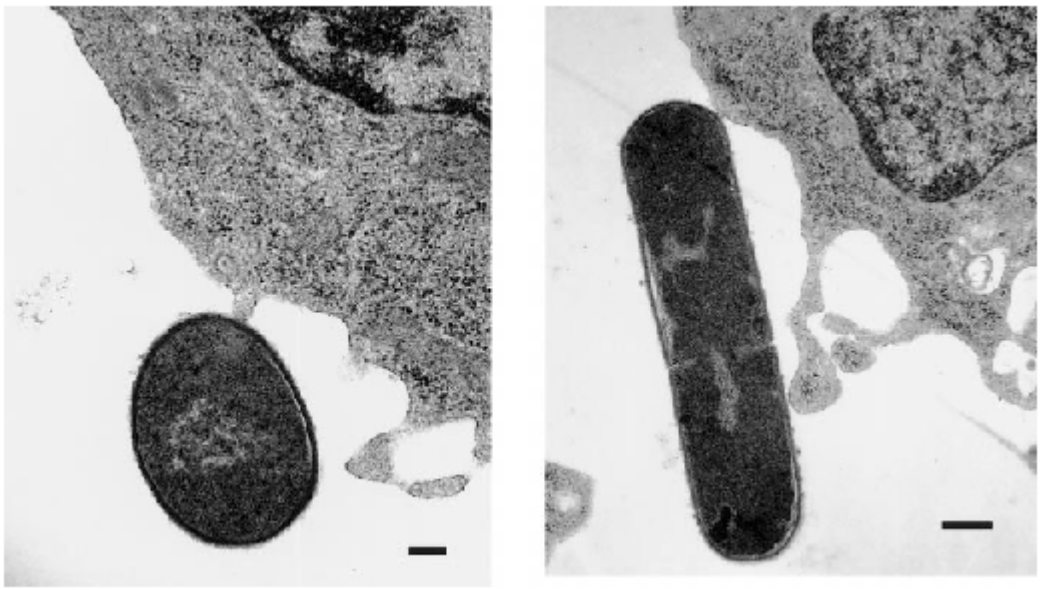

Figure 2-9. J774-33 cells bind to *C. perfringens* at distinct "patchy" areas.
TEMs of J774-33 cells treated with 1 μg/ml cytochalasin D and then infected with *C. perfringens* under aerobic conditions. The areas of attachment of the plasma membrane to the bacteria occurred at distinct areas or "patches". Bars: Left panel, 200 nm, right panel, 500 nm.

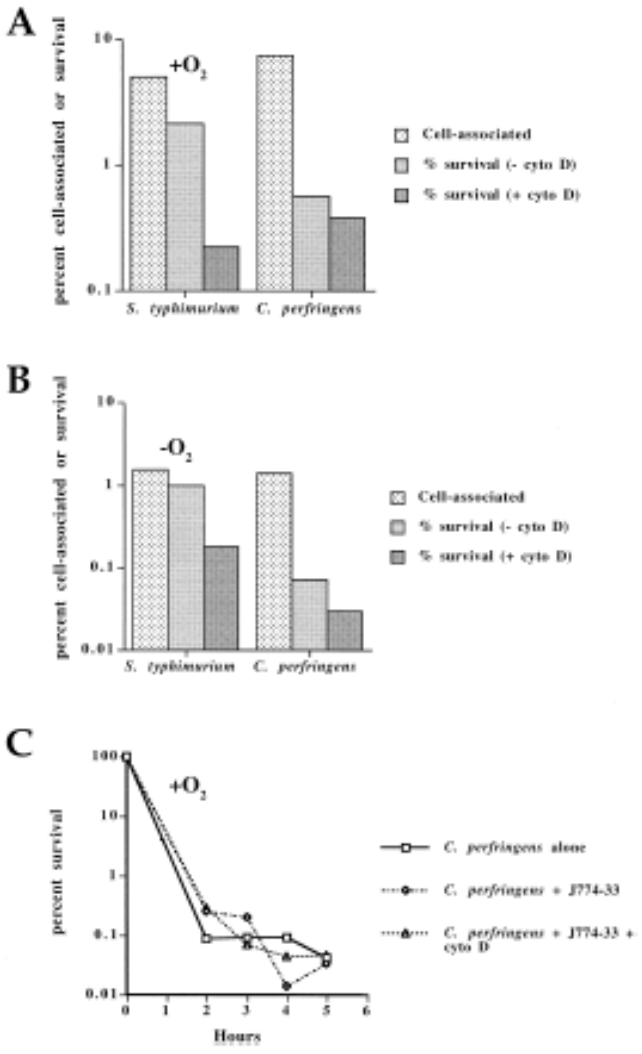

Figure 2-10. Invasion assay for J774-33 cells infected with *C. perfringens* and *S. typhimurium*.

A. Aerobic invasion assay, performed as described in Experimental Procedures. + Cyto D: One μg/ml cytochalasin D was added to the assay.

B. Same as A, except the assay was performed under anaerobic conditions.

C. Survival of *C. perfringens* incubated with 5 μg/ml penicillin G (all curves), J774-33 cells and 1 μg/ml cytochalasin D, as indicated in the figure.

C. perfringens (compare the levels of survival to that shown in Fig. 2-2A). However, *C. perfringens* survival was not significantly affected by the addition of cytochalasin D, despite the evidence presented above that cytochalasin D inhibited the uptake of *C. perfringens*. A similar pattern was also seen under anaerobic conditions (Fig. 2-10B). Since the invasion assays shown in Fig. 2-10 A and B, were carried out for only 1 hour, J774-33 cells were infected with *C. perfringens* in the presence of penicillin G for up to 5 hours. However, no significant difference in bacterial killing was seen whether cytochalasin D was added or not (Fig. 2-10C). These results suggest that *C. perfringens* was killed by the penicillin G even when it was inside J774-33 cells, indicating the bacterium either lyses the cells or makes them leaky to penicillin.

***C. perfringens* can also escape the phagosome in mouse peritoneal macrophages.**

Both activated and unactivated mouse peritoneal macrophages were used to determine if the results we observed with cell line J774-33 would also be seen using primary cells. Mouse peritoneal macrophages were infected with *C. perfringens* under aerobic conditions for 60 min and then processed for TEM. Representative images are shown in Fig. 2-11. Some of the intracellular bacteria appeared to have broken down the phagosomal membrane in both activated and unactivated macrophages (Fig. 2-11A and 10B). However, in contrast to what we saw with J774-33 cells, the majority of the bacteria (~70%) appeared to remain inside the phagosome (Fig. 2-11C and 2-11D). This suggested peritoneal macrophages were probably more effective at killing *C. perfringens* under aerobic conditions than were the J774-33 cells. Nevertheless, it appears some *C. perfringens* cells can still escape the phagosome and, presumably, survive inside the

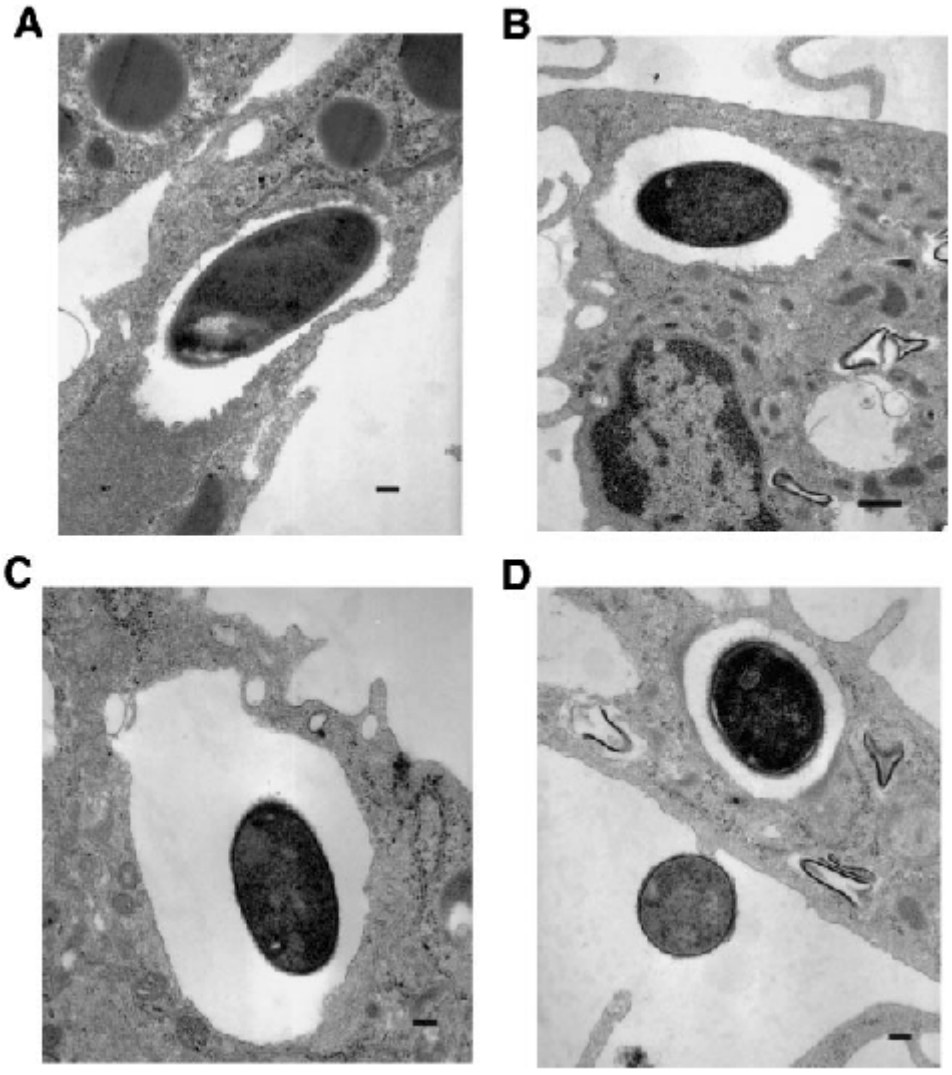

Figure 2-11. *C. perfringens* can escape the phagosome of mouse peritoneal macrophages.
TEMs of activated (A and C) and unactivated (B and D) mouse peritoneal macrophages infected with *C. perfringens* under aerobic conditions.
A and B. Intracellular bacteria with degraded phagosomal membranes.
C and D. Intracellular bacteria still inside an intact phagosome.

peritoneal macrophages. In contrast, using light microscopy, we observed that peritoneal macrophages infected with *C. perfringens* under anaerobic conditions were extremely sensitive to the cytotoxic effects of *C. perfringens*, especially activated macrophages (data not shown).

Activated J774-33 and mouse peritoneal macrophages are more sensitive to *C. perfringens* dependent cytotoxicity, but not because they are phagocytosed more than activated macrophages.

J774-33 Macrophages were grown to near confluency in 96-well tissue culture plates and then activated by the addition of *Escherichia coli* LPS and murine IFN-γ. Peritoneal macrophages were isolated from C3HeB/FeJ mice, transferred to 96-well tissue culture plates at a concentration of $3x10^5$ macrophages per well, and activated with *E. coli* LPS and murine IFN-γ. Cells were then infected at an MOI of 5:1 for 60 min under aerobic and anaerobic conditions. The experiment was done in the presence and absence of cytochalasin D. *C. perfringens* dependent cytotoxicity was determined using the LDH assay described previously. Activated J774-33 macrophages were more sensitive to *C. perfringens* dependent cytotoxicity than non-activated macrophages under both aerobic and anaerobic conditions (Fig. 2-12 Top row). The same trend, although more pronounced, was observed with mouse peritoneal macrophages (Fig. 2-12 Bottom row). When cytochalasin D was added to J774-33 cells and peritoneal macrophages, they were still sensitive to *C. perfringens* suggesting that cytotoxicity occurs whether or not the bacteria are inside the cells.

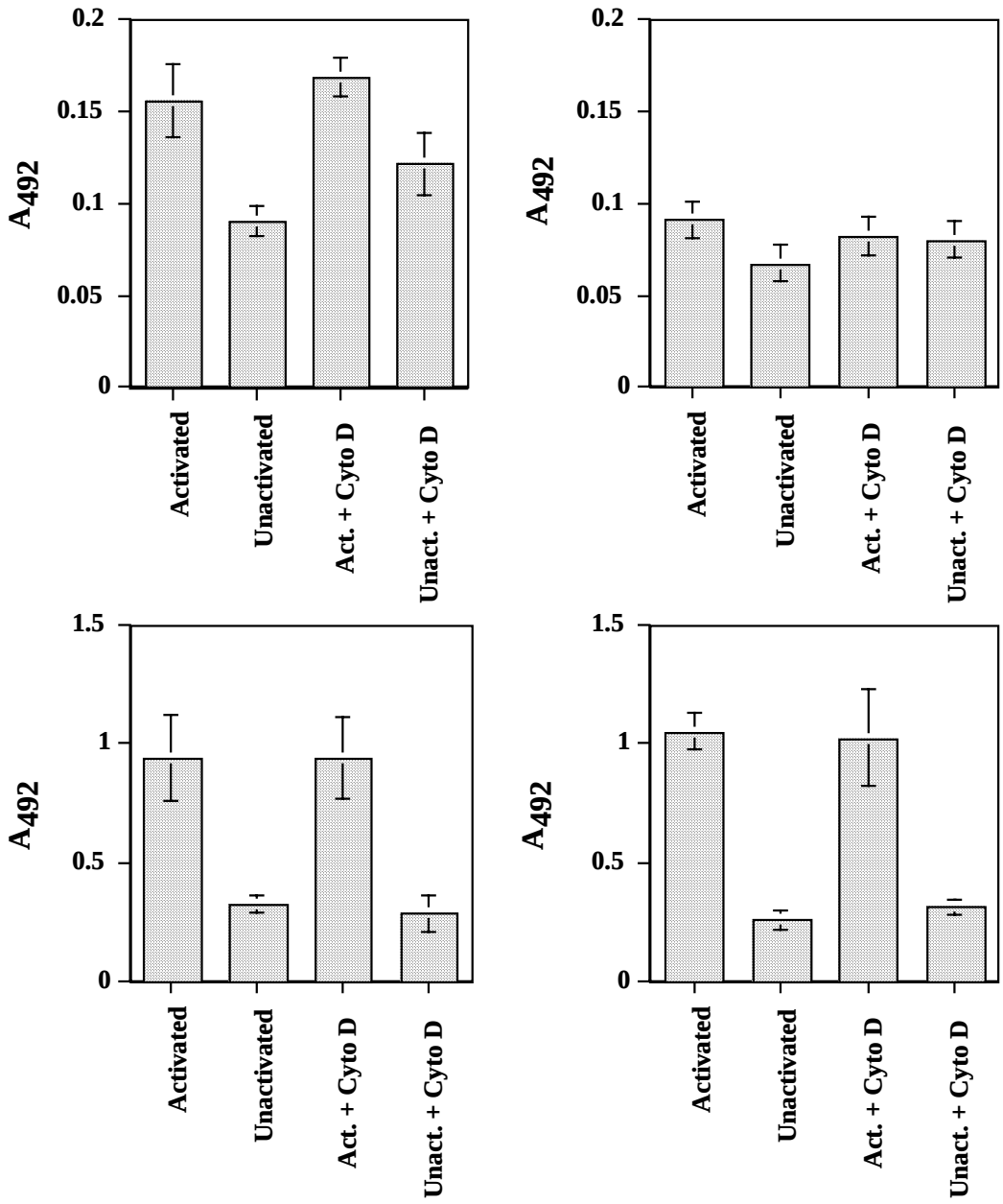

Figure 2-12. ***C. perfringens*** **dependent cytotoxicity towards macrophages.** The Cytotox 96 cytotoxicity assay was used to determine the cytotoxicity of *C. perfringens* to activated and unactivated J774-33 cells (top row) and mouse peritoneal macrophages (bottom row).

DISCUSSION

A clone of the well-characterized mouse macrophage-like cell line, J774A.1, was isolated that exhibited a very high level of phagocytosis for *C. perfringens* and *B. subtilis*. The J774-33 cells were able to effectively take up and kill *B. subtilis* under both aerobic and anaerobic conditions (Fig. 2-2B and 2-2D). Therefore, we believe the clone is a useful model for studying how *C. perfringens* interacts with macrophages under a variety of conditions.

C. perfringens is usually considered to be an exclusively extracellular pathogen that secretes powerful cytotoxins that lyse cells and break down connective tissue (152). We have presented evidence in this report that the anaerobic pathogen *C. perfringens* also has the ability to escape the phagosomal compartment. Other intracellular pathogens, including bacterial species of the genera *Listeria*, *Shigella*, and *Rickettsia*, as well as the protozoan, *Trypanosoma cruzi*, have been shown to escape the phagosome and multiply in the cytoplasm of infected cells (31, 63). We believe *C. perfringens* should now be added to that list. Whether *C. perfringens* actually grows when it escapes into the cytoplasm, as do the other intracellular pathogens listed above, could not be determined, because we were unable to develop a method for killing extracellular bacteria that does not also kill intracellular *C. perfringens*. The most likely explanation for this effect is that the bacteria make the cell permeable to penicillin G, the selective agent in the invasion assay (Fig. 2-10). Similar effects were seen with *L. monocytogenes* and *B. subtilis*, when the *C. perfringens pfoA* gene was expressed in these heterologous host bacteria (94, 157).

We have demonstrated that *C. perfringens* can survive in the presence of a phagocytic cell line even under aerobic conditions (Fig. 2-2A). We hypothesize that *C. perfringens* survives because it can escape the phagosome into the cytoplasm. This is based on three major lines of evidence: (1) Electron micrographs indicate intracellular *C. perfringens* are intact (Fig. 2-4); (2) there is no phagosomal membrane around the majority of intracellular *C. perfringens*, while *B. subtilis* is nearly always surrounded by a phagosomal membrane (Fig. 2-4 and 2-5); (3) the late-endosome lysosomal marker LAMP-1 failed to co-localize with intracellular *C. perfringens*, whereas it did co-localize with while *B. subtilis* (Fig. 2-3 and Table 2-1).

C. perfringens produces two toxins that could potentially break down phagosome membranes and release the bacterium: PLC (a phospholipase and sphingomyelinase) and PFO, a pore-forming cytotoxin. PFO has already been shown to be effective at releasing another intracellular pathogen, *L. monocytogenes*, from the phagosome, and allowing the non-pathogenic bacterium, *B. subtilis*, to persist in J774 cells (94, 157). It might be expected that *C. perfringens* can escape the phagosome under anaerobic conditions with these toxins. The surprising results are the *C. perfringens* also seems able to escape the phagosome even under aerobic conditions.

There are probably several factors that allow the anaerobe *C. perfringens* to survive in the J774-33 cells under aerobic conditions: (1) *C. perfringens* is an aerotolerant anaerobe; in atmospheric oxygen concentrations it is unable to grow but does not die rapidly ((173), Fig. 2-2A). (2) Another important factor may be that the oxygen concentration inside macrophages is considerably lower than in the extracellular

environment. James et al. (91) measured the oxygen concentration at the macrophage cell surface and in the phagosomal compartment. When the oxygen concentration was 180 μM in the extracellular environment, it was reduced to 141 μM in phagosomes. When the extracellular oxygen concentration was lowered to 84 or 36 μM, the phagosomal concentration dropped to 31.7 and 7.7 μM, respectively. Therefore, the lower oxygen concentration in the intracellular environment may allow the bacterium to be metabolically active. (3) In our aerobic experiments, the J774-33 and peritoneal macrophages were maintained in standard tissue culture conditions, where the cells are attached to the bottom of a well or flask and covered by a thin layer of medium. Clearly, oxygen can diffuse into the medium, since the J774-33 cells grew well (generation time was 12-14 h) in these conditions, but the respiratory activity of the macrophages may deplete the oxygen enough for *C. perfringens* to maintain many metabolic functions, including synthesizing and secreting toxins that allow them to escape the phagosome.

A unique characteristic of *C. perfringens* gangrenous infections is the lack of inflammatory cells in the infected area, while leukocytes are concentrated in the surrounding tissues and blood vessels (leukostasis). A model for this ineffective inflammatory response has been proposed by Stevens and co-workers (see (29) for a review), where PLC induces the synthesis of inflammatory cytokines and cell adhesion molecules leading to dysregulation of the chemtotactic response. Phagocytic cells are then held in leukostasis. The result is a completely ineffective inflammatory response, and is probably a major factor in the morbidity and mortality of the disease (29). However, this model was based on observations made in advanced cases of the disease

and in animal models, where the bacteria had already multiplied to an enormous level in the host tissue (11, 209). We believe that the ability of *C. perfringens* to persist inside macrophages is an important element in the very early stages of the disease. In this scenario, a wound is usually contaminated with just a few bacteria and there are likely to be large numbers of phagocytic cells present (see the Introduction). However, despite being phagocytosed by macrophages, *C. perfringens* would still be able to survive. If the wound becomes ischemic, the bacteria can begin to multiply and cause a clinical case of gas gangrene. If the wound is well vascularized, the bacteria would eventually be cleared from the site.

Finally, the nonpathogenic bacterium *B. subtilis* was taken up and killed by J774-33 cells under anaerobic conditions (Fig. 2-2D). This is another demonstration that macrophages have the capacity for carrying out the phagocytosis and killing of bacteria under anaerobic conditions. This indicates that macrophages may play an important role in controlling diseases that originate in the many anaerobic niches in the body, including the gastro-intestinal tract, the gum line, the skin, and the genito-urinary tract. Since many anaerobic and facultatively anaerobic pathogens live in these environmental niches, this may be an important immune function of macrophages.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strains used in this study were *C. perfringens* strains 13 and NCTC 8798 (both obtained from C. Duncan), *B. subtilis* strain JH642 (obtained from J. Hoch), *Salmonella enterica* subspecies (*S. typhimurium*) group B. *B. subtilis* and *S. typhimurium* were grown in Luria broth (LB) (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter). *C. perfringens* strains were grown in a Coy anaerobic chamber (Coy Laboratory Products, Inc.) in PGY medium (127) (30 g of proteose peptone, 20 g of glucose, 10 g of yeast extract, and 1 g of sodium thioglycolate per liter).

Isolation of a highly phagocytic clone of mouse macrophage-like cell line J774

The J774A.1 cell line was obtained from the American Type Culture Collection (ATCC). J774A.1 cells were grown in Dulbecco's Modified Eagle's Medium with 4.5 g/l of both glucose and L-glutamine (DMEM) (Biowhittaker), 10% (v/v) fetal bovine serum (FBS), and 5 mM sodium pyruvate. J774A.1 cells were cultured in 5% CO_2 at 37°C in 50 ml tissue culture flasks. The clone, J774-33, was identified from single cells deposited in 96-well culture plates, using a single-cell deposition accessory attached to a FACStar^{plus} cell sorter (Becton Dickinson). The clones were expanded from 96-well plates into progressively larger culture vessels. The percentage of macrophages with at least one cell-associated bacterium was determined as follows: J774A.1 cells were cultured on glass slides in 24-well culture plates to near confluency. Each clone was then infected with *C. perfringens* at an MOI of 10:1, and incubated for 1 h at 37 °C under aerobic conditions. The J774A.1 cells were then washed 3 times with phosphate buffered

saline (PBS), fixed with 1.5% paraformaldehyde in PBS for 20 min at room temperature and washed 3 more times with PBS. The coverslips were then examined in a light microscope using phase contrast.

Survival of J774-33 under anaerobic conditions

The survivability of J774-33 cells under anaerobic conditions was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). This assay measures the cytoplasmic enzyme lactate dehydrogenase (LDH), which is released upon cell lysis. The released LDH was then quantitated through a coupled enzymatic assay, which resulted in the conversion of iodo-nitro tetrazolium chloride (INT) into a red formazan precipitate, with a maximum absorbance at 490 nm. The amount of the precipitate is proportional to the amount of LDH released (Promega Inc.). At the start of the assay, J774-33 cells were washed in 5 ml of PBS and then diluted in fresh DMEM to a concentration of approximately 4 $x10^4$ cells/ml. The J774-33 cells were then added to a 96-well plate and grown overnight in 5% CO_2 at 37°C. The plate was placed in the anaerobic chamber 2 h prior to the start of the assay to allow the medium to become anaerobic. At each time point, quadruplicate samples were assayed. The absorbance at 490 nm was read using a Spectra Max 340 96 well plate reader (Molecular Devices, Sunnyvale, CA).

Survival of bacteria with macrophages under aerobic and anaerobic conditions

Growth curves were performed under aerobic and anaerobic conditions for both *C. perfringens* and *B. subtilis*. *B. subtilis* was grown aerobically in 3 ml LB and shaken overnight at 37°C. *C. perfringens* was grown overnight in 3 ml PGY in the anaerobic

chamber at 37°C. The J774-33 cells were inoculated into 24 well tissue culture plates at approximately 3.5 x 10^5 cells/well and allowed to grow in DMEM overnight in 5% CO_2 at 37°C. The next day, both bacterial cultures were inoculated into pre-warmed media and grown for ~ 3 h to obtain mid-logarithmic phase bacteria. The bacteria were washed three times in PBS, re-suspended in 1 ml of PBS, and then used to infect the macrophages. Bacteria were added at an MOI of 10:1, 5:1, or 1:1. *C. perfringens* is a large (1-2 μm wide x 4-10 μm long) non-motile, rod shaped bacterium. In aerobic experiments, we could easily observe by microscopy that the bacteria had settled to the bottom of the wells in a few minutes without any force (i.e., centrifugation) being applied. For anaerobic experiments, the 24 well plate was placed in the anaerobic chamber 2 h prior to infection with bacteria. After the addition of bacteria, the macrophages were lysed at the indicated times and the surviving *C. perfringens* cells were plated on PGY agar medium and incubated anaerobically. To lyse the J774-33 cells, one hour prior to each time point Triton X-100 was added to the respective wells to a final concentration of 0.02% (higher levels of Triton X-100 were toxic to *C. perfringens*). After the infected macrophages were incubated with the Triton X-100 for 1 h, the macrophage suspensions were pipetted vigorously to ensure the release of phagocytosed bacteria. The samples were then serially diluted in PBS and plated on PGY (*C. perfringens*) or LB (*B. subtilis*).

Transmission Electron Microscopy

Electron microscopy experiments were done in collaboration with K. G. Murti, St. Jude Children's Research Hospital and K. Troughton, Dept. of Neuroanatomy, UT

Memphis. For transmission electron microscopy (TEM), J774-33 cells were grown in DMEM to near confluency in 50 ml tissue culture-treated flasks. *C. perfringens* strain 13 cells were grown in PGY medium to mid-log phase and then washed three times in PBS to remove extra-cellular toxins. The bacteria were added at an MOI of 50:1 to the macrophage culture and incubated at 37°C for either 60 or 90 min. For anaerobic experiments the macrophages were given fresh media and then placed in the anaerobic chamber two h before the addition of bacteria. The infected macrophages were washed with PBS and then fixed in either 2.0% or 2.5% glutaraldehyde in PBS for 5 minutes, pelleted by a 1 minute spin at 200 x g and left in the fixative for 1-2 h at room temperature. The pellets were then processed as previously described (137) or by the following procedure: The pellets were post-fixed in 1% osmium tetroxide for 1-2 h, rinsed with water and stained with 2% uranyl acetate in 0.85% NaCl for 1 h. The pellets were then dehydrated in 30%, 50%, 70%, 85%, 95% ethanol for 15 minutes each, followed by three incubations in 100% ethanol for 30 minutes each. The macrophages were infiltrated in 50% ethanol/Spurr epoxy overnight and in 100% Spurr for 8 h, changing the Spurr three times. The plastic was cured at 60°C for two days. One micron sections were cut and stained with 0.1% toluidin blue in 1% sodium borate for light microscopy. Seventy-five nm sections were cut and counterstained with uranyl acetate-lead citrate. These thin sections were viewed and photographed on a JEOL 2000EX electron microscope at 60 kV.

Invasion assays

J774-33 cells were inoculated into a 24 well tissue culture plate at a concentration of approximately 3.5 x 10^5 cells/ml. For anaerobic conditions, the macrophage plates were placed in the anaerobic chamber 2 h prior to infection with the bacteria. *C. perfringens* was prepared from mid-logarithmic phase cultures as described above, spun down and washed three times in sterile PBS, re-suspended in 1 ml of PBS, and added to the appropriate monolayer. Aerobically grown *S. typhimurium* was added directly from a 3 ml overnight culture grown unshaken in LB. For both anaerobic and aerobic conditions, the bacteria were incubated with the macrophages for 1 h at 37°C. Then each well was washed five times with 1 ml of PBS. Anaerobic PBS was used for the anaerobic plates. To determine the number of cell-associated bacteria, 1 ml of distilled water was added to one third of the wells and pipetted vigorously to lyse the J774-33 cells. Anaerobic distilled water was used for the anaerobic plates. The suspensions were then serially diluted in PBS, and then plated to record the number of colony forming units. To the remaining wells, 1 ml of fresh DMEM containing penicillin G was added, at a final concentration of 100 μg/ml for *S. typhimurium*, and 5μg/ml for *C. perfringens*. We had previously determined the minimum inhibitory concentration (MIC) of penicillin G to be 0.75 μg/ml for *C. perfringens* strain 13 and 12.5 μg/ml for *S. typhimurium*. The antibiotic-containing media for *C. perfringens* was made anaerobic before adding it to the wells. The plates were then incubated at 37°C for the times indicated, after which they were washed 3 times with PBS. One ml of distilled H_2O was then added to each well and the J774-33 cells were then lysed, diluted, and plated out, as described above.

Cytochalasin D was added, when indicated, to a final concentration of 1 μg/ml for 30 min before the bacteria were added to each well and was maintained for the duration of the experiment.

Immunofluorescence confocal microscopy

To assay intracellular and extracellular bacteria, 12 mm round glass coverslips were placed in 24 well tissue culture plates, and the J774-33 cells were added and grown in DMEM until nearly confluent. Log phase *C. perfringens* and *B. subtilis* were then added at an MOI of 10:1. The plates were incubated in 5% CO_2 at 37°C for 1 h. When cytochalasin D was used, it was added to each well 30 min before the bacteria were added, at a final concentration of 1μg/ml. The media was removed from the wells and the coverslips were washed once in PBS. The intracellular bacteria and LAMP-1 proteins were localized by immunofluorescence, based on a previously described method (8): The cells were fixed in periodate-lysine-paraformaldehyde (125) containing 5% sucrose at room temperature for 20 min and then washed three times in PBS. The cells were then permeablized by dipping the coverslips in cold (-20°C) methanol for 10 s and washed three times for 5 min with 2% goat serum in PBS (blocking buffer) at room temperature. We discovered that the anti-*C. perfringens* rabbit antibodies cross-reacted with *B. subtilis* whole cells (data not shown). Therefore, to label both *C. perfringens and B. subtilis*, the samples were stained with anti-*C. perfringens* polyclonal rabbit serum (Applied Diagnostics International) diluted 1:100 in blocking buffer and incubated for 1 h at 37°C. Following three 5 min washes in blocking buffer, the cells were stained with anti-LAMP-1 rat monoclonal antibody 1D4B diluted 1:200 in blocking buffer and incubated for 1

hour at 37°C. (The 1D4B monoclonal antibody developed by J. T. August was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). The coverslips were washed three times in blocking buffer for 5 min at room temperature. The cells were then stained simultaneously with Texas Red-X-conjugated goat anti-rat IgG diluted 1:200 and FITC-conjugated goat anti-rabbit IgG diluted 1:200 (Molecular Probes) for 1 h at 37°C. The coverslips were then washed 3 times in PBS and mounted on slides with ProLong anti-fade mounting medium (Molecular Probes). Samples were viewed using a Zeiss LSM 510 laser confocal microscope at excitation wavelengths of 448 and 543 nm, for FITC and Texas Red, respectively. To produce 3-dimensional images, an average of 20 cross-sectional images were captured over a vertical distance of 8-15 μm.

Mouse peritoneal macrophages

Female C3HeB/FeJ Mice were given intra-peritoneal injections of heat killed *Listeria monocytogenes* alone (unactivated), or with *L. monocytogenes* and Interleukin-12 (activated) (130-132). Peritoneal exudate cells were harvested by peritoneal lavage and the macrophages were grown in 50 ml tissue culture flasks. Peritoneal macrophages were grown for two days in DMEM, infected with *C. perfringens* at MOI of 50:1 and processed for electron microscopy as described above.

ACKNOWLEDGEMENTS

We thank William Walker, Saint Jude Children's Research Hospital (SJCRH), for many helpful discussions and isolation of the J774-33 clone. We also thank Janet Gatewood and Richard Cross (SJCRH) for help in isolating the J774-33 clone, K. G. Murti (SJCRH) and Katherine Traughton (UT Memphis) for electron microscopy assistance, Mark Miller (UT Memphis) for providing mouse peritoneal macrophages and Thomas Hatch (UT Memphis) for critical reading of the manuscript.

CHAPTER THREE

Multiple effects on *Clostridium perfringens* binding, uptake and trafficking to lysosomes by inhibitors of macrophage phagocytosis receptors

This paper has been accepted for publication in *Microbiology*

ABSTRACT

Clostridium perfringens is a Gram positive, anaerobic bacterium that is the most common cause of gas gangrene (clostridial myonecrosis) in humans. *C. perfringens* produces a variety of extracellular toxins that are thought to be the major virulence factors of the organism. However, *C. perfringens* has recently been shown to have the ability to survive in a murine macrophage-like cell line, J774-33, even under aerobic conditions. In J774-33 cells, *C. perfringens* can escape the phagosome and gain access to the cytoplasm. Since the receptor that is used for phagocytosis can determine the fate of an intracellular bacterium, we used a variety of inhibitors of specific receptors to identify those used by J774-33 cells to phagocytose *C. perfringens*. It was found that the scavenger receptor, and mannose receptor(s) were involved in the phagocytosis of *C. perfringens*. In the presence of complement, the complement receptor (CR3) was also involved in the binding and/or uptake of *C. perfringens*. Since the receptor inhibition studies indicated the scavenger receptor played a major role in phagocytosis, we performed *C. perfringens* binding studies with a Chinese hamster ovary (CHO) cell line expressing the mouse SR-A receptor. The cell line expressing the SR-A receptor showed a significant increase in *C. perfringens* binding in comparison to the non-transfected CHO cells. In the absence of opsonizing antibodies, the Fc receptor was not used to phagocytose *C. perfringens*. Forcing the macrophages to use a specific receptor by using combinations of different receptor inhibitors led to only a slight increase in co-localization of intracellular *C. perfringens* with the late endosome-lysosome marker LAMP-1. Carbohydrate analysis of *C. perfringens* strain 13 extracellular polysaccharide

confirmed the presence of mannose and negatively charged residues of glucuronic acid, which may provide the moieties that promote binding to the mannose and scavenger receptors, respectively.

INTRODUCTION

C. perfringens is a ubiquitous, anaerobic spore-forming bacterium, found at high levels in the large intestine, on the skin, and in soil and freshwater sediments (173). In humans, *C. perfringens* causes anaerobic cellulitis, gas gangrene, enteritis necroticans (Pigbel), and food poisoning (173). Gas gangrene, or clostridial myonecrosis, is an infection that originates in ischemic tissues in which the blood supply has been cut off due to trauma or circulatory blockages. Once the infection begins, it rapidly spreads to healthy tissues and, if left untreated, the disease is always fatal due to the release of toxins into the bloodstream resulting in severe shock and cardiac stress (10, 208).

As a species, *C. perfringens* makes thirteen different toxins, which are thought to be the major virulence factors of the diseases it causes (172). However, we recently presented evidence that *C. perfringens* can persist inside macrophages, even under aerobic conditions, perhaps by escaping the phagosome and gaining access to the cytoplasm (149). *C. perfringens* was not killed by the cells of a clone, J774-33, of the macrophage-like murine cell line J774A.1, under aerobic or anaerobic conditions, while the non-pathogenic bacterium, *Bacillus subtilis*, was killed by J774-33 cells under both conditions. *C. perfringens* is also capable of surviving in the presence of mouse peritoneal and bone marrow derived macrophages (O'Brien and Melville, unpublished results). Electron microscopic evidence indicated *C. perfringens* can escape the phagosome of J774-33 cells and both activated and unactivated mouse peritoneal macrophages (149). Therefore, the ability to escape the phagosome and persist in the

cytoplasm of macrophages may be important in the earliest stages of a gangrene infection, when bacterial numbers are low and phagocytic cells are present.

One factor that is important in intracellular trafficking and/or survival inside macrophages is the receptor used to phagocytose the bacterium. For example, phagocytosis using the Fc and mannose receptors lead to a strong oxidative burst but phagocytosis using the complement receptor alone avoids the oxidative burst (2). The receptor(s) used by macrophages to phagocytose *C. perfringens* has never been identified. Therefore, we used inhibitors of receptor binding to identify the specific receptors used by J774-33 cells to phagocytose *C. perfringens*. We have found that the scavenger, mannose and complement receptor (CR3), were all involved in the phagocytosis process. We have also determined that *C. perfringens* strain 13, the strain used in these studies, produces a polysaccharide capsule that is likely recognized by phagocytosis receptors on the surface of macrophages.

RESULTS

C. perfringens phagocytosis is mediated by a variety of receptors on J774-33 cells.

Since macrophages are unable to kill *C. perfringens* and the receptor used for phagocytosis can influence the fate of intracellular bacteria, we used specific inhibitors to block receptors known to function during the phagocytosis of bacteria by macrophages. The number of cell-associated bacteria (intracellular and surface-bound) seen with J774-33 macrophages under aerobic conditions with different receptor inhibitors was determined using laser confocal microscopy. To do this, the cytoplasm of the J774-33 cells was stained with Cell-Tracker Green and the bacterial (and J774-33) nucleic acids were stained with propidium iodide. A representative image is shown in Fig 3-1A.

The addition of fucoidin, an inhibitor of the scavenger receptor on macrophages (20), led to the highest level of inhibition of bacterial cell association, 80% (Fig. 3-2A), indicating the scavenger receptor played a major role in the process. The addition of methyl-α-D-mannopyranoside resulted in a 50% reduction in the number of cell-associated bacteria (Fig. 3-2B), while mannan only inhibited attachment and uptake by 20% (Fig. 3-2C). The use of heat-treated serum (HTS), i.e., inactivated complement, led to about a 50% reduction in the number of cell-associated bacteria over the course of 60 min (Fig. 3-2D). The addition of guinea pig complement to the assay using HTS restored most of the binding and internalization that was lost with HTS (Fig. 3-2E). The addition of a blocking antibody to the complement receptor (CR3) resulted in comparable amounts of inhibition when compared to the use of HTS (compare Fig. 3-2F to Fig. 3-2D).

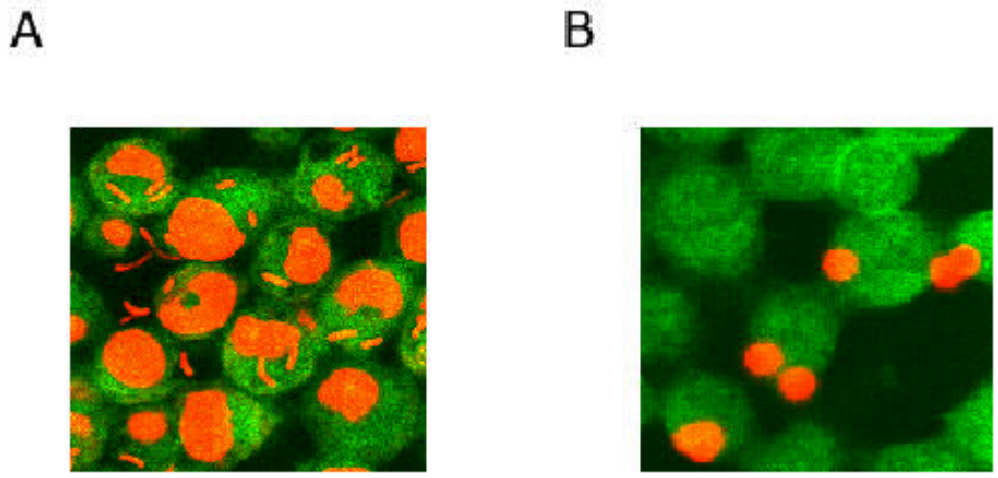

Figure 3-1A. Laser confocal microscopy images of J774-33 cells infected with *C. perfringens*. The cytoplasm of the J774-33 cells was stained with Cell-Tracker green and the bacterial and J774-33 nuclei were stained with propidium iodide. **1B. Laser confocal microscopy images of J774-33 cells with added fluorescent polystyrene microspheres.** The cytoplasm of the J774-33 cells was stained with Cell-Tracker green and the microspheres fluoresce red.

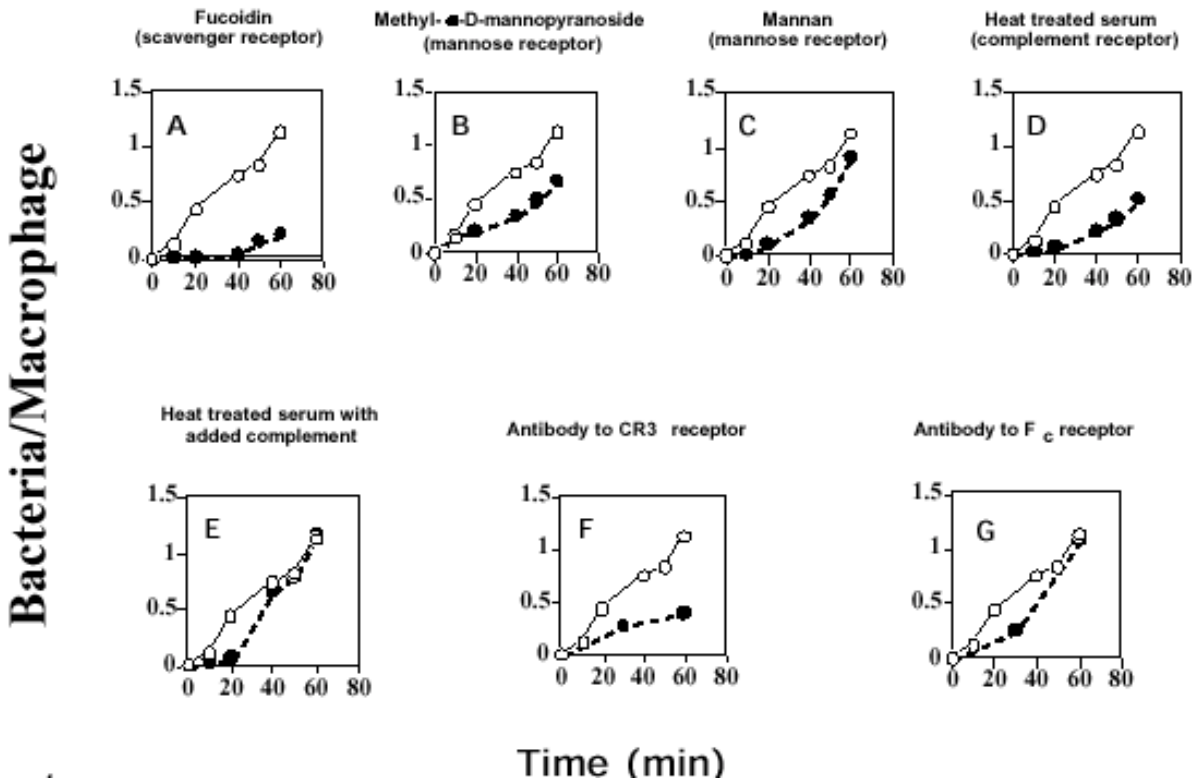

Figure 3-2. Number of cell-associated bacteria detected after infecting J774-33 cells with *C. perfringens* under aerobic conditions in the presence of specific inhibitors of phagocytosis receptors. Cell associated bacteria were defined as those that were either attached to the surface or were intracellular, as determined by laser confocal microscopy (see Methods). A-G: The open circles represent conditions in the absence of inhibitors. The results when the receptor was blocked with the reagent listed above each graph are shown as closed circles. For comparison, the same curve for standard conditions is shown for each of the graphs. At each time point, the number of attached and intracellular bacteria was determined for 100 macrophages. Each inhibitor treatment was tested in at least three independent experiments; representative results are shown.

In contrast, blocking the Fc receptor (FcR) with an FcR-specific antibody led to only a slight decrease in the number of cell-associated bacteria (Fig. 3-2G), indicating the FcR did not play a role in *C. perfringens* uptake in the absence of opsonizing antibodies.

The *C. perfringens* receptor inhibition assays were also performed under anaerobic conditions (Fig. 3-3), because the anaerobic *C. perfringens* has been shown to survive better and is more cytotoxic to macrophages in the absence of oxygen (149). The results were similar to that seen under aerobic conditions, with two exceptions: (1) The addition of methyl-α-D-mannopyranoside led to a significantly greater level of inhibition of *C. perfringens* attachment and internalization than was seen under aerobic conditions (compare Fig. 3-3B and 3-2B), and (2) The addition of guinea pig complement to the HTS did not fully restore the binding of *C. perfringens* during the first 50 min of the assay (Fig. 3-3E),

Binding of *C. perfringens* to SR-A transfected CHO cells.

Additional evidence that the scavenger receptor SR-A was involved in the binding and uptake of *C. perfringens*, was obtained using mouse SR-A transfected CHO cells. The percent of cell-associated bacteria per coverslip in the presence and absence of fucoidin was determined (Fig 3-4). We found an average of 25 cell-associated bacteria per coverslip when SR-A transfected CHO cells were infected with *C. perfringens*. The average number of cell-associated bacteria decreased to 7 when fucoidin was added to transfected CHO cells (Fig. 3-4). This indicated that the scavenger receptor was involved in the binding of *C. perfringens* and that fucoidin could decrease the number of cell-associated bacteria. Wild-type CHO cells infected with *C. perfringens* in the presence

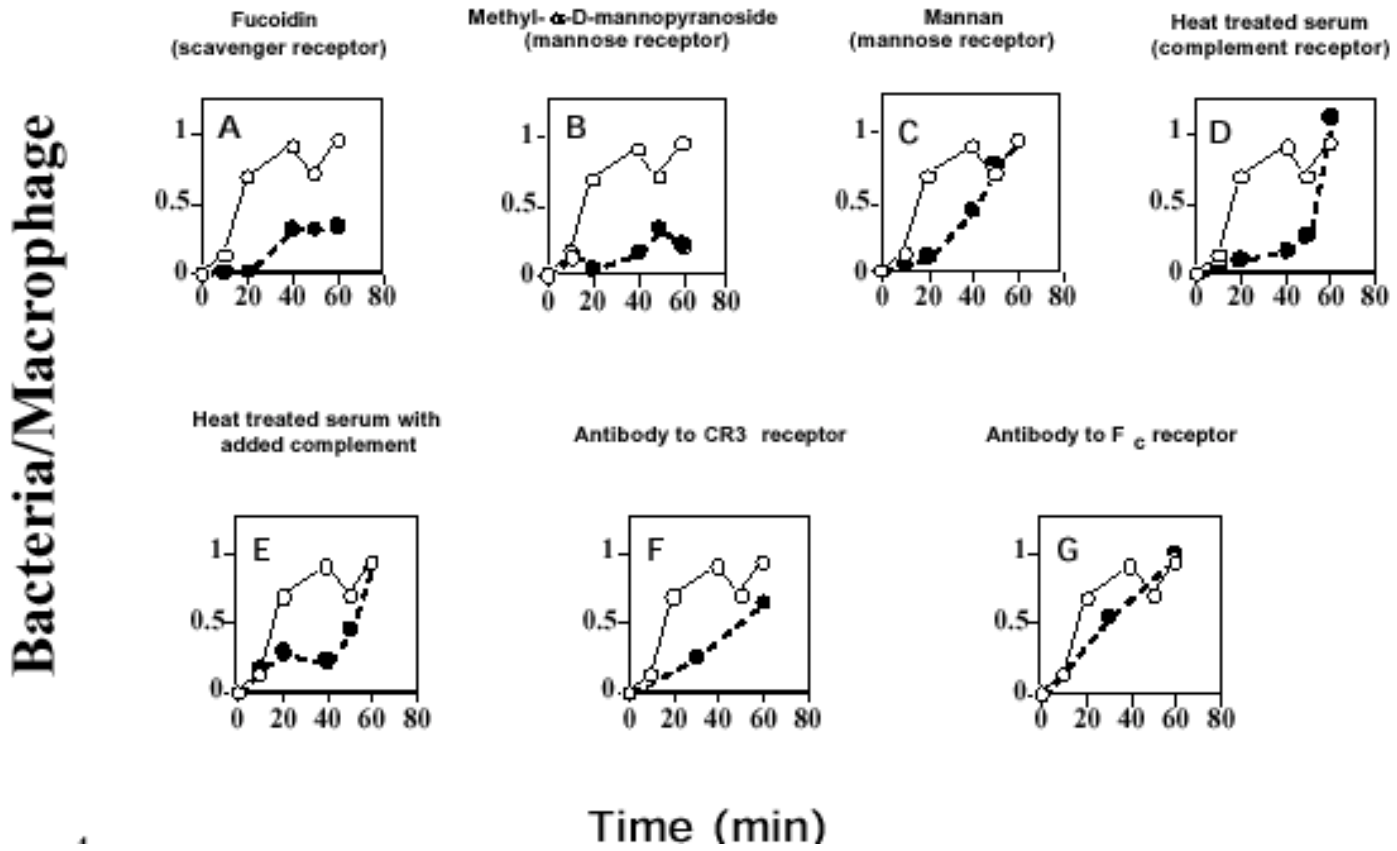

Figure 3-3. Number of cell-associated bacteria detected after infecting J774-33 cells with *C. perfringens* under anaerobic conditions in the presence of specific inhibitors of phagocytosis receptors. A-G: The open circles represent conditions in the absence of inhibitors. The results when the receptor was blocked with the reagent listed above each graph are shown as closed circles. For comparison, the same curve for standard conditions is shown for each of the graphs. At each time point, the number of attached and intracellular bacteria was determined for 100 macrophages. Each inhibitor treatment was tested in at least three independent experiments; representative results are shown.

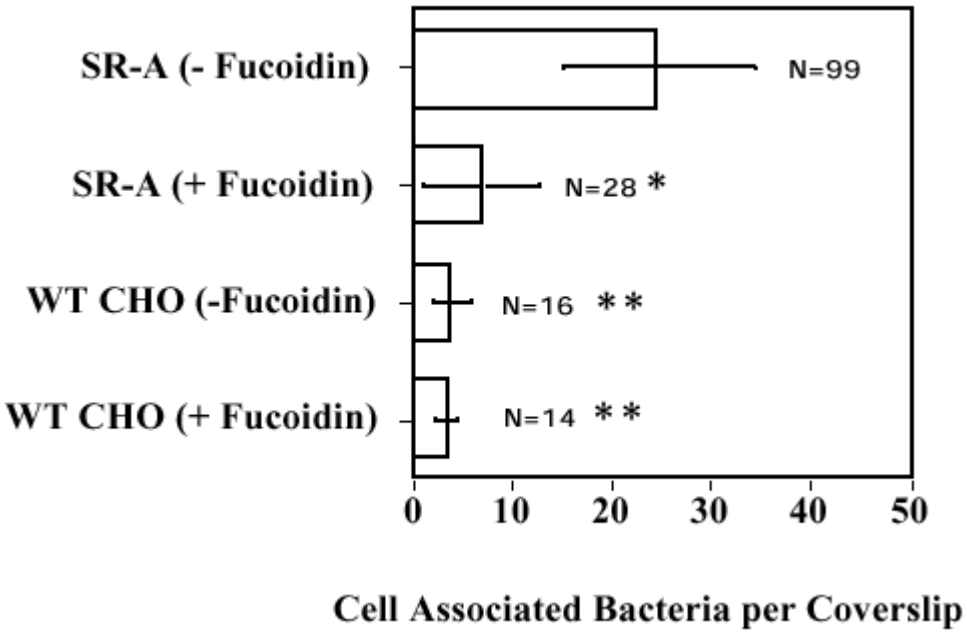

Figure 3-4. Number of cell-associated bacteria detected after infecting CHO and SR-A transfected CHO cells under aerobic conditions for 60 min in the presence and absence of fucoidin. The data is represented as the number of cell-associated bacteria/coverslip. Results shown are the mean (+/- SD) of at least three independent experiments. Statistically significant from SR-A transfected CHO cells in the absence of fucoidin, using the Students unpaired *t*-test (two tailed); *, $P < 0.05$; **, $P < 0.01$.

and absence of fucoidin gave an average of 4 and 3.5 cell-associated bacteria per coverslip, respectively. The lack of inhibition seen in the presence of fucoidin, is consistent with the fact that there are no SR-A receptors on CHO cells.

Efficient internalization of *C. perfringens* requires the simultaneous activity of the mannose, CR3, and scavenger receptors.

The data in Fig. 3-2 and Fig. 3-3 represent the numbers of cell-associated bacteria present during the course of the assay, including intracellular bacteria and bacteria bound to the surface of the macrophage. To differentiate between binding to a receptor and subsequent internalization by the macrophage, the percentage of intracellular to extracellular (i.e., on the macrophage surface) was determined using laser confocal microscopy for the aerobically infected cells at 60 min (Fig. 3-5). Addition of the receptor inhibitors led to a decrease in the efficiency of internalization by 25-70% for all the inhibitors used, except for the anti-FcR antibody, which showed no decrease in the efficiency of internalization after binding (Fig 3-5). In the absence of complement (i.e. use of HTS), the percent of *C. perfringens* internalization decreased more than 2-fold; the addition of guinea pig complement to the HTS restored uptake to the same level as the untreated control (Fig. 3-5).

Red fluorescent polystyrene microspheres were used as phagocytosis controls to determine if the addition of receptor inhibitors resulted in a generalized inhibition of phagocytosis by the J774-33 macrophages. The cytoplasm of J774-33 cells was stained with Cell-Tracker green and confocal microscopy was used to determine the percent of cell-associated microspheres that were intracellular (a representative image is shown in

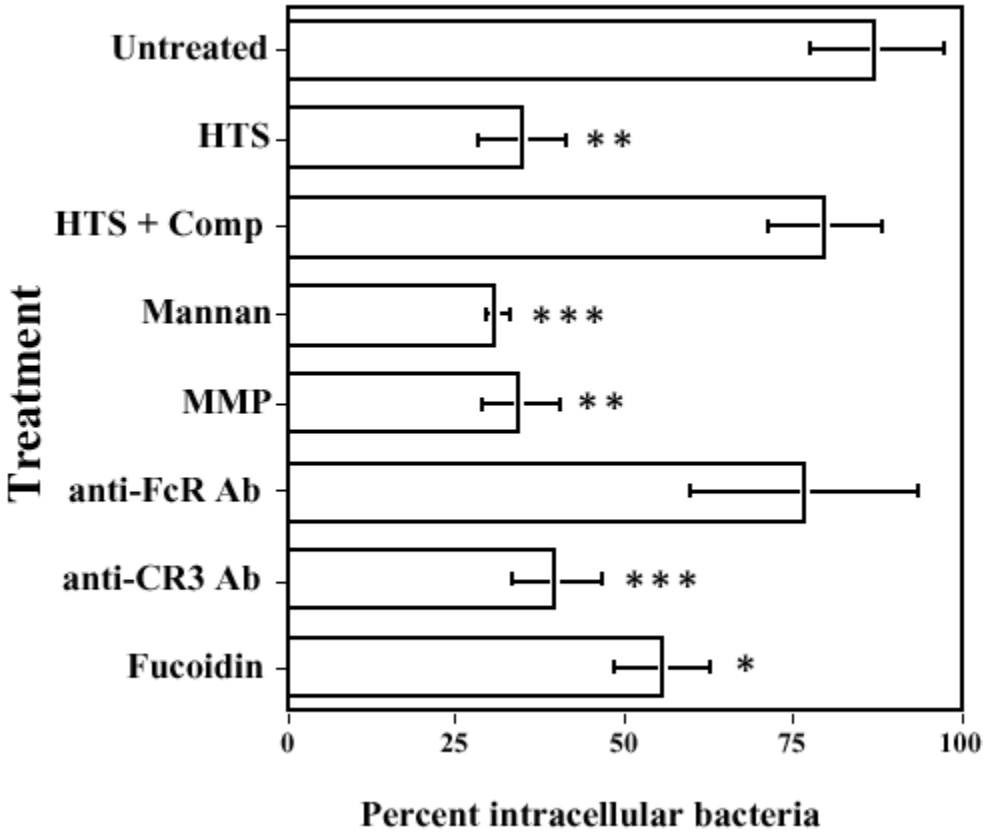

Figure 3-5. The percent of cell-associated *C. perfringens* that were intracellular 60 min after infection of J774-33 cells under aerobic conditions, as determined using laser confocal microscopy. One hundred macrophages were examined. Results shown are the mean (+/- SD) of at least three independent experiments. HTS, heat-treated serum; Ab, blocking antibody; MMP, methyl α-D mannopyranoside; COMP, guinea pig complement. Statistically significant from untreated J774-33 cells using the Students unpaired *t*-test (two tailed); *, P< 0.01; **, P< 0.005; *** P< 0.001.

Fig 3-1B). In the absence of any treatment, 18% of the cell-associated microspheres were intracellular (Fig 3-6), which was about 5-fold lower than the ratio of internal vs. cell-bound for untreated *C. perfringens* (Fig 3-5). In contrast to the results seen with *C. perfringens*, the ratio of internal vs. bound microspheres was unchanged by the addition of any of the receptor inhibitors tested (Fig 3-6), indicating that addition of the inhibitors did not result in a generalized blockage of phagocytosis by the J774-33 cells. Even though there are two mechanisms of phagocytosis (35), both utilize filamentous actin polymerization; this feature of phagocytosis was not affected by the addition of receptor inhibitors.

In order to demonstrate that our inhibitor treatments could block phagocytosis and were specific for the receptors we were testing, microspheres were coated with mouse IgG before addition to the macrophages to stimulate their uptake via the FcR (see Materials and Methods). The addition of a blocking antibody specific to the FcR resulted in a 4-fold decrease in internalization of the IgG-coated beads (Fig. 3-6), indicating that blocking the FcR receptor led to an inhibition of phagocytosis via that specific receptor.

Co-localization with the late endosome-lysosome marker LAMP-1 in the presence of inhibitors of receptors.

We looked to see if the rate of co-localization with LAMP-1, an indicator of phagosome/lysosome fusion, would change in the presence of receptor inhibitors and/or combinations of inhibitors. A mixture of inhibitors was used to force the bacteria to be taken up by a single receptor. For these assays, J774-33 cells were infected with *C. perfringens* and the amount of co-localization with LAMP-1 was measured using laser

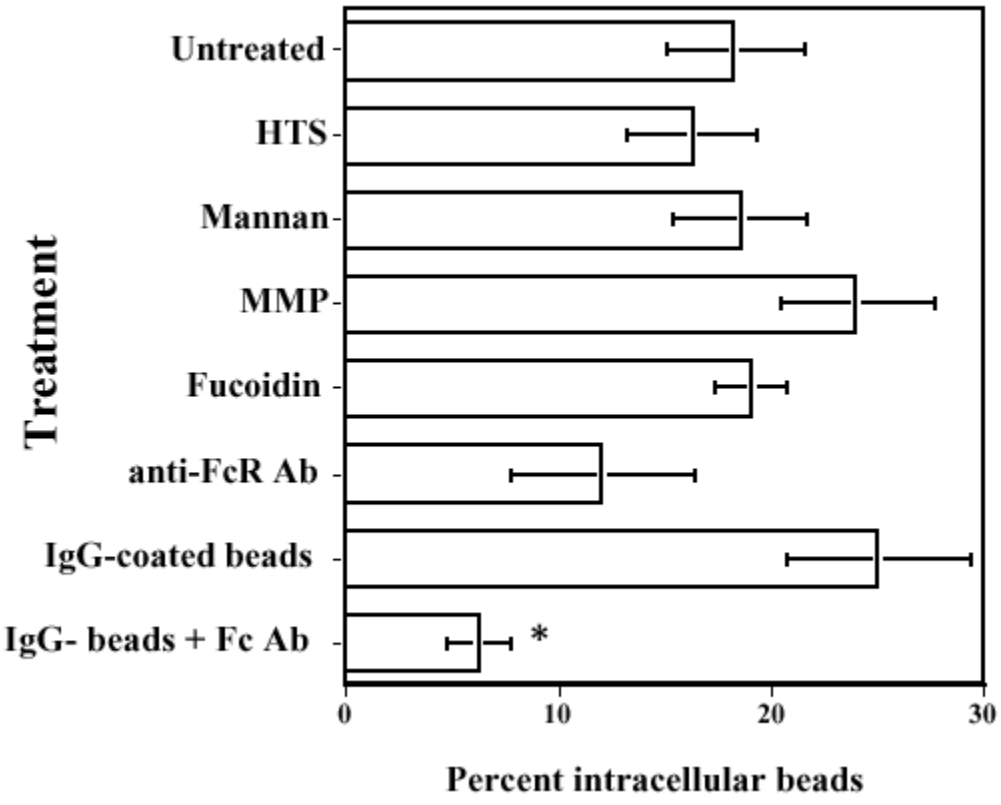

Figure 3-6. The percent of cell-associated fluorescent microspheres (beads) that were intracellular 60 min after addition to J774-33 cells under aerobic conditions, as determined using laser confocal microscopy. One hundred macrophages were examined. Results shown are the mean (+/- SD) of at least three independent experiments. HTS, heat-treated serum; Ab, blocking antibody; MMP, methyl α-D mannopyranoside. Statistically significant from the IgG coated beads using the Students unpaired *t*-test (two tailed); *, $P < 0.005$.

confocal microscopy (Fig. 3-7). The non-pathogenic bacterium *B. subtilis* was used as a positive control, and was found to co-localize with the LAMP-1 marker 63% of the time, while only 9% of the *C. perfringens* (with untreated J774-33 cells) co-localized with LAMP-1 (Fig. 3-7). Of the single inhibitors added, only the addition of fucoidin (i.e., functional CR3 and mannose receptors) gave a statistically significant increased level of LAMP-1 co-localization in comparison to untreated J774-33 cells (Fig 3-7). Combinations of two inhibitors were added to permit the uptake of *C. perfringens* to occur by only a single receptor; however, no significant difference was seen in the amount of co-localization with LAMP-1 as compared to the untreated J774-33 cells (Fig. 3-7). Although the addition of fucoidin gave increased levels of co-localization, the combination of fucoidin and either methyl α-D mannopyranoside or heat-treated serum did not show a statistically significant increase in co-localization with LAMP-1. Possibly, the complement and mannose receptors need to act together to achieve the most effective internalization of bacteria. When *C. perfringens* was opsonized with antibodies, a statistically significant increase in the number of *C. perfringens* co-localized with LAMP-1 was seen in comparison to the untreated J774-33 cells. Interestingly, while fucoidin treatment or opsonization of *C. perfringens* gave a statistically significant increase in co-localization efficiencies, the levels were only one-third of that seen with the *B. subtilis* control (Fig 3-7).

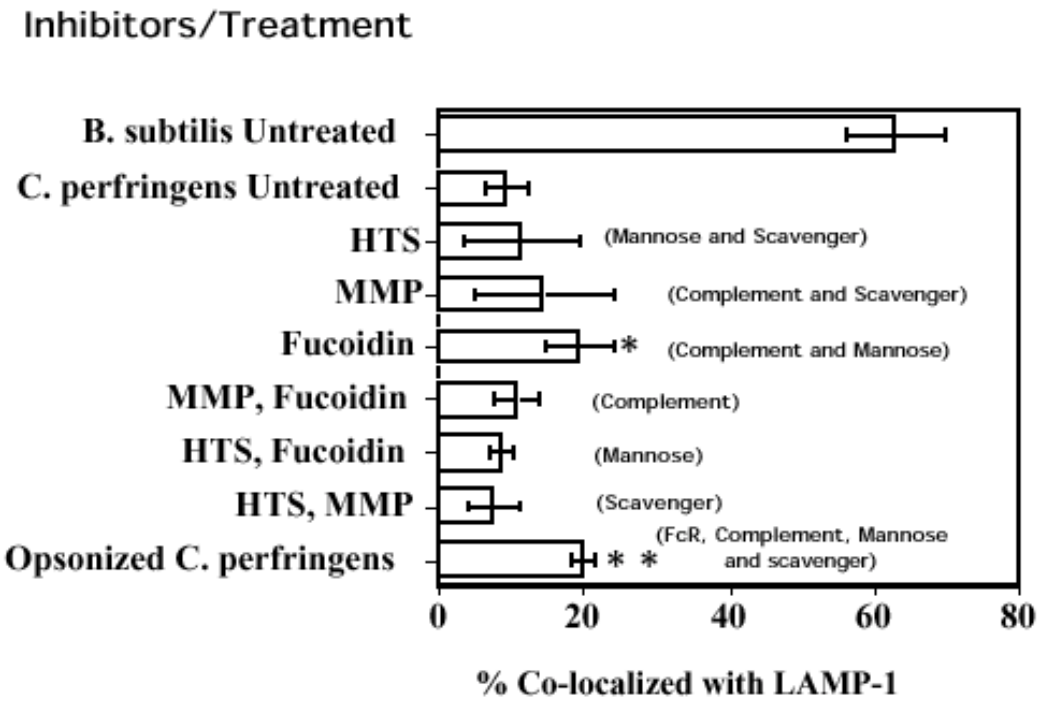

Figure 3-7. The percent of intracellular *C. perfringens* that co-localized with the late endosome-lysosomal marker protein LAMP-1 60 min post-infection with *C. perfringens* under aerobic conditions. For comparison, the results after infection with the non-pathogenic bacterium, *B. subtilis*, are also included. The unaffected receptors shown for each condition are listed in parentheses. Between 19 and 121 intracellular bacteria were examined for LAMP-1 co-localization (for some inhibitor experiments, few intracellular bacteria were detected). Results shown are the mean (+/- SD) of at least three independent experiments. HTS, heat-treated serum; MMP, methyl α-D mannopyranoside; opsonized, *C. perfringens* cells were opsonized with rabbit anti-*C. perfringens* serum. Statistically significant from untreated *C. perfringens* using the Students unpaired *t*-test (two tailed); *, $P < 0.01$; **, $P < 0.005$.

Electron microscopy of macrophage-bound *C. perfringens* shows macrophages bind to an extracellular matrix on the surface of the bacteria.

We have presented evidence that *C. perfringens* can bind to at least three different receptors on macrophages. Therefore, we wanted to visualize the interactions of *C. perfringens* with these receptors on the surface of J774-33 cells. To increase the number of surface bound *C. perfringens*, attachment to the macrophages was allowed to occur at a temperature (4°C) in which phagocytosis was inhibited. After binding of the bacteria to the macrophage had occurred, the cells were shifted to 37°C and fixed at later time points. The contact points between the macrophages and the bacteria were visualized by transmission electron microscopy. Representative images at 0, 10, and 20 min after being transferred to 37°C are shown in Fig. 3-8. At 0 min, all of the bacteria were seen as extracellular, indicating the low temperature was effective at blocking uptake of the bacteria, but not binding to the surface. By 20 min, nearly all of the cell-associated bacteria examined were intracellular, indicating that phagocytosis proceeded once the macrophages were transferred to the warmer temperature. Interestingly, very few of the intracellular bacteria had escaped the phagosome after 20 min. This is in contrast to our previously reported results (149) and this may be due to the exposure of the macrophages and the bacteria to a low temperature of 4°C. The macrophages bound the bacteria at a fibrous layer external to the bacterial cell wall (arrows in insets of Fig. 3-8).

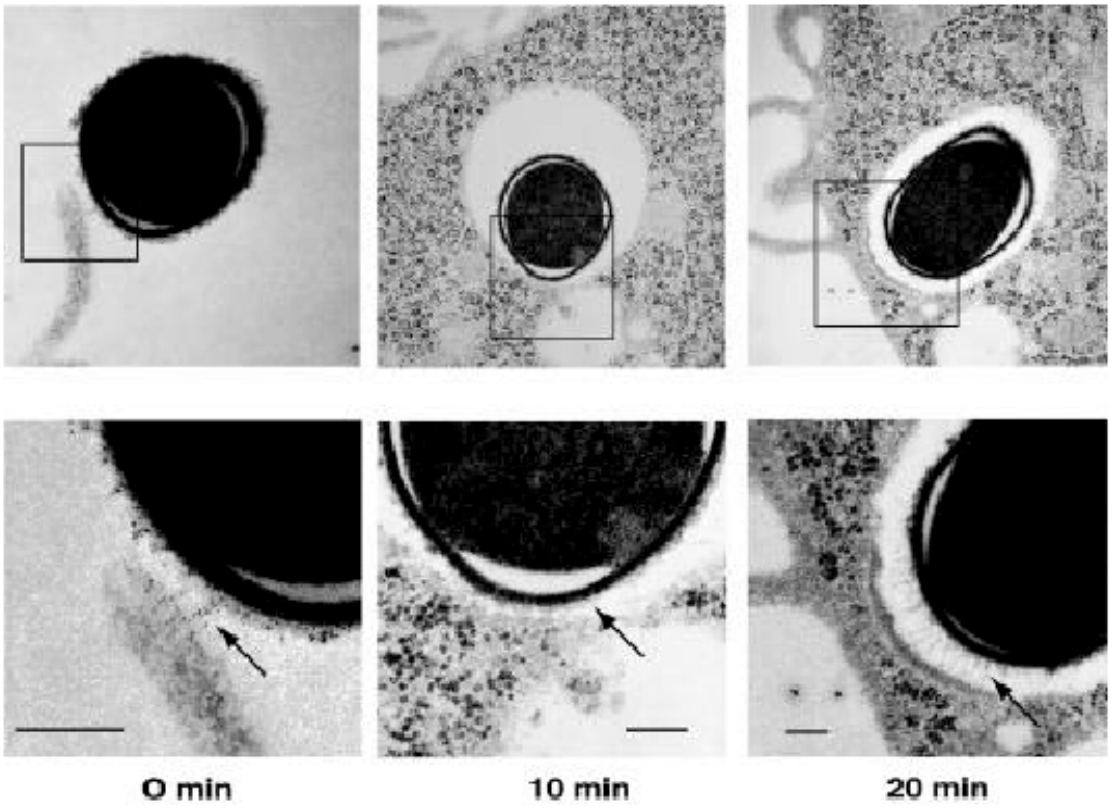

Figure 3-8. Transmission electron microscopy images of J774-33 cells infected with *C. perfringens*. Binding of the bacteria was synchronized by infecting the macrophages at 4°C, and then transferring the infected macrophages to 37°C. At the times indicated, the macrophages were fixed and processed for electron microscopy. Representative images are shown for each time point. Boxes in upper panels show regions magnified in the panels shown in the bottom row. The arrows point to an extracellular matrix material outside of the electron-dense cell wall of *C. perfringens*. Bars: 200 nm.

Composition analysis of the *C. perfringens* strain 13 extracellular polysaccharide indicate the presence of residues that may interact with phagocytic receptors.

Polysaccharide capsule of *C. perfringens* was purified and subjected to carbohydrate composition analysis (see Materials and Methods). After analysis, it was found that the polysaccharide contained six sugar residues: glucose (38.6%), galactose (23.1%), mannose (19.5%), glucuronic acid (9.5%), N-acetyl-galactosamine (8.7%) and trace amounts of N-acetyl-glucosamine (0.6%). All of these sugar residues have been found in the bacterial capsule of another strain of *C. perfringens* (97), indicating the extracellular fibrous layer on strain 13 is probably a capsular polysaccharide.

DISCUSSION

The results of these studies indicate that the scavenger, mannose, and CR3 receptors (in the presence of complement) were used for binding and phagocytosis of *C. perfringens* by J774-33 cells (Fig. 3-2 and 3-3). J774-33 cells were used in this study since they readily bind and phagocytose *C. perfringens* and other Gram-positive bacteria (149). J774 cells are known to express the scavenger receptor (51) and the Fc receptor (122) and some clones express the mannose receptor (61). They also express the complement receptor, CR3 (73), but not CR1 or CR2 (122).

At least on of these receptors has the capacity to bind two different receptors. For example, besides binding C3bi, CR3 has a lectin-binding site that recognizes α-methylpyrannosides, among other carbohydrates (54). Therefore, in the experiments where methyl-α-D-mannopyranoside was added, there may have been inhibition of binding to both the mannose receptor and CR3.

The FcR was not used for phagocytosis of *C. perfringens* by J774-33 cells in the absence of opsonizing antibodies. *C. perfringens* infections are usually of sudden onset after exposure to the bacterium; times as short as six hours after wounding have been reported for the onset of gas gangrene (204). Therefore, it is questionable whether the humoral immune response plays a significant role in most myonecrotic infections, since it takes several days for high levels of antibodies to be produced.

The data in Figs 3-2 and 3-3 depicts the effects receptor inhibitors had on the total number of cell-associated bacteria, including attached and intracellular bacteria. In order to determine if the inhibitors affected uptake in a manner independent of binding to the

receptor, we ascertained the percent of cell-associated bacteria that were intracellular 60 min post-infection (Fig. 3-5). All of the inhibitors tested, except for the anti-FcR antibody, resulted in a decreased level of uptake of cell-associated bacteria. However, even when the total number of cell-associated bacteria was only slightly affected, as with the addition of mannan (Fig. 3-2), the internalization of the bacteria in the presence of mannan was still affected (Fig. 3-5). This suggests that while the scavenger, mannose, and complement receptors are all involved in the binding and internalization of *C. perfringens*, the most effective internalization of the bacteria occurred when all of the receptors were involved in the phagocytosis process. The total amount of inhibition seen with the addition of inhibitors in separate experiments adds up to greater than 100% (Fig. 3-2 and 3-3), indicating there was some lack of specificity in their effects on the specific receptors we have identified as taking part in binding *C. perfringens*.

Of the three receptors used for phagocytosis of *C. perfringens* the scavenger receptor was the most important. The most dramatic decrease in the number of cell-associated bacteria was seen when J774-33 cells were infected in the presence of the scavenger receptor inhibitor fucoidin. To examine the role of the SR-A scavenger receptor plays in the phagocytosis of *C. perfringens*, we used CHO cells transfected with the murine SR-A receptor. We found that binding of *C. perfringens* to CHO cells was enhanced greater than 8-fold in the presence the SR-A receptor (Fig. 3-4). When fucoidin was added the number of cell-associated bacteria decreased greater than 3-fold. This indicates fucoidin can act as a specific inhibitor of *C. perfringens* binding. This

supports our J774-33 inhibitor studies that showed the scavenger receptor is important in the binding and uptake of *C. perfringens*.

Phagocytosis control experiments. Polystyrene microspheres were used as phagocytosis controls in these assays. The fact that the uptake of the microspheres was not affected by the addition of inhibitors while *C. perfringens* was (Fig. 3-5 and 3-6), showed that phagocytosis in general was not affected by the addition of inhibitors. As a second control, we showed that uptake of IgG-coated microspheres could be blocked by the addition of anti-FcR antibodies, indicating that this receptor is efficiently inhibited by the addition of a blocking antibody. The FcR receptor was used because it was not used for the internalization of *C. perfringens* (Fig. 3-5), while all the other receptors tested were involved in the phagocytosis process. Therefore, the results in Fig. 3-6 showing the anti-FcR antibody could block uptake of IgG-coated microspheres, reinforces the results seen in Figs. 3-2 and 3-3 showing the FcR was not used for phagocytosis in the absence of opsonizing antibodies.

LAMP-1 co-localization studies. The addition of fucoidin and opsonization of *C. perfringens* showed statistically significant increases in the levels of co-localization with the late endosome-lysosome marker LAMP-1 in comparison to the untreated J774-33 cells. However, the biological significance of this increase is questionable, since the non-pathogenic bacterium, *B. subtilis*, was found to co-localize with LAMP-1 at a rate three times higher than that observed with fucoidin treatment or with the opsonized *C. perfringens* (Fig. 3-7). Since we have demonstrated in a previous report that J774-33 cells can kill *B. subtilis*, but not *C. perfringens* (149), and intracellular *B. subtilis* co-

localized with LAMP-1 at a high (63%) level, the lower level of co-localization seen with the fucoidin treatment and opsonized *C. perfringens* probably does not reflect a significant increase in killing efficiency.

The capsule of Strain 13 may be the target of phagocytosis receptors on J774-33 cells. What do these receptor experiments tell us about the surface properties of *C. perfringens*? We have obtained high magnification electron microscopy images of *C. perfringens* attached to J774-33 cells that show the contact points on the macrophage surface appear to bind to an extracellular matrix material located immediately outside the gram positive cell wall of *C. perfringens* (Fig. 3-8 left panels). This is similar in appearance to the distinct patchy areas of bacteria and macrophage contact we described in an earlier report (O'Brien & Melville, 2000). The extracellular matrix on the surface of strain 13 was also seen when the bacteria were internalized by macrophages (Fig. 3-8 middle and right panels). For unknown reasons, the fibrous material can vary in length and appearance (Fig. 3-8).

Many strains of *C. perfringens* are known to produce an extracellular capsule (38); (97), but strain 13, the strain used in these experiments, did not show evidence of a capsule when examined with the India ink negative stain (S. Melville, unpublished results). However, the extracellular fibrous material seen in high magnification electron micrographs of bacteria attached to the outer membrane of J774-33 cells (Fig. 3-8 left panels), indicated this strain might possess a tightly bound capsule on its surface. To determine if a capsule was present, extracellular polysaccharide from strain 13 was purified and characterized.

The purified polysaccharide from strain 13 cells contained glucose, galactose, mannose, glucuronic acid, N-acetyl-galactosamine, and N-acetyl-glucosamine. This is the first description of a capsule being associated with *C. perfringens* strain 13. Also, the residues found in the polysaccharide of strain 13 are the same residues found in the capsule polysaccharide of *C. perfringens* strain ATCC 12919 (97), indicating the material from strain 13 is most likely a polysaccharide capsule. The presence of mannose and glucuronic acid would be consistent with residues that would bind the mannose and scavenger receptors, respectively, which we have identified as receptors involved in the binding and phagocytosis of *C. perfringens*. For example, the mannose in the capsule is likely the residue that the mannose receptor recognized on the surface of the bacteria. Glucuronic acid would impart an overall net negative charge to the capsule polysaccharide oligomers, which are characteristic of one group of substrates bound by the scavenger receptor (68). Further evidence that strain 13 can synthesize a capsule was provided by the recent publication of the complete genome sequence of strain 13, in which many genes that could be involved in capsular biosynthesis were identified (http://w3.grt.kyushu-u.ac.jp/CPE/).

Interestingly, the capsule of strain 13 does not appear to inhibit phagocytosis by macrophages, unlike capsules in other Gram-positive bacteria such as *Streptoccocus pneumoniae* and *Bacillus anthracis*. In fact, J774-33, mouse peritoneal, and bone marrow derived primary macrophages phagocytose the encapsulated form of strain 13 very efficiently (Fig. 3-2 and 3-3) and (149). Therefore, the capsule of *C. perfringens* strain 13 may not play an anti-phagocytic role in the host. Since we have reported that *C.*

perfringens can persist in the presence of macrophages despite being phagocytosed (149), it may actually be advantageous for *C. perfringens* to be readily phagocytosed by a macrophage. Because *C. perfringens* is not killed by the process, the intracellular environment may provide more nutrients and a lower oxygen concentration than is found outside of the macrophage (91), which would be advantageous to an anaerobic bacteria.

MATERIALS AND METHODS

Bacterial Strains and mammalian cell lines.

The bacterial strains used in this study were *C. perfringens* strain 13 (obtained from C. Duncan) and *Bacillus subtilis* JH642 (obtained from J. Hoch). *C. perfringens* was grown in a Coy anaerobic chamber (Coy Laboratory Products) in PGY medium (30 g of proteose peptone, 20 g of glucose, 10 g of yeast extract and 1 g of sodium thioglycollate per liter) (127). *B. subtilis* was grown in Luria broth (LB) (10 g of tryptone, 5 g of NaCl and 5 g of yeast extract per liter).

J774-33 cells, a highly phagocytic clone of J774A.1 cells, were used in these assays (149). J774-33 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l of both glucose and L-glutamine supplemented with 10% FBS and 1% sodium pyruvate, in an atmosphere of 5% CO_2 at 37 °C. Chinese Hamster Ovary cells (CHO) and CHO cells transfected with the SR-A receptor were obtained from M. Krieger (108). CHO cells were grown in Ham's F-12 growth medium containing 1% penicillin/streptomycin, 3% newborn calf lipoprotein deficient serum, 250 μM mevalonate, 40 μM mevinolin and 3 μg of AcLDL per ml.

Phagocytosis receptor inhibition assays.

C. perfringens bacteria were grown to log phase and then washed three times in phosphate buffered saline (PBS) and added J774-33 cells grown in 24-well tissue culture plates, containing 12 mm round glass coverslips at an multiplicity of infection (MOI) of 6-16:1. CHO cells were harvested with PBS containing 10 mM EDTA and 1% trypsin

and seeded into 24-well tissue culture plates containing coverslips and allowed to grow to confluency before infecting with *C. perfringens* at an MOI of 10:1. Inhibitors were added 30 min prior to the addition of the bacteria. The inhibitors were added at a final concentration of: 1 mg/ml mannan, 7 μg/ml fucoidin, 2% w/v methyl α-D mannopyranoside, 1:200 dilution of rat anti-mouse antibody against the complement receptor CR3 (CD11b) and a 1:200 dilution of rat anti-mouse antibody against the Fc receptor (CD16/CD32) (Pharmingen). Complement was removed from the serum by heat-treating FBS (56 °C for 30 min). Complement activity was restored by adding a 1:100 dilution of pooled normal guinea pig complement (Cedarlane) back to the heat inactivated serum. For anaerobic experiments, J774-33 cells were placed in an anaerobic chamber 2 h prior to the start of the experiments to allow the media to become anaerobic. For all the experiments, the bacteria were added and at various time points, the coverslips were washed twice for 5 min in PBS and then fixed in 2.5% paraformaldehyde for 20 min at room temperature. The J774-33 cells were then permeabilized by dipping the coverslips in cold (-20 °C) methanol for 10 s. To visualize the macrophage cytoplasm, coverslips were stained for 45 min at 37 °C with Celltracker Green CMFDA (Molecular Probes). To visualize the bacteria and J774-33 nuclei, coverslips were stained for 10 min in the dark at room temperature with the nucleic acid stain, propidium iodide (Molecular Probes). Coverslips were mounted on slides with ProLong Anti-Fade mounting medium (Molecular Probes). Macrophages were examined using a Zeiss LSM 510 laser confocal microscope. To produce three-dimensional images using the confocal microscope, an average of 20 cross-sectional images were captured over a vertical distance of 8-15 μm.

The bacteria were identified as cell-associated if they were either attached to the surface of the macrophage or inside the macrophage.

Phagocytosis of polystyrene microspheres.

To determine if the receptor inhibitors affected the ability of J774-33 cells to phagocytose inert particles, we used 2 μm fluorescent carboxylate-modified microspheres (FluoSpheres, Molecular Probes) as a control for phagocytosis receptor inhibition assays. These microspheres are made from polystyrene and contain a red fluorescent dye. To reduce the nonspecific binding of the beads, bovine serum albumin was added at a concentration of 50 μg/ml in PBS containing the beads and shaken overnight at room temperature to coat the beads. The beads were then washed in PBS and added to J774-33 cells at a final concentration of 0.0025% bead volume/ml. Phagocytosis receptor inhibition assays were then carried out for 60 min and processed as described above for bacteria.

To show that the decrease in phagocytosis in the presence of various inhibitors was due to specific inhibitor effects, we used 2 μm fluorescent microspheres coated with purified mouse IgG as a control. The covalent coupling of purified mouse IgG (Chemicon) to the fluorescent microspheres was performed as described by Molecular Probes. Inhibition assays were done as described above using mouse IgG coated microspheres, in the presence or absence of rat anti-mouse antibody against the Fc receptor (CD16/CD32) at a 1:200 dilution.

Co-localization of *C. perfringens* and the late endosome-lysosome marker LAMP-1.

To determine if the presence of various inhibitors affected the co-localization of intracellular *C. perfringens* with the late endosome-lysosome marker glycoprotein LAMP-1, co-localization assays were performed as previously described (149). *C. perfringens* cells were opsonized by the addition of a 1:10 dilution of rabbit anti-*C. perfringens* serum (149) to bacteria in PBS. It was shown recently that rabbit IgG binds tightly to murine Fc receptors (148). For opsonization, the bacteria-antibody solution was placed on a rotator at room temperature for 30 min, washed 3 times with PBS, incubated for 30 min in PBS, then washed once more.

Electron microscopy.

J774-33 cells were grown in DMEM until nearly confluent in 50 ml tissue culture-treated flasks and the flasks were then incubated at 4°C. *C. perfringens* strain 13 cells, grown to mid-log phase and washed three times in PBS, were then added at a multiplicity of infection (MOI) of ~10:1 and incubated at 4°C for 30 min to allow attachment to occur. The infected macrophages were placed in an incubator (5% CO_2 at 37°C), and at times 0, 10, and 20 min after being transferred to the 37°C incubator, the infected macrophages were fixed with 2.5% glutaraldehyde. Samples were processed for electron microscopy as previously described (149). Thin sections were viewed and photographed on a JEOL 2000EX electron microscope at 60 kV.

Isolation of capsular polysaccharide.

The isolation of capsular polysaccharide from strain 13 was done according to Lee and Cherniak (111). To summarize, 4 L of bacteria were grown overnight, harvested, and dried using increasing concentrations of ethanol and lastly, acetone. Cells were boiled for 5 min and then stirred in an ice-water bath for 3 h. After centrifugation, the supernatants were removed and carbohydrates were precipitated overnight by adding two volumes of 95% ethanol. The precipitate was then harvested by centrifugation and washed in increasing concentrations of ethanol and, acetone. The pellet was resuspended in 0.01 M Tris-0.1 M NaCl buffer, pH 7.5, and was extracted with chloroform-butanol (9:1, v/v) until no protein was present at the solvent interface. Samples were then loaded onto a Q-Sepharose ion exchange column to separate nucleic acids from the sample. The polysaccharide was then eluted using a linear gradient of 0-1.0 M NaCl. Fractions from the Q-Sepharose column were tested for the presence of hexoses using an anthrone assay (52). Fractions containing hexoses were lyophilized and used for glycosyl composition analysis.

Glycosyl composition analysis.

To determine the sugar composition of the capsular material isolated from *C. perfringens*, glycosyl composition analysis was performed according to York et. al (235). Briefly, the sample was hydrolyzed with 1.0 M methanolic-HCl for 16 hours at 80°C. The released methylglycosides were dried down and N-acetylated using methanol and acetic anhydride (1:1, v:v) for 15 min at 45°C. The acetylated sample was trimethyl-sialylated (TMS) with Tris-Sil and resolved on a 30m DB-1 column (0.25mm x 0.25mm,

i.d., J&W Scientific, Folsom, CA) in a Hewlett Packard 5985 GC-MS system using myo-inositol as an internal standard. The following temperature conditions were used: an initial temperature of 160°C, then raised to 200°C at 2°/min, and increased to 260°C at 10°/min. To aid in the identification of the sugars, a standard of derivatized methylglycosides was run alongside the samples.

Media and reagents.

Mannan, fucoidin, mevalonate, mevinolin and methyl α-D-mannopyranoside and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO.). Rat anti-mouse antibody against the complement receptor CR3 (CD11b) and rat anti-mouse antibody against the Fc receptor (CD16/CD32) were purchased from Pharmingen. DMEM with 4.5 g/l of both glucose and L-glutamine supplemented with 10% FBS and 1% sodium pyruvate, Newborn calf serum and Ham's F-12 growth medium were all from Biowhittaker (Walkersville, MD). AcLDL was obtained from Biomedical Technologies Inc. (Stoughton, MA). Anti-*C. perfringens* rabbit polyclonal antibodies were obtained from Applied Diagnostics International. Anti-LAMP-1 rat monoclonal antibody 1D4B was developed by J. T. August and was obtained from the Developmental Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. Texas Red-X-conjugated goat anti-rat IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were both obtained from Molecular Probes.

ACKNOWLEDGEMENTS

We thank Katherine Traughton (UT Memphis) for electron microscopy assistance, David Popham for critical reading of the manuscript, and the Complex Carbohydrate Research Center (CCRC) (University of Georgia, Athens, Georgia) for the analysis of *C. perfringens* polysaccharide. CCRC is supported in part by the Department of Energy-funded (DE-FG09-93ER-20097) Center for Plant and Microbial Complex Carbohydrates.

CHAPTER FOUR

Perfringolysin O and alpha toxin mediate escape from the phagosome and are required for survival of *Clostridium perfringens* in host tissue

This paper has been submitted to *Cellular Microbiology*

ABSTRACT

Clostridium perfringens is a Gram-positive anaerobic pathogen that causes a variety of diseases in humans, including gas gangrene (clostridial myonecrosis). We reported previously that *C. perfringens* could escape the phagosome and survive in the presence of the mouse macrophage-like cell line J774-33, even under aerobic conditions. Two membrane-active toxins are thought to be important in the pathogenesis of gas gangrene, alpha toxin (PLC) and perfringolysin O (PFO), encoded by the *plc* and *pfoA* genes, respectively. We found that *C. perfringens* survival in the presence of J774-33 cells is dependent on PFO, but not PLC. *C. perfringens* survival in the presence of mouse peritoneal macrophages is dependent on both PFO and PLC. *C. perfringens* escape from the phagosome of J774-33 cells and mouse peritoneal macrophages is mediated by either PFO or PLC. In a mouse gangrene model, using conditions designed to mimic those found in the early stages of infection, we found that both PFO and PLC were necessary for *C. perfringens* to survive *in vivo* at doses 1,000 times lower than those required to initiate a gangrene infection. This is the first report of a role for PFO in survival of *C. perfringens* under *in vivo* conditions.

INTRODUCTION

Clostridium perfringens is a Gram-positive, spore-forming, non-motile, anaerobic pathogen. *C. perfringens* causes a variety of diseases in humans, including gas gangrene (clostridial myonecrosis) (197), enteritis necroticans (Pigbel) (80), acute food poisoning (93), and antibiotic-associated diarrhea (1, 44, 84). *C. perfringens* is a ubiquitous bacterium that commonly contaminates wounds. If these wounds become ischemic due to disruption of the arterial blood supply, *C. perfringens* can grow and the clinical signs of gangrene can appear as soon as 6 h after trauma or surgery but the incubation period can also be as long as several days (204). Once initiated, the disease spreads rapidly through healthy tissues, leading to shock and death if not treated. Shock, which is a common and frequent occurrence in gangrene infections, is most likely due to extracellular toxins produced by *C. perfringens* (10, 208). The two main toxins contributing to gas gangrene infections are thought to be PLC (alpha toxin), which is a phospholipase C and sphingomyelinase (220), and perfringolysin O or PFO (theta toxin), which is a member of the cholesterol-dependent cytolysin family of toxins (228).

A hallmark of clinical gas gangrene infections is the lack of phagocytic cells at the site of infection (29). Even though only a relatively small number of *C. perfringens* cells infect a wound, these bacteria are able to multiply and cause a gangrene infection. This occurs in the presence of phagocytic cells of the host immune system, primarily polymorphonuclear cells (PMNs) and lesser amounts of monocytes and macrophages. In order for *C. perfringens* to survive, it must be capable of avoiding or subverting the bactericidal activity of these phagocytic cells. The fact that gangrene infections develop

to a clinically apparent stage indicates these phagocytic cells often fail to kill *C. perfringens* effectively.

An early report demonstrated that PMNs can kill *C. perfringens* under aerobic and anaerobic conditions (120). Therefore, we have focused our recent efforts at characterizing *C. perfringens* interactions with macrophages. Bacterial pathogens such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Salmonella typhimurium* enter macrophages and modify the vacuolar maturation process to ensure their survival (2). Other intracellular bacteria, including species of *Shigella*, *Rickettsia*, and *Listeria*, can escape the phagosome and gain access to the cytosol where they replicate (63). We believe that *C. perfringens* falls into the latter group of pathogens. This is based on several lines of evidence. Most significantly, we report that *C. perfringens* can escape the phagosome of J774-33 cells and mouse peritoneal macrophages, even under aerobic conditions (149). In addition, when a strain of the non-pathogenic bacterium *Bacillus subtilis* was transformed with the *pfoA* gene of *C. perfringens*, *B. subtilis* was able to lyse the phagocytic vacuole and grow in the cytoplasm of J774 cells (157). When the *hly* gene, which encodes Listeriolysin O (LLO) of *Listeria monocytogenes,* was replaced by the *pfoA* gene (which encodes PFO) of *C. perfringens*, *L. monocytogenes* was able to escape from the macrophage vacuole and replicate in the cytoplasm (94). This strain showed a significant increase in cytotoxicity, and however, a reduced virulence in an *in vivo* mouse model of infection (94).

PFO is a cholesterol-dependent cytolysin, which is secreted from the bacterium as a highly water-soluble monomeric protein that oligerimizes to form large pores upon

contact with cholesterol-containing membranes (192). Up to 50 of these individual monomers can partake in forming these pore complexes (150), which range from 20-30 nm in diameter (82).

PLC is a monomeric protein (109, 133), comprised of two domains (16). Titball et al (222) showed an N-terminal domain made up of amino acids 1-249 retained PLC activity, did not have hemolytic or lethal activities, and showed a reduced sphingomyelinase activity. A C-terminal domain from amino acids 247-370 possessed no enzymatic or lethal activity. However, when the two fragments were mixed together, hemolytic activity was partially restored, suggesting that the C-terminus is responsible for conferring hemolytic, and perhaps lethal activities to the toxin (219).

Purified PLC is lethal when injected into mice (208). Additional evidence for the essential role of PLC in gangrene infections comes from experiments using strains of *C. perfringens* that have been genetically altered by inactivating the *plc* gene. When mice were injected into the hind leg muscles with 1 x 10^9 cells of a *C. perfringens* strain carrying an inactive *plc* gene, a marked decrease in virulence was seen in comparison to the wild-type strain (11, 209).

As with PLC, purified PFO is lethal when injected into mice (208). The role PFO plays in gangrene infections, however, is more subtle than that of PLC. When mice were infected with 1 x 10^9 cells of a *pfoA* mutant strain of *C. perfringens*, only small decreases in virulence indicators were seen (11, 209). However, PFO has been shown to be important in the development of leukostasis in the region near the site of infection (26, 56, 209). Awad et al. (12), created a *pfoA*/*plc* mutant and found that most of the

characteristics of a gangrene infection were absent when mice were injected with 1 x 10^9 bacteria of this mutant. When the *plc* and *pfoA*/*plc* mutants were complemented with either *plc* or both *pfoA* and *plc*, respectively, the typical pathological characteristics of a gangrene infection reappeared. When the *pfoA*/*plc* mutant was partially complemented with a plasmid carrying only a functional *pfoA* gene, increased coagulative necrosis was seen in comparison to the *pfoA*/*plc* mutant, but most of the pathological parameters were similar to those seen with the *pfoA*/*plc* strain (12). Therefore, a definitive role for PFO in the initiation of gangrene infections has yet to be demonstrated when large doses (i.e., 1 x 10^9) of *C. perfringens* are used as the inoculum.

Here, we present evidence that escape from the macrophage phagosome is mediated by both PLC and PFO but that PFO also plays a role in *C. perfringens*-mediated cytotoxicity to macrophages. We believe PFO-dependent effects are important in the early stages of a gangrene infection since PFO is needed for survival in mouse tissue when the infectious dose is 1,000-fold lower than that needed to cause a gangrene infection, conditions designed to mimic those encountered early in the infectious process.

RESULTS

Construction and characterization of *pfoA* and *pfoA/plc* strains

We have previously reported that J774-33 cells are unable to kill wild-type *C. perfringens*, even under aerobic conditions (149). *C. perfringens* persistence in the presence of macrophages may have been due to their ability to escape the phagosome. Two toxins that *C. perfringens* produces, PFO and PLC, disrupt membrane integrity. PFO is a thiol-sensitive pore-forming toxin in the same family as LLO from *L. monocytogenes*. PLC is a type C phospholipase and sphingomyelinase. To determine if these toxins are involved in mediating escape from the phagosome, we used homologous recombination techniques to inactivate the *pfoA* gene (which encodes PFO) and obtained a *plc* strain (PLC-) of *C. perfringens* stain 13 from A. Okabe (98). The PLC- strain was used as a host to create a *pfoA/plc* strain using the same technique used to make the *pfoA* strain (see Materials and Methods). PFO activity in the *pfoA* and *pfoA/plc* strains was measured using filtered supernatants from stationary phase cultures and tested for their ability to lyse sheep red blood cells (Table 4-1). The *pfoA* strain, DOB3, showed a 105-fold decrease in PFO activity, while the *pfoA/plc* strain, DOB4, exhibited a 4,850-fold decrease in PFO activity (Table 4-1). To avoid overproduction of PFO due to a multicopy gene effect, a promoterless version of the *pfoA* gene was cloned on an *C. perfringens-Escherichia coli* shuttle vector and assayed for its ability to complement PFO activity in strain DOB3 (see Materials and Methods). While the *pfoA* complemented strain, DOB3(pSM260), showed a 5-fold lower PFO activity level than did the wild-type strain (Table 4-1), it still had 20-fold higher PFO activity than the *pfoA* strain.

Table 4-1. PFO activity of *C. perfringens*.

Strain	Phenotype	Hemolytic Units[a]	WT/Mutant
Strain 13 (wild-type)	PFO^+/PLC^+	0.1842 +/- 0.01	1
DOB3	PFO^-/PLC^+	0.00176 +/- 0.000045	105
DOB3 (pSM260)	PFO^+/PLC^+	0.036 +/- 0.01	5
DOB4	PFO^-/PLC^-	0.000038+/- 0.0000034	4850

a) PFO activity was measured by the ability of *C. perfringens* strains to lyse sheep red blood cells. PFO levels were determined by measuring the release of hemoglobin using a spectrophotometer set at 550 nm. A hemolytic unit is the difference in the absorbance at 550 nm between the sample and blank per μg of protein present in each sample. DOB3 and DOB4 showed decreased levels of red cell lysis, indicating that little PFO was being expressed by these strains. DOB3-pSM260 showed 20-fold higher levels of red cell lysis than DOB3 indicating that pSM260 is capable of partially complementing DOB3. Results shown are the mean (+/- SD) of quadruplicate samples measured in each of three independent experiments.

PFO is necessary for survival in the presence of J774-33 macrophages under aerobic conditions

To determine if PFO, PLC, or both toxins, are necessary for the survival of *C. perfringens* in the presence of J774-33 cells under aerobic and anaerobic conditions, we performed 24 h bacterial survival experiments using the wild-type (strain 13) and our PFO- and PLC- deficient strains of *C. perfringens*, DOB3 (*pfoA*), PLC-, and DOB4 (*pfoA/plc*) (Fig. 4-1A). Strains 13, PLC- and DOB3(pSM260) were able to grow in the presence of the macrophages after 3 h, with strain PLC- showing the highest levels of growth (Fig. 4-1A). In the presence of J774-33 cells, strains DOB3 and DOB4 exhibited 3-4-log lower level of survival 9 h post infection compared to that of wild-type *C. perfringens*. When the *C. perfringens* strains were incubated aerobically in media alone, with no macrophages present, all the strains except for DOB4 died by 24 h and the DOB4 strain had less than 0.0001% survival (Fig. 4-1B). A large decrease in survival efficiency after 1 h of incubation occurred in the presence and absence of macrophages (Fig. 4-1A and 4-1B) and was due to an experimental artifact in which the *C. perfringens* cells became temporarily sensitive to the effects of the 0.02% Triton X-100 added to the wells to lyse the macrophages (unpublished results).

J774-33 cells were also infected with the wild-type and mutant strains of *C. perfringens* under anaerobic conditions. Either in the presence or absence of macrophages, all the bacterial strains grew exponentially (data not shown) and the macrophages were completely lysed by 5 h after infection, as we previously described (149).

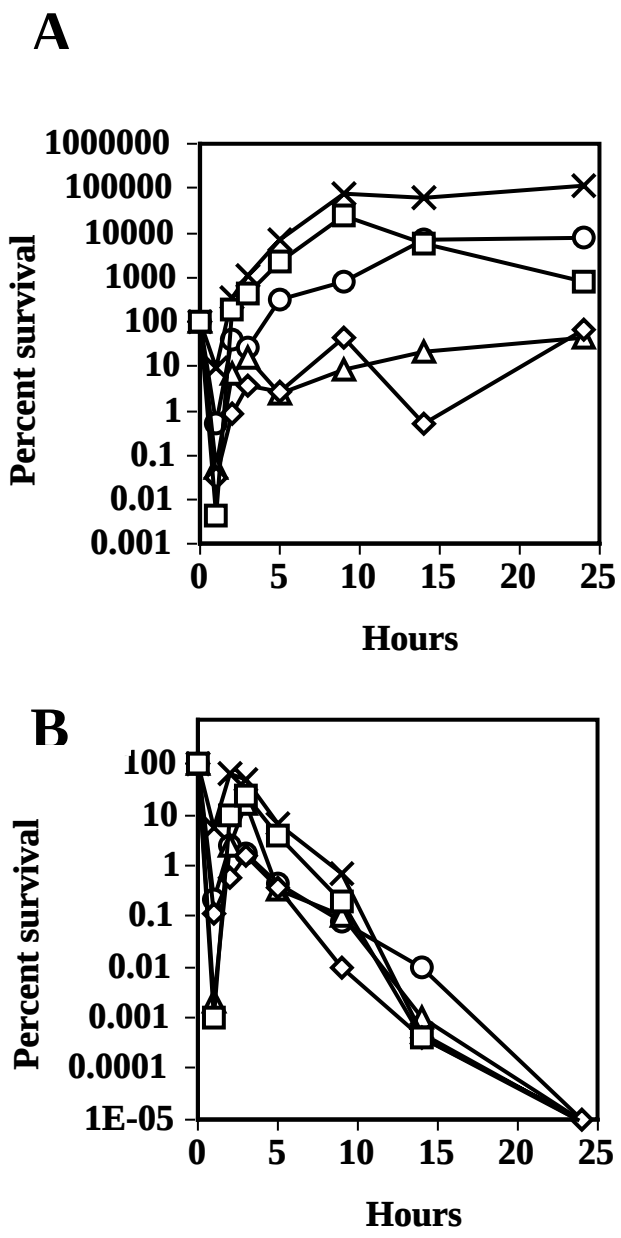

Figure 4-1. Survival of *C. perfringens* strain 13, DOB3, DOB3 (pSM260), DOB4, and the PLC- strains incubated with J774-33 cells under aerobic conditions. Survival is expressed as the percentage of original innoculum of 1.5 x 10^6 bacteria/well. A) *C. perfringens* incubated with J774-33 cells. B) *C. perfringens* incubated in the absence of J774-33 cells. Strain13, squares; DOB3, triangles; DOB3 (pSM260), circles; PLC-, cross hairs; DOB4, diamonds. Each time point represents the mean value of at least three independent experiments. For clarity, error bars representing the SD values are not shown.

Both PFO and PLC are necessary for the persistence of *C. perfringens* in the presence of mouse peritoneal macrophages

Fluid thioglycollate medium (FTG)-elicited mouse peritoneal macrophages were infected with *C. perfringens* under aerobic conditions and bacterial survival in the presence and absence of the macrophages was determined (Fig. 4-2). In the presence of peritoneal macrophages, *C. perfringens* strain 13 survived and actually grew to 500% of the level of the original inoculum over the course of 24 hours (Fig. 4-2A). However, when peritoneal macrophages were infected with the DOB3 and PLC- strains of *C. perfringens*, a marked decrease in survival occurred (Fig. 4-3A). Strains DOB3(pSM260) and DOB4 showed an intermediate level of survival. These results suggest that both PFO and PLC are needed for the survival of *C. perfringens* in mouse peritoneal macrophages. The reasons strain DOB4 survived slightly better than strains DOB3 and PLC- are not known.

In the absence of macrophages, the bacteria died rapidly after the first 4 hours of incubation (Fig. 4-2B). Under anaerobic conditions, the bacteria grew at logarithmic rates after an incubation period of 1-3 hours irrespective of the presence of macrophages (data not shown).

Both PFO and PLC mediate escape from the phagosome of J774-33 cells and mouse peritoneal macrophages

To determine if PFO and/or PLC were involved in the ability of *C. perfringens* to escape the phagosome of J774-33 cells and mouse peritoneal macrophages, we tested the mutants for their ability to lyse the phagosomal membrane. Electron micrographs of

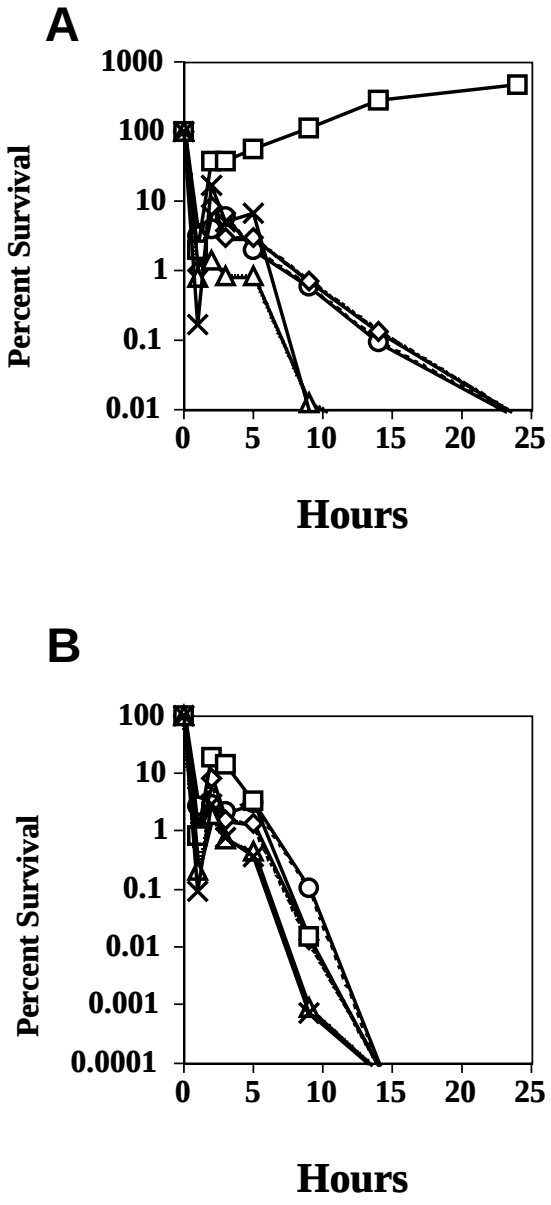

Figure 4-2. Survival of *C. perfringens* strain 13, DOB3, DOB3 (pSM260), DOB4, and the PLC- strains incubated with FTG-elicited mouse peritoneal macrophages. Survival is expressed as the percentage of original innoculum of 5 x 10^5 bacteria/well. A) *C. perfringens* incubated with J774-33 cells. B) *C. perfringens* incubated in the absence of J774-33 cells. Strain 13, squares; DOB3, triangles; DOB3 (pSM260), circles; PLC- , cross hairs; DOB4, diamonds. Each time point represents the mean value of at least three independent experiments. For clarity, error bars representing the SD values are not shown.

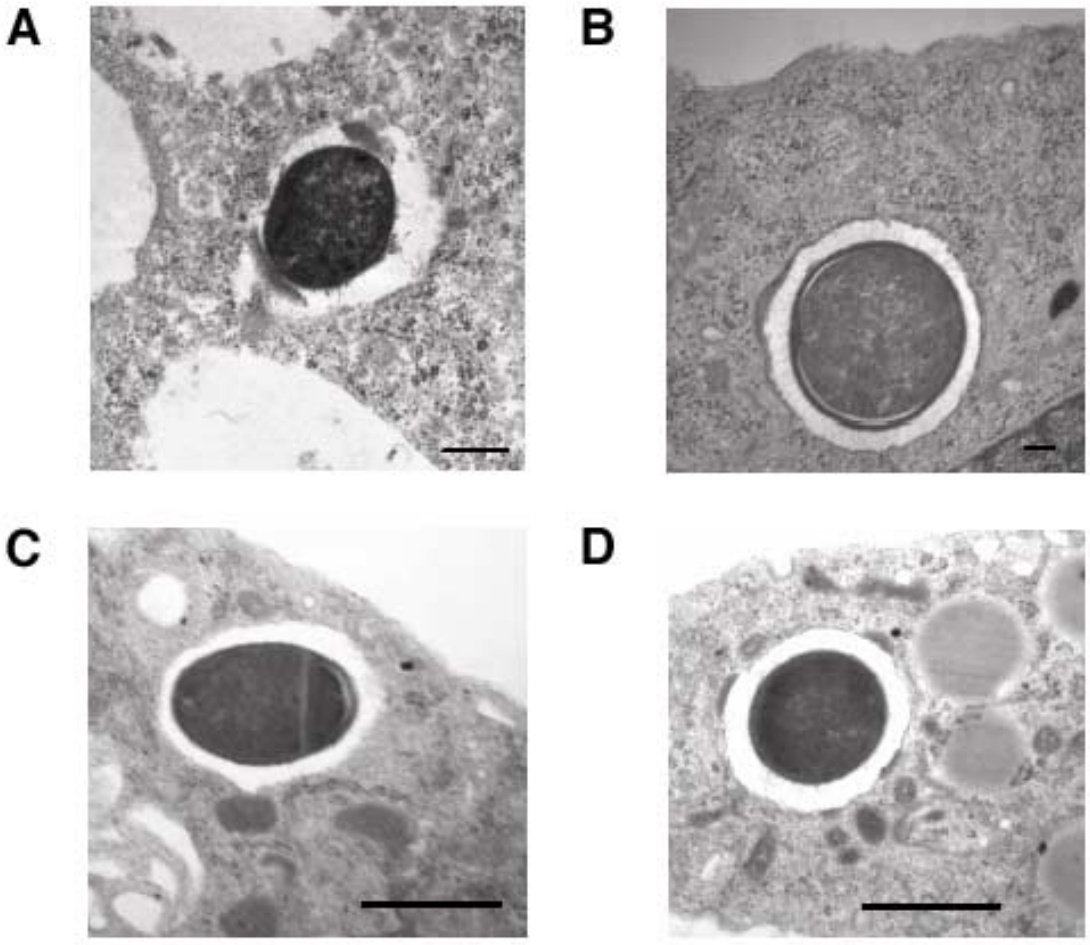

Figure 4-3. Transmission electron microscopy images of J774-33 cells and mouse peritoneal macrophages infected with *C. perfringens* strain13. Images show *C. perfringens* in the cytoplasm of J774-33 cells (A) and in the phagosome of J774-33 cells (B). *C. perfringens* in the cytoplasm of mouse peritoneal macrophages (C) and in the phagosome of mouse peritoneal macrophages (D). Cells were infected for 60 min, fixed and processed for electron microscopy. Bars: A and B, 200 nm, C and D, 1 μm.

infected cells were examined, and the number of intracellular bacteria that were either in the phagosome or in the cytoplasm was determined. Representative micrographs are shown in Fig. 4-3. In J774-33 cells, we found that 53% of wild-type *C. perfringens* strain 13 were in the cytoplasm, and 28% were in membrane bound phagosomes while the remainder of the bacteria were in partially degraded phagosomes (Table 4-2). In contrast, we observed that 56-58% of intracellular DOB3 and PLC- *C. perfringens* remained in a membrane bound phagosome, but 26-28% of the bacteria were still able to escape the phagosome (Table 4-2). However, with strain DOB4, only ~3% of the intracellular bacteria were found in the cytoplasm while 86% of the bacteria were found inside a membrane bound phagosome (Table 4-2). Strain DOB3(pSM260) exhibited similar results to that seen with DOB3, perhaps due to incomplete complementation of the PFO activity seen with this strain (Table 4-1). In contrast, the PLC- complemented strain, PLC-(pJIR418α), was able to escape at a similar rate as that seen with the wild-type strain 13 (Table 4-2).

The percentage of bacteria that escaped the phagosome of mouse peritoneal macrophages was less than that seen in J774-33 cells, similar to results we reported earlier (149). While 31% of wild-type *C. perfringens* escaped into the cytoplasm, 53% of the wild-type bacteria resided in a phagosome. The mutant strains were found in the cytoplasm 19-21% of the time (Table 4-2), while 62-72% of the intracellular mutant bacteria were clearly in phagosomes (Table 4-2). In contrast to the results seen with J774-33 cells, strain DOB4 was just as efficient at escaping the phagosome as were the single mutant strains, DOB3 and PLC- (Table 4-2). For all *C. perfringens* strains, the

Table 4-2. The percentage of intracellular *C. perfringens* bacteria in different intracellular locations in J774-33 cells and peritoneal macrophages.

	J774-33[a]		Peritoneal[a]	
Strain	**In phagosome**	**In cytoplasm**	**In phagosome**	**In cytoplasm**
Strain 13	28	53	53	31
DOB3	58	26	72	21
DOB3(pSM260)	56	30	ND	ND
PLC-	56	30	68	19
PLC-(pJIR418a)	25	66	ND	ND
DOB4	86	3	62	20

a) Electron microscopy was used to determine if intracellular bacteria were in the phagosome or cytoplasm. The numbers for each sample do not add up to 100%, since some of the intracellular bacteria were in a partially degraded phagosome. ND: Not determined.

lower rates of phagosomal escape seen with peritoneal macrophages corresponded with the decreased survival rates seen with peritoneal macrophages in comparison to the J774-33 cells (compare Fig. 4-1 and Fig. 4-2).

The absence of PFO or PLC had only a minor effect on the co-localization of *C. perfringens* with LAMP-1 in J774-33 cells

In a previous report, we noted that *C. perfringens* was able to escape the phagosome and avoid co-localization with the late endosome-lysosomal marker LAMP-1 (149). Since the three mutant strains showed reduced levels of phagosomal escape, we used immunofluorescence microscopy to determine if the intracellular bacteria from the mutant strains co-localized with LAMP-1 at a higher level than that seen with the wild-type strain (see Materials and Methods). J774-33 macrophages were infected for 60 min with either *B. subtilis*, or wild-type and mutant *C. perfringens* strains and were examined using a laser confocal microscope. *B. subtilis* was used as a positive control for phagosomal-lysosome fusion (149). The results are shown in Fig. 4-4. Sixty-three percent of the intracellular *B. subtilis* co-localized with LAMP-1. However, only 9% of wild-type *C. perfringens* cells co-localized with LAMP-1. None of the strains of *C. perfringens* examined were found to co-localize with LAMP-1 at a significantly different level than that of wild-type *C. perfringens* except for the *plc*/*pfoA* strain, DOB4. While DOB4 co-localized with LAMP-1 18% of the time, this level was still 3.5-fold less than that observed with *B. subtilis* (Fig. 4-4). These results suggest that the low level of LAMP-1 co-localization observed with intracellular *C. perfringens* was not due to the

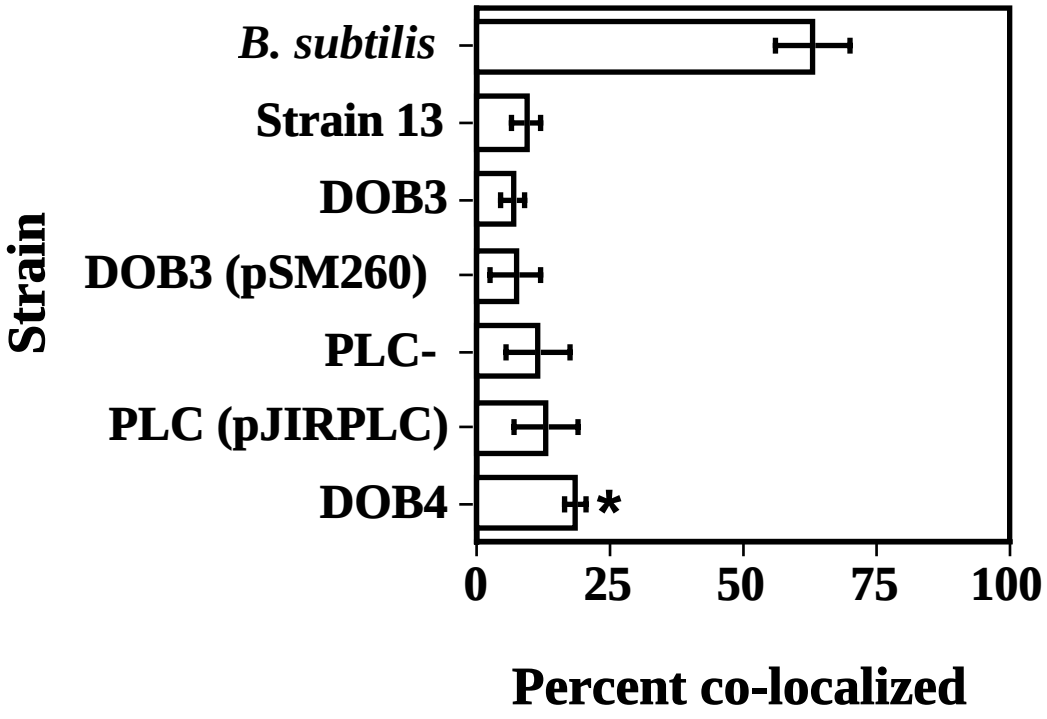

Figure 4-4. Co-localization of intracellular *Bacillus subtilis* and *C. perfringens* strains with the late endosome/lysosome marker LAMP-1 in J774-33 cells. *C. perfringens* was visualized using a primary antibody against whole cells and a FITC-conjugated secondary antibody. LAMP-1 was visualized using a primary monoclonal antibody and a Texas Red-conjugated secondary antibody. Laser confocal microscopy was used to determine the extent of co-localization (see Experimental Procedures). Shown are the mean +/- SD of at least 100 intracellular bacteria in each of three independent experiments. * Statistically significant from strain 13 using the Students unpaired *t*-test (two-tailed); $P < 0.05$.

functions of PFO or PLC and escaping the phagosome was not the primary mechanism of avoiding LAMP-1 co-localization.

C. perfringens strains expressing PFO are more cytotoxic to J774-33 and mouse peritoneal macrophages under aerobic conditions.

Besides allowing escape from the phagosome, another role that PFO and PLC may play in defense against phagocytic cells is their direct cytotoxic effects. Purified PFO is cytotoxic to human PMNs (26, 207) and we observe significant cytotoxicity towards J774-33 and mouse peritoneal macrophages using recombinant PFO (unpublished results). In contrast, relatively high concentrations of PLC were needed for cytotoxicity to PMNs (207) and murine macrophages (unpublished results). To determine if whole cells of *C. perfringens* were cytotoxic to J774-33 cells and mouse peritoneal macrophages, we measured cytotoxicity to these cells in the presence of *C. perfringens* using the Cytotox 96 assay (Promega). This assay measures release of the cytoplasmic enzyme lactate dehydrogenase (LDH) by cells that have lost the integrity of their cytoplasmic membrane. We infected J774-33 cells and mouse peritoneal macrophages for 5 h and compared the cytotoxicity exhibited by wild-type *C. perfringens* and strains that lacked PFO, PLC, or both toxins (Fig. 4-5). J774-33 cells were more sensitive to *C. perfringens*-dependent cytotoxicity than peritoneal macrophages (compare Fig. 4-5A to Fig. 4-5B, where the MOI was 1:1 for both types of macrophage). Both wild-type *C. perfringens* strain 13 and the PLC- strain were more cytotoxic to J774-33 cells (Fig. 4-5A) and mouse peritoneal macrophages (Fig. 4-5C) than was DOB3 or DOB4, which lack PFO. These results suggested that PFO was the toxin most

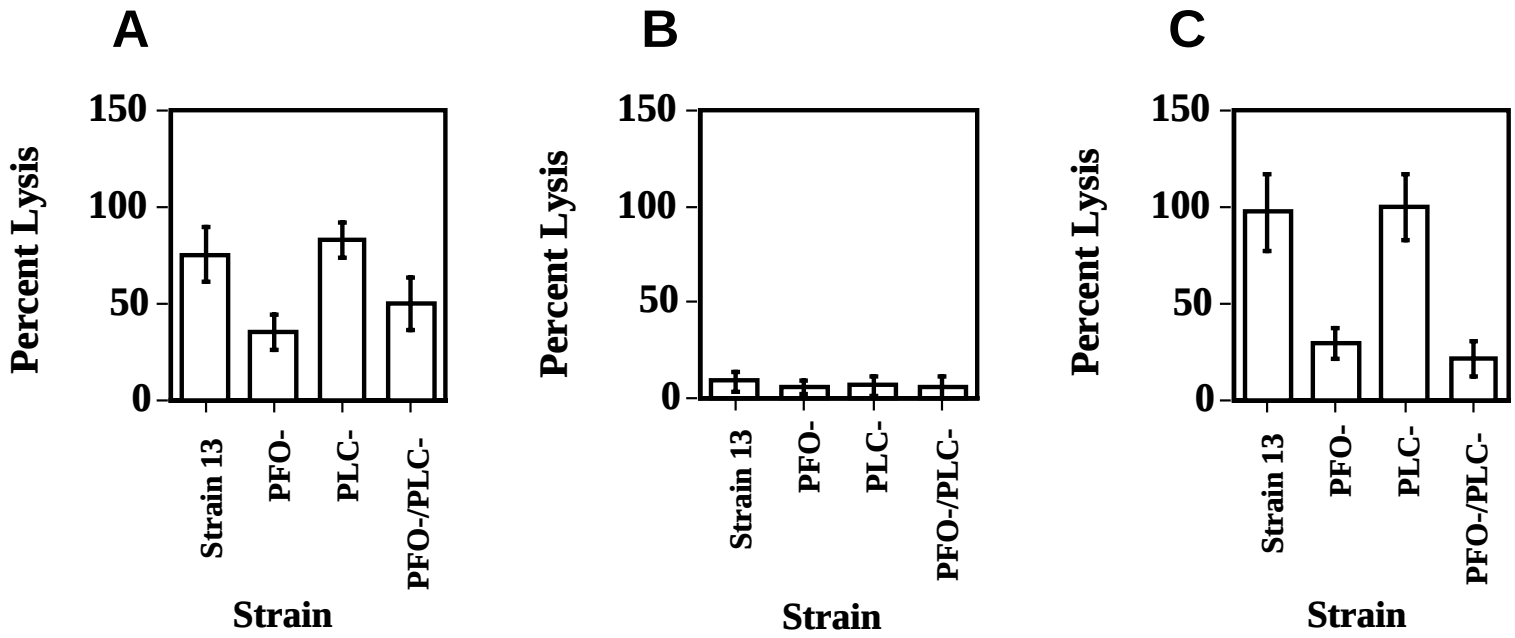

Figure 4-5. ***C. perfringens*** **dependent cytotoxicity on macrophages after 5 h of infection under aerobic conditions.** A) Cytotoxicity towards J774-33 cells incubated with *C. perfringens* strain 13, DOB3, DOB4, and PLC- strains at an MOI of 1:1. B) Cytotoxicity towards mouse peritoneal macrophages incubated with *C. perfringens* strain 13, DOB3, DOB4, and PLC- strains at an MOI of 1:1 and C) MOI 10:1. The means +/- SD of at least three independent experiments performed on quadruplicate samples are shown.

responsible for the *C. perfringens*-dependent cytotoxicity seen with infected macrophages.

PLC and PFO are necessary for survival of *C. perfringens in vivo* at sub-lethal doses

Since our hypothesis is that *C. perfringens* interacts with tissue macrophages in the earliest stages of a gangrene infection, we wanted to simulate these conditions *in vivo* using a mouse model. Therefore, we used infectious doses (1×10^6) that were 1,000-fold less than those needed to initiate a gangrene infection (1×10^9) and examined the survival rates of wild-type and mutant *C. perfringens* strains in mouse tissue. Mice were injected in the femoral muscle and the colony forming units (cfu) of bacteria in the muscle tissue were measured over a period of 24 h (see Materials and Methods). We observed that wild-type *C. perfringens* cfu were found, on average, to be 3 logs higher than DOB3 and PLC- after 24 h (compare Fig. 4-6A to Figs. 4-6B and 4-6C). Strain DOB4 was even more severely limited in its ability to survive *in vivo* since no cfu could be detected in the tissues of the mice infected with DOB4 after 14 h (Fig. 4-6D). These results suggest both PFO and PLC are needed for persistence in mouse tissues when delivered in sublethal doses. Additional experiments have shown that wild-type *C. perfringens* can be detected in mouse muscle tissue up to 72 h post-infection with 1×10^6 bacteria (unpublished results).

As a control, we injected mice in the hind leg with high doses ($\sim 1 \times 10^9$ bacteria per mouse) of *C. perfringens* strains 13, DOB3, DOB4, and PLC-, to ensure our mutant phenotypes were consistent with previously published studies (11, 12, 56, 209). All mice injected with the wild-type and DOB3 strains of *C. perfringens* showed clear signs of

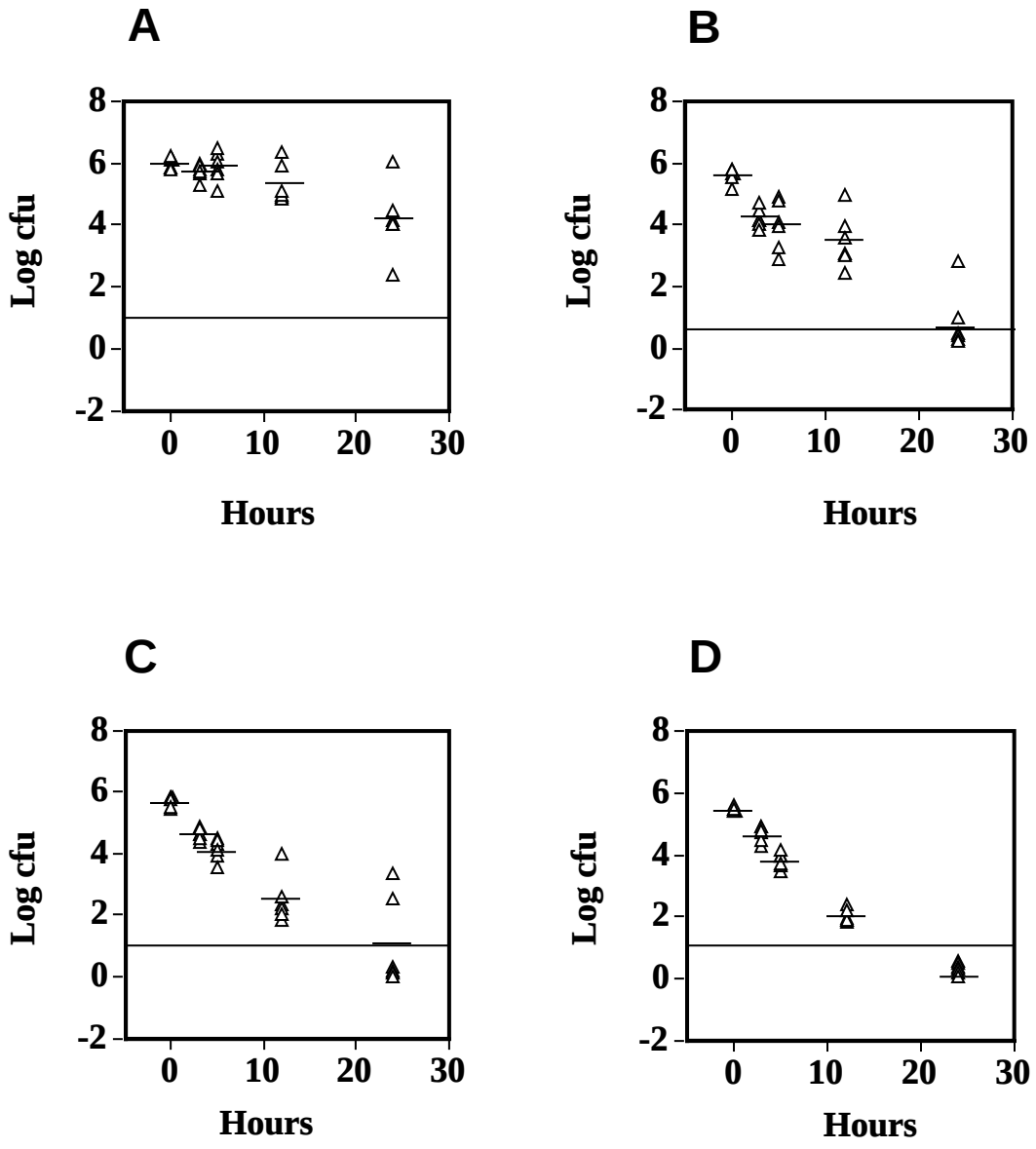

Figure 4-6. In vivo survival of *C. perfringens* injected into the hind leg muscles of mice. Thirty-six mice were injected in the left femoral muscle with 1 x 10^6 bacteria/mouse and 6 mice were sacrificed at each time point shown. Each triangle represents the number of cfu obtained from one mouse. Dashes represent the mean value of the number of cfu recovered from the six mice analyzed at each time point indicated. The horizontal line represents the lowest level of detection. A) strain 13, B) DOB3, C) PLC-, D) DOB4. Results shown are the mean of the log cfus (+/- SD) of 6 mice per time point.

gangrene between 7-12 h post-infection (Table 4-3). Mice injected with strains PLC- and DOB4 never developed signs of gangrene (Table 4-3). These results are similar to those reported by other groups, in which PLC was needed to initiate a gangrene infection in the mouse model when high doses of bacteria were administered to the mice (11, 12, 56, 209).

Table 4-3. Infections of mice with high doses of *C. perfringens* strains.

Strain	**# Mice Survived[a]/ # Mice**
Strain 13 (wild-type)	0/5[b]
PLC-	5/5
DOB3 (PFO-)	0/5[b]
DOB4 (PFO-/PLC-)	5/5

a) After 36 h of infection with ~10^9 bacteria/mouse

b) Mice showing clear signs of gangrene were euthanized in accordance with humane animal care practices. All Wild-type mice were euthanized 8 1/2 h after being injected. DOB3 mice were euthanized between 8 1/2 and 11 1/2 h, PLC- and DOB4 mice were euthanized 36 h after being injected.

DISCUSSION

The primary host immune defense against *C. perfringens* in the earliest stages of a post-traumatic infection is likely to be the phagocytic cells of the innate immune system, mainly PMNs and monocytes/macrophages. In later stages of the infection, when the bacteria have multiplied to the extent that a clinical case of gangrene is evident, there is a profound lack of phagocytes in the immediate vicinity of the bacteria (29). A lack of oxygen in the wound area may play a role in the growth of the anaerobic *C. perfringens*. PMNs and monocytic cells, however, have well-documented bactericidal and fungicidal activities under anaerobic conditions (105, 201, 218) so additional factors must be important in the initiation of a gangrene infection. Indeed, in a 1974 report, using an unidentified strain of *C. perfringens*, Mandell demonstrated that human PMNs were capable of killing *C. perfringens* under aerobic and anaerobic conditions (120). Unpublished data from our laboratory indicates that human PMNs can kill *C. perfringens* in a tumbling tube assay if complement is present in the medium.

In a previous report, we demonstrated that *C. perfringens* could persist in the presence of J774-33 macrophages and could escape the phagosome in both J774-33 and peritoneal macrophages (149). In this report, we examined the role of the membrane-active toxins PFO and PLC in *C. perfringens* interactions with macrophages and the persistence of the bacteria *in vivo*. To accomplish this, homologous recombination methods were used to make a *pfoA* mutant in both a wild-type (strain 13) and *plc* mutant strain (strain PLC-), giving us the single mutants and *pfo*/*plc* double mutant strain.

Under aerobic conditions, peritoneal macrophages were more bactericidal to *C. perfringens* than J774-33 cells (compare Fig. 4-1 and 4-2). Interestingly, while PFO alone is necessary for survival of *C. perfringens* in the presence of J774-33 cells, both PFO and PLC were needed for survival in the presence of peritoneal macrophages. This suggests these toxins play a different role for survival of the bacterium in each macrophage type. For the J774-33 cells, either PLC or PFO could mediate escape from the phagosome (Table 4-2), but only PFO was cytotoxic (Fig. 4-5). Therefore, PFO-dependent cytotoxicity effects correlated more closely with bacterial survival than phagosomal escape in J774-33 cells, indicating killing of these cells was more important than escaping the phagosome for survival of the bacteria. For peritoneal macrophages, survival of *C. perfringens* correlated more closely with phagosomal escape than cytotoxicity. PFO or PLC could mediate escape from the phagosome but only PFO was cytotoxic. Since both PFO and PLC are necessary for survival in the presence of peritoneal macrophages, this indicated phagosomal escape probably played a more important role in survival than did cytotoxicity.

The use of an invasion assay would be valuable in studies examining the survival potential of intracellular *C. perfringens* bacteria following phagocytosis by macrophages. In a previous report, we described attempts to develop a survival assay for *C. perfringens*/macrophage interactions, but these were unsuccessful since both intracellular and extracellular bacteria were killed by the addition of penicillin (149). One explanation for this killing of intracellular bacteria was that PFO made the host cells leaky to the antibiotic (94, 157)

With the exception of strain DOB4, the percentage of each bacterial strain tested that could escape the phagosome was lower in peritoneal macrophages than J774-33 cells and the difference in escape efficiency of the wild-type in comparison to the mutant strains was also lower in the peritoneal macrophages (i.e., 24-27% lower in J774-33 cells compared to 10-12% lower in peritoneal macrophages) (Table 4-2). Despite the lower escape efficiency observed in peritoneal macrophages, we believe that phagosomal escape is important in explaining the large increase in survival efficiency seen with the wild-type strain in comparison to the mutant strains (Fig. 4-2). Data in support of this is shown in Table 4-2, where 68-72% of the *pfoA* and *plc* strains clearly resided inside a phagosome, while only 53% of the wild-type were clearly in phagosomes. One reason for the low efficiency of phagosomal escape seen with peritoneal macrophages may be the time interval (i.e., 1 h) in which the macrophages were fixed and examined using an electron microscope. At the 1 h time point in the survival assay shown in Fig. 4-2A, in which the MOI was 1:1, there was little difference seen in bacterial survival between the wild-type and mutant strains of *C. perfringens*. Judging from the results shown in Fig. 4-2A, if the macrophages were examined at later times in the electron microscope (e.g., 9 h post-infection) a larger difference between the wild-type and mutants may have been observed. However, peritoneal macrophages infected at a MOI of 10:1, which was necessary to observe sufficient intracellular bacteria in the electron microscope (data not shown), for periods longer than 3 h showed significant lysis, even under aerobic conditions (see Fig. 4-5 C and unpublished data), making the quantification of phagosomal escape efficiency quite difficult at these later times.

FTG-elicited peritoneal macrophages were significantly less sensitive to PFO-mediated cytotoxicity than were J774-33 cells (Fig. 4-5). The mechanisms behind this difference are unknown. However, if the peritoneal macrophages or J774-33 cells were first activated by the addition of interferon-γ and *E. coli* lipopolysaccharide (LPS), they became extremely sensitive to *C. perfringens*-dependent lysis, so much so that they lost any bactericidal effects on *C. perfringens* under aerobic conditions (unpublished results). This suggests that factors induced in the activated macrophage make the cell more susceptible to *C. perfringens*-dependent lysis. For example, their ability to produce reactive oxygen and reactive nitrogen intermediates.

Both PFO and PLC can mediate escape from the phagosome in macrophages (Table 2). In a previous report, we observed that intracellular *C. perfringens* did not co-localize with the late endosome-lysosomal marker LAMP-1 in J774-33 cells (149). Since we also observed that *C. perfringens* was able to escape the phagosome and reside in the cytoplasm of these cells, we suggested this may be the reason for the lack of co-localization with LAMP-1. However, strains DOB3 and PLC-, which showed significantly reduced levels of phagosomal escape in comparison to the wild-type strain (Table 4-2), did not show a significant decrease in LAMP-1 co-localization (Table 4-2). Strain DOB4, of which only 3% of the intracellular bacteria were able to escape into the cytoplasm, did show a statistically significant increase in LAMP-1 co-localization in comparison to strain 13, but this level was still 3.5-fold less than that seen with the non-pathogenic positive control bacterium, *B. subtilis*. This leads us to question whether the increased level of co-localization seen with strain DOB4 is biologically significant. If

not, there must be additional factors besides phagosomal escape that prevent LAMP-1 co-localization. One possibility is the presence of other enzymes (toxins) made by *C. perfringens*, including sialidases, endo- and exoglycosidases, and proteases (9, 33, 171, 173). These enzymes could affect maturation of the phagosomal compartment in which the bacteria are residing by degrading membrane-associated glycoproteins. Another possibility is that a combination of sialidases, glycosidases and proteases may lead to degradation of the lumenal portion of the LAMP-1 glycoprotein. This would give a false indication that LAMP-1 was not present in the phagosomes since the epitope recognized by the monoclonal antibody used in these studies, 1D4B, seems to recognize a lumenal epitope of LAMP-1 (J. T. August, personal communication).

Since our hypothesis is that *C. perfringens* interacts with phagocytic cells early in the infection process, we set up experimental conditions to simulate the early stages of an infection. This was done by inoculating mice with strain 13 and mutant *C. perfringens* strains at a dose (1×10^6) 1,000-fold less than that needed to initiate a gangrene infection (1×10^9). We then measured how long the bacteria could persist in the mouse muscle tissue. Surprisingly, we found that after 24 h ~5% of the original inoculum of strain 13 bacteria was still alive (Fig. 4-6A). In addition, viable cells of strain 13 could be detected in the mouse tissues up to 72 h post-infection with an inoculum of 1×10^6 bacteria (unpublished data). These results indicate vegetative *C. perfringens* cells can persist in the host tissues for a significant period of time after inoculation into a wound area. Some cases of gangrene actually occur one to several days after trauma or surgery (204). The persistence of *C. perfringens* in the wound area may be an important factor in these

delayed cases of post-traumatic or post-surgical gangrene infection. A possible scenario is that a traumatic wound becomes contaminated with *C. perfringens* cells, but remains well vascularized and aerobic. *C. perfringens* can persist but not multiply in this environment. Subsequently, the blood supply to the area becomes compromised, perhaps by natural clotting factors or *C. perfringens*-dependent functions (27, 28). The resulting hypoxia may permit the surviving *C. perfringens* cells to proliferate and initiate a clinically evident gangrene infection.

In comparison to strain 13, strains DOB3 and PLC- exhibited a three-log decrease in the number of cfu found in the mouse muscle tissue after 24 h (Fig. 4-6 B and C). The level of persistence was about the same for both strains DOB3 and PLC-, suggesting that PFO and PLC are equally important for the persistence of *C. perfringens* in host tissues. This is the first time that a definitive role for PFO in survival of *C. perfringens in vivo* has been demonstrated. Strain DOB4, lacking both PFO and PLC, was even more compromised in its ability to survive *in vivo* than strains DOB3 and PLC-, since no live bacteria could be detected in the muscle tissue after 14 h (Fig. 4-6D). This suggests PFO and PLC play an additive role in *in vivo* survival. In previous studies using *pfoA* and *plc* mutant strains, a high dose (~1 x 10^9) bacteria was used to infect the mice (11, 12, 56, 209) and only subtle effects were observed when the *pfoA* mutant strain was compared to the wild-type strain. The results presented here suggest PFO may be essential only when the ratio of bacteria to phagocytic cells is low, and infections using large doses of bacteria overwhelm the host immune cells and disguise the role of PFO in the infection process.

In summary, we have identified a new role for PLC and PFO in *C. perfringens* pathogenesis: escaping the phagosomal membrane in macrophages. This adds a new feature to the multiple roles proposed for PLC in gangrene infections (see (206) for a review). We hypothesize that PFO plays an important role in bacterial defense against phagocytic cells early in the infection. We have identified two functions for PFO that are probably important in defense against host cell mediated killing have been identified in this report: phagosomal membrane lysis and cytotoxicity to the host cells (Table 4-2 and Fig. 4-5). Which function is more important in the infection process? Stevens and co-workers have suggested that cytotoxicity and upregulation of adhesion proteins on the surface of PMNs are an important component of PFO activity (26), while Portnoy and co-workers suggested that the ability of PFO to kill the macrophage after being phagocytosed may be an important function for PFO in *C. perfringens* infections (157). Our data suggest both functions are important. The ability to escape the phagosome is probably important in defense against macrophages while PFO-mediated cytotoxicity towards macrophages depends on the concentration of bacteria in the local environment and the extent to which the macrophages have become activated. Cytotoxicity and disruption of PMN bactericidal functions by PFO may be more important in defense against these cells than is the ability to escape the phagosome.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 4. *B. subtilis* and *E. coli* were grown in Luria-Bertani medium (LB) (10 g of tryptone, 5 g of NaCl, 5 g of yeast extract per liter). *C. perfringens* strains were grown in a Coy anaerobic chamber (Coy Laboratory Products) in PGY medium (30 g of proteose peptone, 20 g of glucose, 10 g of yeast extract and 1 g of sodium thioglycollate per liter) (127).

A highly phagocytic clone J774-33 (149), from the parent cell line J774A.1 macrophage like cell line, was used in these experiments. J774-33 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g l^{-1} of glucose and L-glutamine (Biowhittaker), 10% (v/v) fetal bovine serum (FBS), 5 mM sodium pyruvate and incubated in 5% CO_2 at 37°C.

Plasmid constructs and DNA manipulations

To create a *pfoA* mutant, the *pfoA* gene was amplified by PCR with oligonucleotides ODOB3 (5'–GGAACTGCTAGCATAATTGGAATCCCAG- 3'), located 400 bases upstream of the *pfoA* transcriptional start site and ODOB2 (5'-GACTAGCTAGCATCAGTTTTTAC- 3'), located 29 bases downstream of the *pfoA* stop codon. The PCR product was then digested with *Hind*III and *Spe*I, giving a 905 bp fragment internal to the *pfoA* gene, which was ligated to pSM300, digested with *Hind*III and *Xba*I, to form pSM250. pSM300 is a plasmid carrying an *ermBP* gene which confers erythromycin resistance on both *E. coli* and *C. perfringens* but an origin of

replication that functions only in *E. coli*. Early stationary phase *C. perfringens* strain 13 cells were subjected to electroporation in a 2 mm cuvette with 50 μg of pSM250 and grown on PGY plates with 30 μg/mL of erythromycin. Three erythromycin resistant colonies were isolated and all exhibited a PFO- phenotype on sheep blood agar plates (i.e., lacked the inner zone of hemolysis characteristic of *C. perfringens*). All subsequent work was done with one of these isolates, DOB3. To make a *pfoA*/*plc* mutant, a *pfoA* gene disruption was introduced into strain PLC-, a *plc*- derivative of *C. perfringens* strain 13 (obtained from A. Okabe (98)), as described above for the *pfoA* strain. Transformants were plated on PGY plates containing chloramphenicol and erythromycin. Several chloramphenicol/erythromycin resistant colonies were seen; one of these (DOB4) was characterized further. Cells of strain DOB4 were plated on sheep blood agar plates, and the colonies exhibited a complete lack of both the inner (i.e., PFO) and outer (i.e., PLC) zones of hemolysis.

To complement the *pfoA* strain, the *pfoA* gene was amplified by PCR using primer ODOB1 (5' –GATTTACTTAACAAATGAGGGG- 3'), which begins 53 bases upstream of the *pfoA* ATG start codon and primer ODOB2. The PCR product was cloned into pGEM-T Easy making pSM259. This clone contained the entire coding sequence and ribosomal binding site for the *pfoA* gene but lacked the previously identified promoters of the *pfoA* gene (17). pSM259 was digested with *Sac*I and *Sph*I and the *pfoA* gene-containing fragment was ligated into pJIR750, also digested with *Sac*I and *Sph*I, forming pSM260. DOB3 was transformed by electroporation with pSM260. A promoterless version of the *pfoA* gene was chosen to prevent overexpression of the *pfoA* gene on a

multicopy plasmid, which would make subsequent experiments more difficult to interpret. Positive transformants were struck onto sheep-blood agar plates and compared to wild-type strain 13 to determine if two zones of hemolysis were present. As expected, positive transformants yielded two zones of hemolysis. The PLC- mutant and plc complemented mutant (PLC-(pJIR418α)), obtained from A. Okabe, were plated on sheep-blood agar and egg yolk agar plates to confirm the lack of PLC activity.

Southern hybridization analysis

Southern hybridization analysis was done on the *pfoA* and *pfoA/plc* mutants. Chromosomal DNA from strain 13 and DOB3 was isolated and digested with *Eco*RI or *Xho*I and *Eco*RI. Digests were run on a 0.8% agarose gel and transferred to a nylon membrane (Sambrook). DOB3 blots were hybridized with the internal *pfoA* fragment from pSM250 or with an *ermBP* gene probe. After *Eco*RI digest, the *pfoA* gene probe was predicted to hybridize to a 10.3 kb fragment (i.e. a 6.7 chromosomal region plus 3.6 kb for pSM300). Instead, the probe hybridized to a ~13 kb fragment. One explanation for the larger than predicted hybridization band, was that the plasmid integrated as a dimer (Fig. 4-7). Therefore, we performed a second set of restriction digests with *Eco*RI and *Xho*I and used an *ermBP*-specific probe. If pSM250 integrated as a dimer, the *ermBP* probe was predicted to hybridize to bands 3.6 kb and 6.3 kb in size (Fig. 4-7). This was the pattern observed indicating pSM250 had integrated as a dimer and disrupted the chromosomal *pfoA* gene. Probes were labeled and hybridized according to the protocol of the NEBlot Phototope Kit (New England Biolabs).

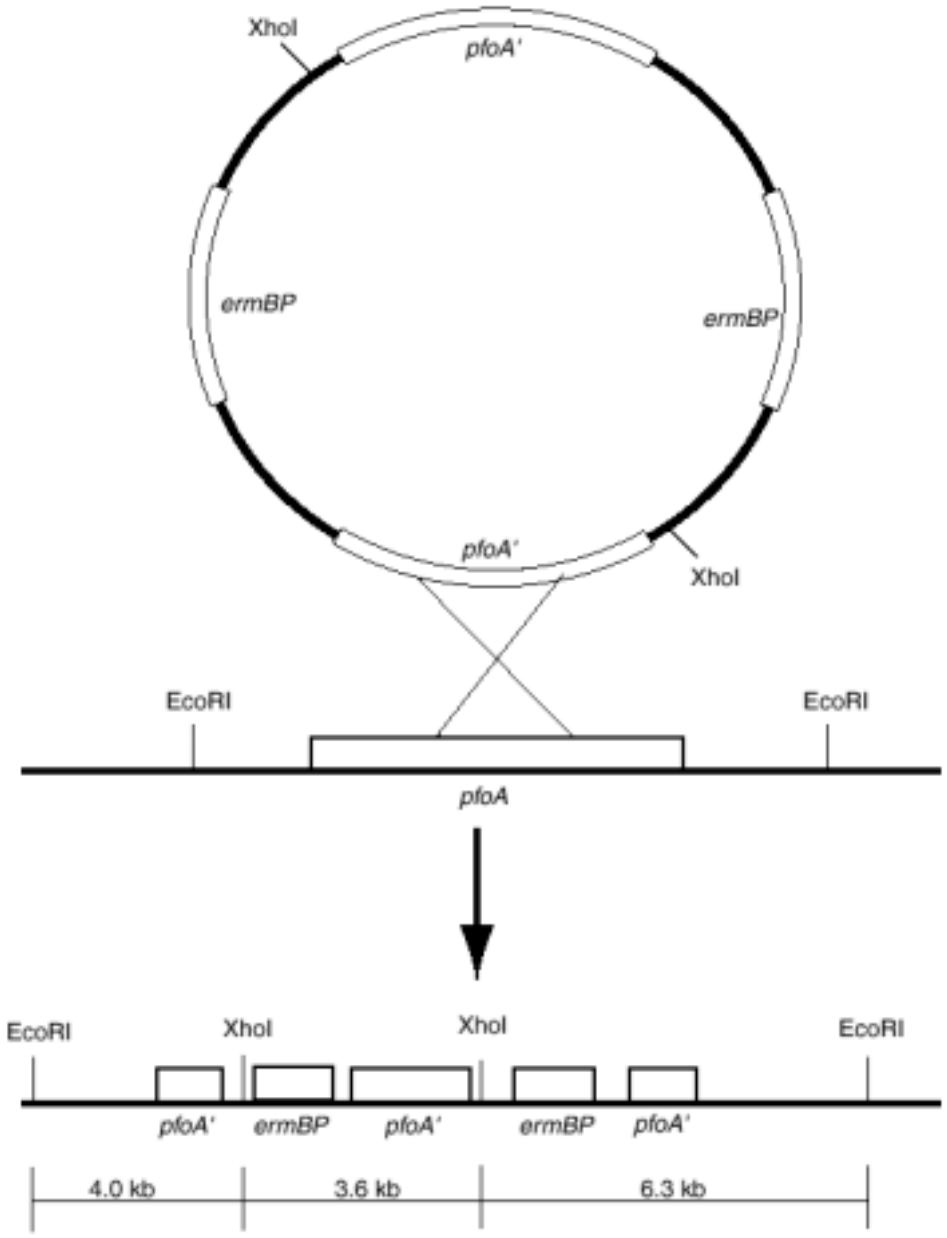

Figure 4-7. A schematic diagram illustrating the integration event of pSM250 into the chromosome of the *pfoA* gene of *C. perfringens* strains 13 and PLC-. Southern blot analysis showed pSM250 integrated into the chromosome in the doublet form illustrated in the figure. *Eco*RI and *Xho*I fragments used for the Southern hybridization analysis are shown at the bottom of the figure.

Enzyme assays

The *pfoA* and *pfoA*/*plc* strains of *C. perfringens* were grown to stationary phase and the supernatants were collected, filtered, and used to measure PFO production. Red blood cell buffer (2 mM EDTA, 20 mM dithiothreitol, 64 mM KH_2PO_4, 156 mM NaCl, pH 6.8) containing 50% red blood cells (RBC) was added to an equal volume of filtered supernatant. The mixture was incubated at 37°C for 30 min under anaerobic conditions. Reactions were centrifuged at 1000 rpm for 5 min. A spectrophotometer set at 550 nm was used to detect the release of hemoglobin from the RBC. Protein assays, using the BioRad Protein Assay Kit, were performed on each sample. A hemolytic unit was defined as the difference in the absorbance at 550 nm between the sample and blank per microgram of protein present in each sample.

Bacterial survival in the presence of macrophages under aerobic and anaerobic conditions

Bacterial survival assays were performed in the presence of J774-33 cells under aerobic and anaerobic conditions as previously described (149) with the exception that DOB3, DOB4, and DOB3pSM260 were incubated in the presence of 30 μg/mL erythromycin for the duration of the experiment. Briefly, J774-33 cells were inoculated into 24-well tissue culture plates at approximately 3.5 x 10^5 cells/well and allowed to grow in DMEM overnight in 5% CO_2 at 37°C. *C. perfringens* was grown overnight in 3 ml PGY in the anaerobic chamber at 37°C. The next day, bacterial cultures were inoculated into pre-warmed media and grown for ~ 3 h to obtain mid-logarithmic phase bacteria. The bacteria were washed three times in PBS, re-suspended in 1 ml of PBS, and

then used to infect the macrophages. Bacteria were added at a multiplicity of infection (MOI) of ~ 1:1. After the addition of bacteria, the macrophages were lysed at the indicated times, and the surviving *C. perfringens* cells were plated on PGY agar medium and incubated anaerobically. To lyse the J774-33 cells, Triton X-100 was added to the respective wells to a final concentration of 0.02%. After the infected macrophages were incubated with the Triton X-100 for 1 h, the macrophage suspensions were pipetted vigorously to ensure the release of phagocytosed bacteria.

Co-localization of *C. perfringens* and the late endosome-lysosomal marker LAMP-1

Co-localization studies of intracellular *C. perfringens* and the late endosome-lysosomal marker LAMP-1, were performed as previously described (149). Briefly, 12 mm round glass coverslips were placed in 24-well tissue culture plates, and J774-33 cells were added and grown in DMEM until nearly confluent (1.5-2 days). Log phase *C. perfringens* were then added at an MOI of 10:1. The plates were incubated in 5% CO_2 at 37°C for 1 h. The cells were then fixed in periodate-lysine-paraformaldehyde (125) containing 5% sucrose at room temperature for 20 min and then washed three times in PBS. The J774-33 cells were permeablized by dipping the coverslips in cold (-20°C) methanol for 10 s and washed three times for 5 min with 2% goat serum in PBS (blocking buffer) at room temperature. Both *C. perfringens and B. subtilis* were stained with anti-*C. perfringens* polyclonal rabbit serum (Applied Diagnostics International) diluted 1:100 in blocking buffer and incubated for 1 h at 37°C. *B. subtilis* cells cross react with anti-*C. perfringens* antibodies (149). Following three 5 min washes in blocking buffer, the cells were stained with anti-mouse LAMP-1 rat monoclonal antibody

1D4B diluted 1:200 in blocking buffer and incubated for 1 hour at 37°C. (The 1D4B monoclonal antibody developed by J. T. August was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). The coverslips were washed three times in blocking buffer for 5 min at room temperature. The cells were then stained simultaneously with Texas Red-X-conjugated goat anti-rat IgG diluted 1:200 and FITC-conjugated goat anti-rabbit IgG diluted 1:200 (Molecular Probes) for 1 h at 37°C. The coverslips were then washed 3 times in PBS and mounted on slides with ProLong anti-fade mounting medium (Molecular Probes). Samples were viewed using a Zeiss LSM 510 laser confocal microscope at excitation wavelengths of 448 and 543 nm, for FITC and Texas Red, respectively. To produce 3-dimensional images, an average of 20 cross-sectional images were captured over a vertical distance of 8-15 μm.

Transmission electron microscopy

To determine if mutants lacking PFO or PLC were capable of escaping the phagosome of J774-33 cells, transmission electron microscopy was performed as previously described (149). Briefly, J774-33 cells were grown in DMEM to near confluency in 50 ml tissue culture-treated flasks. *C. perfringens* strains were grown in PGY medium to mid-log phase and then washed three times in PBS to remove extra-cellular toxins. The bacteria were added at an MOI of 10:1 to the macrophage culture and incubated at 37°C for 60 min. The infected macrophages were washed with PBS and then fixed in 2.5% glutaraldehyde in PBS for 5 minutes, pelleted by a 1 minute spin at

200 x g and left in the fixative for 1-2 h at room temperature. The pellets were then processed as previously described (137). Between 23 and 42 intracellular bacteria were counted for each sample and were judged to be to be inside the phagosome or in the cytoplasm. Some intracellular bacteria appeared to be in partially degraded phagosomes (not shown). Samples were viewed and photographed on a JEOL 2000EX electron microscope at 60 kV.

Survival of *C. perfringens* incubated with mouse peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages were isolated from C3Heb/Fe mice as previously described (130) and transferred to 24-well tissue culture plates. Nonadherent cells were washed off with Medium C (RPMI 1640 with 10% FBS, 10 mM HEPES buffer, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin) and adherent cells were incubated overnight in Medium C in 5% CO_2 at 37°C. Each well of the 24-well tissue culture plates contained 6 x 10^5 macrophages per well. Macrophages were washed 2 times with, and incubated until infected, in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l of both glucose and L-glutamine, 10% (v/v) FBS and 5 mM sodium pyruvate. After growth in PGY, log phase *C. perfringens* were washed three times in PBS and added at an MOI of ~ 1:1 to aerobically and an MOI of 0.5:1 to anaerobically treated macrophages. Lower MOIs were used for the anaerobic conditions because higher numbers were found to be rapidly lethal to the macrophages under anaerobic conditions. Plates containing macrophages were incubated either aerobically or anaerobically in 5% CO_2 at 37 °C during the entire assay. For anaerobic assays, plates with macrophages were incubated in an anaerobic

chamber 2 h prior to infection to ensure anaerobic conditions. The macrophages were lysed at increasing times post infection, and the surviving bacteria diluted and plated on PGY medium and incubated under anaerobic conditions. Triton X-100 was added to the wells at a final concentration of 0.02% for 1 h to lyse the macrophages.

In vivo* survival of *C. perfringens

Female Balb/c mice were used for the *in vivo* survival experiments. Mid-logarithmic phase *C. perfringens* cells were injected into the femoral muscle of the left leg at ~ 10^6 bacteria/mouse. Before injections, the mice were anesthetized by placing them in an enclosed container containing isofluorane for ~ 30 seconds. At the indicated time points the mice were euthanized and the left hind leg removed. The femoral muscle was then dissociated using a metal sieve to release the bacteria from the muscle. The bacterial suspensions were diluted and plated on PGY medium under anaerobic conditions to determine the number of cfu. Six mice were used for each time point in the experiment.

To deliver a lethal dose of *C. perfringens*, mice were treated as described above and injected in the femoral muscle with ~ 1 x 10^9 bacteria per mouse. Once the onset of gangrene was observed, the mice were euthanized to minimize any unnecessary suffering in accordance with Virginia Tech animal care practices. Mice that did not show signs of gangrene were euthanized 36 h after the start of the experiment. Experimental protocols involving mice were examined and approved by the Virginia Tech Institutional Animal Care and Use Committee.

C. perfringens-dependent cytotoxicity to J774-33 and mouse peritoneal macrophages

C. perfringens dependent cytotoxicity to J774-33 cells was determined using the Cytotox 96 Cytotoxicity assay (Promega) as previously described (149). J774-33 cells were rinsed with DMEM and diluted into 96-well tissue culture plate to a final concentration of 5 x 10^4 cells ml^{-1}. Mouse peritoneal macrophages were harvested as described above and added to 96-well tissue culture plates at a final concentration of 5 x 10^4 cells ml^{-1}. Macrophages were grown overnight in 5% CO_2 at 37°C. *C. perfringens* was prepared from mid-logarithmic phase cultures as described above and added at the MOI indicated in Fig. 4-5. For the cytotoxicity experiments, macrophages were infected for 5 h. Samples were then processed and the absorbance at 570 nm was measured using a Spectra Max 340 96 well plate reader (Molecular Devices).

Table 4-4. Strains and plasmids used in the study.

Strain/Plasmid	Relevant characteristics	Source or reference
E. coli		
DH10B	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) F80d *lacZ* ΔM15 *lacX74 deoR recA*l *araD139* (*ara, leu*)7697 *galU galK λ-rpsL endAl nupG*	Gibco/BRL Corp.
B. subtilis		
Strain JH642	*TrpC2 pheA1*	J. Hoch
C. perfringens		
strain 13	PFO+, PLC+	C. Duncan
DOB3	PFO-, PLC+	This study
DOB4	PFO-, PLC-	This study
PLC-	PFO+, PLC-	A. Okabe (Kameyama *et al*., 1996)
Plasmids	Parent/genotype	
pGEM-T Easy		Promega Corp.
pSM300	*E. coli* origin of replication, erythromycin resistance	This study
pSM250	pSM300/ 905 bp internal fragment of *pfoA*	This study
pSM259	pGEM-T Easy/ promoterless *pfoA*	This study
pJIR750	*C. perfringens-E. coli* shuttle vector	(Bannam and Rood, 1993)
pSM260	pJIR750/ promoterless *pfoA* from pSM259	This study
pJIR418-α	*C. perfringens-E. coli* shuttle vector/ *plc* from strain 13	(Ninomiya *et al.*, 1994)

ACKNOWLEDGEMENTS

We thank Katherine Traughton (UT Memphis) and John Varga for electron microscopy assistance; Bambi Jarrett, Michael Woodman and Brandon Black for assistance with the *in vivo* mouse studies, and David Popham and Donna Pate for critical reading of the manuscript.

CHAPTER FIVE

The Role of Polymorphonuclear Leukocytes and Macrophages in Host Defense Against *Clostridium perfringens*

ABSTRACT

Upon entry into a wound, *C. perfringens* encounters host phagocytic cells. Both macrophages and PMNs can phagocytose *C. perfringens*, but the role, if any, these cells play a role in host defense, *in vivo*, is uncertain. By eliminating a component of the immune system, either macrophages or PMNs, we can determine what role that component plays in an infection. In this study, we used a liposome-selective macrophage-elimination technique to reduce monocytic cells (macrophages and monocytes) in BALB/c mice. We reduced PMNs in mice using a monoclonal antibody, RB6-8C5, specific for PMNs. The reduction of macrophages from the muscle tissue made mice ~10-fold more susceptible to *C. perfringens* infections, suggesting that macrophages do play a role in preventing gangrene infections from occurring although previously no macrophage killing has been observed *in vitro*. These results increase our knowledge of what role macrophages play in the initial stages of a gangrene infection, and suggest this could be an area for new adjunctive therapies.

INTRODUCTION

Clostridium perfringens is a Gram-positive anaerobic bacterium that is the most common cause of gas gangrene (clostridial myonecrosis) in humans. As a species, *C. perfringens* produces at least 13 different toxins, which are thought to be the major virulence factors in causing disease (172, 197). Of these toxins, the theta (θ, PFO) and alpha (α, PLC) toxins are important in the pathogenesis of gas gangrene. If left untreated, gas gangrene is always fatal due to the severe shock that follows the release of toxins into the bloodstream (10, 208).

We are interested in what happens during the initial stages of an infection, when bacterial numbers are low and phagocytic cells are present, because once gangrene has occurred, there is a marked decrease in the amount of immune cells at the site of an infection. Therefore, by studying the interaction between *C. perfringens* and phagocytic cells prior to the onset of gangrene, we can establish what role these cells play in an infection. The mechanism most often proposed is that the toxins produced by *C. perfringens* destroy the phagocytic cells present at the site of an infection. There is evidence that PFO is cytotoxic to leukocytes at high concentrations (29, 207). We believe the leukocyte toxicity effect to be relevant only at the later stages of an infection when the wound becomes more anaerobic, which in turn allows the bacteria to become more metabolically active than at the initial stages, when the wound is probably still somewhat aerobic. Another reason for the lack of PMNs at the site of infection is that PFO can alter the chemotactic migration and morphology of polymorphonuclear leukocytes (207). PFO and PLC also interfere with the migration of leukocytes by

upregulating the expression of intercellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1) on endothelial cells, a function normally done by interleukin-8 (IL-8). Bryant and Stevens supported this by demonstrating that alpha toxin induced the expression of both ICAM-1 and ELAM-1, as well as the secretion of IL-8 in human umbilical vein endothelial cells (HUVEC). Theta toxin only increased the expression of ICAM-1 but neither ELAM-1 nor IL-8 (30). This is most likely responsible for the accumulation of PMNs in blood vessels surrounding the wound site, a phenomenon known as leukostasis.

Not much is known about what role macrophages play in a gangrene infection. We reported that *C. perfringens* could survive in the presence of J774-33 cells (149), mouse peritoneal, and bone marrow-derived primary macrophages (unpublished data), *in vitro* under aerobic conditions. Other work done in the lab has shown that PMNs are capable of killing *C. perfringens* under aerobic and anaerobic conditions when complement is present (unpublished data). The role that PMNs play *in vivo* has not been elucidated.

To answer what role phagocytic cells play in *C. perfringens* infection, we used methods to eliminate macrophages and PMNs from the host. We injected BALB/c mice with a monoclonal antibody, RB6-8C5, which reacts with the Gr-1 surface antigen of murine granulocytes (167), but not with lymphocytes or monocytes. Liposomes containing dichloromethylene diphosphonate (clodronate, CL_2MBP) deplete host macrophages (231) with no damage to the architecture or presence and function of other phagocytic cells (41, 138, 161). Therefore, we injected BALB/c mice with Cl_2MDP

encapsulated in liposomes to deplete tissue macrophages. These liposomes deliver the drug directly to the cytosol of macrophages, leading to disruption of their metabolism in an unknown manner, and death (231). Using these model systems, we can determine if either of these cell types are involved in the pathogenesis of *C. perfringens*.

RESULTS

Reduction of blood monocytes and tissue macrophages

To assess the level of macrophage depletion in CL_2MDP liposome-treated mice, we collected the femoral muscle of the mice made 24 h after injection with the CL_2MDP-liposomes and PBS-liposomes. One mm thick muscles were sent to Histo-Scientific Research Laboratories for the production of sections 5 μm. Using imunofluorescence microscopy, we stained the tissue sections with anti-mouse CD68, an antibody specific for tissue macrophages, and determined that there was a 66% decrease in the amount of macrophages in the CL_2MDP-treated mice as compared to the PBS-treated mice (data not shown).

Flow cytometry was also performed to assess the level of macrophage depletion in peripheral blood. These results were inconclusive due to the low specificity of our antibody for peripheral blood monocytes and macrophages. We have received another antibody which is specific for peripheral blood monocytes and macrophages and should make this assay much more quantitative.

Effects of macrophage depletion in *C. perfringens* infections

Having ascertained the efficiency of macrophage depletion, we investigated the role of macrophages in the development of gas gangrene. Six BALB/C mice were given i.v. injections of CL_2MDP-liposomes 24 h prior to infection with *C. perfringens*. Control mice were given PBS liposome i.v. injections prior to being challenged with *C. perfringens*. Mice were infected in the femoral muscle with varying concentrations of *C. perfringens* ranging from 1 x 10^6 bacteria/mouse to 1 x 10^9 bacteria/mouse, a non-lethal

dose and a lethal dose respectively. The susceptibility of these mice to *C. perfringens* was determined by the onset of gangrene symptoms, which include limping, swelling, and blackening of the hind leg and foot.

Mice treated with CL_2MDP-liposomes were more susceptible to *C. perfringens* infections at lower doses than were the group that received PBS-liposomes (Figure 5-1). Both groups that received a lethal dose of *C. perfringens* showed clear signs of gangrene between 6 and 7 h post infection. However, four of the six mice mice treated with CL_2MDP-liposomes that received 1 x 10^8 bacteria/mouse, showed signs of gangrene between 13 and 14 h post infection, while mice that received PBS liposomes showed no signs of gangrene throughout the experiment. The two remaining mice in the CL_2MDP-liposomes treated group that did not show signs of gangrene, did have a noticeable limp as well as a disheveled look. Two mice in the PBS-liposome control group also had a minor limp.

Reduction of peripheral polymorphonuclear leukocytes

Prior to injection with PBS or RB6-8C5, peripheral blood from five BALB/C mice was collected and examined for the number of PMNs/cu mm. Mice were then injected with 100 μg of RB6-8C5 antibody or 100 μL of PBS and examined again for the number of PMNs/cu mm 1, 3, and 5 days post infection (Fig. 5-2). Prior to treatment with PBS or RB6-8C5, control mice had 1226 PMNs/cu mm and experimental mice had 914 PMNs/cu mm. One day after injection, control mice showed no decrease in PMNs, while the RB6-8C5 injected mice showed a marked decrease to 42 PMNs/cu mm, a > 97% decrease in concentration. By the third day after injection, the level of PMNs in the

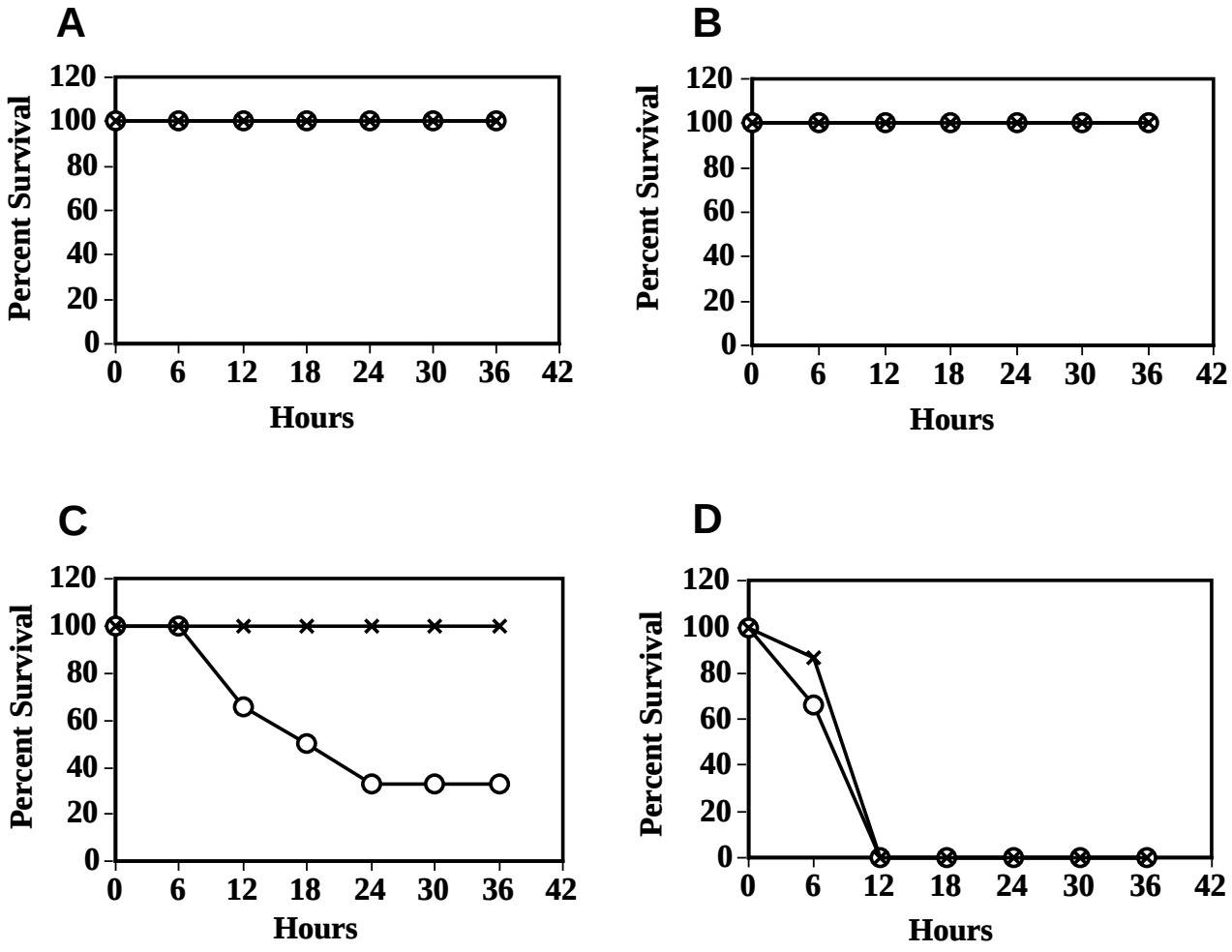

Fig 5-1. The effect of clodronate on mice infected with varying doses of *C. perfringens*. Mice were depleted of their tissue macrophages by injection with liposomes containing clodronate 24 h prior to infection with *C. perfringens*. Circles represent clodronate-liposome treated mice. Cross hairs represent PBS-liposome treated mice. *C. perfringens* infectious doses were A) 1×10^6 B) 1×10^7 C) 1×10^8 D) 1×10^9.

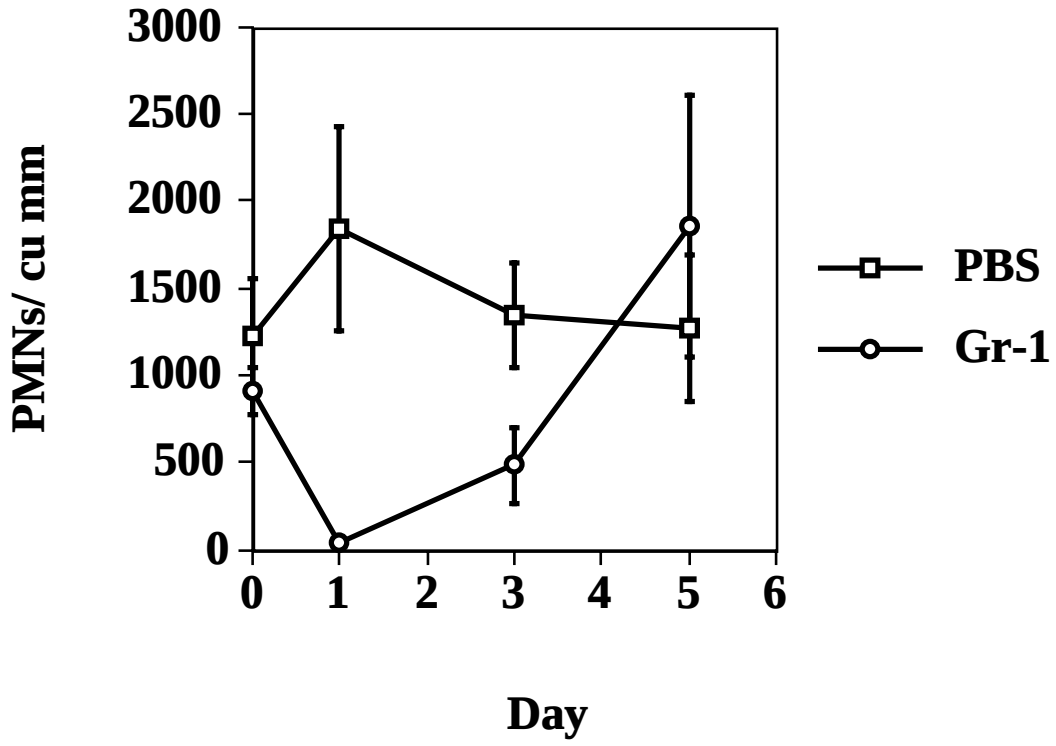

Figure 5-2. Polymorphonuclear leukocytes were depleted from BALB/c mice upon injection with RBC-8C5 (Gr-1). Mice showed a dramatic decrease in the number of PMNs/cu mm one day after injection with RBC-8C5. The number of PMNs/cu mm were restored to normal levels by day five after injection with RBC-8C5.

RB6-8C5 injected mice began to increase and by day five PMN levels were completely restored to pre-treatment levels. Control mice showed no difference on days three and five after infection. These results suggest that RB6-8C5 is sufficient to deplete peripheral blood PMNs.

DISCUSSION

In these studies, we show for the first time that injected liposome-encapsulated clodronate eliminates muscle tissue macrophages. Using this procedure, we have established the importance of macrophages in a *C. perfringens* infection. We have shown that macrophages are unable to kill *C. perfringens* even under aerobic conditions *In vitro* (149). *C. perfringens* was also capable of persisting for 72 h *in vivo* when mice were infected at a sub-lethal dose of 1 x 10^6 bacteria (unpublished data). Therefore, we originally thought macrophages were providing a niche for the bacteria to reside in until conditions became sufficient for growth. This niche would also provide refuge from PMNs that have been shown to kill *C. perfringens* (unpublished data). However, our data suggests differently that this is not the case, and that macrophages are involved in bacterial killing. When macrophages were eliminated from mice, the onset on gangrene occurred with 10-fold fewer bacteria, as compared to PBS treated mice. This suggests that macrophages seem to be involved in bacterial killing, however, due to the minor 10-fold decrease; we cannot rule out that some macrophages may be unable to kill *C. perfringens*. To understand the role PMNs play, we still need to eliminate them from mice and challenge those mice with *C. perfringens*. Due to our *in vitro* data, we believe that by knocking out the PMNs, we will see a more dramatic decrease in the number of bacteria needed to cause gangrene.

We believe that once *C. perfringens* enters a wound, it is phagocytosed by resident macrophages, where some are killed and some escape the phagosome and persist until conditions become right for growth. At the same time, PMNs are recruited to the

wound area and kill the bacteria they are able to phagocytose. However, due to the lack of total killing and the increasing anaerobic conditions, the remaining *C. perfringens* can grow and secrete toxins killing the macrophages they reside in. The released toxins can then diffuse through the tissue preventing the influx of new PMNs to the site of infection, allowing the bacteria to grow unrestrained ultimately leading to gangrene.

MATERIAL AND METHODS

Bacterial strains and growth conditions

The strain used in this study was *C. perfringens* strain 13 (obtained from C. Duncan). *C. perfringens* strain 13 was grown in a Coy anaerobic chamber (Coy Laboratory Products, Inc.) in PGY medium (30 g of proteose peptone, 20 g of glucose, 10 g of yeast extract, and 1 g of sodium thioglycolate per liter) (127).

Preparation of liposomes

Clodronate-liposomes were prepared as previously described (231). Briefly, 33mg/mL of phosphatidylglycerol (DSPG) (Avanti), 33 mg/mL of cholesterol (Sigma), and 100mg/mL of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (Avanti) were dissolved in 10 mL of chloroform and the chloroform was removed by reverse-phase evaporation. The resulting phospholipid film was dissolved in 10 ml of chloroform containing 0.6 M clodronate (CL_2MBP). The suspension was kept at room temperature under N_2 gas for 2 h. The suspension was then sonicated for 3 min and stored under N_2 at 4˚C overnight. Liposomes were then washed in phosphate buffered saline (PBS) 3 times by centrifugation at 25,000 x g for 30 min. The liposomes were resuspended in 4 ml PBS and kept under N_2 at 4˚C until they were ready for use.

Control liposomes were made in the same way except PBS was used in place of clodronate.

Depletion of tissue macrophages

To deplete the tissue macrophages of mice, 100µl of CL_2MBP-liposomes or PBS-liposomes were administered i.v. into the tail vein of the mice. To assess the depletion of

macrophages, the mice were sacrificed 24 h post-infection and the femoral muscle of the mouse removed. Muscles were then soaked in neutral-buffered formalin for ~36 h and placed in 70% ethanol. Samples were then sent to Histo-Scientific Research Laboratories (HSRL) (Woodstock, Virginia) for sectioning. Flow cytometry was also used to assess the degree of macrophage depletion.

Immunohistochemistry of paraffin sections

Tissue samples were examined for the depletion of macrophages by immunofluorescence as previously described (166). Briefly, 5 μm sections that had previously been imbedded in paraffin were incubated in 10% normal goat serum in PBS for 10 min. Sections were then incubated with rat anti-mouse CD68 IgG specific for tissue macrophages (Serotec) and diluted 1:200 in 0.2% saponin and 10 % normal goat serum in PBS for 6 h at 4˚C. Samples were then washed 3 times with PBS and stained with Oregon green goat anti-rat IgG diluted 1:200 (Molecular Probes), for 1 h at 4˚C. Samples were then washed 3 times in PBS and sealed using ProLong anti-fade mounting medium (Molecular Probes). Samples were viewed using an Olympus Provis AX70 fluorescence microscope.

Flow cytometry

Two-hundred μl of anticoagulated blood was incubated for 15 min at room temperature in PharmM lyse (BD Biosciences) to remove the red blood cells. Cells were spun at 200 x g for 5 min and then washed in wash buffer (0.1% sodium azide, 1.0% fetal bovine serum in PBS) and centrifuged at 350 x g for 5 min and resuspended at a final concentration of 2 x 10^7 cells/ml. Macrophages were then labeled with rat anti-mouse

F4/80 IgG (Serotec) (0.5μg per 100 μl of wash buffer) and incubated for 40 min at 4˚C. Cells were washed 3 times in wash buffer and stained with Oregon green goat anti-rat IgG (Molecular Probes) (0.5μg per 100 μl of wash buffer) for 40 min at 4˚C. Cells were washed twice in wash buffer and resuspended in 500 μL of wash buffer for flow cytometry.

Depletion of polymorphonuclear leukocytes

Mice were injected i.p. with 100 μg of the monoclonal antibody RB6-8C5 (Cedarlane) specific for Gr-1 surface antigen present on murine granulocytes but not on monocytes or lymphocytes (167). The antibody was administered 1 day before being challenged with *C. perfringens*. To verify that the PMNs were being depleted, blood smears were taken from control mice 1, 3, and 5 days post injection, stained using Hema 3 Stain Set (Fisher), and the total number of PMNs was determined. Control mice received a similar injection of PBS. Mice receiving bacterial inoculations were checked prior to their inoculation for the lack of PMNs as described above.

Experimental infection

Female Balb/c mice were used for the *in vivo* survival experiments. Prior to infection with *C. perfringens*, mice were depleted of either their macrophages or PMNs as described above. Briefly, 1 h prior to the start of the experiment, 0.1 mg of bupronorphine per kg of mouse was injected sub-cutaneously. *C. perfringens* cells were grown to mid-logarithmic phase and injected into the femoral muscle of the left leg at ~ 1 x 10^9, 10^8, 10^7, and 10^6 bacteria/mouse. Before injections were made, the mice were anesthetized by placing the mice in a container containing isofluorane for ~ 30 seconds.

Once the onset of gangrene was observed, the mice were euthanized to eliminate any unnecessary suffering. Any mice that did not show signs of gangrene were euthanized 36 h after the start of the experiment. Six mice were used for each time point.

CHAPTER SIX

Significant strain variability is seen in the sialidase-encoding genes of *Clostridium perfringens*

ABSTRACT

Clostridium perfringens is a common cause of disease in humans and animals. The bacterium produces up to thirteen different toxins, which are believed to be the main virulence factors in the disease process. One of these toxins is sialidase, which cleaves off terminal sialic acid residues from sialoglycoconjugates. Sialidase is thought to provide a carbohydrate, in the form of free sialic acid, as a nutrient and also interferes with the normal cellular functions of cells missing their sialic acid residues. *C. perfringens* is unusual in that it has two sialidases, NanI and NanH, which are secreted and cell-associated, respectively. Eleven strains of *C. perfringens* were examined to determine if they carried the *nanI* and/or *nanH* genes; four strains were identified that lack the *nanI* gene but only one strain, strain 13, lacked the *nanH* gene. The four *nanI*- strains were all from the Hobbs serotype group of *C. perfringens* strains, isolated in Great Britain in the 1950s, and known to have caused large-scale outbreaks of food poisoning. The transcription start sites for the *nanI* gene were mapped by primer extension of RNA isolated from *C. perfringens* strain 13 grown on medium with or without added sialic acid. Three putative start sites were identified and all showed significant levels of induction in the presence of sialic acid. A comparison of the promoter regions of other sialic acid-associated genes identified a conserved 17 bp motif that is present in multiple copies in the *nanH*, *nanI*, and *nanE* promoter regions. The *nanH* genes from two *C. perfringens* strains were cloned and expressed in *E. coli* or *C. perfringens*. Interestingly, the *nanH* gene product from strain 6234 LI, a type C porcine enteritis isolate, was found in the extracellular medium and cell-associated when it was expressed in both *E. coli* and

C. perfringens, while the *nanH* gene product from strain NCTC 8798 was always found to be cell-associated.

INTRODUCTION

Clostridium perfringens commonly causes clostridial myonecrosis (gas gangrene), acute food poisoning and antibiotic-associated diarrhea in humans, as well as a wide range of enteric diseases in domestic livestock (144). As a species, *C. perfringens* produces at least 13 different toxins, which are thought to be the major virulence factors in causing disease (172). One of these toxins, the enzyme sialidase (or neuraminidase) cleaves off terminal sialic acid residues from sialoglycoconjugates (187). *C. perfringens* is an unusual bacterium in that it synthesizes two different sialidase enzymes. The larger (73 kD) sialidase, called NanI, is secreted from the cell and exhibits a broad substrate specificity (171). *C. perfringens* also synthesizes a smaller (43 kD) cell-associated sialidase, called NanH (170, 171, 224). The role of the cell-associated enzyme was initially puzzling, since the substrate for sialidase is usually a complex carbohydrate that cannot be transported into the cell. However, more recent work shows NanH has a marked preference for cleaving sialic acid attached to short oligosaccharides (169), indicating it acts on low molecular weight oligosaccharides that may be transported into the cell.

At present, it is unknown whether *C. perfringens* sialidase is a virulence factor in enteric diseases of animals, although a role for sialidases in acquiring nutrients for the bacterium is proposed (46). Free sialic acid is taken up and can be used as a carbohydrate source by *C. perfringens* (171). Previous reports by us (236) and that of Traving et al. (223) have described the cloning and sequencing of the *nanE*/*nanA* bicistronic operon of *C. perfringens*, which encodes the genes for a putative N-acetylmannosamine-6-P

epimerase (*nanE*) and sialic acid lyase (*nanA*), both of which are involved in the breakdown and utilization of sialic acid (236). A role for sialidases is known in other bacterial pathogenesis systems. For some bacteria, removal of sialic acid by sialidase activity may play a role in pathogenesis by providing a location for bacterial attachment to the remaining glycoconjugate residues (64, 100). The neuraminidase of *Streptococcus pneumoniae* was recently identified as essential for virulence in a mouse model, using signature tagged mutagenesis as a method to identify virulence factors (155). In addition, a direct nutritional role for sialic acid metabolism has been demonstrated for an animal model of *Bacteroides fragilis* abscess formation (67).

The gene encoding the large sialidase (*nanI*) is cloned and sequenced from strain A99 (224), while the *nanH* gene is cloned and sequenced from strains A99 (170) and ATCC 10543 (39). The *nanI* gene product shows the greatest amount of sequence identity to other large sialidases from diverse *Clostridium* spp. and less (28%) to the small sialidase of *C. perfringens* (169). In a genetic screen of 2,659 isolates of *C. perfringens* from human and animal diseases, all but one strain, JIR323, a laboratory derivative of strain 13, hybridized to a *nanH*-specific gene probe (49). This suggests that the *nanH* gene product is important in the survival of *C. perfringens* in association with animal species. We report here that there is a population of strains, isolated in Great Britain and associated with large-scale outbreaks of acute food poisoning, that lack the *nanI* gene, suggesting that the extracellular sialidase is not essential for this group of strains.

RESULTS

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

In order to study the regulation of the *nanI* gene in different strains of *C. perfringens*, a technician in the lab, Veronica Stirewalt, used PCR techniques to amplify an internal segment of the *nanI* gene to use as a molecular probe for Southern and northern blots. Degenerate primers designed to anneal to regions of the *nanI* coding sequence (224) 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 available in our laboratory were also tested and found to display an interesting pattern: Four CPE+ strains designated as Hobbs serotypes 3, 6, 9, and 13, isolated from food poisoning outbreaks in Great Britain in the 1950s (210), lack the *nanI* gene (Figure 6-1, top). Each strain lacking the *nanI* gene also lacked the NanI enzymatic activity (Figure 6-1, 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 lacking *nanI* are CPE+ and the 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 (Figure 6-1).

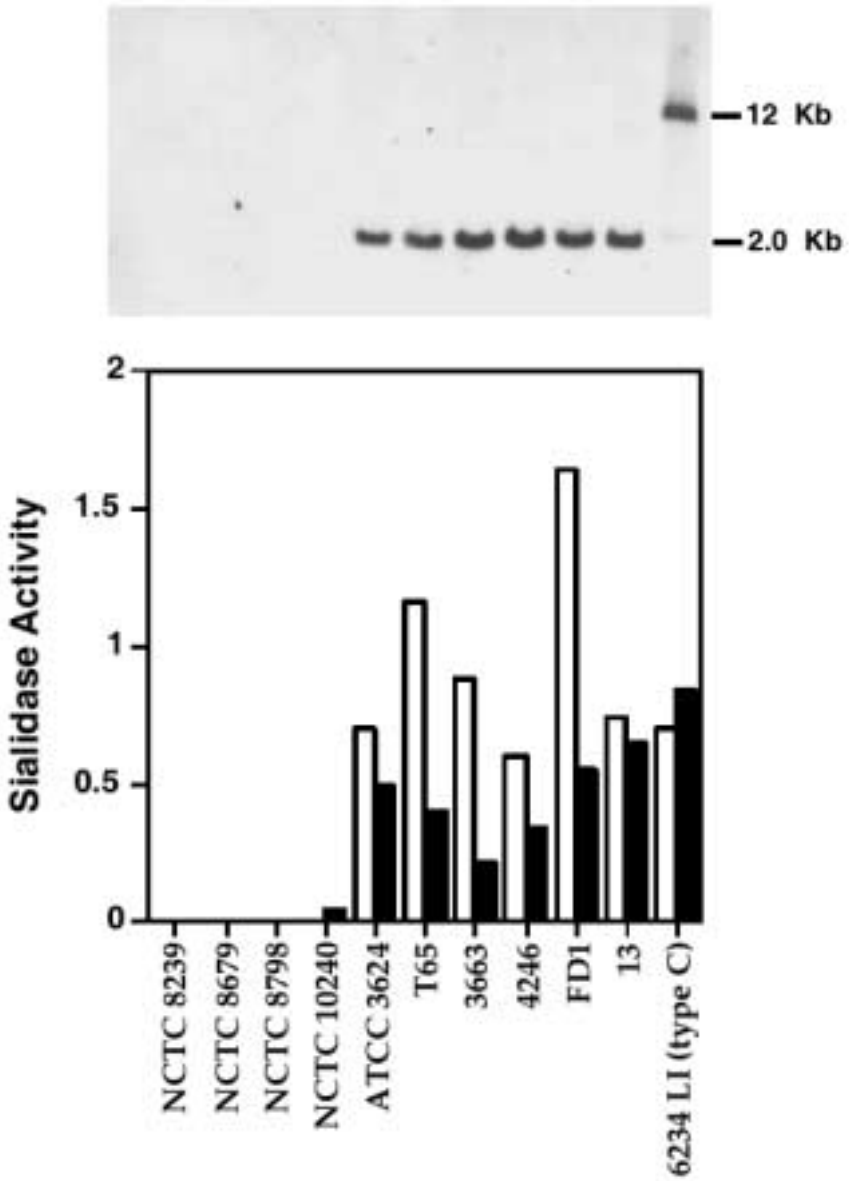

Figure 6-1. Top: Southern blot of chromosomal DNA isolated from the corresponding strains shown at the bottom of the figure. The DNA was digested with HindIII and probed with a *nanI*-specific DNA fragment (see Methods). The numbers on the right show the sizes of two of the hybridizing bands, as estimated using lambda phage DNA digested with EcoRI and HindIII. **Bottom:** Sialidase enzyme activity (μmoles 4-methylumbelliferyl-α-D-N-acetylneuraminic acid hydrolyzed/min/mg protein) using the NanI-specific enzyme assay for the strains listed at the bottom. Open bars represent bacteria grown in PY plus 1 mg/ml sialic acid, solid bars were grown in PY only.

Some of the strains containing *nanI* showed a 1.5 to 3-fold induction, when grown in the presence of sialic acid (Figure 6-1), while others, including strain 13, showed little difference in activity between the different growth conditions.

Only strain 13 lacks the *nanH* gene.

A previous study using DNA hybridization techniques reported that of 2,659 *C. perfringens* strains tested, all contained the *nanH* gene except JIR323, a derivative of strain 13 (49). Strain 13 and 10 other strains were assayed by using Southern blots and enzyme activities for the presence of the *nanH* gene (Figure 6-2). Southern blots were performed by Veronica Stirewalt while the enzyme assays were performed by myself. Only strain 13 lacked the *nanH* gene. Since strain 13 lacks the *nanH* gene, it is likely that the low level of activity seen with this strain is due to a small amount of NanI that is still associated with the cell.

In contrast to the results seen with NanI, most of the strains containing *nanH* actually showed a higher level of activity in the absence of sialic acid, especially strain FD-1, which was 3-fold higher when grown in PY alone (Figure 6-2). The one notable exception to this pattern was seen with strain NCTC 8798, which exhibited a low but inducible NanH activity (Figure 6-2).

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

Nees and Schauer (142) reported that sialidase activity was inducible by the addition of a sialic acid to the growth medium. To determine whether this induction occurs at the level of transcription and to identify the promoters of the *nanI* gene,

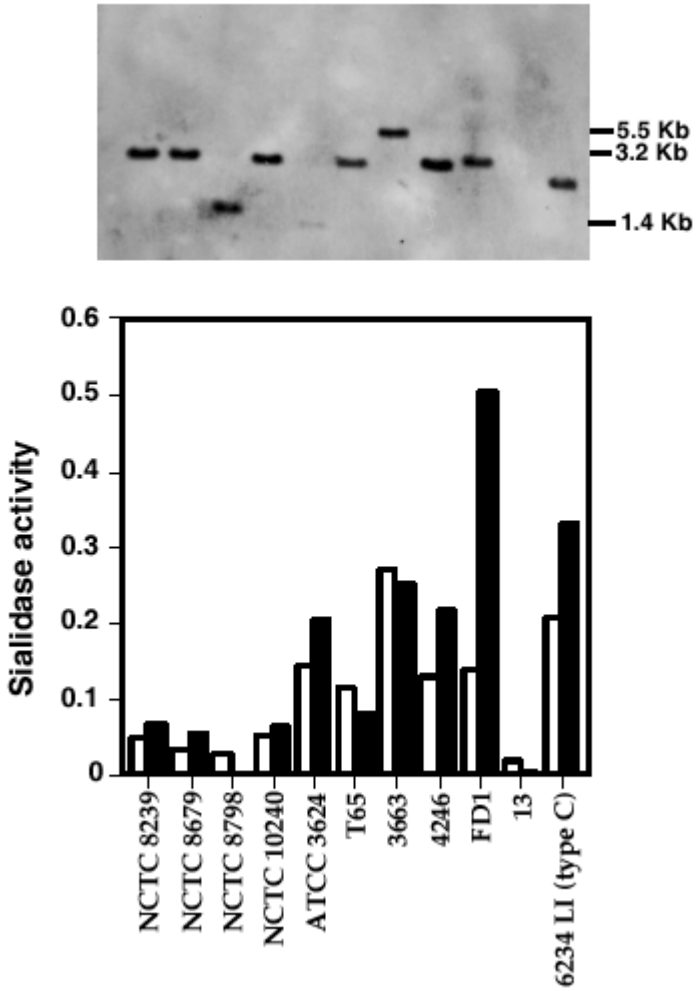

Figure 6-2. Top: Southern blot of chromosomal DNA isolated from the corresponding strains shown at the bottom of the figure. The DNA was digested with HindIII and probed with a *nanH*-specific DNA fragment (see Methods). The numbers on the right show the sizes of some hybridizing bands, as estimated using lambda phage DNA digested with EcoRI and HindIII. **Bottom:** Sialidase enzyme activity (μmoles 4-methylumbelliferyl-α-D-N-acetylneuraminic acid hydrolyzed/min/mg protein) using the NanH-specific enzyme assay for the strains listed at the bottom. Open bars represent bacteria grown in PY plus 1 mg/ml sialic acid, solid bars were grown in PY only.

Veronica Stirewalt performed primer extension analysis with RNA extracted from strain 13 growing with or without sialic acid as a carbon source. She identified three 5' ends that may represent promoters in the region upstream of the *nanI* gene (Figure 6-3). These 5' ends are located 66, 163, and 308 bp upstream of the start of the *nanI* coding sequence (Figure 6-3, (224). In addition, all of these potential promoters were induced by the presence of sialic acid in the growth medium (Figure 6-3). However, this does not agree with our results using direct measurements of NanI activity in strain 13, where there was an insignificant amount of induction seen in the presence of sialic acid. We do not have an explanation for this difference, but it may be due to post-transcriptional regulation of the *nanI* gene product.

Using primer extension and northern blot analyses numerous attempts were made by Veronica Stirewalt to identify the *nanH* promoter in strain SM101, but were unsuccessful. No signal could be detected in either type of experiment. This may be due to low levels of expression of the *nanH* gene in this strain, as evidenced by the low levels of NanH activity (Figure 6-2).

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

To confirm that the 5' ends we detected in the primer extension experiment were actual promoters, we fused a fragment containing the 664 bp of the intergenic region between *nanI* and the upstream ORF 1 (224) 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

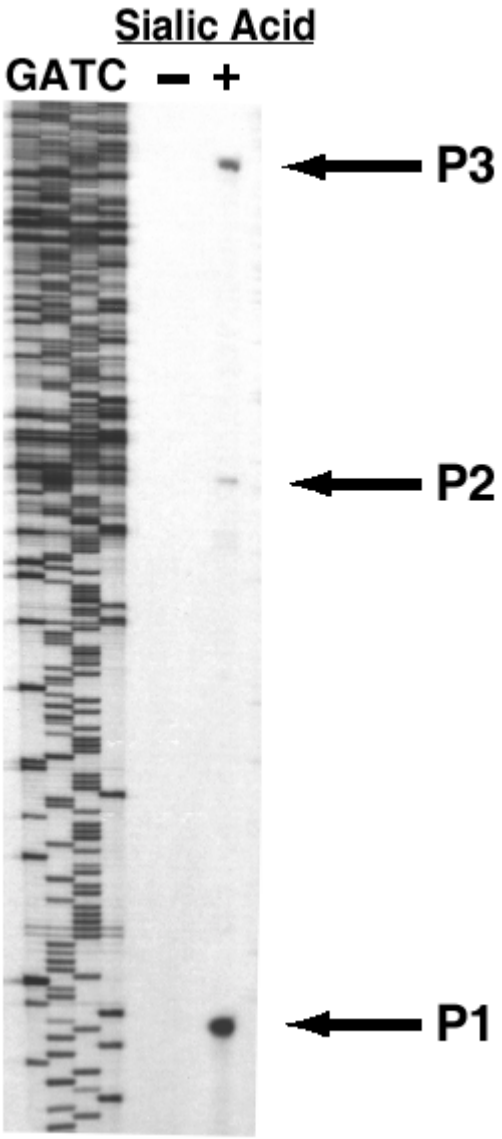

Figure 6-3. Primer extension results on RNA isolated from *C. perfringens* strain 13 using a primer specific for *nanI* mRNA. The cells were grown on PY (- sialic acid) or PY plus 1 mg/ml sialic acid (+ sialic acid). The GATC lanes show the results of sequencing using the same primer used for the primer extension experiments.

plasmid pSM218 (236). The *nanI* promoter region did show a significant level of transcriptional activity, but this activity was induced only 2-fold by sialic acid (Figure 6-4). 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 occurred with another sialic acid-inducible promoter upstream of the *nanE/A* operon in *C. perfringens* (236).

The *nanH* gene products from two strains of *C. perfringens* exhibited differing patterns of secretion in *C. perfringens* and *E. coli*.

The *nanH* gene product is a cytoplasmic enzyme because it is cell-associated and the N-terminus of the coding sequence lacks a recognizable signal sequence (171). During the course of this study, the *nanH* gene was cloned from two different strains, the type A strain NCTC 8798 and the type C strain 6234 LI, and transformed on multicopy plasmids into *E. coli*. Surprisingly, NanH activity was detected in both the extracellular medium and cell-associated extracts when the *nanH* gene from strain 6234 was analyzed, while only cell-associated activity was seen with the *nanH* gene from NCTC 8798 (Figure 6-5). As a control, the *nanI* gene product from strain 13 was also detected in both the extracellular medium and associated with the cells (Figure 6-5), as previously described (224). To determine the location of the *nanH* gene products in *C. perfringens*, strain 13 and strain SM101 (a derivative of NCTC 8798 (246) were transformed with shuttle plasmids carrying the two *nanH* genes (strain 6234 LI is not transformable (unpublished results)). Strain SM101 showed the same pattern as that seen in *E. coli*, i.e., the *nanH* gene product from strain 6234 LI was detected both in the growth medium and

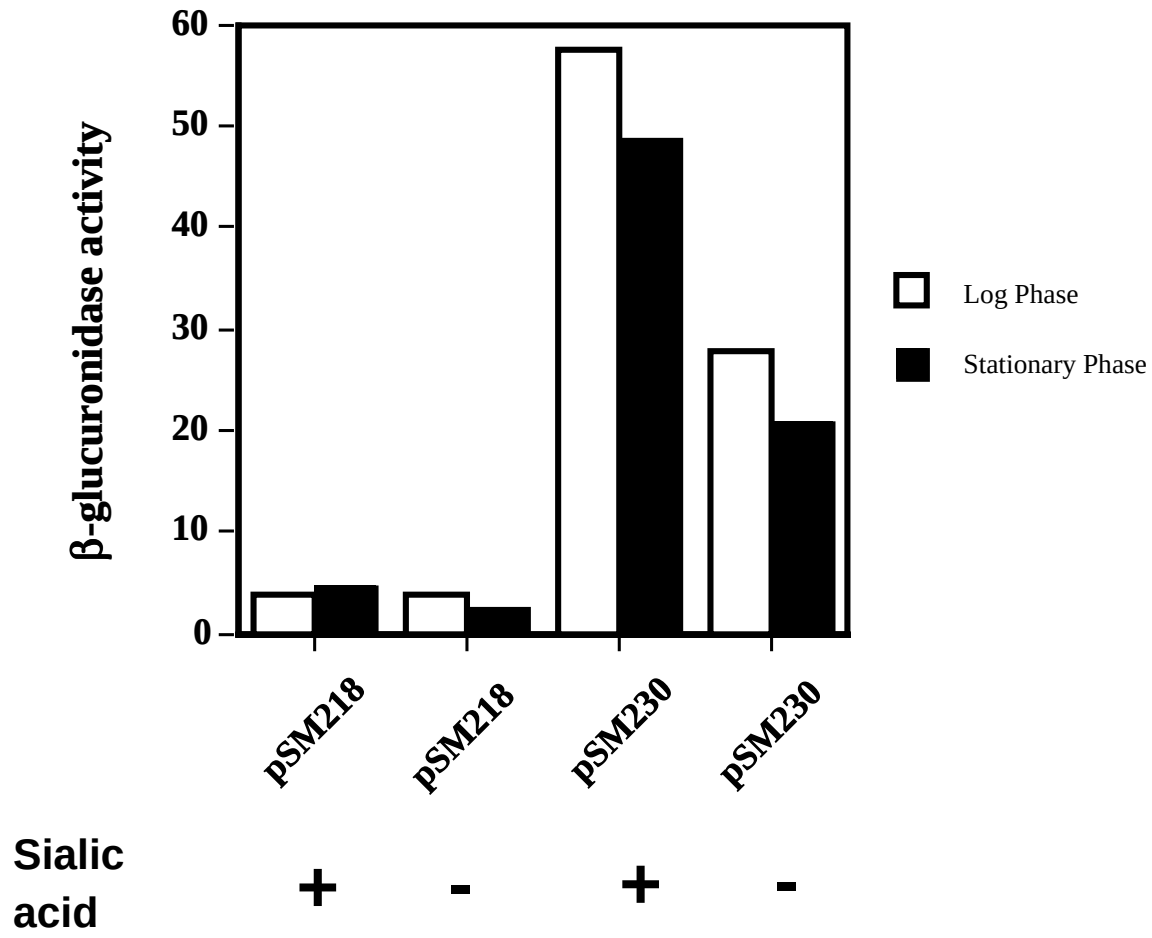

Figure 6-4. β-glucuronidase activity of *C. perfringens* strain SM101 carrying plasmids pSM218 or pSM230. Cells were grown in PY with or without 1 mg/ml sialic acid, as indicated. pSM218 is our transcriptional vector control with no insert and pSM230 is pSM218 with the *nanI* promoter from strain 13.

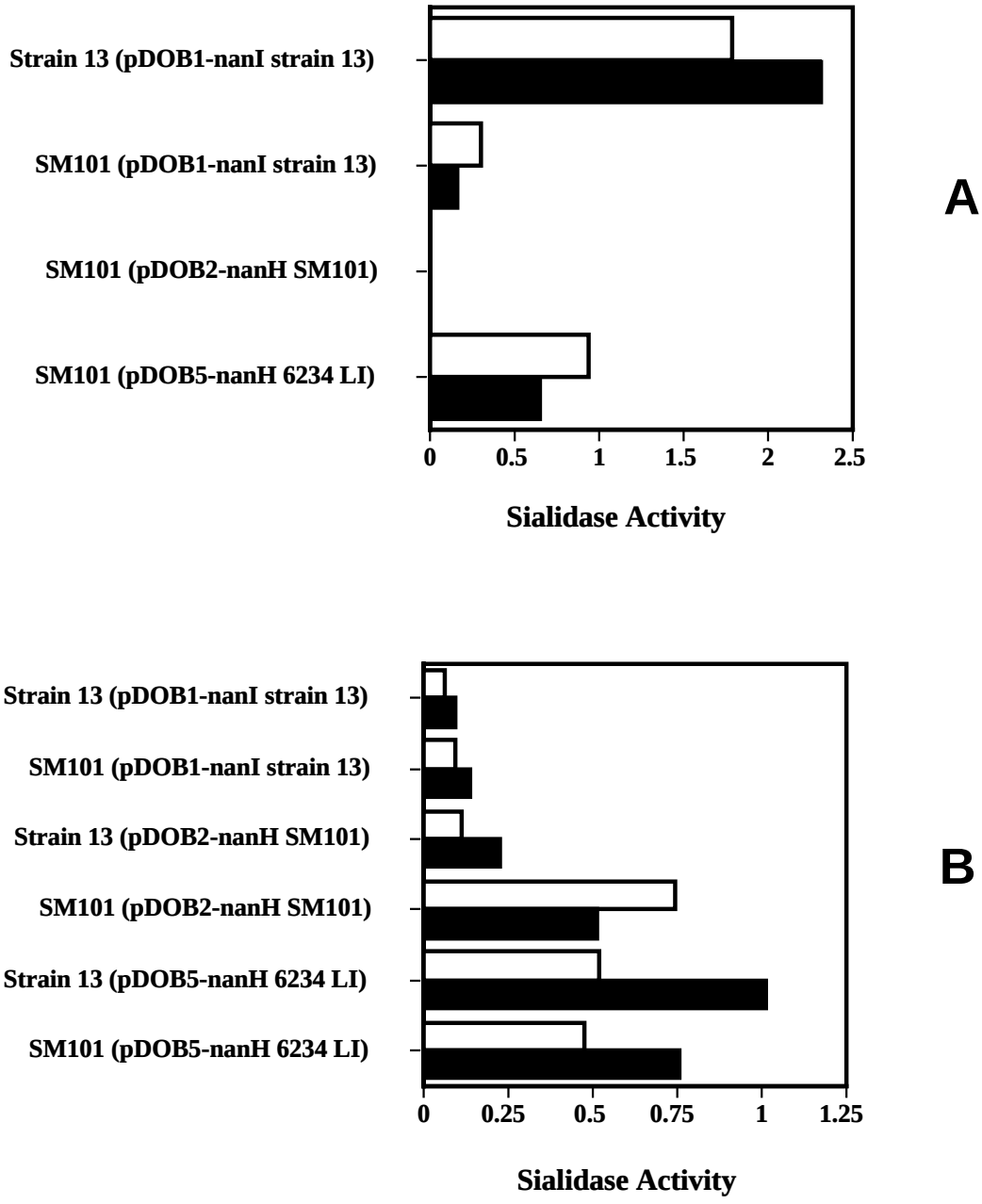

Figure 6-5. **Sialidase activity of sialidase genes expressed in *E. coli*.** Activity units are μmoles 4-methylumbelliferyl-α-D-N-acetylneuraminic acid hydrolyzed /min/mg protein. On the Y-axis are listed the plasmids carrying the *nanH* or *nanI* genes, with the source strain in parentheses. A: Culture supernatants analyzed with the NanI-specific enzyme assay. B: Cell extracts analyzed with the NanH-specific enzyme assay. Open bars show the activities with cells grown in LB plus 1 mg/ml sialic acid, solid bars show the activity from cells grown in LB alone.

associated with the cells (Figure 6-6). Strain 13, which lacks a *nanH* gene of its own (Figure 6-2), showed increased cell-associated sialidase activity when both *nanH* genes were expressed (Figure 6-5B). Since strain 13 produces extracellular NanI (Figure 6-1) which cannot be discriminated from NanH activity under the conditions tested, it was not possible to determine if either *nanH* gene product was secreted by this strain.

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* (236), we examined the sequences of the promoter regions to determine if there were any conserved sequence elements. Interestingly, we discovered a conserved 17 bp element that was present in 12 total copies in the promoter regions of *nanI*, *nanH*, and *nanE* (Figure 6-7). There are identical copies present in the *nanE* and *nanI* promoters (Figure 6-7). These conserved elements in the *nanI* and *nanE* promoter are in the regions known to be the start sites of transcription for these promoters (Figure 6-3 and (236), suggesting they may function as a recognition sequence for a transcriptional regulator.

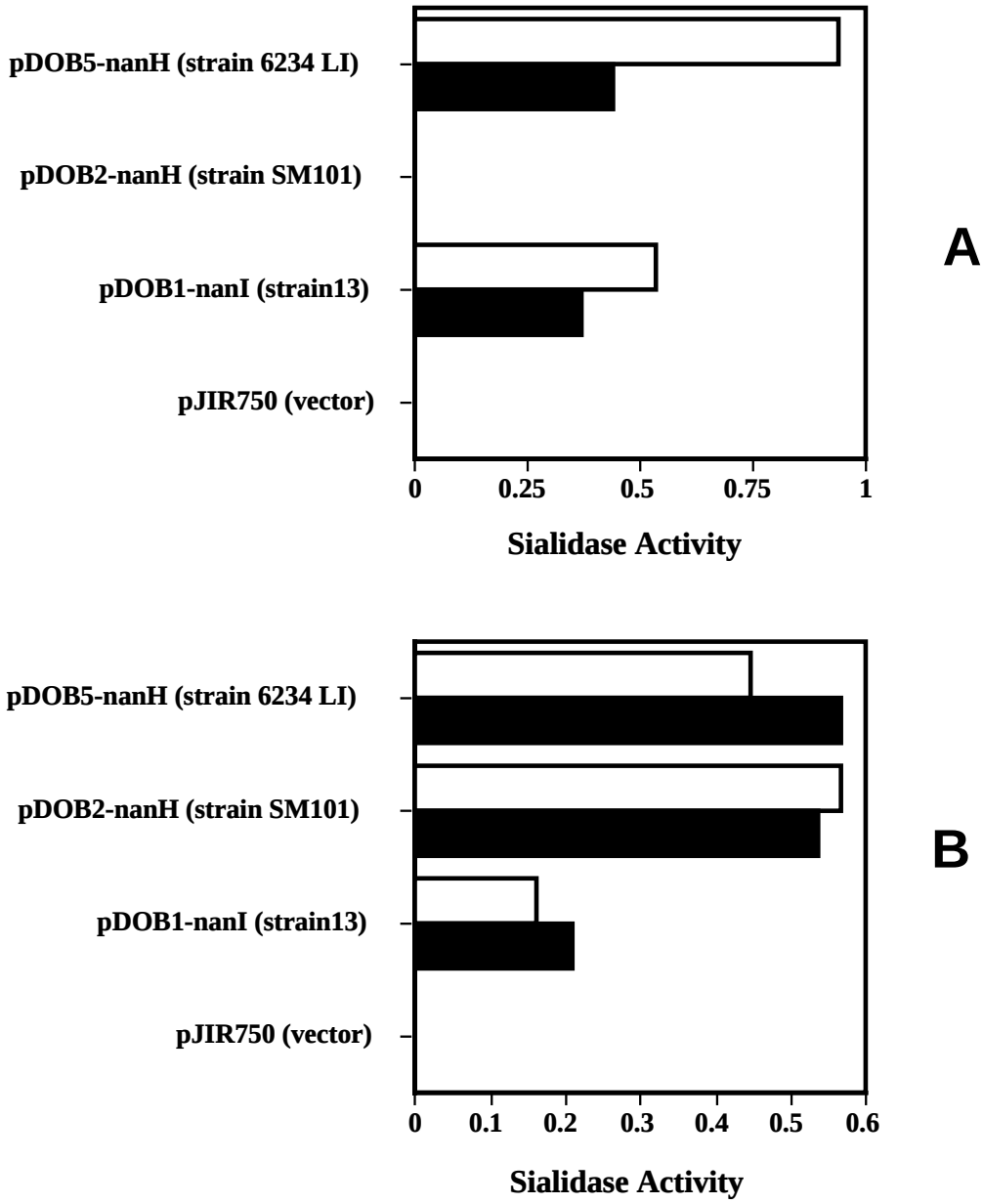

Figure 6-6. Sialidase activity of sialidase genes expressed in *C. perfringens*. Activity units are μmoles 4-methylumbelliferyl-α-D-N-acetylneuraminic acid hydrolyzed /min/mg protein. On the Y-axis are listed the strains harboring the plasmids carrying the *nanH* or *nanI* genes, with the plasmid name and source strain in parentheses. A: Culture supernatants analyzed with the NanI-specific enzyme assay. B: Cell extracts analyzed with the NanH-specific enzyme assay. Open bars show the activities with cells grown in PY plus 1 mg/ml sialic acid, solid bars show the activity from cells grown in PY alone.

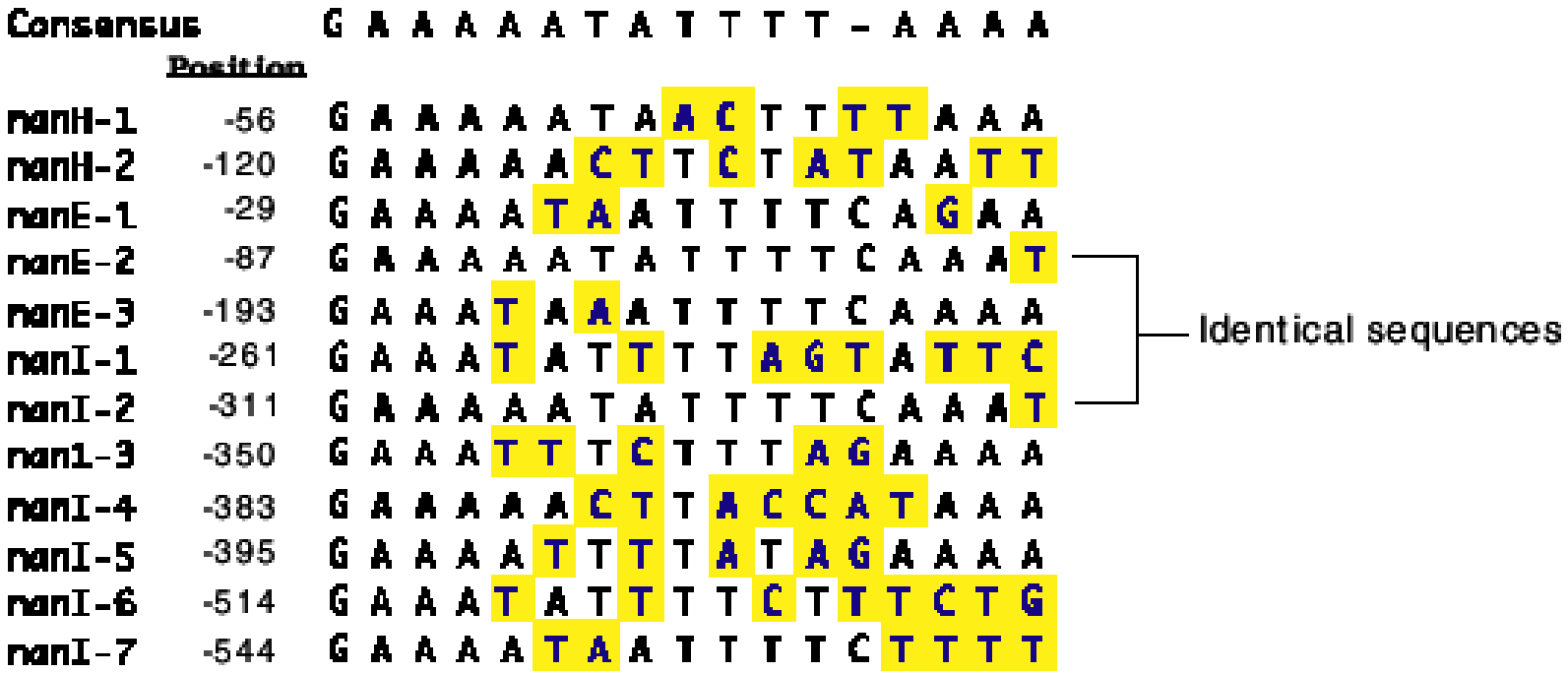

Figure 6-7. Alignment of conserved 17 bp elements located in the promoter regions of sialic acid-related genes or operons. The position refers to the distance from the "A" bp in the initiator methionine residue for each gene shown to the left. Shaded bases show bases that differ from the consensus sequence, shown at the top.

DISCUSSION

Eleven strains of *C. perfringens* were analyzed to determine which sialidase-encoding genes they carried. Four strains, all of which were isolated in Great Britain as the cause of separate outbreaks of food poisoning, were found to lack the *nanI* gene and the corresponding enzyme activity (Figure 6-1). These strains, all Hobbs serotype strains, have a chromosomal copy of the *cpe* gene, yet *cpe* does not seem to be the determining factor in the lack of a *nanI* gene, since strains 3663 and 4246 are CPE+ yet have the *nanI* genes. It seems the Hobbs strains are all related by a common ancestor that lost (or never had) the *nanI* gene.

Interestingly, a chromosomal map was made on one member of this group, NCTC 8798, and it was found to have a chromosome considerably smaller (3.1 Mbp) than a class of type A gangrene causing strains of *C. perfringens* (3.6 Mbp) (34, 101, 102). Cole and co-workers have identified 10 regions of length polymorphisms in different strains of *C. perfringens*, and NCTC 8798 usually exhibits smaller chromosomal segments in these regions (34, 101, 102). The *nanI* gene maps adjacent to, but not inside, variable region "d". Evidently, the region containing at least the *nanI* gene is also absent, indicating the variable region may extend to that point in the chromosome, thus accounting for the lack of a *nanI* gene in strain NCTC 8798.

Of the eleven strains we examined, only strain 13 lacks the *nanH* gene and associated enzymatic activity (Figure 6-2). This is in agreement with the results of Daube et al. (49) who found that all 2,659 isolates they examined had the *nanH* gene except a laboratory derivative of strain 13, JIR323. While Daube et al. (49) speculated

that the *nanH* gene of strain JIR323 was lost upon laboratory culture, it seems more likely that strain 13 never had the *nanH* gene. The relative conservation of *nanH* in comparison to *nanI* suggests the *nanH* gene product may play a more essential role in the overall metabolism of the bacterium and its ability to adapt to different environmental conditions.

The cytoplasm of the cell was presumed to be the cellular location of the *nanH* gene product because the activity was cell-associated and the primary gene sequence shows the protein would lack a leader peptide that could be recognized by a SecA-dependent secretion apparatus (171). However, the results illustrated in Figures 6-5 and 6-6 suggest that the some of the *nanH* gene product from strain 6234 LI is secreted from the cells of both *E. coli* and *C. perfringens* strain SM101, while all of the *nanH* gene product from SM101 remains cell-associated. We have sequenced the N-terminal encoding regions of these genes and can find only one significant difference in the amino terminus: The first amino acid after the initiator methionine in the *nanH* gene from 6234 LI is cysteine, while this residue is arginine in the *nanH* gene product from SM101. This may be a significant difference, since Rothe et al. (176) determined that the NanH homolog from *Clostridium sordellii*, has a leader peptide not found in the *C. perfringens nanH* gene but does have a cysteine residue at the same location as that of the *C. perfringens nanH* gene product. The *C. sordellii* NanH enzyme is secreted from the cell, apparently by a lipoprotein processing and secretion mechanism, which has been identified in Gram-positive bacteria (143). Since the *nanH* gene product from strain 6234 LI has the cysteine and was partially secreted by Gram-negative and Gram-positive

bacteria, while the arginine-containing version found in SM101 was not, it may be that the cysteine can still function in some fashion as a mechanism to allow secretion of the protein, although not effectively.

Using primer extension analysis, we were able to identify three 5' ends that represent putative start sites of transcription in the *nanI* promoter region (Figure 6-3). Each of the putative start sites were also inducible by the presence of 1 mg/ml sialic acid in the growth medium. This supports the early observation that the extracellular sialidase activity of *C. perfringens* was inducible by sialic acid (142). We were able to demonstrate that the region upstream of *nanI* has promoter activity (Figure 6-4), but it was not highly inducible by sialic acid, perhaps due to plasmid copy number effects (see Results). While the high A-T content of *C. perfringens* chromosomal DNA, makes it difficult to recognize -10 and -35 promoter elements based on sequence alone (173), we have identified a conserved 17 bp motif in the promoter regions of genes associated with sialic acid metabolism (Figure 6-7). While this sequence lacks dyad symmetry, it may represent the DNA recognition sequence of a transcriptional regulator that is responsible for the sialic acid-dependent induction of the *nanI* and *nanE* promoters.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth media.

Bacterial strains and plasmids used in this report are listed in Table 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 (the same as PGY but lacking glucose) was prepared and stored in a Coy anaerobic chamber (Coy Laboratory Products, Inc.), as previously described (246).

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 (224) was placed upstream of the *gusA* gene in plasmid pSM218 (236), to create plasmid pSM230. 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 (236). The *nanI-gusA* fusion contained the entire promoter region and the first three codons of the *nanI* structural gene.

To construct plasmid pDOB1, the *nanI* gene from *C. perfringens* strain 13 chromosomal DNA was amplified by PCR using OSM90 and OBR4 as primers. OSM90 annealed approximately 660 base pairs upstream of the *nanI* gene while OBR4 annealed approximately 24 base pairs downstream of the stop codon for the *nanI* gene. The PCR product was then ligated to plasmid pCR2.1 (Invitrogen). The *nanI* gene was then

subcloned into plasmid pJIR750 using *SphI* and *EcoRI* as the restriction enzymes. Plasmid pDOB2 was created by digesting pSM311 (see below) with *SacI* and *KpnI* and cloning the entire *nanH* gene and ~300 bp upstream into pJIR750. pDOB5 was created by amplifying the *nanH* gene from *C. perfringens* strain 6234 LI (a type C strain) by PCR using OSM 117 and OSM 118 as primers. The PCR product was then ligated to PCR2.1. The *nanH* gene was then subcloned into pJIR750 using *BamHI* and *PstI* restriction sites of both plasmids. All three plasmids confer chloramphenicol resistance encoded for by pJIR750. The resulting plasmids were then transformed by electroporation into either *C. perfringens* strain 13, *C. perfringens* SM101, or *E. coli* DH10B electrocompetent cells, as previously described (127).

Chromosomal DNA from *C. perfringens* was prepared as follows: Cells grown to late log phase were harvested by centrifugation and then washed and resuspended in buffer containing 100 mM Tris, pH 8.0, and 20 mM EDTA. Lysozyme was added to a final concentration of 3 mg/ml and the cells incubated for 1 h at 37 C. Then an equal volume of lysis buffer (100 mM Tris, pH 8.0, 20 mM EDTA, 2.8 M NaCl, 4% (w/v) CTAB, and 4% (v/v) β-mercaptoethanol) was added and the cells incubated at 55 °C for 45 min. The lysed cells were extracted twice with chloroform:isoamyl alcohol (24:1) and the DNA precipitated by adding an equal volume of isopropanol. The pellet was rinsed 5 times with 70% ethanol, dried, and resuspended in 10 mM Tris, 1 mM EDTA (TE). RNase was added to a final concentration of 10 mg/ml, and the DNA was incubated for 30 min at 37 °C, followed by phenol:chloroform and chloroform extractions. The DNA

was then precipitated, rinsed, and dried down as described above and resuspended in TE buffer.

For Southern blot analyses, 5 μg of chromosomal DNA was digested to completion with *Hind III*, and agarose gel electrophoresis and transfer to a nylon membrane were done as previously described (184). To detect hybridization to the probes, the Phototope Star detection system was used (New England Biolabs). For *nanH*, the probe consisted of a 1.3 kb *XbaI* fragment, which included all of the *nanH* gene except the first 5 codons (data not shown). 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).

The *nanH* gene from strain NCTC 8798 was cloned in the following manner: A Southern blot analysis showed the *nanH* gene was encoded on a 1.8 kb *HindIII* fragment in the chromosome of strain NCTC 8798 (Figure 2). Therefore, chromosomal DNA from NCTC 8798 was digested with *HindIII* and electrophoresed on an agarose gel. Fragments between 1.5 and 2.5 kb were removed from the gel and purified. The fragments were then ligated to pBluescript SK- that had been cut with *HindIII* and phosphotased, to make a library, and transformed into *E. coli* DH10B. This library was probed by colony hybridization with a *nanH* gene fragment made by PCR amplification of the N-terminal and promoter region of the *nanH* gene from strain NCTC 8798, using published sequences for the *nanH* gene of strain A99 (170). One recombinant plasmid, pSM311, was isolated and found to contain a 1.8 kb insert that contained all of the *nanH* gene coding sequence and 242 bp of the upstream region (unpublished results).

Enzyme assays.

For sialidase assays, *C. perfringens* cultures were grown overnight in an anaerobic chamber in 2 mls of PY media, in either the presence or absence of 1 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. The NanH and NanI enzyme activities, were assayed by growing cells as described above. For NanH, the cells were pelleted and washed three times with 1 ml of PBS. Pellets were frozen on dry ice and resuspended in TE containing 10 mg/ml of lysozyme and incubated at 37 °C for 20 min. Five hundred µl of water was added to lyse the bacterial cells and the extract was centrifuged for 5 min to remove cell debris. The NanH-containing supernatant was kept on ice until assayed. For NanI, the overnight cultures were pelleted and the supernatants, which contained NanI, were removed and kept on ice until assayed.

The measurement of NanH and NanI sialidase activity was performed as previously described (169) with the following modification: the fluorogenic substrate 4-methylumbelliferyl-α-D-N-acetylneuraminic acid (MU-Neu5Ac; Sigma) was diluted to a final concentration of 0.1 mM. To determine the amount of released substrate, the fluorescence generated was compared to a standard curve created using pure MU. The released MU was quantified using an Aminco-Bowman Series 2 Luminescence Spectrometer (Spectronic Instruments; Rochester, NY). The data shown in Figures 1, 2, 5, and 6 represent the means of at least three independent measurements of NanH and NanI activity, where the standard deviation was no greater than 30% of the mean values. While the NanH and NanI were assayed at pH values of 6.1 and 5.0, respectively, each

enzymes retains approximately 80% of its maximum activity at the alternative pH values (169), allowing the quantification of both NanH and NanI activity in strains that expressed the enzymes on multicopy plasmids (see Figures 6-4 and 6-5).

β-glucuronidase assays in *C. perfringens* were performed as previously described (127). The means of duplicate samples of a representative experiment are shown in Figure 6-4.

Primer extension experiments.

To determine the start site of transcription for the *nanI* gene, *C. perfringens* strain 13 was grown to mid-log phase on either PY or PY plus 1 mg/ml sialic acid and total RNA was extracted from the cells using the Trisol reagent, as previously described (127). Twenty μg of RNA were used as a template for primer extension reactions, using the Promega Primer Extension System kit, done 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.

Plasmid table 6-1

Strain/Plasmid	Relevant characteristics	Source or reference
E.coli		
DH10B	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) F80d *lacZ* ΔM15 Δ*lacX74 deoR* *recA1* *araD139* Δ(*ara* , *leu*)7697 *galU* *galK* λ- *rpsL* *endA1* *nupG*	Gibco/BRL Corp.
C. perfringens		
NCTC 8239	Cpe+	C. Duncan
NCTC 8679	Cpe+	C. Duncan
NCTC 8798	Cpe+	C. Duncan
NCTC 10240	Cpe+	C. Duncan
ATCC 3624	Cpe-	C. Duncan
T-65	Cpe-	C. Duncan
3663	Cpe+	R. Skjelkvale
4246	Cpe+	R. Skjelkvale
FD-1	Cpe-	C. Duncan
13	Cpe-	C. Duncan
6234 LI	type C	J. G. Songer
SM101	High frequency electroporation derivative of NCTC 8798	(Zhao and Melville, 1998)
Plasmids	Parent/genotype	
pBluescript SK-		Stratagene Corp.
pJIR750	*C. perfringens-E. coli* shuttle vector	(Bannam and Rood, 1993)
pSM218	Transcription vector	(Zhao and Melville)
pSM230	pSM218 with the nanI promoter from strain 13	This study
pSM311	pBluescript SK-/ *nanH* from NCTC 8798	This study
pDOB1	pJIR750/ *nanI* from strain 13	This study
pDOB2	pJIR750/ *nanH* from strain NCTC 8798	This study
pDOB5	pJIR750/ *nanH* from strain 6234 LI	This study

ACKNOWLEDGEMENTS

This work was supported by a grant (#98-02844) from NRICGP/USDA awarded to S.B.M.

CHAPTER SEVEN

Ongoing Research

1. The Role of Phagosomal Escape in the Survival of *Clostridium perfringens in vivo*

A. INTRODUCTION

Thus far, we have evidence that *C. perfringens* is not killed by any type of macrophage. However, what effect the bacteria have on macrophages, has only been briefly studied. Experiments were conducted using mutants that do not produce either PFO or PLC, or either toxin. We found that J774-33 cells and mouse peritoneal macrophages were more sensitive to strains that possessed PFO at a multiplicity of infection (MOI) of 10:1. However, J774-33 cells were also sensitive to PFO expressing strains at an MOI of 1:1, while mouse peritoneal macrophages were not. This cytotoxicity is independent of whether the bacteria are inside the macrophages or not. We found that both PFO and PLC can mediate escape from the phagosome in J774-33 cells and mouse peritoneal macrophages. When J774-33 cells were infected with a *pfoA* mutant, *C. perfringens* escaped the phagosome, was not cytotoxic, and did not survive as well as wild-type *C. perfringens*. This led to the theory that escape as well as cytotoxicity might play a role in the ability of *C. perfringens* to survive in J774-33 cells. This is reinforced by data showing that a *plc* strain of *C. perfringens* can survive in the presence of J774-33 cells, can escape the phagosome, and is cytotoxic. These results suggest that cytotoxicity may play a role in the ability of *C. perfringens* to survive in J774-33 cells along with the ability to escape the phagosome. A similar trend was seen with mouse peritoneal macrophages. Only wild-type *C. perfringens* was capable of surviving while the *pfoA* and *plc* strains were not; PFO expressing strains were only cytotoxic at higher MOIs (10:1), whereas the survival assays were done at an MOI of 1:1.

These two mutants were still capable of escaping the phagosome, although at lower levels than wild-type *C. perfringens*. We believe this lowered level of escape from the phagosome is the reason for their death *in vitro*, and led us to believe the ability of wild-type *C. perfringens* to survive *in vitro* was due only to its ability to escape the phagosome.

In vivo experiments were performed using 1000-fold less bacteria than is needed to initiate a gangrene infection. We again found that wild-type *C. perfringens* was capable of persisting in a mouse for 36 hours, while *pfoA* and *plc* strains did not. Although our *in vitro* experiments were done at an MOI of 1:1, where the bacteria are not very cytotoxic, we cannot determine the MOI of our *in vivo* experiments and therefore, cannot rule out the possibility that cytotoxicity plays a role in their survival *in vivo*. Since our *in vitro* mouse peritoneal macrophage survival data correlated with our *in vivo* survival data, and that cytotoxicity plays no role in the survival of *C. perfringens in vitro* at an MOI of 1:1, we want to determine if cytotoxicity is needed for survival *in vivo* or if only escape is sufficient for survival.

To determine if the ability of *C. perfringens* to survive *in vivo* is due to its ability to escape the phagosome or because it is cytotoxic to host immune cells, we will infect mice with a *pfoA/plc* strain of *C. perfringens* expressing forms of PFO or wild-type LLO that still allow escape but are not cytotoxic. This will allow us to eliminate the cytotoxic effects *C. perfringens* has on immune cells and let us examine what role only escape plays in survival. We obtained two strains of *Listeria monocytogenes* from Daniel Portnoy that have had the LLO encoding *hly* gene removed, and replaced with two

mutant forms of the *pfoA* gene. One strain contains a mutated *pfoA* gene that has an amino acid change (Leu-462 to Phe) rendering PFO active at a pH of 5.6, instead of the normal pH of 7.4. This activity at a lower pH results in the toxin only being active once the phagosome has become acidified. Therefore, when the phagosome is broken down by PFO and the toxin enters the more neutral cytoplasm, it will become inactivated and unable to lyse the cell. The other strain contains an amino acid change (His-438 to Tyr), decreasing the half-life of PFO by half. This shortened half-life still allows time for PFO to break down the phagosomal membrane, but not enough time for the cell membrane to be lysed. We also obtained a strain of *L. monocytogenes*, devoid of its chromosomal *hly* gene, that contains a plasmid encoding a wild-type *hly* gene. *In vitro* cytotoxicity assays will be performed to confirm that these strains are not cytotoxic to macrophages. The mutant forms of PFO as well as LLO will be cloned and used to complement our *pfoA/plc* strain of *C. perfringens*. We have chosen to use a *pfoA/plc* strain of *C. perfringens* because it shows very little cytotoxicity towards macrophages *in vitro* and cannot escape the phagosome as well as wild-type *C. perfringens*.

Using these *L. monocytogenes* strains containing the mutant forms of PFO, Jones has shown these mutants still allow the escape of *L. monocytogenes* from the phagosome, but are not as cytotoxic to macrophages as a strain that carries the wild-type form of PFO (95). The LLO protein of *L. monocytogenes* is active at a pH of 5.6 and is not cytotoxic to macrophages, but it still allows escape from the phagosome (140).

B. RESULTS

This work is currently underway, and most plasmid constructs needed for this experiment have been constructed. Due to the fact that our *pfoA/plc* mutant strain contains two antibiotic markers, chloramphenicol and erythromycin, we first needed to construct a plasmid conferring resistance to a third antibiotic. We chose to use the tetracycline gene from Tn*916*. In order to do this, we removed the *tet*(M) gene from pVB101, a pUC-4K derivative, using *Eco*RI and blunt ended the gene. A *C. perfringens* replicative plasmid, pJIR751, which had the *ermBP* gene removed by restriction digest with *Spe*I and *Sca*I was also blunt ended and ligated to the *tet*(M) gene.

To isolate the mutated *pfoA* genes from *L. monocytogenes*, we constructed primers to the C- and N-terminal ends of the *pfoA* gene and amplified the gene using PCR. These genes were under the control of the *hly* promoter. Therefore, to put the mutant *pfoA* genes under the control of the *pfoA* promoter, another set of primers were used to amplify the wild-type *pfoA* gene including the promoter from *C. perfringens*. Both mutations in the *pfoA* gene were in the C-terminus of the gene. To move the mutation to our wild-type *pfoA* gene, we digested all the fragments with *Nde*I, a restriction site located towards the middle of the gene. The mutated fragments were then ligated to the wild-type gene also cut with *Nde*I. Sequencing confirmed that the mutation now existed in our *pfoA* gene under the control of its own promoter. These genes were then cloned into the plasmid which contained the *tet*(M) gene and transformed by electroporation into the *pfoA/plc* mutant strain of *C. perfringens*.

The next step will be to infect mice with this strain and determine if these mutant strains are capable of surviving or persisting in the mouse. To do this, mice will be injected in the femoral muscle and at various time points the mice will be sacrificed and the femoral muscle extracted. The muscle will be dissociated using a metal sieve and plated for colony forming units. These strains will also be tested for their cytotoxicity *in vitro* using a cytotoxicity assay. This is a colorimetric assay that relies on the release of lactate dehydrogenase from dying cells. The amount of LDH released can be correlated to the amount of cytotoxicity towards the macrophages. Electron microscopy will also be performed to demonstrate that the bacteria are capable of escaping the phagosome of macrophages. Ultimately, these experiments will tell us is if the ability of the bacteria to persist and grow in a host is due to its ability to escape the phagosome of macrophages, or if cytotoxicity plays a role in survival as well.

2. The Role of PilT and PilC in the Pathogenesis of Gas Gangrene

A. INTRODUCTION

Upon examination of the sequenced genome of *C. perfringens*, we found the presence of two *pil* genes, *pilC* and *pilT*. *pilT* is a putative twitching motility gene while *pilC* is thought to be involved in fimbrial assembly. Both of these gene products are considered parts of type IV pili. Type IV pili are usually found on the surface of Gram-negative bacteria (124, 211) and because these genes are present in a Gram-positive bacteria, and the fact that *C. perfringens* has no twitching motility, we are interested in the function of these gene products.

Some functions for *pilT* and *pilC* in other organisms include: promoting the attachment of bacteria to specific receptors on host cells during colonization (53), the uptake of DNA (126), and twitching motility (81). The type IV pilus biogenesis system is also closely related to type II secretion pathways (146). In *Pseudomonas aeruginosa* *pilT* is required for *in vitro* adherence and cytotoxicity to epithelial cells, and is required for virulence *in vivo* (45). For these reasons, we believe these genes may be involved in toxin secretion in *C. perfringens*, or they could possibly facilitate the binding of *C. perfringens* to host phagocytic cells. This could possibly explain a mechanism as to how toxins are secreted by *C. perfringens*, or if the proteins encoded for by the *pil* genes are necessary to cause disease. Another possibility is that these gene products could aid in the uptake of the bacteria into host phagocytic cells by allowing intimate contact to occur between *C. perfringens* and the phagocytic cells. This is a possibility because *C.*

perfringens survives better in the presence of macrophages under aerobic conditions and phagocytosis may be required for this survival.

To answer these questions, we plan to inactivate the *pilT* and *pilC* genes using recombinational mutagenesis. Southern blot hybridization will be used to confirm that the wild-type *pil* genes have been disrupted. We will then test for the ability of these mutants to secrete the PFO and PLC toxins. One method is to plate the mutants on egg yolk agar plates as well as blood agar plates to see if the zones of hemolysis consistent with each toxin are present or not. We will then assay for the hemolytic activity of PFO and PLC by measuring the release of hemoglobin from sheep red blood cells. Electron microscopy will then be performed to determine if any phenotypic differences can be seen as compared to wild-type *C. perfringens*. Finally, we will inject these mutants into a mouse to see what role if any these genes play in the ability of *C. perfringens* to cause gangrene.

B. RESULTS

This project is in its early stages of development and has not yielded many results thus far. We have constructed the suicide plamids needed to inactivate the *pil* genes in *C. perfringens*. This was done by amplifying an internal fragment to either *pilT* or *pilC* and inserting it into a suicide plasmid. The internal gene fragment will allow for a recombinational event to occur between the plasmid and the chromosomal copy of the gene. Many attempts have been made to make a *pilT* and *pilC* mutant. However, none of the transformants we have tested have yielded the correct banding patterns as analyzed by Southern hybridization analysis.

CHAPTER EIGHT

OVERALL CONCLUSIONS

The overall goal of my research was to define the interactions of *C. perfringens* with host phagocytic cells during the initial stages of a gangrene infection. The main questions we focused on were: (1) Is *C. perfringens* phagocytosed by macrophages? (2) Does *C. perfringens* survive in the presence of macrophages and if so, how does *C. perfringens* survive? (3) Finally, what factors are required for *C. perfringens* survival? Our first objective was to determine if macrophages could phagocytose and kill *C. perfringens*. We believed macrophages could not efficiently kill *C. perfringens* based on the reasoning that gangrene would not occur if macrophages could kill them. Knowing other pathogens can survive in the presence of macrophages, we determined if *C. perfringens* was capable of surviving in a similar manner. For example, we wanted to determine if *C. perfringens* was cytotoxic to the macrophages, due to *C. perfringen's* vast toxin production, or does *C. perfringens* prevent phagolysosome fusion, or does it escape the phagosome into the cytoplasm where it can replicate? We approached these questions in multiple ways including *in vitro* studies using J774-33 cells, mouse peritoneal and bone marrow derived primary macrophages, as well as multiple *in vivo* experiments.

Using light microscopy, we found that *C. perfringens* was phagocytosed by macrophages in an actin-dependent manner, and that *C. perfringens* itself played no active role in the phagocytosis process. We learned from our *in vitro* survival experiments that *C. perfringens* could survive in the presence of all three types of macrophages we tested under anaerobic as well as aerobic conditions. The fact that *C.*

perfringens could survive under aerobic conditions was interesting since *C. perfringens* is an anaerobe.

This led to the next question of how does *C. perfringens* survive in the presence of macrophages. One theory was that the bacteria were escaping the phagosome, thereby allowing the bacteria access to the cytoplasm prior to phagosomal fusion with lysosomes. Using immunofluorescence microscopy and an antibody against the late endosome/lysosome marker glycoprotein LAMP-1, we found that phagosomes containing *C. perfringens* did not co-localize with LAMP-1. This suggested that *C. perfringens* was preventing phagosome/lysosome fusion from occurring. Due to the ability of *C. perfringens* to produce two membrane-lysing toxins, PFO and PLC, we hypothesized that *C. perfringens* might be breaking down the phagosomal membrane, allowing the bacteria to enter the cytoplasm. Electron microscopy of intracellular bacteria supported this hypothesis. After 60 minutes post-infection, more than half of the macrophages contained *C. perfringens* located in the cytoplasm rather than in a phagosome. This explained the lack of phagosome/lysosome fusion observed in previous experiments. We hypothesized that the bacteria were simply escaping the phagosome prior to phagosome/lysosome fusion.

Since the receptor used to phagocytose bacteria can affect its intracellular fate, we determined which receptors were utilized to phagocytose *C. perfringens*. Using specific receptor inhibitors for the receptors present on macrophages, we were able to block one or multiple receptors to determine if that receptor was involved in the uptake of *C. perfringens*. We determined that *C. perfringens* was taken up by multiple receptors

including the scavenger, mannose, and complement receptors. We used multiple receptor inhibitors to force the bacteria to be taken up by only one receptor, and determined if that affected the ability of phagosomes containing *C. perfringens* to fuse with lysosomes. We found that only the Fc receptor gave a slight increase in the co-localization of phagosomes with lysosomes. Still, we attributed this to the ability of *C. perfringens* to escape the phagosome before lysosomes fusion had occurred. Glycosyl composition analysis of the capsule of *C. perfringens* confirmed the presence of residues that would be consistent with those used in the uptake by the receptors we examined.

We then switched our attention to how *C. perfringens* is capable of surviving in the presence of macrophages. As mentioned earlier, *C. perfringens* produces two membrane-lysing toxins, PFO and PLC. Other bacteria such as the *Rickettsia* species, *Shigella* and *Listeria monocytogenes*, can lyse phagosomal vacuoles to ensure their survival in the presence of macrophages. One way *L. monocytogenes* accomplishes phagosomal escape, is by using a cytolysin called Listeriolysin O (LLO). We believed PFO could be important in *C. perfringens'* escape from the phagosome since PFO is in the same family of cholesterol-dependent cytolysins as LLO. *L. monocytogenes* also possesses two phospholipase C toxins, a broad specificity phospholipase that cleaves most phospholipids, as well as a phosphatidyl inositol specific phospholipase (PI-PLC), both of which are necessary for *L. monocytogenes* escape and survival in host cells. PI-PLC is responsible for escaping from the phagosome (196). This suggested that the PLC produced by *C. perfringens*, might also play a role in escape from the phagosome and ultimately survival.

Therefore, we used mutations in the *pfoA* and *plc* genes to determine what role these toxins played in the ability of *C. perfringens* to survive in macrophages. Using recombinational mutagenesis, we inactivated the *pfoA* gene, and obtained a *plc* strain of *C. perfringens* from A. Okabi's group in Japan. Using his *plc* strain of *C. perfringens*, we created a *pfoA/plc* mutant strain of *C. perfringens*. We found that only PFO was needed for survival in the presence of J774-33 cells, while both PFO and PLC were necessary for survival in mouse peritoneal macrophages at a multiplicity of infection (MOI) of ~ 1:1.

We then determined if these toxins were necessary for survival because they allowed *C. perfringens* to escape the phagosome. We found that in both cell types, both PFO and PLC could allow escape from the phagosome. The fact that both PFO and PLC could allow escape in J774-33 cells was very interesting because only PFO was needed for survival. This suggested to us that PFO was functioning in some other fashion. Since PFO is cytotoxic to macrophages and leukocytes, we tested whether our strains were cytotoxic to J774-33 cells and mouse peritoneal macrophages. We found that PFO expressing strains were more cytotoxic to both cell types. However, J774-33 cells were more sensitive at a lower MOI (1:1), while mouse peritoneal macrophages were only affected by a higher MOI (10:1). This led to the conclusion that escape from the phagosome as well as cytotoxicity, played a role in the ability of *C. perfringens* to survive in the presence of J774-33 cells. Furthermore, only escape is responsible for survival in the presence of mouse peritoneal macrophages.

We then assayed for the ability of phagosomes containing these mutants to fuse with LAMP-1. We found that only the *pfoA/plc* strain co-localized with LAMP-1, at a slightly higher level than wild-type, *pfoA*, and PLC- strains of *C. perfringens*. Since the *pfoA/plc* strain could not survive in the presence of J774-33 cells, and it was found in the phagosome twice as much as wild-type, we believed LAMP-1 co-localization should have been much higher. The fact that these mutants did not fuse with LAMP-1 at higher levels, was surprising to us. This suggested that something interesting was taking place in phagosomes containing *C. perfringens*. One reason for this observation is that *Trypanosoma cruzi*, and most likely *C. perfringens,* can desialylate known vacuolar constituents, i.e., lysosomal membrane glycoproteins using its neuraminidases (74). Desialylated membranes therefore become more accessible to membrane lysing toxins [246], most probably PLC and PFO. This could prevent LAMP-1, and other lysosomal markers, from fusing with phagosomes containing *C. perfringens*. This mechanism is plausible because these neuraminidases are most active at an acidic pH, much like the environment of a mature phagosome.

Much work has exists using the mouse model, but this research has only focused on the later stages of gas gangrene infections. We believe it is important to understand what is happening in a gangrene infection before the actual onset of clinical gangrene occurs, because of the significant morbidity and mortality resulting from most *C. perfringens* infections. The mouse model is problematic in that in order for *C. perfringens* to cause gangrene, ~ 1 x 10^9 bacteria need to be injected into the mouse. This large innoculum allows for the production of massive quantities of PFO and PLC,

possibly masking the effects that these toxins might have at low concentrations during the early stages of a *C. perfringens* infection, when bacterial numbers are low and phagocytic cells are present. It is evident that PLC is the main player in gangrene infections, but our *in vitro* data suggest that PFO is important as well.

We therefore determined which toxin was necessary for survival at an infectious dose 1000-fold lower than needed to cause a gangrene infection. We infected mice with 1×10^6 bacteria/mouse and determined that both PFO and PLC were needed for survival. This directly correlated with our *in vitro* survival data in mouse peritoneal macrophages. Interestingly, we have identified a role for PFO *in vivo*. PFO is required for survival, at least during the initial stages of an infection, a function never seen before. However, we cannot conclude if this survival is due to the ability of the bacteria to escape the phagosome or because the bacteria are cytotoxic to host cell present at the site of infection. This will be determined as described in Chapter 7.

As of now, there are two main models to explain why *C. perfringens* is capable of surviving in a host, both of which are dependent on the ability of the bacteria to produce PFO and PLC. One theory is that once *C. perfringens* is phagocytosed by macrophages, it can persist in the cytoplasm by escaping the phagosome and reside there until conditions become right (i.e. the tissue becomes ischemic) for the bacteria to grow and progress into gangrene. This theory is supported by several lines of evidence. First, the oxygen concentration inside macrophages is lower than in surrounding tissue (91). Also, there are a number of nutrients inside the macrophage that the bacteria could utilize. Finally, while inside the macrophages, there is protection from the lethal effects of

polymorphonuclear leukocytes (PMNs). PMNs can kill *C. perfringens* under aerobic and anaerobic conditions (120) but complement must be present (unpublished data-Woodman and Melville). We are currently testing this model by eliminating either the PMNs, or macrophages from mice, to determine what role either PLC or PFO play in an infection (Chapter 5). Preliminary data suggests that macrophages do play a minor role in preventing gangrene from occurring. This is based on the fact that it took 10-fold fewer bacteria to cause a gangrene infection in mice devoid of their monocytes and macrophages as compared to an untreated control.

A second theory could be that PFO secreted by *C. perfringens* is preventing the influx of PMNs to the site of infection. The effects of PFO on PMN migration have been shown by Stevens et al. (207). The lack of PMNs at the site of infection would allow *C. perfringens* to overcome the less lethal macrophages and cause disease. The effect of PFO on PMN migration and their ability to kill *C. perfringens in vitro* is currently under investigation in our lab.

At this point, we believe that aspects of both of the theories outlined above are correct. We believe that once *C. perfringens* enters a wound, it is phagocytosed by resident macrophages where some are killed and some escape the phagosome and persist until conditions become favorable for growth. At the same time, PMNs are recruited to the wound area and kill the bacteria they are able to phagocytose. However, due to the lack of total killing and the increasing anaerobic conditions, the remaining *C. perfringens* can grow and secrete toxins, killing the macrophages harboring bacteria. The released toxins can then diffuse through the tissue preventing the influx of PMNs to the site of

infection, allowing the bacteria to grow unrestrained, ultimately leading to a gangrene infection.

This work has granted us a better understanding as to how *C. perfringens* survives during the early stages of infection. We have elucidated additional roles PLC and PFO play in infections and, to a certain extent, what role host cells play in defending against *C. perfringens* infections. We have generated enough preliminary data to possibly provide new methods of preventing infections from progressing to gangrene in a clinical setting. This work also expanded our knowledge about the contributions of the toxins to virulence, potentially leading to the development of new adjunctive therapies that could target the immune system for the treatment of gangrene infections.

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Education:

Graduate (1998-2003)
Virginia Polytechnic Institute and State University-Blacksburg, VA (2001-2003)
University of Tennessee-Memphis, Memphis, TN (1998-2001)
Principle Investigator: Dr. Stephen Melville. Area of research: *Clostridium perfringens* interactions with host phagocytic cells.
Doctorate of Philosophy earned in Microbiology

Undergraduate (1994-1998)
Florida State University-Tallahassee
Bachelor of Science earned in Biology, with an interdisciplinary minor earned in Chemistry, Physics, and Mathematics

Professional Experience:

Laboratory Graduate Teaching Assistant, University of Tennessee, Memphis
Fall 1999 to Spring 2000
Duties included teaching medical and dental students the basics of microbiology

Invited speaker for the Microbiology Club of Virginia Tech Spring 2002

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Seminars and Posters Presented:

1. Perfringolysin O and Alpha toxin are Required for Survival of *Clostridium perfringens* in Host Tissue
Poster presented at the General ASM Meeting, Washington D.C., May 2003.

2. Perfringolysin O and Alpha toxin are Required for Survival of *Clostridium perfringens* in Host Tissue
Poster presented at the Clostridium Pathogenesis Meeting, Cape Cod, Massachussetts, April 2003
3. Perfringolysin O and Alpha toxin are Required for Survival of *Clostridium perfringens* in Host Tissue
Poster presented at the Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, VA, February 2003
4. *Clostridium perfringens* interaction with host phagocytic cells
Slide presentation given at the Department of Biology, Virginia Tech, Blacksburg, September 2001.
5. *Clostridium perfringens* interaction with host phagocytic cells
Slide presentation given at the Regional ASM Meeting in Virginia, Richmond, VA, September 2001.
6. Identification of receptors involved in the phagocytosis of *Clostridium perfringens* by J774-33 macrophages
Poster presented at the General ASM Meeting, Orlando, FL, May 2001.
7. Interactions of the anaerobic pathogen *Clostridium perfringens* with macrophages under aerobic and anaerobic conditions
Poster presented at the Kentucky-Tennessee ASM Branch Meeting, Memphis, TN, April 2001.
8. *Clostridium perfringens* virulence factors and mechanism of phagocytosis
Slide presentation given at the Department of Microbiology and Immunology, University of Tennessee, Memphis, December 2000.
9. Identification of receptors involved in the phagocytosis of *Clostridium perfringens* by J774-33 macrophages
Poster presented at the Midwest Pathogenesis Meeting, Columbus, OH, October 2000.
10. The anaerobic pathogen *Clostridium perfringens* can escape the phagosome of macrophages under aerobic conditions
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Publications:

David K. O'Brien and Stephen B. Melville. (2000) The anaerobic pathogen *Clostridium perfringens* can escape the phagosome of macrophages under aerobic conditions. *Cellular Microbiology* **2**(6), 505-519.

David K. O'Brien and Stephen B. Melville. Multiple effects on *Clostridium perfringens* binding, uptake and trafficking to lysosomes by inhibitors to macrophage phagocytosis receptors. *Microbiology.* In Press.

David K. O'Brien and Stephen B. Melville. Perfringolysin O and alpha toxin are required for the escape from phagosomes and enhance the survival of *Clostridium perfringens* in host tissue. Manuscript in preparation.

David K. O'Brien, Veronica L. Stirewalt and Stephen B. Melville. Significant strain variability is seen in the sialidase-encoding genes of *Clostridium perfringens.* Manuscript in preparation.

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