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Bovine Footrot (Acute Interdigital Phlegmon): The Effects of Bacterial Etiologic Agents on Polymorphonuclear Neutrophil Function

by

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Abstract

Bovine Footrot (acute interdigital phlegmon; AIP) is a commonly occurring anaerobic bacterial infection in cattle worldwide. These studies investigated the effects of three anaerobic bacteria associated with AIP (*Porphyromonas levii*, *Fusobacterium necrophorum*, and *Prevotella intermedia*) on bovine polymorphonuclear neutrophil (PMN) function (movement, phagocytosis, and oxidative metabolism) *in vitro*. All three bacteria were chemotactic for bovine PMN with *Fusobacterium necrophorum* demonstrating the highest chemoattraction. To our knowledge, this is the first study to demonstrate that *Porphyromonas levii* is not readily phagocytosed by bovine PMN and this anti-phagocytic property may in part be associated with its possession of a capsule. Also, high titre anti-*Porphyromonas levii* serum and specifically IgG from this serum enhanced the ability of bovine PMN to phagocytose *Porphyromonas levii*. Finally, *Prevotella intermedia* failed to induce an oxidative response in bovine PMN. The above inhibitions of PMN function may enhance bacterial synergism at the focus of infection in AIP, thus promoting colonization, proliferation and pathogenesis of AIP by these three anaerobes.

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To:

Mom, Dad,

Tony, Donnie, Paul, Bonnie,

and especially Michele

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List of Abbreviations

AIP	Acute interdigital phlegmon
BBA	Brucella blood agar
BHI	Brain heart infusion broth
BPI	Bactericidal/permeability-increasing protein
BSA	Bovine serum albumin
C3b	Complement factor 3b
C5a	Complement factor 5a
CD14	PMN surface receptor that binds LBP
CFU	Colony forming units
CR1	PMN surface receptor for C3b complement protein
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
Fc	Fragment of immunoglobulin that crystallizes
FcR	PMN surface receptor for Fc region of immunoglobulin
FMLP	N-formyl-methionyl-leucyl-phenylalanine
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
IgG	Immunoglobulin G
kDa	Kilodalton
KVLB	Kanomycin, vancomycin laked blood agar

List of Abbreviations (continued)

LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NBT	Nitroblue tetrazolium
NV	Neomycin / vancomycin
O_2^-	Superoxide anion
OH^-	Hydroxyl radical
PBS	Phosphate buffered saline
PMA	Phorbol-12 myristate-13 acetate
PMN	Polymorphonuclear neutrophil
SEM	Standard error of mean
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
rpm	Revolutions per minute
TEM	Transmission electron microscopy
TEMED	N,N,N',N' - tetramethylethylenediamine
UV	Ultraviolet
v/v	Volume to volume ratio
w/v	Weight to volume ratio

Literature Review

A. Bovine Footrot

Acute interdigital phlegmon (AIP) (bovine footrot) is a commonly occurring anaerobic bacterial infection in cattle (1,2). AIP has a worldwide distribution with greatest significance in the intensive beef and dairy industries, possibly due to the strenuous conditions under which the cattle are kept (1,2). Incidence of the infection appears to vary depending on climate, weather, housing, breed, and age (1,2). Highest levels of occurrence are seen in wet weather or under conditions of high soil moisture; however, stony ground and sharp gravel also predispose animals to this condition (2). Since in North America spring and autumn are the seasons of the year that produce the highest rainfall, there is an increasing trend of AIP occurrences at these times (1). In feedlots, spring is the time in which manure is scraped out and removed from the pens, thus disrupting the microorganisms present in the manure.

1. Economic Implications

AIP has significant economic implications in both the dairy and beef fattening industries and also has relevance in the cow-calf operations and in pastured cattle (1,2). Economic losses in the dairy industry can be attributed mainly to the decrease in milk yield and discarded milk due to withdrawal times of the antibiotic used for treatment of the cows (1,2,3). In the beef (feedlot) industry the economic significance is seen in decreased weight gain due to lameness (1,2,3). Other financial concerns arise with the cost of antibiotic treatment of the disease and euthanasia of cattle unresponsive to various

treatments. The infection can cause a decrease in male libido and therefore reduced breeding efficiency which will also have decreased economic ramifications in cow-calf operations and pastured range cattle (2).

2. Clinical Signs

AIP is a necrotizing infection of the interdigital region of the foot. It is rare that more than one foot is affected at the same time (1,2). The disease has a rapid onset and leads to severe lameness, inflammation of the distal limb, pain and swelling (see Figure 1A) (1,2,3). An interdigital lesion can be associated with the infection and a purulent discharge is often present (see Figure 1B) (1,3). If AIP is neglected or left untreated then the swelling and inflammation can extend proximally up the limb. In some cases infection can result in fibrosis of the ligaments and tendons, septic arthritis and permanent lameness (1,2). The main immunopathological features of acute interdigital phlegmon are tissue edema and localized infiltration of polymorphonuclear neutrophils (PMN) into the subcutaneous tissues (4). Little is known about the role that phagocytic cells, such as PMN, play in the resolution of this anaerobic infection.

3. Bacterial Etiology of AIP

Multiple anaerobic bacteria and certain synergistic associations between bacterial species may be responsible for acute interdigital phlegmon (1,2,5). Historically, *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* have been described as the primary etiologic agents of bovine footrot (6). *Dichelobacter* [*Bacteroides*] *nodosus*, the etiologic agent of ovine footrot, has also been isolated in Australian cattle with footrot (7,8). Our laboratory has consistently isolated *Porphyromonas levii* from tissue biopsies

Figure 1A. Photograph of a feedlot steer showing clinical signs of bovine footrot (acute interdigital phlegmon) in its front right leg. Note the severe swelling, inflammation and lameness.

Figure 1B. Photograph of a foot of a feedlot steer infected with bovine footrot (acute interdigital phlegmon). Note the interdigital lesion and purulent discharge.

A

B

of animals with bovine footrot (9). To support the possibility of these anaerobic bacteria being associated with this disease, Gibbs (1996) found *Bacteroides (Porphyromonas) levii* the most commonly isolated organism from typical cases of bovine footrot in cattle from the United Kingdom (10). Metzner *et al.* (1996) also recovered *B. levii* from skin biopsies of digital dermatitis lesions in cattle in Germany (11). Finally, Dietz et al. (1995) found *B. levii* in all examined samples of digital dermatitis (12). Since *F. necrophorum* is an opportunistic pathogen (1,2) and seems to be able to act synergistically in mixed infections (1), the possibility arises that *Porphyromonas levii* may play a primary role in AIP and possibly as a synergistic partner in this infection. *Prevotella intermedia* also has been isolated from tissue biopsies collected by our laboratory and its involvement in this disease remains unknown.

4. Treatment

Immediate antibiotic treatment of acute interdigital phlegmon is necessary for rapid resolution of the infection. Parenteral administration of antibiotics and sulfonamides have been used quite extensively (1,2). In the United Kingdom, local antibiotic therapy has been promoted for treatment of this infection (5). Several antibiotics including ceftiofur sodium and oxytetracycline have been evaluated and proven efficacious (13). It has been suggested that ethylenediamine dihydriodide, added to feed, is effective in preventing bovine footrot although the mechanism of this effect is unknown (14,15). Other antibiotics such as amoxicillin, penicillin G and sodium sulfadimidine are also used for treatment of cattle infected with AIP (1,2,16). AIP may also be treated and prevented

through the use of formaldehyde or copper sulfate foot baths (1,2). Unfortunately, increasing numbers of infected cattle are unresponsive when treated with routine antibiotics, (5) thus placing an increased demand on researchers to determine the bacterial pathogenesis of this disease.

B. Anaerobic Bacteria

Anaerobic bacteria are important pathogens in a wide variety of infections in both humans and animals (17). They play an important role in many commonly encountered categories of infection including; skin and soft tissue infections, septic osteomyelitis, as well as pleuropulmonary, intra-abdominal and genital tract infections (18). The severity of anaerobic and mixed (anaerobic/aerobic) infections can vary from inapparent or subclinical to infection resulting in mortality (18). The involvement of anaerobes in infectious processes is readily overlooked, particularly when they are present in mixed culture (18). Clinical signs that indicate the probable involvement of anaerobes at the site of infection are: abscess formation, tissue necrosis, and a foul smelling discharge (17,18).

1. Oxygen Environments

Anaerobes can be categorized into various groups according to the environmental atmosphere in which they thrive most prominently (17). Facultative anaerobes, such as *Clostridium tertium*, are capable of surviving in either the presence or absence of oxygen (17). Microaerotolerant anaerobes, including *Campylobacter spp.* and some *Clostridium spp.* are capable of growth in an environment containing no higher than 5% oxygen (17).

Finally, obligate anaerobes are bacteria that grow only in an anaerobic environment (containing no oxygen) (17). Examples of obligate anaerobes include *Bacteroides spp.*, *Fusobacterium spp.*, and *Porphyromonas spp.* (17,18). It is these obligate anaerobes that are associated with acute interdigital phlegmon (AIP), attacking the soft tissue area of the cattle foot (1,2).

2. Virulence Factors

Anaerobes produce or possess a variety of virulence factors, such as endotoxins, fimbriae, capsules, and exotoxins, that are thought to be involved in the pathogenicity of disease (17). Endotoxins, molecules residing in the outer membrane of the bacterium (19) are an identifying feature of gram negative bacteria. This endotoxin, or lipopolysaccharide (LPS), plays an important role in stimulating macrophages which initiates the complex process of inflammation (20). Fimbriae protrude from the bacterial surface and allow for organisms such as *Dichelobacter* [*Bacteroides*] *nodosus* to adhere to cell surfaces and initiate colonization (17). Polysaccharide capsules such as those possessed by *Bacteroides spp.* and *Porphyromonas spp.* are thought to promote abscess formation (21) and serve an anti-phagocytic function (17). Finally, bacteria are able to release exotoxins that are very harmful to the host. Examples of such exotoxins include; leukotoxins (*F. necrophorum*), immunoglobulin proteases that directly inhibit the host's immune response, neurotoxins, cytotoxins that act towards specific cells, collagenases, lipases, and hemolysins (17). All of these different virulence factors help in the survival and growth of infectious bacteria.

3. Anaerobic Bacteria in AIP

3.1. Classification / Synonyms

Specifically for this research, we examined three obligate anaerobic bacteria associated with AIP *(Porphyromonas levii, Fusobacterium necrophorum*, and *Prevotella intermedia*). The taxonomic classification of these bacteria appears to be an on-going process as the methodology for speciation is refined. They are initially described as nonspore-forming anaerobic gram negative rods and are all grouped in the same family (Bacteroidaceae) (22). There are actually 13 genera in this family of which only *Bacteroides* and *Fusobacterium* are of veterinary significance (22). The Bacteroides subgroup has been divided into three major phylogenic clusters: Bacteroides, Prevotella (containing *P. intermedia*), and Porphyromonas (containing *P. levii*) (23). *Fusobacterium necrophorum* is of course under the Fusobacterium genus (22). Paster *et al.* has performed 16S rRNA sequence comparative analysis on all the bacteria located in the phylogenic clusters to attempt to construct a phylogenic tree (23). These results place *P. levii* [*Bacteroides levii*] (24) within the Porphyromonas cluster because of its similar physiologic characteristics to others located in that cluster (23).

The classification of these bacteria is even more interesting when one looks at the bacteria historically associated with AIP (specifically *Bacteroides melaninogenicus*) and the anaerobes recently isolated in our laboratory from cases of AIP. In 1970, *Bacteroides melaninogenicus* was divided into three subspecies; *asaccharolyticus*, *intermedius*, and

melaninogenicus (25). After 1977 more subspecies were added to each of these three subspecies. Then in 1990, the bacteria located in the same subspecies group as *asaccharolyticus* were given a new genus name (*Porphyromonas*) and *B. levii* was subsequently placed within this group (25). The bacteria located within the *intermedius* group were also renamed (*Prevotella*), and *B. intermedius* was then referred to as *P. intermedia*. Finally the *melaninogenicus* group was also renamed as *Prevotella* and *B. melaninogenicus* was referred to as *P. melaninogenica* (25). Therefore, what was once classified as *B. melaninogenicus* 30 years ago can now be placed into one of three genera and one of 17 species (25). Classification of *Fusobacterium necrophorum* has changed very little over this same time. This bacterium has been classified into two subspecies; *necrophorum* (biotype A) and *funduliforme* (biotype B) based on DNA homology, haemagglutination and chemical composition of LPS (26,27).

3.2. *Porphyromonas levii*

Porphyromonas levii is a pigmented gram negative pleomorphic rod. It has been isolated from bovine footrot biopsy specimens and characterised in our laboratory using standard bacteriologic techniques and gas liquid chromatography (9). As mentioned before, *P. levii* is of animal origin (25) and is novel in its association with AIP. *P. levii* recently has been frequently isolated from cattle in Europe infected with AIP (10,12). Collighan and Woodward (1997) used 16S rRNA gene sequencing to identify *P. levii* from digital dermatitis lesion biopsies of cattle in the United Kingdom (28). Consistent isolation of *P. levii* from cattle infected with bovine footrot helps support the possibility

that this bacteria plays a primary role in the pathogenicity of AIP. *P. levii* and other anaerobes have been implicated in summer mastitis of cattle (22) and *P. levii* has also been isolated from the bovine rumen (25). Isolates phenotypically, but not genotypically, resembling *P. levii* have been recovered with increasing frequency from human infections of the skin and soft tissue (25). Jousimies-Somer *et al.* looked at *Bacteroides levii*-like organisms isolated from human sources and demonstrated through preliminary genetic analysis that these isolates are distinct from the bovine *P. levii* (29).

3.3. *Fusobacterium necrophorum*

Fusobacterium necrophorum is a long (up to 10 μm) pleomorphic gram negative rod that is found in the alimentary tract and on mucosal membranes (17,22). It is found in various necrotic disease conditions such as hepatic abscesses and AIP in cattle (22,27). Infections are frequently mixed, as it is believed to be a secondary invader (22). A water soluble exotoxin (leukotoxin) appears to be a primary virulence factor in the pathogenesis of fusobacterial infections (22,26,27). Biotype A , most frequently isolated in pure culture from bovine liver abscesses, produces more leukotoxin than biotype B which predominates in ruminal contents and ruminal lesions (22,27,30). Scanlan *et al.* demonstrated that biotype A was more pathogenic than biotype B using leukotoxins from three bovine strains of *F. necrophorum* (30). Scanlan *et al.* was also able to show that the leukotoxins from each strain were heat stable, pH stable and their cytotoxic activity was not affected by enzymatic treatment with trypsin, protease, α-amylase, lipase, DNAase, or

RNAase (30). Other studies have shown that culture supernatant of *F. necrophorum* was toxic to polymorphonuclear neutrophilic leukocytes (PMN) from cattle and sheep, but not to those from pigs and rabbits (27).

3.4. *Prevotella intermedia*

Prevotella intermedia [*Bacteroides intermedius*] is the final nonspore-forming obligate anaerobe that was used in our experimental design. *P. intermedia* is a short gram negative rod (17). Traditionally, *P. intermedia* has been implicated in the pathogenesis of periodontal diseases and is found in the gingival crevice of diseased humans (31,32). The virulence of *P. intermedia* is thought to be due to a combination of factors including; lipopolysaccharide, adhesins, hydrolytic enzymes, polysaccharide capsule and the ability to bind IgG Fc fragments (32). Due to its importance in periodontal diseases, studies have been performed on its ability to be phagocytosed by human PMN under various fibrin meshworks (31). Also, Shinzato and Saito (1994) demonstrated that *P. intermedia* inhibited bactericidal activity of human neutrophils (33). To date little is known about its association or involvement in the pathogenesis of AIP.

4. Bacterial Synergism

The types of infections produced by anaerobic gram negative rods, such as AIP, are often of mixed etiology, with two or more species being present (34). However, anaerobic bacterial synergism has been described only in a few instances (34). Interestingly, Price *et al.* has implied a synergistic effect in the growth of *P. intermedia*

when associated with *F. necrophorum* (34). It was reported that a growth factor, whose characteristics were indistinguishable from bacterial LPS, produced by *F. necrophorum* enhanced the *in vitro* growth of *P. intermedia* (34). A different study characterized the acute phase of the host response to mixed infection with *Bacteroides melaninogenicus* and *Fusobacterium necrophorum*. An acute to chronic infection progressed in mice with this mixture of anaerobic bacteria, whereas no infection resulted when either organism was injected alone (35). It has also been reported that enhancement of growth of *Streptococcus constellatus* was demonstrated when cultured with *P. intermedia* or culture filtrate of *P. intermedia* (33). This research gives support to the possibility of bacterial synergism in AIP.

C. Polymorphonuclear Neutrophils (PMN)

PMN (neutrophils) have been recognized as an essential component of a host's defense against infection since Metchinkoff's pioneering work at the turn of the century (36). Polymorphonuclear neutrophils represent a primary line of defense in the host's cellular immunity against invading microorganisms (20,37,38). The importance of PMN in the resolution of acute bacterial infection or tissue necrosis (39) is evident. Surprisingly, little attention has been paid to this cell type in the pathogenesis and resolution of AIP. An evaluation of the response of PMN *in vitro* towards the bacteria we suggest as being important in the pathogenicity of AIP may lead to an increased understanding of the *in vivo* response of PMN to resolve this infection. An inhibition of

PMN function in either mobility, phagocytosis, or bactericidal activity by one or several bacteria may lead to an explanation towards the pathogenicity of AIP.

PMN originate from pluripotent stem cells in the bone marrow, of which its predecessors make up 60% of the leukocytes being developed, and are continuously discharged in vast numbers into the blood stream (20). The half life of a PMN circulating in the blood stream is 6-8 hours and once entered into tissues, they remain functional for 1-2 days (38). Senescent PMN are removed by splenic macrophages in circulation, resident tissue macrophages in tissue or discharged from mucosal surfaces (38).

PMN are the first cells recruited to the site of a bacterial infection. Once microbes have invaded the host's tissue, circulating PMN are activated by chemotactic factors and adhere to the endothelial cells located near the site of infection. PMN then migrate through the endothelial barrier (diapedesis) and follow the chemotactic factor gradient to the site of injury (37).

Three important functions must be performed by the PMN in order to eliminate the foreign bacteria from the site of infection. The first mechanism involves migration (chemotaxis/chemokinesis) of the PMN to the microbe. The second mechanism (phagocytosis) allows the PMN to engulf the microbe and the final function is bactericidal activity which enables the PMN to kill and degrade the microbe (20,37,38).

1. Migration

PMN mobility may be placed into several distinct categories to define directional migration response. Random migration is used to describe cell movement without any

chemical stimulus (37). Chemotaxis is defined as directed migration of a cell towards an increasing chemical gradient (38). Chemokinesis is defined as the speed of migration of a cell determined by substances in the environment (37). In order for migration to occur, PMN require a chemoattractant that will bind to specific receptors on its surface membrane. Several potent chemoattractants include interleukin-8, $C5_a$ (complement derived), leukotriene B_4, and N-formyl-methionyl-leucyl-phenylalanine (FMLP - a bacterial byproduct) (40). These chemicals can be released from either the invading bacteria (FMLP) or from the inflammatory cells (IL-8, C5a, LTB_4) already present at the site of infection (40). Chemoattractant-stimulated PMN become polarized with a lamellipodium in the front of the cell and a uropod at the rear (38). PMN locomotion involves the extension of the lamellipodium in the direction of the increasing chemoattractant gradient, attachment to the substrate, detachment of the uropod and retraction toward the cell body (37). The specific mechanisms controlling these events remain unknown.

The most commonly used methods for assessing chemotactic ability of PMN are under-agarose chemotaxis (41) and the Boyden chamber technique (42). The later of these two was used for our experiments. In this technique a reservoir of cells is separated from a reservoir containing a chemoattractant by a filter through which the PMN migrate. Chemotaxis is a measure of the distance PMN migrated through the filter toward the chemoattractant over time. To measure chemokinesis, the PMN are completely surrounded by the chemoattractant and serum and measurements are taken by the distance

migrated through the filter over time (38). Chemokinesis does not determine the direction of migration of cells but instead determines the speed in which these cells move under a certain stimulus (38).

Little work has been done on the migrating properties of bovine PMN. Olson (1990) has tested the chemotactic activity of PMN from adult cattle versus young cattle using the under-agarose method. It was demonstrated that PMN from older cattle may respond more efficiently to chemoattractants than PMN from younger or newborn cattle and this could possibly be due to a greater number and variety of membrane receptors located on the PMN surface of older cattle (43).

Mobility properties of PMN from other species towards bacteria have also been studied. Crude cell extracts of *Bacteroides gingivalis* and *Bacteroides intermedius* were proven to inhibit human PMN migration (44). If this phenomenon is true with bovine PMN, then does *Prevotella intermedia* or other bacteria play an important role in the pathogenesis of AIP by stopping the ability of PMN to reach the site of infection? (44). In contrast, other studies showed the supernatant of *B. fragilis* moderately stimulated chemotaxis in human PMN (45) and rabbit peritoneal PMN showed some chemotaxis to *Bacteroides spp* (46). By experimentally evaluating the chemotactic/chemokinetic response of bovine PMN *in vitro* towards the three anaerobic bacteria associated with AIP one may find a possible mechanism in which the bacteria evade the host's cellular response against AIP.

2. Phagocytosis

Following cell movement to a focus of infection, the second function a PMN must perform is phagocytosis of the bacteria. In order for phagocytosis to occur the PMN must completely engulf the microbe. Like chemotaxis, the surface membrane of the PMN is an important component of the phagocytic process (37). Phagocytosis begins with sequential receptor-ligand binding between the PMN and the bacterium. Pseudopodia are produced and eventually surround the bacterium to form a phagocytic vacuole (phagosome) (37).

Several studies have been performed on the phagocytic abilities of bovine PMN. Silva and Jain (1989) have attempted to describe the effects of glycolytic and cytoskeletal inhibitors on phagocytic activities of bovine PMN against various bacteria (47). Their results demonstrated the importance of these factors in cellular functions such as chemotaxis and phagocytosis (47). Silva *et al.* (1989) looked further into the phagocytic properties of immature bovine neutrophils against *E. coli* and demonstrated that promyelocytes and myeloblasts are not phagocytically active but phagocytosis was seen in all PMN precursors from myelocytes to segmented neutrophils (48). Other studies have devised methods of quantitating *in vitro* phagocytosis of ^{32}P-labeled *Staphylococcus aureus* by bovine PMN in a large number of samples (49). Paape and Miller (1988) demonstrated sources of variation introduced into a phagocytosis assay as a result of the isolation of neutophils from bovine blood (50). The ability of *Bacteroides spp.* to impair phagocytosis not only of themselves but also of accompanying bacteria, has been

suggested to be an important factor in the development of mixed infections (51,52). Is this phenomenon true for the Bacteroides species present in AIP? All the above mentioned studies clearly give support to the importance of phagocytosis by bovine PMN in resolving infections such as AIP.

There is a further need to evaluate the effects of the specific bacteria involved in AIP and their by-products on PMN-mediated phagocytosis. An *in vitro* evaluation of this phenomenon could lead to a better understanding of the host's cellular response, specifically PMN, *in vivo* to bacteria associated with AIP.

3. Bactericidal Activity

Once PMN have phagocytosed bacteria they still need to degrade the microbe. This final function of the PMN in clearing a bacteria from the site of infection is referred to as bactericidal activity. After the bacterium is trapped inside the PMN, the phagosome fuses with a lysosome to form a phagolysosome and now degradation of the bacterium may begin (40). There are two main arms of microbicidal activity of PMN: oxygen-dependent mechanisms and oxygen-independent mechanisms (38). Oxygen-dependent degradation of bacteria relies on toxic molecules produced as a result of the respiratory burst and will be discussed first. Oxygen-independent mechanisms rely principally on a number of bactericidal proteins that are preformed constituents of cytoplasmic granules in PMN (40).

3.1 Oxygen Dependent Metabolism

The initial step in the oxygen-dependent pathway called the, "respiratory burst", occurs when NADPH oxidase in the phagolysosome membrane is activated and reduces O_2 to superoxide (O_2^-) (37,53). NADPH oxidase activation is enhanced by prior exposure of the cells to lipopolysaccharide (LPS) released from gram negative bacteria (40). Superoxide is then reduced to hydrogen peroxide (H_2O_2) by superoxide dismutase at the cell surface within the phagolysosome (53). H_2O_2 is a powerful oxidant but reacts sluggishly with biological materials and bacteria contain enzymes that detoxify hydrogen peroxide (catalase or sulphhydryl-peroxidase) (37). Myeloperoxidase, released from the primary granules, reacts with H_2O_2 in the presence of a halide such as chlorine to form hypochlorous acid (HOCl) (53). Further reactions of HOCl and ammonia (NH_4+) allow for the production of monochloramine which is a highly bactericidal compound (37,40). Finally, reduction of H_2O_2 occurs through a Haber-Weiss reaction that forms a highly reactive hydroxyl radical (OH^-) (37,40). All the reactive oxygen metabolites produced can freely degrade the bacteria trapped in the phagolysosome.

Common markers used for determining oxidative metabolism in PMN are through the use of nitroblue tetrazolium reduction (NBT) (54), myeloperoxidase activity (38) and cytochrome c activation (37). NBT was used as the basis for our experimental design. Once the NBT solution is phagocytosed it quickly reacts with the oxygen radicals present and forms insoluble (blue) formazan crystals that can be visualised under light microscopy (37,54). This method was used to determine the oxidative activity of bovine PMN when

exposed to the bacteria associated with acute interdigital phlegmon. Any reduced oxidative function of bovine PMN by the bacteria in this experiment *in vitro* may suggest a possible mechanism used by the bacteria to evade the host's PMN response to AIP *in vivo*.

Several studies have been performed using NBT as a means of determining oxygen-dependent bactericidal abilities of bovine PMN. Along with phagocytic research, Silva *et al.* (1989) looked at NBT reduction properties of mature and immature bovine PMN (48). Other research has examined the role of protein kinase-C activation, an important intermediate step in stimulation of NADPH oxidase and superoxide anion production, in phorbol 12-myristate 13-acetate (PMA) stimulated PMN from newborn and adult cattle (55). Finally, Watson *et al.* (1995) found that the generation of reactive oxygen species by bovine PMN in the response to *Pasteurella haemolytica* is highly dependent on the presence of opsonins and is greatly enhanced in live versus killed bacteria (56).

3.2. Oxygen Independent Metabolism

Oxygen-dependent mechanisms are not the only method by which PMN can kill bacteria. PMN also demonstrate antimicrobial activity that is oxygen-independent (40). These mechanisms utilize bactericidal proteins released from cytoplasmic granules into the phagolysosome (20). The proteins include; lysosomal hydrolases, bactericidal /permeability-increasing protein (BPI), defensins and lactoferrin to name but a few (20,40). Both the primary (azurophil) and secondary (specific) granules of PMN contain

various hydrolases that possess antimicrobial activity (20). Cathepsin G, a neutral protease, is released from primary granules along with elastase and collagenase (20). Bactericidal/permeability-increasing protein is a cationic protein released from primary granules and is potently bactericidal toward many gram negative bacteria due to the interaction of BPI and LPS (37,40). BPI inserts into the outer membrane of the bacterial envelope, disrupts the LPS layer and increases the permeability of the membrane to other bactericidal factors (37). Also, BPI activates phospholipases and enzymes that degrade bacterial peptidylglycans (20,40). Another group of cationic proteins released from primary granules are the defensins. Defensins are less potent in bactericidal activity than BPI, but make up 30%-50% of the granule protein and therefore their overall activity is probably highly significant (20,40). The precise mechanism of microbicidal activity of the defensins is unclear but they have been shown to permeabilize *E. coli*, presumably by inserting into the cell membrane (40). Defensins are also chemotactic for monocytes suggesting a role of local accumulation of monocytes during acute inflammatory reactions (20,40). Finally, lactoferrin is an iron-binding glycoprotein contained in secondary granules that competes with bacteria for iron therefore inhibiting bacterial growth (20,40).

The efficiency of PMN to degrade bacteria increases when oxygen-dependent and oxygen-independent mechanisms work in conjunction with one another (20). The ability of bovine PMN to degrade bacteria at the site of injury is an important function for PMN trying to resolve an anaerobic infection such as AIP.

D. Opsonins

Phagocytosis of bacteria by PMN can be enhanced through a process called opsonization. Opsonins bind to bacterial surfaces and are recognized by receptors on the surface membrane of PMN (20,37). The PMN can then readily attach to the opsonin and begin phagocytosis. The major opsonins are IgG and complement protein $C3_b$ (20,37,38). PMN contain a F_c receptor (F_cR) that binds the F_c portion of the immunoglobulin and a CR1 receptor that binds $C3_b$ (a fragment of complement protein C3) (20,37,38). Another possible serum opsonin is lipopolysaccharide binding proteins (LBP), an acute phase reactant that binds to lipopolysaccharide on gram negative bacteria. This will then enhance bacterial binding through attachment to the CD14 receptor on the surface of the PMN (20,37). The availability of various opsonins in serum is an important part of the efficiency of PMN to phagocytose bacteria.

1. Immunoglobulins

Immunoglobulins are a key factor in the host's immune response to foreign antigens (57). Immunoglobulins are molecules that specifically recognize antigen and function both as receptors for antigen on the lymphocyte (B-cell) membranes and as secreted products of the plasma cell (58). B-lymphocytes, which are precursors to the plasma cell, present an antibody on its surface so that it will easily recognize a specific antigen. Once this binding of an antigen to a B-cell surface antibody occurs, the cell can begin proliferating in order to produce more antibodies reactive to that antigen (57). Plasma cells, terminally differentiated cells of the proliferating B-lymphocyte lineage, will

synthesize and secrete replicate antibodies throughout the circulation so that they may bind the foreign antigen and form antibody-antigen complexes. These complexes are then removed from circulation through phagocytosis by both macrophages and PMN (57). PMN bind the F_c domain of the immunoglobulin which allows the antibody/antigen complex to be engulfed (20,59). When immunoglobulins bind to the surface of bacteria and allow for easy engulfment by the phagocytes they are then referred to as opsonins (20,57). The response of increased production of antibodies (immunoglobulins) by B-lymphocytes to a specific foreign antigen (bacteria) is the main component of specific immunity.

1.1 Structure

Immunoglobulins are composed of two heavy chain (55,000 daltons) polypeptides and two light chain (25,000 daltons) polypeptides bound together by disulfide bridges and non-covalent bonds (57). The immunoglobulin itself is divided into two protein regions; a Fc (fragment that crystallizes) domain that is involved in immune regulation and two Fab domains that carry the antigen binding sites (57). These two domains are divided by a hinge region which allows lateral and rotational movement of the two antigen binding fragments (20,57,58). Immunoglobulins are subdivided, according their different heavy chains, into five isotypes; IgM, IgE, IgA, IgD, and IgG.

1.2. Immunoglobulin G

Immunoglobulin G (IgG), a 150 kDa monomeric polypeptide, is the most abundant isotype in serum, constituting about 80 % of the total serum immunoglobulin (58). In

bovine serum at least three IgG subclasses differing in net electrical charge and individual immunological specificity are defined (IgG_1, IgG_{2a}, IgG_{2b}) (60). IgG_1 transported into exocrine fluids constitutes greater than 90 % of the antibody in colostrum which is ingested by the newborn calf (60,61). IgG_2, which constitutes half of the total serum IgG, is most prominent in PMN opsonization and antibody dependent cell cytotoxicity (61). McGuire *et al.* (1979) has demonstrated that bovine peripheral blood monocytes and neutrophils readily adhered to, and phagocytosed, erythrocytes coated with IgG_2 but not those coated with IgG_1 (62). Also, antibody responses to bacterial polysaccharides may be predominately or exclusively of the IgG_2 subclass (6). Howard *et al.* (1980) has shown that bovine PMN from peripheral blood and milk possessed surface receptors for bovine IgG_2 but not for bovine IgG_1 (63). Specifically, IgG_{2a} has been shown to be the most important antibody in PMN phagocytosis and antibody-dependent, cell-mediated cytotoxicity in cattle (64). Another way in which IgG_2 can assist in the host immune response to invading bacteria is through activation of the complement pathways (20). IgG is able to activate the classical pathway, through C1 (complement protein) binding to Fc domains of two IgG molecules, and also activate the alternative pathway by C3b-IgG complexes binding to target particles (20). Finally, research has demonstrated that both bovine IgG_1 and IgG_2 antibody-antigen complexes were able to fix bovine complement *in vitro* and that IgG_1 might be slightly more efficient than IgG_2 (62). Whether these antibodies (IgG_2) or other opsonins increase the ability of bovine PMN to phagocytose the bacteria present in AIP is not known.

An experimental evaluation *in vitro* of the effect that serum antibodies, specifically IgG, have on antibody-mediated PMN phagocytosis of the three bacteria associated with AIP could expose new information into the relationship between the host's cellular and humoral responses. The interaction of these responses are an extremely important part of the host's ability to resolve the infection.

E. Hypotheses

PMN are the first cells recruited to the site of a bacterial infection and they are found histologically in tissue biopsies of cattle feet infected with bovine footrot. Interestingly, little is known about the role that PMN play in the resolution of this anaerobic infection. The aim of these studies was to evaluate bovine PMN function *in vitro* when associated with three anaerobic bacteria (*Porphyromonas levii*, *Fusobacterium necrophorum*, and *Prevotella intermedia*) we suggest are of importance in acute interdigital phlegmon. Based on what has been described in the literature (44), stating that *B. intermedius* can inhibit human PMN mobility, we hypothesize that one or more of the bacteria mentioned above, or their by-products, will inhibit the mobility of bovine PMN *in vitro*. We also postulate that PMN-mediated phagocytosis of one or more of the bacteria *in vitro* will be obstructed by the bacteria themselves or their by-products. Based on what was described by Shinzato and Saito (1994) on bactericidal activity, we hypothesize that one or more of the bacteria, or their by-products, will not induce an oxidative metabolic response in bovine PMN *in vitro*. Based on textbook knowledge of serum

immunoglobulins and opsonisation and the interaction between IgG_2 and bovine PMN described by McGuire *et al.* (64), we hypothesize that specific antibodies raised against a bacteria will increase the phagocytic ability of bovine PMN (through opsonisation) to engulf that bacteria *in vitro*.

F. Objectives

The objectives used to accept or reject the above hypotheses were:

1) To examine mobility (chemotaxis / chemokinesis), phagocytosis (light microscopy) and oxidative metabolism (NBT reduction) of bovine PMN when in association with three anaerobic bacteria involved in AIP (*Porphyromonas levii, Fusobacterium necrophorum,* and *Prevotella intermedia*).

2) To determine the effect of high titre bovine serum on antibody-mediated phagocytosis of *P. levii, F. necrophorum,* and *P. intermedia* by bovine PMN.

3) To determine which opsonin (IgG or C3b) is acting as the primary opsonin in bovine serum responsible for increased phagocytosis that may be seen in high titre bovine serum phagocytosis experiments.

Materials and Methods

A. Polymorphonuclear Neutrophil (PMN) Purification

1. Bovine Blood Collection

Peripheral blood was collected by jugular venipuncture from Holstein steers. The animals ranged from 6 months to 1 year in age. Animals were group housed at the University of Calgary farm and restrained using a squeeze chute and halter. The blood collection apparatus was composed of a 21 gauge, 1.5 inch vacutainer needle, a vacutainer collar and five (8 mL draw) acid citrate dextrose (2X-ACD) vacutainer tubes (Becton Dickinson, New Jersey NY, USA). The tubes were inverted several times to mix the anti-coagulant with the blood. All animal handling procedures and protocols involving experimental animals were reviewed and approved by The Life and Environmental Sciences Animal Care Committee at the University of Calgary.

2. Granulocyte Purification

The bovine blood was pooled into a 50 mL polypropylene centrifuge tube. The tube was balanced and centrifuged at 900 x g for 20 minutes with no braking. Using a sterile plastic transfer pipet, the plasma and buffy coat layers were removed and the erythrocyte pack, containing the granulocytes, was decreased to a 10 mL volume level. Twenty milliliters of cold hypotonic solution (5.6 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, in pyrogen-free water, pH 7.2) was added to the cell pack to lyse the erythrocytes. The tube was capped and then inverted and/or swirled gently, to minimize foaming, for one minute. After mixing, 10 mL of hypertonic solution (5.6 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, 0.46

M NaCl, in pyrogen-free water, pH 7.2) was added to restore isotonicity to the solution. The solution was then mixed as described above. The tube was balanced and centrifuged at 900 x g for 10 minutes. The supernatant was decanted and the lysing and restoring steps performed again as described above. After centrifugation, the supernatant was discarded and the pellet resuspended in 10 mL of sterile pyrogen-free phosphate buffered saline (PBS) (4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.14 M NaCl, pyrogen-free water, pH 7.2). The solution was centrifuged as described above, the supernatant decanted and the final pellet resuspended in 4 mL of pyrogen-free PBS. Resuspension of the granulocytic pellet, after each centrifugation, was performed by gentle aspiration with a sterile plastic transfer pipet. All solutions were filter sterilized (0.2 μm, 47 mm) (Sigma, Oakville Ont, Canada).

3. Granulocyte Enumeration and Viability

The original granulocyte cell suspension was diluted by adding 1 mL of cell suspension to 9 mL of pyrogen-free PBS. Fifty microliters of this diluted cell suspension was added to 250 μL of 0.1% (v/v) trypan blue and 200 μL of PBS. A hemacytometer was used to count the number of granulocytes present. To quantitate the cells in the original suspension, the number of cells counted on the center grid of the hemacytometer was multiplied by 1×10^4 to obtain the number of cells/mL and then multiplied by 100 to account for the dilution factors. This enumeration was performed in triplicate to each granulocyte suspension and counts expressed as mean cells per mL. Preparations were standardized to a concentration of 1×10^7 cells/mL.

Concurrent with cell enumeration procedures, granulocyte viability was assessed using trypan blue exclusion. On each hemacytometer grid, 100 cells were examined and categorized as viable or non-viable. Non-viable cells appeared light blue due to inclusion of the trypan blue dye. Viability assessments were performed in triplicate and data expressed as mean cells viable per 100 PMN. Granulocyte viability had to be greater than 95% to allow the cells to be used in experiments.

4. Cell Differential Count

One hundred microliters of the diluted granulocyte suspension was added to the cytospin chamber consisting of a funnel, absorbent paper and a microscope slide. The cell suspension was centrifuged in a Cytospin (Shandon Southern Products Ltd, Astmoor, England) at 1500 x g for 10 minutes. The slide was left to air dry and then stained with a Diff Quik staining set (Baxter Healthcare Corporation, Miami FL, USA) using staining time intervals as follows: 1) fixative (1.8 mg/mL Triarylmethane dye, 100% PDC in methyl alcohol) - 6 seconds, 2) solution 1 [1 g/L Xanthene dye, 100% PDC, buffer and sodium azide (0.01%)] - 10 seconds, 3) solution 2 (1.25 g/L Thiazine dye mixture, 100% PDC, 0.625 g/L azure A and 0.625 g/L methylene blue, buffer) - 3 seconds. The slides were then mounted with Cytoseal (Sigma). The granulocytes were examined under a light microscope at a magnification of 400 X. Cells were categorized as either; neutrophils (PMN), eosinophils or other (basophils, monocytes, lymphocytes). Neutrophils contained a dark blue, multilobed nucleus and a grey cytoplasm. One hundred cells were counted in each of 3 different fields and the mean determined. The granulocyte suspension must have

contained greater than 95% neutrophils to allow the cells to be used in experiments.

B. Anaerobic Bacteria

1. Species Identification

Three different anaerobic bacteria were compared throughout the experiments described below. *Fusobacterium necrophorum* (ATCC 27852), was purchased commercially and this strain of bacteria was isolated from an ovine foot with footrot. *Porphyromonas levii* and *Prevotella intermedia* were isolated from tissue biopsies of actual cases of bovine footrot and classified in our laboratory using standard methodology. Secondary cultures were grown on Brucella blood agar (BBA) plates from the original isolates and single colonies stored at –85°C in sterile CultureSwab transport systems (Difco Laboratories, Detroit MI, USA).

2. Growth

Each bacterial isolate was grown at 37°C in a Bactron Anaerobic / Environmental Chamber (Sheldon Manufacturing Inc., Cornelius OR, USA) in an atmosphere composed of CO_2 (5%), H_2 (5%), and N_2 (95%). *F. necrophorum* was grown on neomycin (100 mg/L) / vancomycin (7.5 mg/L) (NV) anaerobic agar medium (Dalynn, Calgary AB, Canada). *P. levii* was grown on kanamycin (100 mg/L) / vancomycin (7.5 mg/L) laked blood agar (KVLB) medium (Dalynn). *P. intermedia* was grown on BBA medium supplemented with 0.1% (w/v) hemin (Difco) and 0.1% (v/v) vitamin K (Difco). Secondary cultures were thawed weekly and streaked onto the appropriate plates. When

needed, single colonies of each bacterial isolate were transferred from the agar media to 40 mL brain heart infusion (BHI) broths (0.05% hemin, 0.01% vitamin K, and 0.01% (w/v) resazurin (Sigma) and allowed to grow to logarithmic phase (*F. necrophorum* – 1 day, *P. levii* – 4 days, *P. intermedia* – 2 days). Bacterial broths were tested for purity by streak plating on BBA media.

3. Preparation

When the bacterial broths reached their logarithmic growth phase, they were transferred to 40 mL sterile plastic centrifuge tubes and centrifuged at 3000 x g for 10 minutes. The supernatants were decanted and the pellets resuspended in 30 – 40 mL of sterile pyrogen-free PBS. The bacteria were centrifuged again at 3000 x g for 10 minutes. Decanting of supernatants and resuspension of pellets were performed two more times as described above. The final pellet was resuspended in 1-4 mL of pyrogen-free PBS depending on the amount of bacterial pellet present.

4. Quantification

Ten fold serial dilutions, up to 10^{-8}, were performed on each bacterial isolate using 900 μL dilution blanks containing pyrogen-free PBS. Dilutions were compared visually to MacFarland standards (Sigma) (0.5-MacFarland for *P. levii* and *P. intermedia*, 4-MacFarland for *F. necrophorum*). These dilutions were approximately equivalent to 1.5 X 10^7 bacteria/mL. Twenty microliters of each dilution tube was spot plated onto BBA plates and incubated at 37°C under the anaerobic atmosphere described above. Viable bacterial numbers (CFU/mL) were determined from the BBA plates to verify visual

comparisons with MacFarland standards.

C. Immunization Protocol

1. Bacterial Preparation

Porphyromonas levii, *Prevotella intermedia* and *Fusobacterium necrophorum* were individually used to immunize three separate Holstein steers. All 3 steers had low pre-existing titres to each bacteria used for immunization as assessed by ELISA (see section D). Bacteria were grown to logarithmic phase in BHI (0.01% hemin and 0.05% vitamin K) broth under anaerobic conditions (95% N_2, 5% CO_2, 5% H_2) at 37°C. The bacterial broths were streak plated on BBA medium to test for purity. The bacteria were washed and centrifuged (3000 x g for 10 minutes) three times in sterile PBS. To kill the bacteria, the cells were left in 10% (v/v) formaldehyde in PBS for 48 hours. The bacteria were once again centrifuged and washed three times as described above. The final pellet was resuspended in 1-2 mL of sterile pyrogen-free PBS, dependent on the amount of pellet present. To lyse the bacteria, the suspensions were snap frozen with liquid nitrogen, thawed and snap frozen a second time. Bacterial lysate were checked for viability by steak plating on BBA medium and incubating under anaerobic conditions described above. A modification of the Bradford protein assay (65) (Bio Rad protein microassay, Bio-Rad Laboratories, Richmond CA, USA) was used to determine the concentration of bacterial protein present in each solution. The bacterial suspensions were diluted to 1 mg/mL protein in PBS and stored at –85°C until required.

2. Antigen Preparation

Five hundred microliters of each killed and lysed bacterial suspension (antigen) (1 mg/mL) was added to 500 µL of Imject Alum adjuvant (45 mg/mL) (Pierce, Rockford IL, USA). The suspensions were then mixed, until the solutions were emulsified using two 3 mL syringes per preparation connected by female leur locks.

3. Immunization Procedures

One (2 months old) and 2 (6 months old) Holstein steers were used for the immunization procedures. All injections were given subcutaneously into the lateral region of the neck. Pre-immune blood samples, used for experimental controls, were collected from each animal prior to immunization and the serum stored at -20°C until required. Each calf was injected with one of the three antigen/adjuvant suspensions. The bacterial isolate and corresponding cattle identification are as follows: *P. levii* – Yellow 15, *P. intermedia* – Yellow 21, *F. necrophorum* – Blue 2215. The cattle were group housed at the University of Calgary farm and general husbandry was performed by technicians at the Animal Resource Center. All procedures were performed in accordance with the Canadian Council on Animal Care: Guide to the use of Experimental Animals (66). Injection sites were monitored daily and any abnormalities reported to a university veterinarian. The cattle developed no abnormalities to the injections. Booster injections were given 21 days later of the same antigen/adjuvant combination, as described above. Seven days after the boost blood samples were collected and serum antibody titres determined by ELISA (see section D). Calves were boosted every seven days, blood

samples taken and antibody titres determined until adequate levels were reached for each of the bacterial isolates.

4. Blood Collection and Serum Separation

The jugular vein was again designated the site of collection from the Holstein steers. Blood collection was performed as described in section A.1 with the exception that SST Gel and Clot Activator vacutainer tubes were used (Becton Dickinson). Specimens were centrifuged at 1000 x g for 20 minutes. Serum was decanted and aliquoted into 1 mL microcentrifuge tubes and stored at –20°C.

D. Enzyme Linked Immunosorbant Assay (ELISA)

ELISA was used to determine the antibody titres of sera from the cattle immunized against one of *P. levii, P. intermedia,* or *F. necrophorum.* Ninety six well Falcon Pro-Bind Assay Plates (Becton Dickinson) were used for each assay. Each bacterial isolate was grown in BHI, washed and lysed (see sections B 2-3, C 1). Lysed bacteria (0.2 mg protein/mL) were used as the antigen for coating the plates. One hundred microliters of each specific antigen was pipetted into the appropriate wells. The plate was covered with a lid and incubated for 1 hour at 37°C. The plate was then washed 3 times with 0.1% (v/v) Tween 20 in PBS (150 sec soak) using a Maxline microplate washer (Molecular Devices Corporations, Menlo Park CA, USA). Three hundred microliters of blocker, 10% (w/v) skim milk powder (Lucerne, Lucerne Foods Ltd, Vancouver B.C., Canada) in PBS, was added to each well. The plate was then incubated and washed, as described

above. Bovine sera collected from the Holstein calves were added to the wells. Doubling dilutions starting at 1/10 or 1/100 were used to dilute these primary antibody sera. One hundred microliters of each dilution was added to the appropriate wells. The plate was incubated for 1 hour and washed as described above. Affinity purified rabbit anti-bovine IgG conjugated to horse radish peroxidase (Sigma) (diluted 1:2000 in 5% (w/v) skim milk PBS) was used as the secondary antibody. Fifty microliters was pipetted into each well. The plate was then incubated for 1 hour and washed 4 times with the same method previously described. Two hundred microliters of substrate, O-Phenylenediamine Dihydrochloride (Sigma) (0.6 mg/mL) containing 30% (v/v) hydrogen peroxide (Sigma) (40 µL/100 mL buffer) added just prior to use, was added to each well. The plate was incubated for 30 minutes at 37°C. The reaction was stopped by adding 50 µL of 2.5M H_2SO_4 to each well. The plates were read for absorbance on a Thermomax microplate reader (Molecular Devices Corporation) at 490 nm. Results were analysed using Softmax version 2.32 software (Molecular Devices Corporation).

Antibody titres in these bovine sera were expressed as the reciprocal of the highest dilution that gave an absorbance reading closest to, but not below, the control value. All experiments were performed in duplicate to assure consistency of the ELISA.

E. Protein Assay

1. Protocol

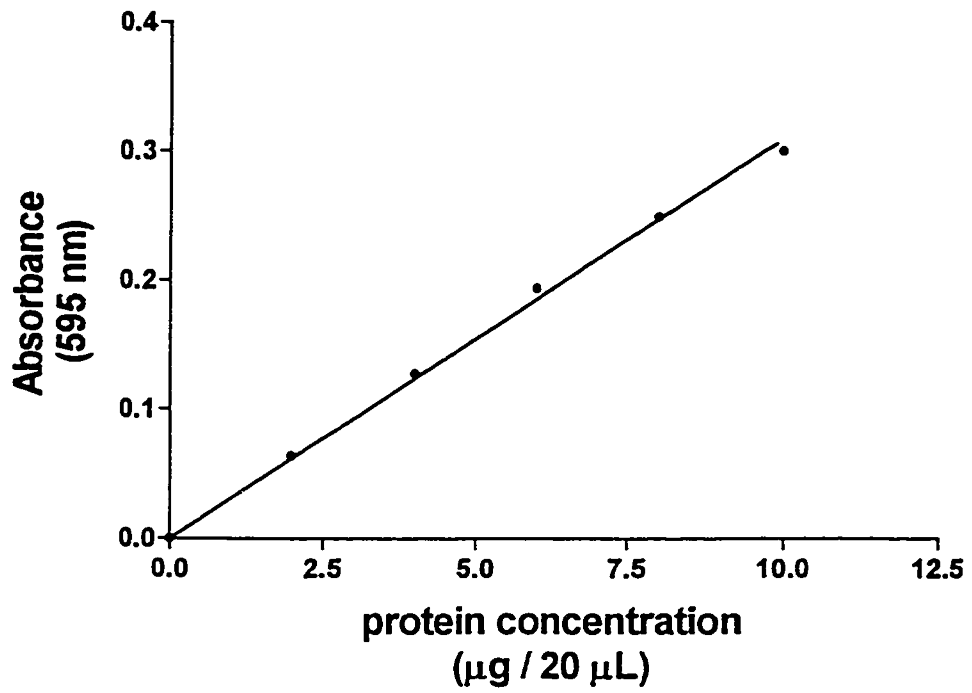
A modification of the Bradford assay (65) protein microassay was used to

determine the amount of bacterial or immunoglobulin protein present in solutions. Bovine serum albumin (BSA) (Sigma) (concentrations from 0 μg to 10 μg) was used to determine the standard curve for protein concentration. The protein being analysed (bacteria or immunoglobulin) was diluted 1/10 and 1/100 in microcentrifuge tubes with PBS. Then, 1580 μL of PBS was added to each cuvette, followed by 20 μL of the appropriate diluant (neat, 1/10, 1/100). PBS (1.6 mL) was added to the last cuvette to be used as the blank. Dye reagent concentrate (Bio-Rad laboratories, Hercules Ca, USA) (400 μL) was then added to each cuvette. The cuvettes were covered in parafilm and mixed by inversion. The solutions were left for 15 minutes at room temperature. The optical density of each solution was read on the UV-1201 UV-VIS spectrophotometer (Mandel Scientific Company Ltd, Japan) at a wavelength of 595 nm and results recorded.

2. Protein Concentration

A standard curve of absorbance (595 nm) versus protein concentration (μg/20μL) was graphed using BSA standard solutions (see Figure 2). The amount of protein present was determined by plotting the specific absorbance of the protein dilution that could be accurately read off the standard graph. To determine the concentration of protein in the original solution, the amount of protein recorded (μg/20 μL) was multiplied by 50 to convert the units to μg/mL. It was then multiplied by the corresponding dilution used (i.e. 100 for 1/100 dilution). The protein samples were diluted to equivalent concentrations (0.1 mg/mL bacteria, 5 mg/mL immunoglobulin) and stored in 5 mL cryogenic vials (Nalge Company, Rochester NY, USA) at –85°C until required for further assays.

Figure 2. Standard curve for Bradford protein microassay using bovine serum albumin standard solutions and reading absorbance at 595 nm. $r^2 = 0.999185$.



0.4
0.3
0.2
0.1
0.0
Absorbance
(595 nm)
0.0
2.5
5.0
7.5
10.0
12.5
protein concentration
(μg / 20 μL)

F. PMN Cell Movement

1. PMN and Bacterial Preparation

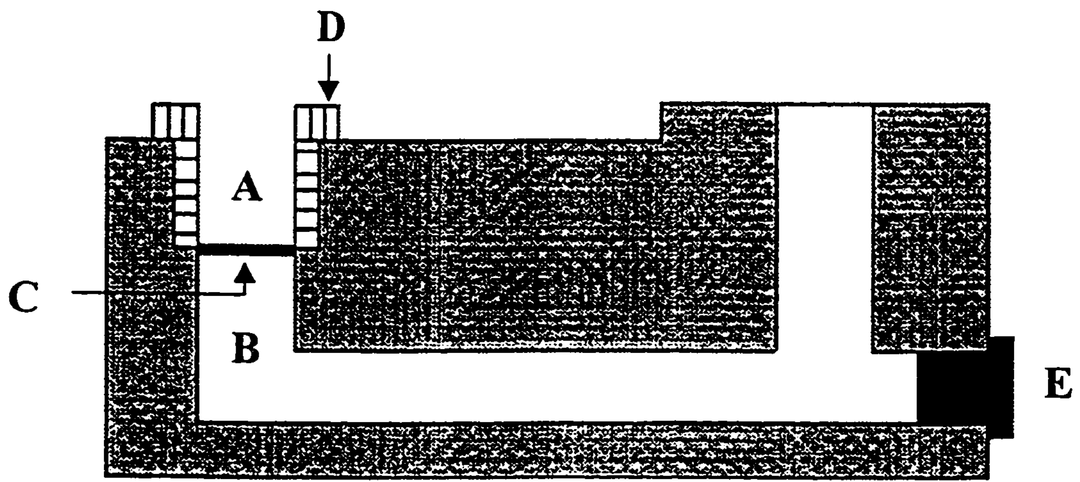
PMN were purified to a working concentration of 1.0 X 10^6 cells/mL as described in section A. PMN viability and purity were both greater than 95%. Bacteria were grown, isolated, and re-suspended at a working concentration of 1.0 X 10^7 CFU/mL as described above (section B). A 10:1 ratio of bacteria : PMN was used because of previous laboratory protocols performed in our research group and protocols from published papers (42). Bacteria were assessed for purity and CFU performed to verify bacterial numbers. All cell movement studies were performed in Boyden chambers (see Figure 3). Three micrometer millipore membrane filters (Millipore, Bedford MA, USA) were placed between the upper and lower chambers of the Boyden chamber apparatus and the lid was screwed firmly in place. The bung was fitted tightly into the side of the apparatus. The Boyden chambers, PBS, casein, chemokinesis rest solutions, chemokinesis stimulated solutions and bacteria were incubated at 37°C in 5% CO_2 for 30 minutes in a US Autoflow CO_2 Water -Jacketed Incubator (Nuaire, USA).

2. Stimulants

2.1 Chemotaxis

Approximately 2 mL of each solution was added to fill the lower chambers of each apparatus as follows: 1) PBS (negative control), 2) Casein (Sigma) (2.5 mg/mL) (positive control), 3) *F. necrophorum*, 4) *P. levii*, 5) *P. intermedia*, 6) All three bacteria (0.33 X 10^6 of each bacteria). After filling the lower chamber, 100 µL of PMN was placed on top

Figure 3. Boyden chamber apparatus used for PMN cell movement. **A** = upper chamber, **B** = lower chamber, **C** = membrane filter (through which PMN migrate), **D** = lid, and **E** = bung.


D
A
C
B
E

of the membrane filter in the upper chamber and PBS was added to fill the remainder of the chamber.

2.2 Chemokinesis

Each solution (5% (v/v) stimulant and 2.5% (v/v) calf serum in PBS) was added to both the lower and upper chambers. The stimulants were as follows: 1) PBS (negative control), 2) *Escherchia coli* lipopolysaccharide (Sigma) (2.5 mg/mL) (positive control), 3) *F. necrophorum*, 4) *P.levii*, 5) *P. intermedia*, 6) All three bacteria (0.33 X 10^6 of each organism). Prior to addition of solutions to the upper chamber in each apparatus, a 100 μL volume of PMN was added.

The Boyden chambers were then placed into a tupperware dish and incubated at 37°C in 5% CO_2 for 1 hour. Following this incubation, the solutions in the upper and lower chambers were discarded and the lid was removed. The membrane filters containing the migrated PMN were then gently removed from the upper chamber using a pair of curved forceps. The membranes were placed in a staining basket. The Boyden chambers were soaked in Nutri-clean (Nutri-Metics International, Calgary AB, Canada) and later rinsed repeatedly with distilled water, air dried and stored until the next experiment.

3. Staining

The membranes were stained using reagents in the following order: 1. Absolute ethanol (5 minutes), distilled H_2O (2 minutes), Hematoxylin (5 minutes) (Fisher Chemical, Fair Lawn NJ, USA), distilled H_2O (2 x 5 minutes), 70% (v/v) ethanol (2 minutes), 95% (v/v) ethanol (3 minutes), and 20% (v/v) butanol in ethanol (5 minutes). The staining

basket was blotted on paper towel between each step. Each membrane filter was placed into a scintillation vial ¼ filled with Xylene (Sigma). The vials were labeled and stored overnight at room temperature. The next day, the membrane filters were placed onto a microscope slide with Type A immersion oil (R. P. Cargille Laboratories Inc., Cedar Grove NJ, USA) beneath and on top of the membrane and a coverslip was placed on top of the membrane.

4. Migration Distance

Membrane filters were observed under a light microscope at 400 X magnification. The top layer of PMN was marked "zero" on the fine focus knob. The microscope was then focused down through the migrated PMN until the last 3 PMN in the same field of view could be seen. This number was recorded from the fine focus knob. The difference between the last number and the original number (zero) was equal to the distance that the PMN migrated in micrometers. Three different readings from random fields in the center of each filter were obtained. Values were expressed as µm/hr.

G. Phagocytosis

1. PMN and Bacterial Preparation

PMN were purified to a working concentration of 1.0 X 10^6 cells/mL as described above in section A. PMN viability and purity were both greater than 95%. Bacteria were purified to a working concentration of 1.0 X 10^7 CFU/mL as described above in section B. Bacteria were tested for purity and CFU performed to verify bacterial numbers. A 10:1

ratio of bacteria : PMN was used once again because of previous laboratory protocols performed in our research group.

2. Assay Protocol

Three hundred microliters of bacterial suspension (1.0×10^7 CFU/mL) or 1.03 μm polystyrene latex beads (1.0×10^7) (Sigma) were placed into microcentrifuge tubes. Then, 300 μL of either PBS, or corresponding high titre specific antibody bovine serum, heat inactivated (56°C for 30 minutes) high titre specific antibody bovine serum, or low titre specific antibody bovine serum were added to the assay tubes. The solutions were allowed to incubate on a shaker (gyratory shaker-model G2) at 100 rpm for 30 minutes at 37°C. The suspensions were then centrifuged at 3000 x g for 10 minutes. The supernatant was decanted and the bacterial pellet resuspended in 300 μL of sterile pyrogen-free PBS.

A 100 μL volume of PMN (1.0×10^6 cells/mL) was added to each microcentrifuge tube, followed by the addition of 100 μL of resuspended bacterial suspension. The tubes were incubated at 37°C for 15 minutes on a shaker as described above. After incubation the PMN/bacterial suspensions were centrifuged at 800 x g for 10 minutes to pellet out the PMN containing phagocytosed bacteria and leaving free bacteria in the supernatant. The supernatant was discarded and the pellet washed and resuspended in 150 μL of PBS. The PMN were then centrifuged onto microscope slides at 1500 x g for 10 minutes. The slides were allowed to dry and then stained using Diff Quik (Baxter Healthcare Corporation) staining kit as described above (see section A.4). PMN

containing phagocytosed latex beads were left in solution 2 for 4-5 seconds to allow for better contrast in the cytoplasm when observing them under the light microscope. The slides were then air dried and mounted using Cytoseal.

3. Phagocytic Index

The PMN were assessed under light microscopy at 1000 X magnification. On each slide 100 cells (x 3 different fields) were counted and classified as either non-phagocytic (phagocytosing 0 bacteria), or phagocytic (phagocytosing 1 or more bacteria). Three slides were used to evaluate each group and the mean calculated.

H. Oxidative Metabolism

1. Reagents

Specific phosphate buffered saline (PBS+) was used throughout these experiments. PBS+ contained 70 mL of distilled water, 135 mg of glucose and 5 mL of each of the following salts dissolved in pyrogen-free sterile H_2O: NaCl (2.74 M), KCl (54 mM), Na_2PO_4 (162 mM), KH_2PO_4 (29.4 mM), $CaCl_2$ (23.8 mM), and $MgCl_2$ (10 mM) (pH 7.2). Nitroblue Tetrazolium (NBT) stock solutions were made from NBT powder (Sigma) and dissolved in PBS+ to a concentration of 2 mg/mL. The NBT rest solution contained 1 mL NBT stock to 1 µL dimethyl sulfoxide (DMSO) (Sigma) and the NBT stimulated solution contained 1 mL NBT stock to 1 µL phorbol 12-myristate 13-acetate (PMA) (Sigma). To make the working concentration of PMA, 1 mg of PMA was dissolved in 500 µL of DMSO. PMA stock samples were aliquoted (10 µL) and stored at –20°C in the dark until

required for assays. NBT stock solutions were made up freshly on the day of the experiment.

2. PMN and Bacterial Preparation

PMN were purified to a working concentration of 1.0 X 10^6 cells/mL (see section A). PMN viability and purity were both greater than 95%. Bacteria were purified to a working concentration of 1.0 X 10^7 CFU/mL (see section B). Bacteria were assessed for purity and viable cell counts performed to verify bacterial numbers. A 10:1 ratio of bacteria : PMN was used again because of previous laboratory protocols performed in our research group.

3. Nitroblue Tetrazolium (NBT) assay

A 100 μL volume of PMN was added to a 100 μL volume of bacterial suspension and incubated on a shaker (see section G.2) at 37°C for 15 minutes. Also incubated at the same time were glass microscope slides (cleaned prior with 75% (v/v) ethanol), PBS+, and all NBT working solutions. PMN/bacterial suspensions were centrifuged at 800 x g for 10 minutes and the PMN pellet resuspended in 100 μL of sterile pyrogen-free PBS. The PMN (100 μL) were placed on a microscope slide. The slides were incubated for 15 minutes at 37°C in a closed humidified chamber (tupperware dish containing moistened paper towel). The slides were then washed with 1 mL of pre-warmed PBS+. After washing, 1 mL of either stimulated or rest NBT solutions were pipetted onto the microscope slides and allowed to incubate for 20 minutes at 37°C in a closed humidified chamber. The slides were washed once again with 1 mL of PBS+ and left to air dry.

Slides were then fixed with absolute ethanol for 1 minute and allowed to air dry. Using 1% (w/v) safranin (Sigma), the slides were stained for 2 minutes and then rinsed gently with distilled water. Once the slides were dry, they were permanently mounted as described above.

4. Oxidative Activity

The PMN were examined under a light microscope at 1000 X magnification. To determine whether or not the PMN were oxidatively activated they were classified as either positive (containing blue formazan deposits) or negative (without blue formazan deposits). In each of 3 separate fields 100 PMN were evaluated for oxidative metabolism. Three slides were used for each experimental group and the means calculated.

I. Immunoglobulin (IgG) Purification.

1. Sample Preparation

High titre bovine sera from cattle immunized, as described in section C, were used as the sources for IgG. Affinity chromatography (67) was used to purify the immunoglobulins (Immunopure (G) IgG purification kit, Pierce). After the solution buffers were warmed to room temperature the serum was diluted 1:1 with Immunopure (G) binding buffer (Pierce). The diluted serum was then centrifuged at 10,000 x g for 20 minutes and the supernatant transferred to another tube to be used in the column.

2. Protocol

The column was first allowed to equilibrate to room temperature. The

Immunopure (G) Immobilized Protein G column (6% beaded agarose) was then opened by first removing the top cap (to avoid any bubbles entering the column) and the storage solution poured off. The protein G column was then equilibrated with 5 mL of Immunopure (G) binding buffer (pH 5.0). Up to 4 mL of diluted serum was then applied to the column and the sample was allowed to flow completely through the gel. The column was then washed with 10 mL of Immunopure (G) binding buffer. To elute the bound IgG, (6) 1 mL aliquots of Immunopure (G) elution buffer (pH 2.8) was added to the column and the eluate was collected in 1 mL microcentrifuge tubes. The pH of the eluted IgG was quickly raised to neutral (pH 7.0) by the dropwise addition of 1 M Tris buffer (pH 7.5), and checked using pH paper 6-8 (Micro Essential Laboratory Brooklyn NY, USA). The elution of the bound IgG was monitored on the spectrophotometer at an absorbance wavelength of 280 nm. The protein G column was regenerated by washing with 5 mL of elution buffer and then equilibrated again with 5 mL of binding buffer. The column was stored in 0.02% sodium azide in water and the top cap replaced.

3. Immunoglobulin (IgG) Concentration and Quantification

The (6) 1 mL aliquots of eluted IgG were then microconcentrated using Centricon-10 microconcentrators (Millipore). Two milliliters of eluted immunoglobulin was added to the sample reservoir after each centrifugation. The apparatus was then placed into a 40 mL centrifuge tube and centrifuged at 4000 x g for 25 minutes. After all the eluted IgG was centrifuged the renate cup was placed over the filtrate cup and then inverted. The apparatus was spun at 700 x g for 3 minutes to remove any protein from the filter. The

concentrated IgG was then transferred to a cryogenic vial and protein concentration determined using the modified Bradford protein microassay (see section E), and the purified IgG placed in –20°C until required.

J. SDS-PAGE

1. Protein Gel

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was conducted to verify that the Immunoglobulin (IgG) protein eluted from the affinity column was pure. Immunoglobulin samples were diluted 1:1 in SDS reducing buffer (12.5% (v/v) 0.5 M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-β mercatoethanol, 0.125% (w/v) bromophenol blue, in distilled water). Samples were then placed in boiling water for 5 minutes to disrupt the disulfide bonds between the heavy and light chains. Gels were cast with a 15% separating gel (25% 1.5 M Tris (pH 8.8), 0.1% SDS, 15% (w/v) Acrylamide / N,N-Bis-methylene-acrylamide, 0.05% (w/v) ammonium persulfate, 0.5% (v/v) TEMED, in distilled water) and a 4% stacking gel (25% 0.5 M Tris (pH 6.8), 0.1% SDS, 3.9% (w/v) Acrylamide / N,N-Bis-methylene-acrylamide, 0.05% ammonium persulfate, 0.1% TEMED, in distilled water). Samples were loaded into the appropriate wells using a Hamilton syringe (Hamilton Company, Reno NV, USA). High molecular weight Rainbow markers (Life Sciences, Oakville Ont, Canada) were used for estimation of protein size and commercially acquired bovine IgG (Sigma) was used as a control. Separations were conducted in running buffer (0.3% (w/v) Tris base, 1.44% (v/v)

Glycine, 0.1% SDS, in distilled water), for 15 minutes at 150 V and then for 45 minutes at 200 V, using a Power Pac 3000 (BioRad).

2. Coomassie Brilliant Blue Stain

Gels were stained for 30 minutes with 0.1% (w/v) Coomassie Brilliant Blue R-250 in fixative (40% (v/v) methanol and 10% (v/v) acetic acid, 50% dH_2O). After fixing, the gels were destained overnight with 40% methanol, 10% acetic acid and 50% dH_2O. Photographs were taken on the UVP White/UV Transilluminator (UVP, Upland CA, USA). A silver stain (ng protein detection) was experimentally performed on a duplicate gel and similar results were observed to the Coomassie Brillant Blue stain (μg protein detection).

K. Maneval Stain

Porphyromonas levii was grown to logarithmic phase under anaerobic conditions in BHI broth supplemented with 0.05% hemin and 0.01% vitamin K. Bacteria were smeared with 1% (w/v) Congo Red (Sigma) on a microscope slide and allowed to dry. The slide was then counter-stained for 1 minute with 0.05% (w/v) acid fuchin (Sigma) in a 5% (w/v) phenol (Sigma), 20% (w/v) acetic acid and 30% (w/v) ferric chloride solution (Sigma) (68). The slides were then rinsed gently in distilled water. Bacteria were observed under a light microscope at 1000 X magnification. Capsules were verified and photographs taken.

L. Transmission Electron Microscopy

1. Sample Preparation

1.1. *P. levii*

Bacteria were prepared as described above to a final concentration of 1.0 x 10^7 CFU/mL. One milliliter of *P. levii* was incubated in a microcentrifuge tube with 150 µL of anti-*P. levii* bovine serum (titre 12,800) for 1 hour at room temperature (69). The sample was then centrifuged at 3000 x g for 10 minutes. The supernatant was decanted and the bacterial pellet fixed for 2 hours at room temperature with 5% (v/v) glutaraldehyde (Electron Microscopy Sciences, Fort Washington Penn, USA) in 0.1 M cacodylate buffer (Sigma) containing 0.15% (w/v) ruthenium red (Sigma) (pH 7.2). The sample was centrifuged as described above. The sample was then washed 3 times for 10 minutes in 0.1% cacodylate buffer containing 0.05% (w/v) ruthenium red (pH 7.2). After washing, the bacterial pellet was enrobed in 4% (w/v) bacto-agar (Difco). For enrobing, the pellet was mixed with the agar under 60°C oven and drawn up into a glass pipet. Just before the agar solidified the agar encasing the bacteria was squeezed out onto a glass plate in cylindrical cores. The cores were trimmed and placed into scintillation vials. The specimens were then washed 2 times in 0.05% ruthenium red, 0.1 M cacodylate buffer. The agar encased bacteria were stained for 2 hours with 2% (v/v) osmium tetroxide (Polysciences Inc., Warrington PA, USA) diluted with cacodylate buffer. The specimens were then washed 5 times with 0.05% ruthenium red in cacodylate buffer. The specimens were dehydrated in acetone-dH_2O solutions using a series of ascending acetone

concentration steps (30%, 50%, 70%, 90%, 100%, 100% (v/v) solutions for 15 minutes each). The samples were then washed 2 times in propylene oxide for 10 minutes. The agar cores were infiltrated with a 3:1 mixture of propylene oxide and Spurr resin (Electron Microscopy Sciences) for 24 hours followed by infiltration in fresh 100% Spurr resin and left for another 24 hours. Samples were then placed in BEEM capsules (J.B. EM Services Inc., Dorval Que, Canada) containing fresh Spurr resin. The capsules were placed in a 60°C oven and allowed to polymerize for 16-18 hours with the caps open to air. Blocks were removed from the BEEM capsules and trimmed for sectioning (69).

1.2. PMN and *P. levii*

PMN were purified as described above to a final concentration of 1.0 X 10^6 cells/mL. Bacteria were prepared as described above to a final concentration of 1.0 X 10^7 CFU/mL. In a microcentrifuge tube, 300 µL of bacteria was added to 300 µL of anti-*P. levii* bovine serum (titre 12,800) and allowed to incubate on a shaker (see section G. 2) at 37°C for 30 minutes. The suspension was centrifuged at 3000 x g for 10 minutes. The bacterial pellet was resuspended in 300 µL of sterile PBS. Into a different microcentrifuge tube 200 µL of PMN was added to 200 µL of resuspended bacteria and placed on a shaker, as described above, for 30 minutes at 37°C (see section G.2). The suspension was centrifuged at 800 x g for 10 minutes, supernatant decanted and the PMN containing phagocytosed bacteria resuspended in 200 µL of sterile PBS. The sample was transferred to a BEEM capsule and centrifuged at 800 x g for 10 minutes. The PMN pellet on the bottom of the BEEM capsule was fixed with 5% glutaraldehyde in 0.1 M

cacodylate buffer (pH 7.2) for 2 hours. The capsule was centrifuged as described above and the supernatant decanted. The sample was washed (3x) for 15 minutes in cacodylate buffer. The sample was centrifuged after each wash as described above. The sample was then stained with 1% osmium tetroxide diluted in cacodylate buffer for 2 hours. The sample was once again washed and centrifuged (X 3) in cacodylate buffer. The pelleted PMN were dehydrated in a series of increasing acetone concentration steps (30%, 50%, 70%, 90%, 100%, 100%). The BEEM capsules were centrifuged after each step. The sample was washed with a 1:1 solution of acetone and propylene oxide for 15 minutes and centrifuged. The sample was then washed and centrifuged twice with 100% propylene oxide. The PMN pellet was then infiltrated with a 1:1 solution of propylene oxide and Spurr resin for 20 - 30 minutes with the caps open. The capsule was filled with fresh resin and left for 40 minutes. Fresh resin was once again added and the capsule was placed in a 60°C oven overnight (approximately 16 hours) for polymerization to occur (caps open). The blocks were removed from the BEEM capsules and trimmed for sectioning.

2. Sectioning

Ultrathin sections (50-90 nm) of specimens were obtained using a LKB Ultrotome III and collected on copper 400 mesh grids resin (Electron Microscopy Sciences). The specimens were then stained with uranyl acetate (Sigma) (10 mg/mL) and lead nitrate (Sigma) (4 mg/mL). The specimens were viewed with a Hitachi (H-7000) transmission electron microscope (accelerating voltage 50 kV).

M. Data Handling and Statistical Analysis

Frequency distribution was used to analyse all numerical data. Values were plotted for determination of gaussian distribution (parametric versus non-parametric). All percent (%) values were transformed to arcsine prior to statistical analysis (70). Parametric data (oxidative metabolism - NBT) was analysed using Bartlett's test for homogeneity of variances and Tukey-Kramer test for multiple comparisons ($P < 0.05$) (70). Non-parametric data (all other graphs) were analysed using Kruskal-Wallis multiway factorial analysis of variance and Dunn's test for multiple comparisons when three or more means were used to determine statistical significance ($P < 0.05$)(70).

Results

A. Bacterial Comparisons Using PMN Functions

1. PMN Movement

The ability of an invading bacterium to promote an immune response, specifically the chemoattraction of PMN, is important in determining the effectiveness of the host's phagocytes to move towards the site of invasion. An evaluation of PMN movement to the three etiologic agents (*Porphyromonas levii*, *Fusobacterium necrophorum*, and *Prevotella intermedia*) associated with acute interdigital phlegmon (AIP) may give a better understanding of the phagocytic response towards this infection. A comparison of the effect of these bacteria on two types of PMN movement (chemotaxis and chemokinesis) was conducted using Boyden chambers.

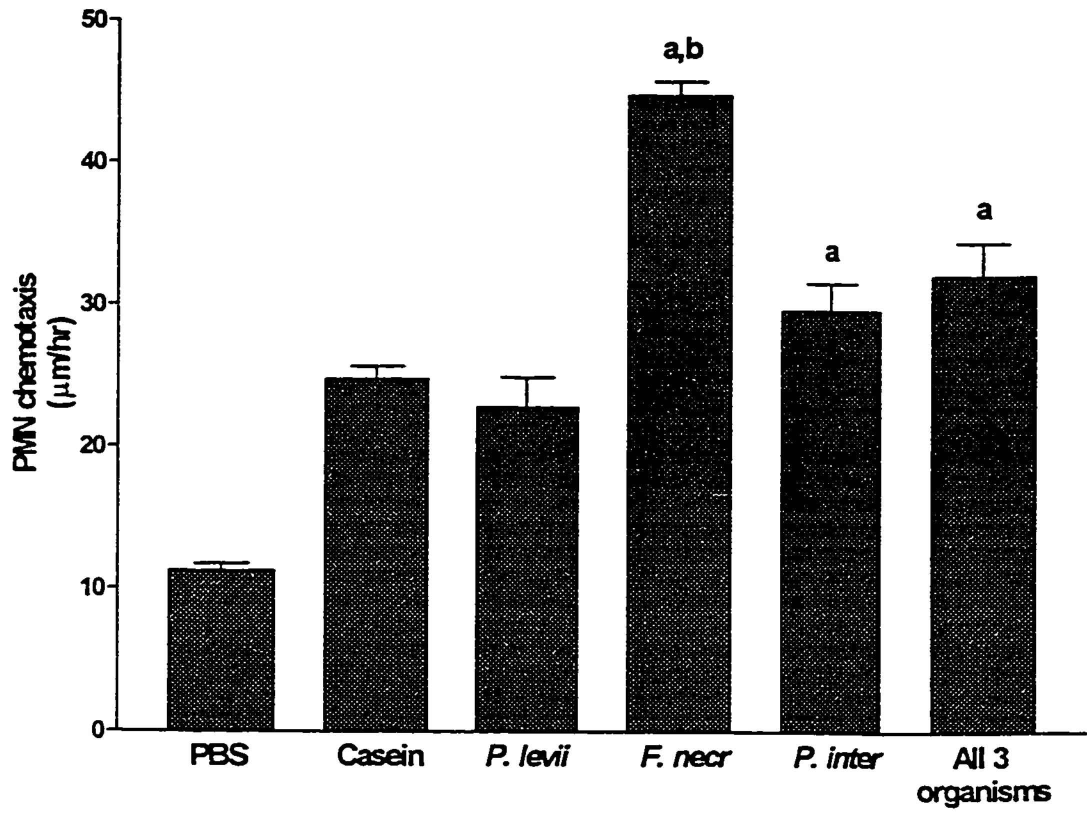
1.1 Chemotaxis

Chemotaxis is defined as directed migration of PMN towards a chemoattractant. Random migration of PMN was observed at 11 µm/hr (PBS) and the positive control for chemotaxis, casein (2.5 mg/mL in PBS), showed an increased movement of PMN of about 14 µm/hr above, but not significantly greater ($P > 0.05$) than, random migration (Figure 4). *F. necrophorum*, *P. intermedia*, and all 3 organisms showed a significant difference ($P < 0.05$) from random migration (PBS). Also illustrated in Figure 4, is the high chemoattractant effect of *F. necrophorum* on PMN, demonstrating a significant difference ($P < 0.05$) from the *P. levii* and casein treatment groups.

Figure 4. Comparison of the chemotactic movement of bovine PMN towards *P. levii, F. necrophorum, P. intermedia*, and all 3 bacteria together (PBS = negative control, casein (2.5 mg/mL) = positive control). Values are expressed as mean distance PMN migrated over time ± SEM (n value is 9 per treatment group).

a = significantly different from PBS ($P < 0.05$).

b = significantly different from casein and *P .levii* ($P < 0.05$).



50
40
30
20
10
0
PMN chemotaxis
(μm/hr)
a,b
a
a
PBS
Casein
P. levii
F. necr
P. inter
All 3
organisms

1.2 Chemokinesis

Chemokinesis (the speed of migration of PMN when exposed to a chemoattractant and serum) was also examined experimentally. PBS served as the control for random migration of PMN and a different stimulant, *Escherchia coli* LPS (125 μg/mL final concentration) was used as a positive control (Figure 5). The LPS and *P. levii* produced the only significant ($P < 0.05$) differences in PMN movement compared to random migration (PBS). The chemokinetic effect of LPS and *P. levii* on PMN was also significantly ($P < 0.05$) higher from that induced by either *F. necrophorum* or *P. intermedia* (Figure 5). All 3 bacteria together also induced a high movement of PMN, but this was only significantly different from *P. intermedia* ($P < 0.05$). It is interesting to note that *P. levii* was the highest chemoattractant of PMN in the chemokinesis experiment as seen in Figure 5, but the lowest chemoattractant in the chemotaxis experiment (Figure 4).

2. PMN Phagocytosis

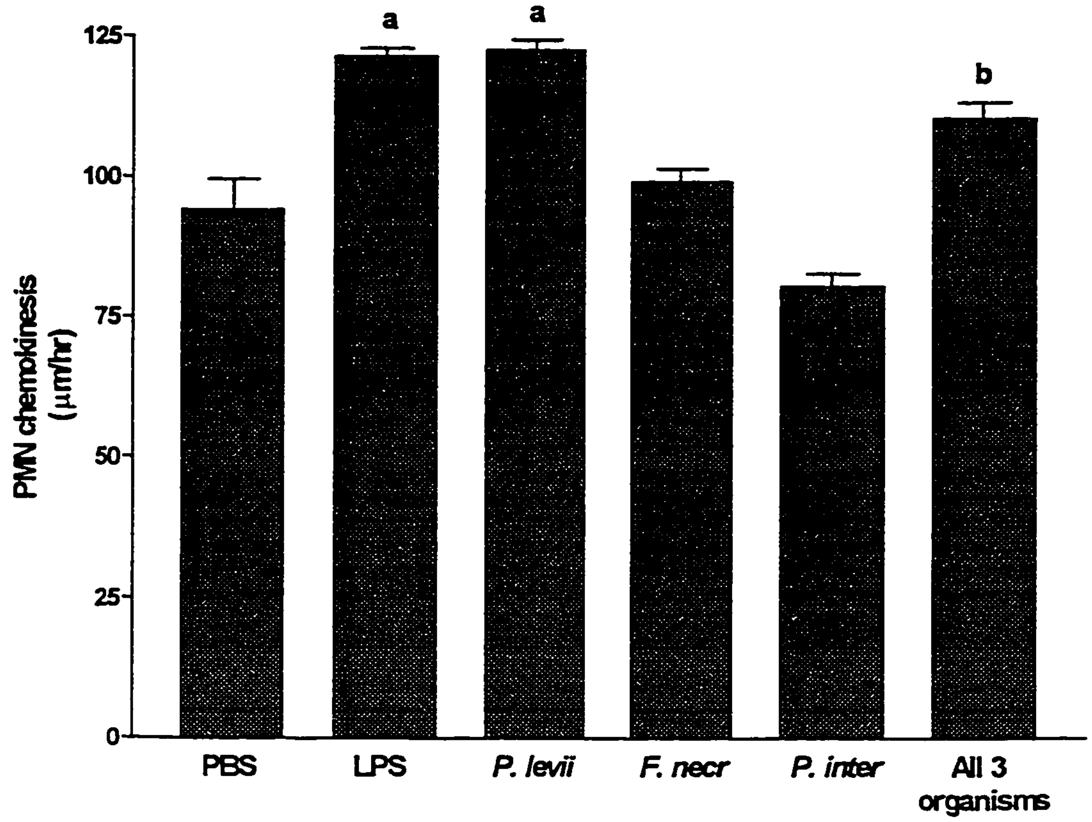
PMN phagocytosis is an important function for the removal of bacteria from the site of infection. Evaluation of the phagocytic abilities of PMN to engulf the bacteria associated with AIP is as important as PMN movement toward these microorganisms.

Phagocytosis of the three anaerobes by bovine PMN was conducted *in vitro*. As seen in Figure 6, 1.03 μm latex beads were used as a control in the experiment giving a baseline PMN phagocytosis of 18 ± 1.25%. *F. necrophorum* was the only treatment group to demonstrate a significant difference from latex beads ($P < 0.05$). The most exciting information demonstrated in these results is the significant difference in

Figure 5. Comparison of the chemokinetic movement of bovine PMN towards *P. levii*, *F. necrophorum*, *P. intermedia*, and all 3 bacteria together (PBS = negative control, LPS from *E. coli* (125 μg/mL) = positive control). Values are expressed as mean distance PMN migrated over time ± SEM (n value is 9 per treatment group).

a = significantly different from PBS, *F. necrophorum* and *P. intermedia* ($P < 0.05$).

b = significantly different from *P. intermedia* ($P < 0.05$).

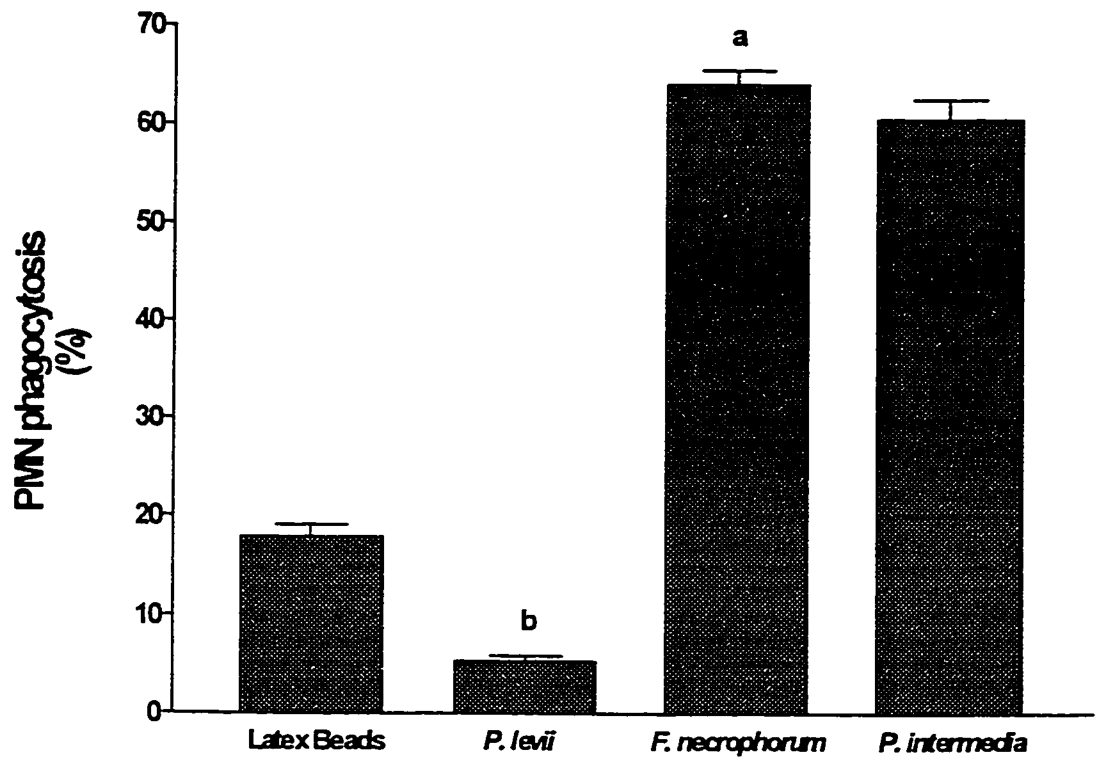


PMN chemokinesis (μm/hr)
125
100
75
50
25
0
a
a
b
PBS
LPS
P. levii
F. necr
P. inter
All 3 organisms

Figure 6. Comparison of phagocytosis by bovine PMN of latex beads (control), *P. levii, F. necrophorum*, and *P. intermedia*. Values are expressed as mean cells phagocytosing bacteria per 100 PMN ± SEM (n value is 9 per treatment group).

a = significantly different from latex beads ($P < 0.05$).

b = significantly different from *F. necrophorum* and *P. intermedia* ($P < 0.05$).



PMN phagocytosis (%)
70
60
50
40
30
20
10
0
a
b
Latex Beads
P. levii
F. necrophorum
P. intermedia

phagocytic uptake of *P. levii* by PMN compared to *F. necrophorum* and *P. intermedia* ($P < 0.05$).

3. Bactericidal Activity of PMN

In Figure 7, PBS served as the negative control giving a baseline activation (6.4 ± 0.95%) for NBT reduction by PMN. All treatment groups induced a significantly higher oxidative response by PMN compared to the negative control (PBS) ($P < 0.05$). The positive control (PMA) induced the highest oxidative activity in PMN which was significantly different from all other groups ($P < 0.05$). Although *P. levii* and *F. necrophorum* did not show a significant difference from each other ($P > 0.05$), they were both significantly higher than *P. intermedia* ($P < 0.05$).

B. Opsonophagocytosis with Immune Serum

When dealing with phagocytosis of bacteria by PMN one must take into consideration the role that opsonins play in helping the PMN remove the bacteria from the site of infection. The effects that the two major opsonins (IgG and C3b) have on PMN phagocytosis could prove detrimental to the efficiency of PMN phagocytosis and therefore must be looked at under experimental conditions. Fetal calf serum (heat inactivated) was experimentally proven to be equivalent to low titre serum in all the immune serum experiments.

Figure 7. Comparison of the oxidative metabolism (NBT reduction) of bovine PMN when exposed to *P. levii, F. necrophorum*, and *P. intermedia* (PBS = negative control, PMA (2.0 μg/mL) = positive control). Values are expressed as mean cells reducing NBT (containing blue formazan deposits) per 100 PMN ± SEM (n value is 18 per treatment group with the exception of *P. intermedia* where n=15).

a = significantly different from PBS ($P < 0.05$).

b = significantly different from PMA ($P < 0.05$).

c = significantly different from *P. levii* and *F. necrophorum* ($P < 0.05$).

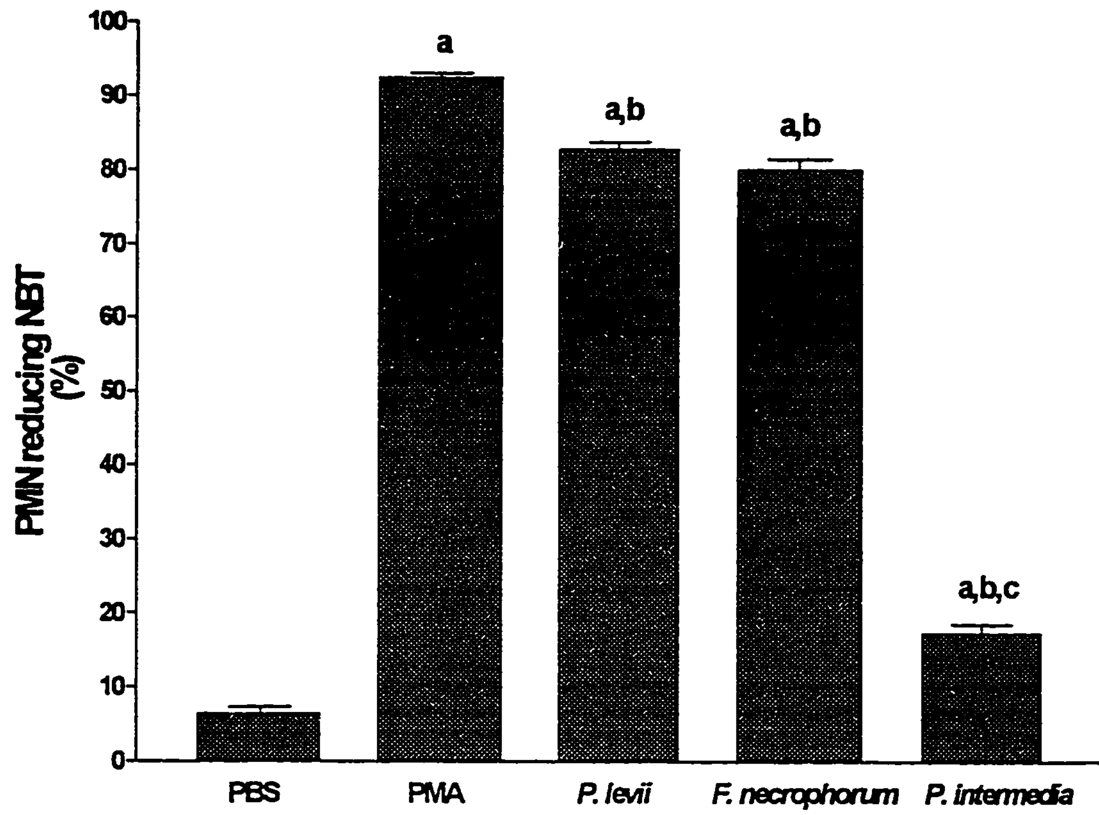
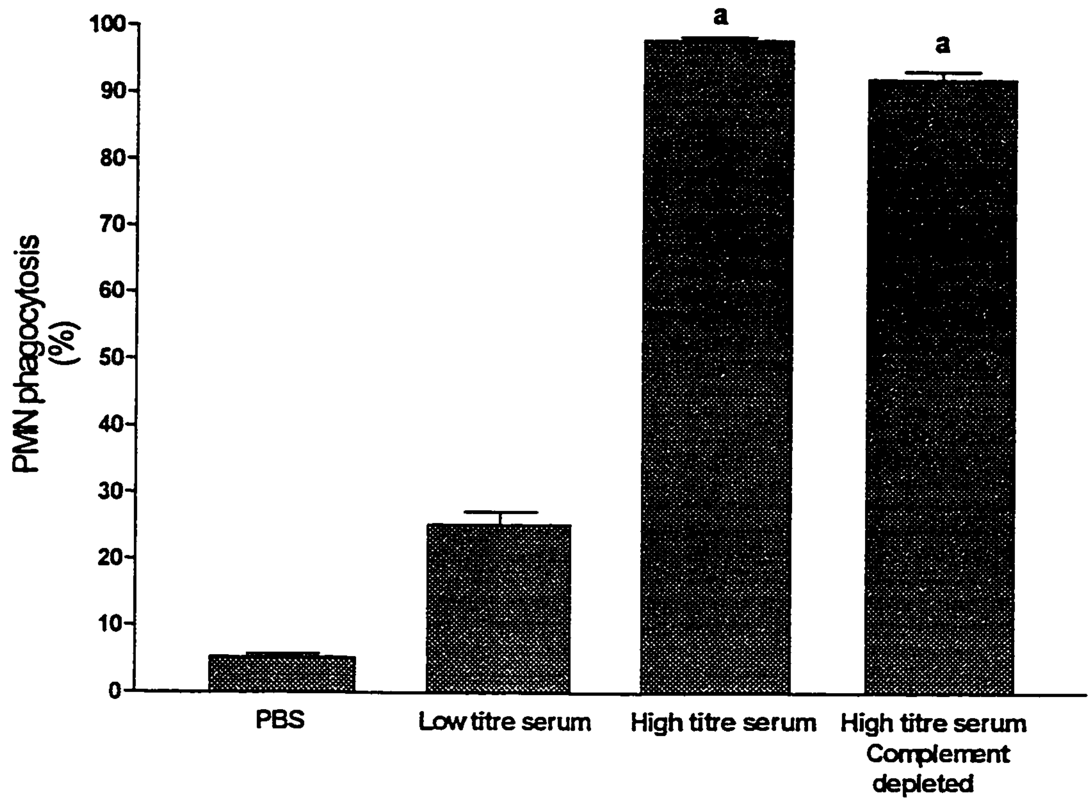

PMN reducing NBT (%)
100
90
80
70
60
50
40
30
20
10
0
a
a,b
a,b
a,b,c
PBS
PMA
P. levii
F. necrophorum
P. intermedia

Figure 8. Examination of phagocytosis of *Porphyromonas levii* by bovine PMN when the bacteria were pre-incubated with PBS (control), low titre (< 40) anti-*P.levii* serum, high titre (1280) anti-*P.levii* serum, or complement-depleted (56°C for 30 minutes) high titre (1280) anti-*P.levii* serum. Values are expressed as mean cells phagocytosing bacteria per 100 PMN ± SEM (n value is 15 per treatment group).

a = significantly different from PBS and low titre serum ($P < 0.05$).


PMN phagocytosis
(%)
100
90
80
70
60
50
40
30
20
10
0
a
a
PBS
Low titre serum
High titre serum
High titre serum
Complement
depleted

1. *Porphyromonas levii*

As originally noticed in Figure 6, *P. levii* has a low phagocytic index (5.3% ± 0.53) when pre-incubated with PBS. Observed in the results (Figure 8) is the significant (P < 0.05) increase from PBS in PMN phagocytosis of *P. levii* once the bacteria are pre-incubated with high titre (1280) anti-*P.levii* bovine serum or complement depleted high titre (1280) anti-*P. levii* bovine serum (P < 0.05). Also shown is the significant difference of these two groups from the low titre (< 40) anti-*P. levii* bovine serum ($P < 0.05$).

2. *Fusobacterium necrophorum*

Fusobacterium necrophorum, pre-incubated with immune or non-immune serum, was also evaluated for PMN phagocytosis (Figure 9). *F. necrophorum* has a high phagocytic index when pre-incubated with PBS (64.1 ± 1.47). Pre-incubation of the bacteria with low titre (< 40) anti-*F. necrophorum* bovine serum or complement depleted high titre (1280) anti-*F. necrophorum* bovine serum caused a significant increase in phagocytosis by the PMN compared to PBS ($P < 0.05$). Complement depleted high titre anti-*F. necrophorum* serum also demonstrated a significant difference from high titre (1280) anti-*F. necrophorum* bovine serum ($P < 0.05$).

3. *Prevotella intermedia*

Finally, *P. intermedia* was evaluated *in vitro* for opsonic enhancement of PMN phagocytosis (Figure 10). *P. intermedia*, like *F. necrophorum*, demonstrated a high phagocytic index when pre-incubated with PBS (60.7 ± 2.01). There was no significant change in phagocytosis when the bacteria were pre-incubated with either low titre (< 40)

Figure 9. Examination of phagocytosis of *Fusobacterium necrophorum* by bovine PMN when the bacteria were pre-incubated with PBS (control), low titre (< 40) anti-*F. necrophorum* serum, high titre (1280) anti-*F. necrophorum* serum, or complement-depleted (56°C for 30 minutes) high titre (1280) anti-*F. necrophorum* serum. Values are expressed as mean cells phagocytosing bacteria per 100 PMN ± SEM (n value is 9 for PBS and low titre serum, 8 for high titre serum, and 6 for complement depleted high titre serum).

a = significantly different from PBS ($P < 0.05$).

b = significantly different from high titre serum ($P < 0.05$).

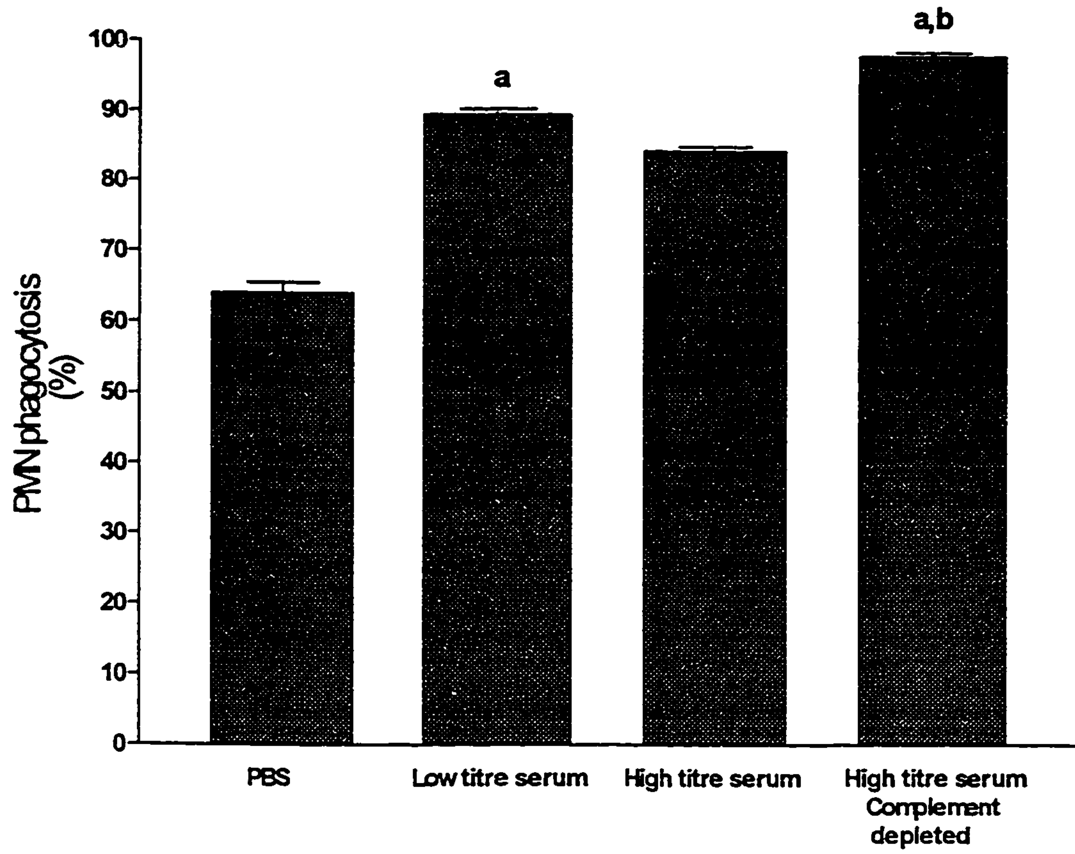
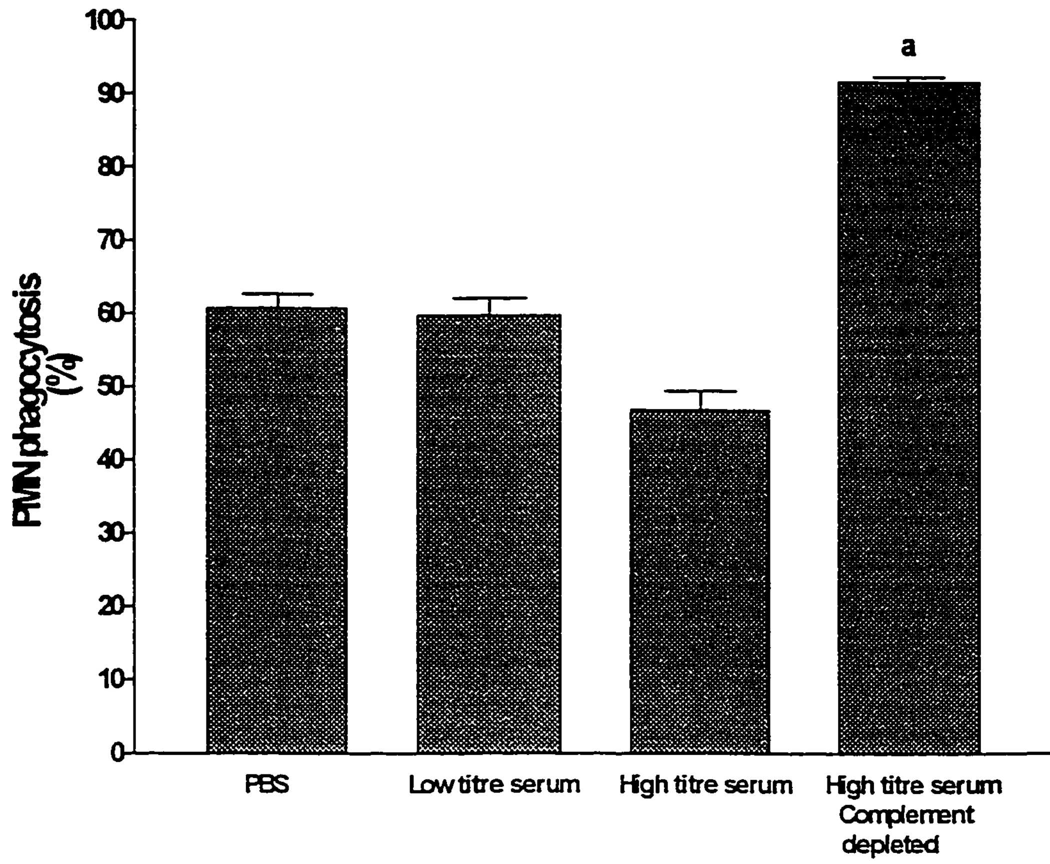

PMN phagocytosis (%)
100
90
80
70
60
50
40
30
20
10
0
a
a,b
PBS
Low titre serum
High titre serum
High titre serum Complement depleted

Figure 10. Examination of phagocytosis of *Prevotella intermedia* by bovine PMN when the bacteria were pre-incubated with PBS (control), low titre (< 40) anti-*P. intermedia* serum, high titre (1280) anti-*P. intermedia* serum, or complement-depleted (56°C for 30 minutes) high titre (1280) anti-*P. intermedia* serum. Values are expressed as mean cells phagocytosing bacteria per 100 PMN ± SEM (n value is 9 per treatment group).

a = significantly different from all other treatment groups ($P < 0.05$).



PMN phagocytosis (%)
100
90
80
70
60
50
40
30
20
10
0
a
PBS
Low titre serum
High titre serum
High titre serum Complement depleted

anti-P. intermedia bovine serum or high titre (1280) anti-*P. intermedia* bovine serum ($P > 0.05$). Interestingly, there was an increase in phagocytosis when *P. intermedia* was pre-incubated with complement depleted high titre (1280) anti-*P. intermedia* bovine serum. This increase was significantly higher than all other groups ($P < 0.05$).

C. Opsonophagocytosis with Purified IgG

1. *Porphyromonas levii*

In order to establish that IgG in bovine serum was the primary opsonin causing increased phagocytosis of *P. levii* by PMN, purified IgG was incorporated into the design of the phagocytosis experiments. IgG was purified from both low and high titre anti-*P. levii* bovine serum using a protein G affinity column and quantified with a Bradford (65) microassay system. In the results shown in Figure 11, latex beads were again used as a control of PMN phagocytosis. As demonstrated in both Figures 6 and 8, *P.levii* is not readily phagocytosed by bovine PMN when incubated with PBS (7.1% ± 0.87). Low titre (< 40) anti-*P. levii* purified IgG (10 mg) and high titre (1280) anti-*P. levii* purified IgG demonstrated a significant increase in phagocytosis from PBS ($P < 0.05$). High titre anti-*P. levii* purified IgG (10 mg) also showed a significant increase in phagocytosis from latex beads ($P < 0.05$).

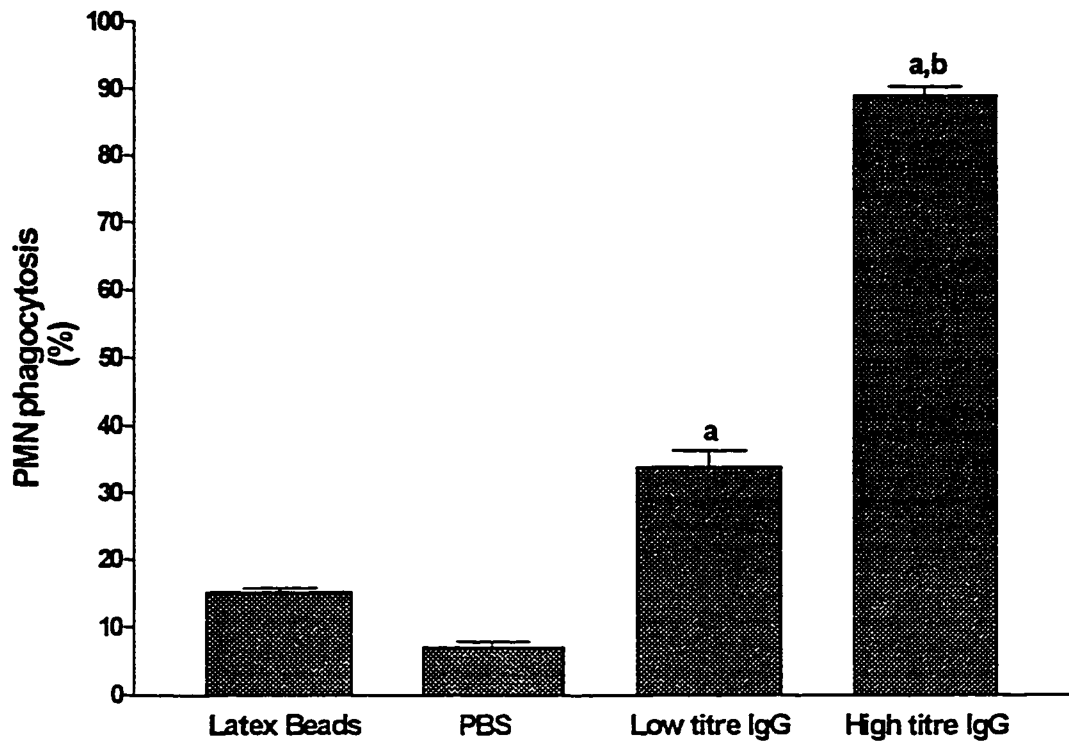
2. Immunoglobulin G Purity.

As seen in Figure 12, a high molecular weight rainbow marker was used for protein size estimation (Lane 1). As shown in lane 2, commercially purified bovine IgG

Figure 11. Examination of opsonophagocytosis of *Porphyromonas levii* and latex beads by bovine PMN when the bacteria or beads were pre-incubated separately with PBS or the bacteria were incubated with either low titre anti-*P.levii* purified IgG (10 mg) or high titre anti-*P.levii* purified IgG (10 mg). Values are expressed as mean cells phagocytosing bacteria per 100 PMN ± SEM (n value is 9 per treatment group).

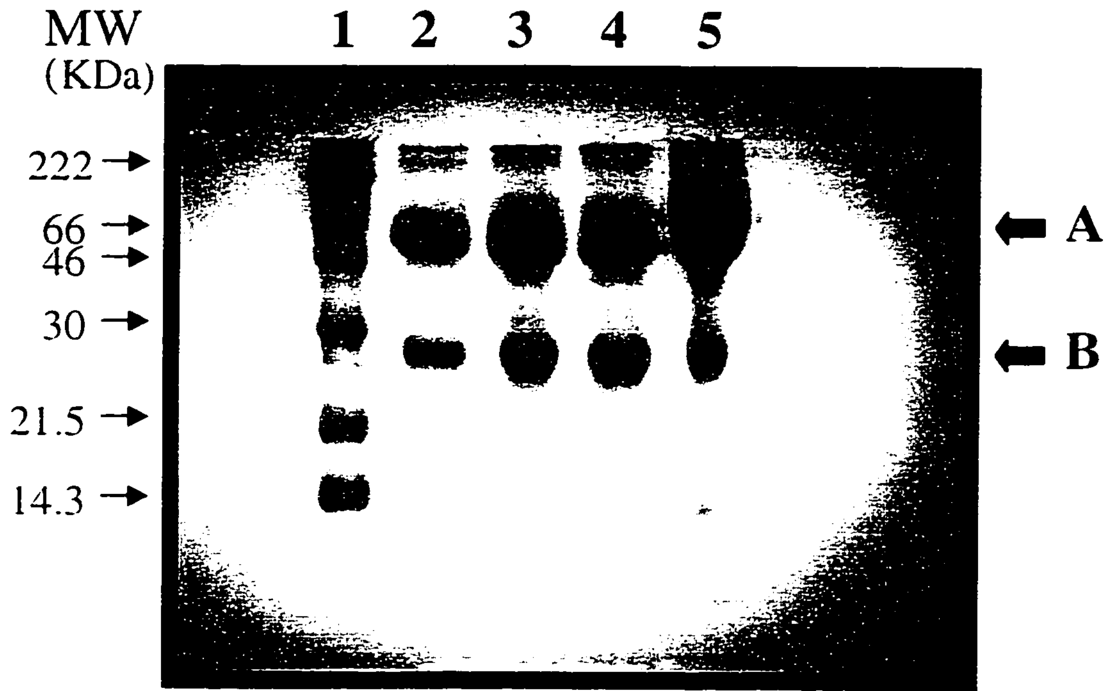
a = significantly different from PBS ($P < 0.05$).

b = significantly different from latex beads ($P < 0.05$).



100
90
80
70
60
50
40
30
20
10
0
PMN phagocytosis (%)
a,b
a
Latex Beads
PBS
Low titre IgG
High titre IgG

Figure 12. SDS - PAGE (15%) of purified IgG proteins. Coomassie Brilliant Blue was used to stain the proteins. **Lane 1** - molecular weight markers (7 μg). **Lane 2** - commercially purified bovine IgG (10 μg). **Lane 3** - high titre anti-*P. levii* bovine IgG purified from serum (10.5 μg). **Lane 4** - low titre anti-*P. levii* bovine IgG purified from serum (10.5 μg). **Lane 5** - bovine serum (1μL of 1/10 dilution). (A = heavy chains, B = light chains).


MW
(KDa)
1
2
3
4
5
222
66
46
30
21.5
14.3
A
B

was used as a control. Both the heavy (A) and light (B) chains were evident. The heavy chain was located between the 66 kDa marker (bovine serum albumin) and the 46 kDa marker (ovalbumin). The light chain was located just below the 30 kDa marker (carbonic anhydrase). Lane 3 contained the high titre anti-*P. levii* bovine IgG purified from serum and lane 4 contained the low titre anti-*P. levii* bovine IgG also purified from bovine serum. Both lanes demonstrated the heavy and light chains clearly and with little contaminating protein. The 5th lane contained bovine serum to act as a comparison to the purified IgG.

D. PMN Phagocytosis of *Porphyromonas levii*

1. Light Microscopy

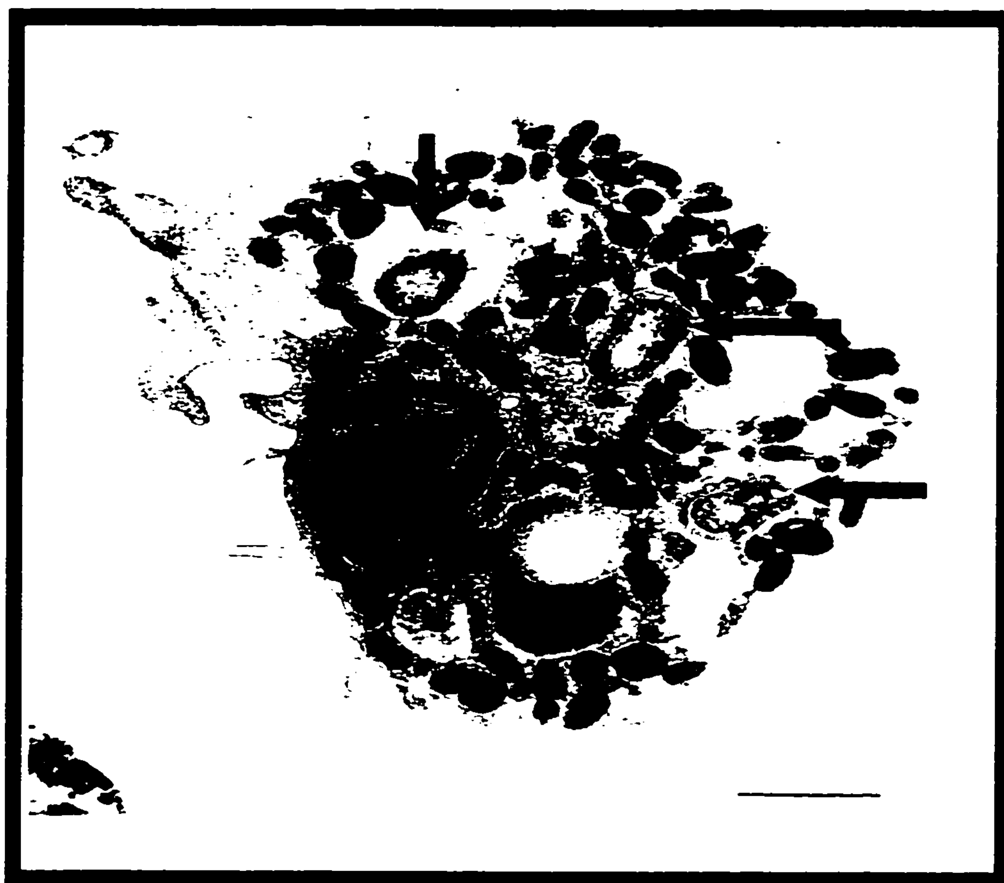
Figure 13 is a direct light microscopic view of bovine PMN phagocytosing *P. levii* (1000 X magnification). Due to the inability of PMN to phagocytose *P. levii* in PBS, the bacteria were pre-incubated with high titre anti-*P. levii* bovine serum for 30 minutes and then incubated with the bacteria for 30 minutes. The five PMN in the picture are highly active since each PMN is phagocytosing greater than 5 bacteria.

2. Transmission Electron Microscopy

Verification that the bacteria in Figure 13 are actually located inside the PMN and are not adhered to the surface membrane was conducted via transmission electron microscopy. Once again PMN were incubated with high titre anti-*P. levii* bovine serum prior to incubation with the PMN. Figure 14 demonstrates an activated PMN that has phagocytosed 3 bacteria (arrows).

Figure 13. Light microscopic view of bovine PMN phagocytosing *Porphyromonas levii* (arrows) (1000 X magnification). The bacteria were pre-exposed to high titre anti-*P. levii* bovine serum and then incubated with the PMN.

Figure 14. Transmission electron micrograph of bovine PMN phagocytosing *Porphyromonas levii* (arrows). The bacteria were pre-exposed to high titre anti-*P.levii* bovine serum and then incubated with the bovine PMN. Bar is equal to 1μm.



E. *Porphyromonas levii* Capsule

It has already been shown in the results that *P. levii* is not readily phagocytosed by bovine PMN. The experimental evaluation of the possibility that *P. levii* possess a capsule that may contribute to the anti-phagocytic properties of *P. levii* was conducted.

1. Maneval Stain

The first step to demonstrate a capsule possessed by *P. levii*, was to perform a light microscopy capsule stain (Maneval). The Maneval stain (68) is a negative stain that allows the background to stain blue, the bacteria to stain red and the capsule to appear clear. Figure 15A is a direct light microscopic view (1000 X magnification) of *P. levii* stained for an extracellular structure. Capsules are clearly evident (arrows). Figure 15B is a higher magnification of the bacteria stained in Figure15A, the clear space (arrow) located around the bacteria is noted as the capsule.

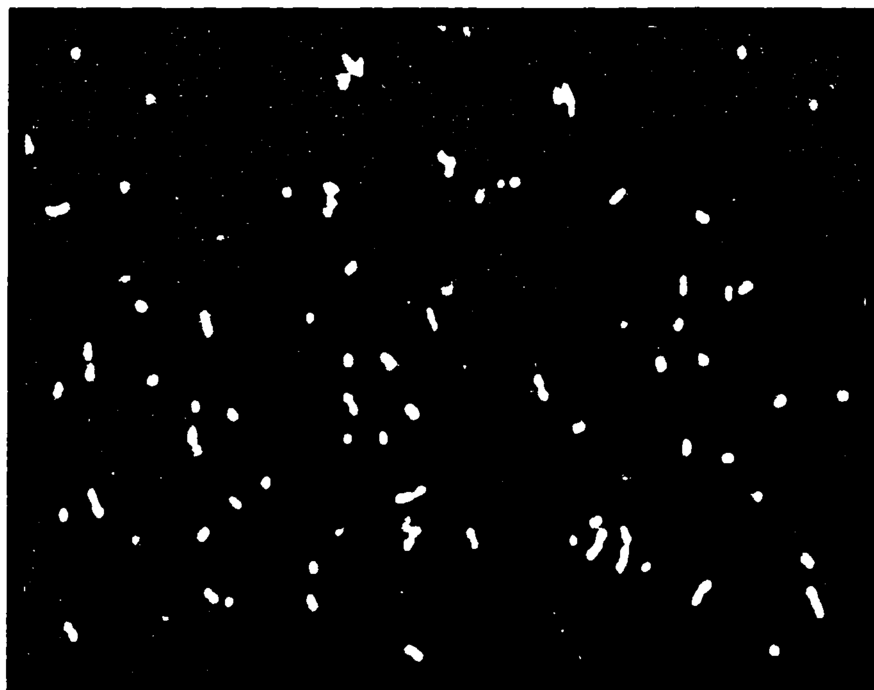
2. Transmission Electron Microscopy

To further support the possibility of *P. levii* possessing a capsule, transmission electron microscopy was performed. Figure 16A demonstrates the capsular structure surrounding the bacteria (arrows). Figure 16B is a higher magnification of one bacterium located in Figure 16A. Once again the capsule is clearly evident (arrow).

Figure 15A. Maneval (capsule) stain of *Porphyromonas levii* viewed by light microscopy. The capsules are the clear spaces (arrows) surrounding the bacteria (1000 X magnification).

Figure 15B. Higher magnification of the negatively stained capsule of *Porphyromonas levii* in Figure 12A. The capsule is visualized as the clear space (arrow) surrounding the red bacteria.

A



B

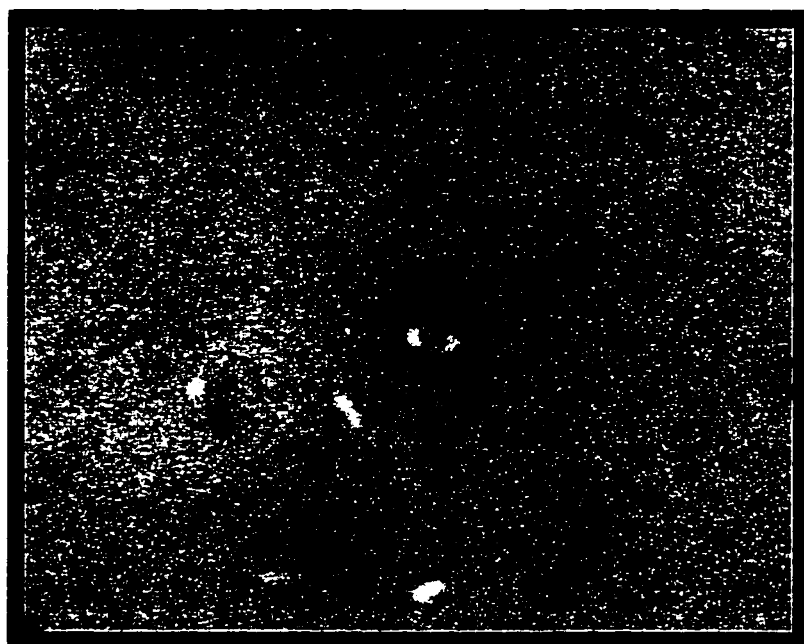
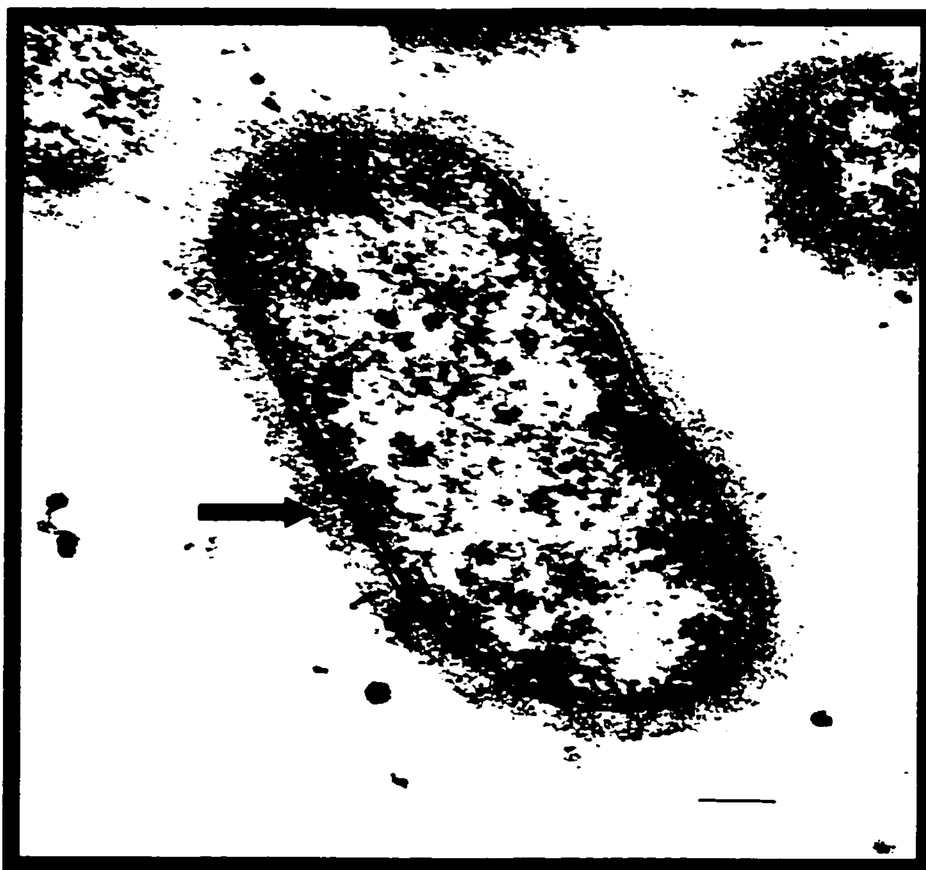


Figure 16A. Transmission electron micrograph of *Porphyromonas levii* verifying a capsular structure surrounding the bacteria (arrows). The bacteria were incubated with high titre anti-*P. levii* bovine serum and stained with ruthenium red. Bar is equal to 1μm.

Figure 16B. Transmission electron micrograph (higher magnification) of one bacteria in Figure 13A. The capsule (arrow) is clearly evident. Bar is equal to 0.1μm.

A

B



Discussion

Acute interdigital phlegmon (AIP) is a commonly occurring anaerobic bacterial infection in cattle (1,2). Polymorphonuclear neutrophils (PMN) are the first cells recruited to the site of a bacterial infection and are therefore important in the resolution of these infections through the elimination of bacteria present at the site of injury (6,18). Histologically, PMN have been shown to be present in tissue biopsies of cattle feet diagnosed with AIP. *Fusobacterium necrophorum* has been historically associated with AIP (6) and we have also cultured two distinct types of gram-negative obligately anaerobic bacteria from AIP cases (*Porphyromonas levii* and *Prevotella intermedia*) (9). Anaerobic bacterial infections, like AIP, are often of mixed etiology with two or more species being present (34). Synergism between the three bacteria at the site of injury in AIP could possibly lead to an alteration in the host's immune response and thus allow for the establishment of an infection. It is therefore important to investigate the functional properties of bovine PMN needed to remove the bacteria stated above from the site of injury. It has been postulated that an inhibition in the functional ability of bovine PMN to remove these bacteria from the site of injury could lead to an ineffectiveness of PMN to resolve this infection. If PMN are unable to remove these bacteria from the localized area, due to a functional disorder caused by the bacteria or their by-products, then the PMN are no longer useful in the resolution of this infection and will only contribute to the tissue necrosis seen in AIP.

To address the objectives of these studies, that will allow us to accept or reject the hypotheses presented, the effects that *P. levii*, *F. necrophorum*, and *P. intermedia* had on bovine PMN function (mobility, phagocytosis, and oxidative metabolism) were investigated *in vitro*. All three bacteria were chemotactic for bovine PMN, with *F. necrophorum* demonstrating the highest chemoattraction of the three bacteria studied. The results suggest that PMN mobility is not inhibited by any of the three bacteria or their by-products; therefore, PMN are likely recruited to the site of injury. This is consistent with what has been observed histologically in tissue biopsies of bovine footrot.

Porphyromonas levii is not readily phagocytosed by bovine PMN and this anti-phagocytic property may in part be associated with its possession of a capsule. The results stated suggest that *P. levii* can obstruct PMN phagocytic function *in vitro*, which could explain why this bacteria or others through synergism are allowed to colonize and proliferate *in vivo* to establish AIP. High titre anti-*P. levii* bovine serum, and specifically IgG from this serum, enhances the ability of bovine PMN to phagocytose *P. levii in vitro*. These results imply that specific anti-*P. levii* antibodies are important in assisting the PMN to remove these bacteria, which have successfully evaded phagocytic uptake, from the site of injury. Thus, another mechanism (humoral immunity) is shown to be an important part of the host's defense against AIP.

Finally, it was demonstrated that *P. intermedia* failed to induce an oxidative metabolic response in bovine PMN *in vitro*. These results suggest a failure to induce bactericidal activity by PMN against *P. intermedia* and possibly other bacteria through

synergism that may occur at the site of injury in AIP. This functional disorder by PMN may inhibit their ability to remove the bacteria from the site of injury and therefore allow the bacteria to colonize and establish the infection.

A. PMN Movement

The effects that bacteria or bacterial by-products have on PMN movement can be detrimental to the number of PMN recruited to the site of injury. In these studies, PMN movement (chemotaxis and chemokinesis) was assessed through Boyden chamber techniques (42). As shown with Figure 4 and Figure 5, all three bacteria were chemotactic for bovine PMN *in vitro*. The results demonstrated that one or more bacteria, or their by-products, will inhibit the mobility of bovine PMN *in vitro* and therefore our hypothesis can be rejected. This is significant because by rejecting the hypothesis we give support to the fact that PMN have the potential to be recruited to the site of injury and therefore are important in the resolution of AIP. It is proposed that a direct inhibition of PMN chemotaxis is not the reason for the colonization of bacteria and establishment of infection in AIP. As seen in Figure 4, we have demonstrated that *Fusobacterium necrophorum* is the most potent chemoattractant of the three anaerobes examined. Since equal numbers of bacteria were used for each group, the large cellular size of *F. necrophorum* compared to *P. levii* and *P. intermedia* may be a potential explanation of these results as it has the ability to release more chemoattractant chemicals (LPS, FMLP) from each bacterial cell. An extracellular leukotoxin has been reported to be released by *F. necrophorum* (27);

however, we were unable to document similar leukotoxic activity in these experiments. *F. necrophorum* has already been implicated in synergistic behavior within infections since it is an opportunistic pathogen and is often seen as a secondary invader (22). Price and McCallum (1986) showed that the growth of *P. intermedia* was enhanced in the presence of *F. necrophorum* (34). We suggest that the primary role of *F. necrophorum* in AIP is not via direct inhibition of PMN function, but instead through its secondary ability to cause tissue necrosis. It may rely on the synergistic effects of other bacteria, possibly *P. levii* and *P. intermedia*, to decrease the host immune response and allow for bacterial proliferation and subsequent tissue destruction that is associated with AIP. This proposal is significant since *F. necrophorum* has traditionally been described as one of the main etiologic agents involved in bovine footrot (6) and we are suggesting that it plays a secondary role in AIP.

It was also demonstrated that *P. levii* displayed the highest chemokinetic response by bovine PMN compared to *F. necrophorum* and *P. intermedia* (Figure 5). *P. levii* was shown in our results to be a poor chemoattractant for bovine PMN, but a potent stimulant of PMN chemokinetic movement once serum was added to the experiment. Therefore, serum factors present at the site of injury could enhance the migrational properties of PMN toward this bacterium. These results suggest what may be occurring *in vivo* in AIP. Serum factors are present in the tissue fluid at the site of injury and thus allow for increased recruitment of PMN. Finally, we could not demonstrate an inhibition of bovine PMN movement by *P. intermedia*, similar to the inhibition of human PMN migration by

Bacteroides intermedius that has been described in the literature (44). We suggest that the role *P. intermedia* plays in AIP is not through inhibiting PMN migration as proposed with human PMN but by disrupting a different PMN function that will be discussed later.

B. PMN Phagocytosis

PMN-mediated phagocytosis is important in the initial response to, and clearance of, invading bacteria at the site of injury. We have demonstrated that *P. levii* was not phagocytosed by bovine PMN. From the results stated we can accept the hypothesis that PMN-mediated phagocytosis of one or more bacteria (*P. levii*) *in vitro* will be obstructed by the bacteria themselves or their by-products. These results are similar to what has been described in the literature. Various types of *Bacteroides spp*. impair PMN phagocytosis (51,52), possibly through the expression of a polysaccharide capsule or the depletion of serum complement. To our knowledge, this is the first report of *P. levii* expressing anti-phagocytic properties. The results are of great significance when examining the pathogenicity of AIP. The anti-phagocytic properties of *P. levii* described in our investigations could possibly lead to an inability of PMN to remove not only *P. levii*, but possibly other bacteria from the site of injury.

Relating back to what is occurring *in vivo*, this loss of phagocytic function by PMN may be in part responsible for the colonization of bacteria and establishment of AIP. Future research could concentrate on determining the precise factors, either cellular or soluble, responsible for this reduced phagocytosis. Phagocytosis assays combining culture

supernatant from logarithmic growth phase *P. levii* and PMN phagocytosing latex beads or other bacteria may determine whether it is a soluble factor that is released from *P. levii* that inhibits phagocytic function. This would also demonstrate the synergistic behavior, that we have postulated earlier, is responsible for AIP. One cell associated factor addressed within these studies was the possession of a bacterial capsule by *P. levii*. Using a Maneval stain (Figure 15) as an initial experiment, and then verifying with transmission electron microscopy (Figure 16), a moderate (50 nm) capsule was demonstrated. As suggested by other investigators using *Bacteroides spp.* (21), bacterial capsules can obstruct phagocytosis by PMN. We believe that this capsule of *P. levii* may be an important factor in the anti-phagocytic properties demonstrated in these studies; however, there may be other equally significant factors present. By inhibiting capsular synthesis or genetically altering *P. levii* such that it is unable to produce a capsule and then including this non-capsular *P. levii* in similar phagocytosis experiments, one may be able to determine if the capsule has anti-phagocytic properties.

C. PMN Bactericidal Activity

Bactericidal activity is the final function of PMN used to eliminate a bacteria from the site of injury. We examined oxidative metabolism as a measure of this activity. The results indicated in Figure 7 demonstrate that *P. intermedia* failed to induce an oxidative metabolic (NBT reduction) response in bovine PMN. These results allow us to accept the hypothesis that one or more bacteria, or their by-products, will not induce an oxidative

response in bovine PMN *in vitro*. This is another phenomenon of great significance for researchers attempting to explain the pathogenicity of the bacteria involved in AIP. We suggest that in actual cases of AIP, *P. intermedia* is not degraded by bovine PMN and may in fact inhibit PMN from degrading other bacteria (*F. necrophorum* and *P. levii)* at the site of injury. This may directly lead to the colonization, proliferation and pathogenesis of AIP. The above results are in agreement with Shinzato and Saito (1994) who demonstrated that culture filtrate of *P. intermedia* inhibited bactericidal activity of human neutrophils (33). Additional research on the mechanisms involved in this failure of *P. intermedia* to induce an oxidative metabolic response in bovine PMN should investigate the culture supernatant of *P. intermedia* and its effect on bovine PMN bactericidal activity against the other bacteria involved in AIP. Furthermore, the bacterial factors involved in this phenomenon should be isolated and characterized. Inhibition of non-oxidative bactericidal mechanisms of bovine PMN by *P. intermedia* have not been examined and such experiments should also be undertaken. *P. intermedia* is potentially involved in synergistic associations with other bacteria involved in AIP. It not only grows more quickly and to a greater extent when associated with *F. necrophorum*, but its presence also provides increased growth to bacteria such as *Streptococcus constellatus* (33). *P. intermedia* shares similar properties to *Streptococcus uberis* (a cause of bovine mastitis) such that they both possess a capsule which does not obstruct phagocytosis, but they both fail to induce an oxidative metabolic response in bovine PMN (71). This mechanism for

disrupting PMN function is very important in the development of a bacterial infection such as AIP.

D. Opsonophagocytosis

1. Immune Serum

Opsonization is the process through which antibody (IgG) and complement (C3b) bind to antigens to enhance phagocytosis of the antigen by PMN and macrophages (20). Opsonins in serum are of significant importance for the enhancement of PMN phagocytosis of bacteria at the site of injury (37). Immunoglobulin G (specifically IgG_2) and C3b are the primary opsonins present in bovine serum that enhance this recognition and phagocytic uptake of bacteria by bovine PMN (20, 37). The results shown in Figure 8, suggest that *P. levii* is not efficiently phagocytosed by PMN *in vitro* and that the presence of high titre anti-*P. levii* bovine serum can enhance phagocytosis of this bacterium by PMN to high levels. The results presented allow us to accept the hypothesis that specific antibodies raised against a bacteria (*P. levii*) will increase the ability of bovine PMN to engulf these bacteria *in vitro*. The acceptance of this hypothesis is very meaningful because it suggests that the development of antibodies to *P. levii* by the host will help in the removal of these bacteria from the site of injury and possibly the resolution of AIP. This is also very significant to a researcher attempting to develop a vaccine against the bacteria responsible for AIP in that it demonstrates that an immune response

generated towards *P. levii* could serve practical importance in the resolution or prevention of the infection.

2. Purified IgG

Interestingly, pre-incubation with complement depleted high titre anti-*P. levii* serum also resulted in a significant ($P < 0.05$) increase in phagocytosis of this bacterium by bovine PMN (Figure 8). These results suggests that heat labile complement factors had only minimal involvement as opsonins in this system and that specific IgG was the primary factor involved in this enhancement of phagocytosis of *P. levii* by PMN. In order to verify that IgG was the primary opsonin in serum responsible for the increased phagocytosis of *P. levii*, IgG was purified from serum using affinity column chromatography. These experiments demonstrated that when *P. levii* was pre-incubated with purified high titre anti-*P. levii* IgG, a significant ($P < 0.05$) increase in phagocytosis of *P. levii* by bovine PMN was observed compared to the controls (Figure 11). The results presented give further support for the acceptance of the hypothesis that specific antibody raised against these bacteria will increase the phagocytosis of those bacteria by bovine PMN. These data verify that immune serum, and specifically anti-*P. levii* IgG, is responsible for this effect and lends support to the literature suggesting IgG is the major opsonin present in bovine serum (63). This is of primary significance in AIP as it demonstrates that the production of IgG specific to *P. levii* is very important in allowing the PMN to remove these bacteria from the site of injury and possibly resolve the infection.

In context of the importance of IgG as an opsonin of this bacterial species, other work in our laboratory may provide an explanation for these anti-phagocytic properties of *P. levii*. Recently we have described an immunoglobulin protease produced by *P. levii* that specifically cleaves IgG_2 (72). This potential virulence factor may possibly alter the opsonization ability of IgG. We suggest that although IgG specific for *P. levii* will enhance PMN-mediated phagocytosis, the production of such an IgG_2 protease *in vivo* could inhibit the phagocytic response of these cells in the focus of infection. Such a protease could also inhibit the phagocytic uptake of other bacteria (*F. necrophorum, P. intermedia,* or others) present at the site of injury and thus provide another mechanism of synergism within acute interdigital phlegmon. Future research should concentrate on combining antibody-mediated PMN phagocytosis of *P. levii* with the IgG_2 protease production by *P. levii*. The verification that this protease is inhibiting PMN phagocytosis of *P. levii* and other bacteria is important in describing the pathogenicity of AIP.

E. Limitations

As with all research there are limitations on the technical, interpretive, and conclusive parameters of the work performed. The common limitations for all research are time and money, for which substantial amounts are needed to answer the scientific questions at hand.

In our research there were many technical limitations in the protocols used. All experiments were performed *in vitro* and therefore only an assumption can be made that

the same mechanisms are being used by the host *in vivo*. Physiological conditions can only be estimated in an experiment due to the number of variables that are present *in vivo*. Several limitations in the protocols used can be identified. Sensitivity of the phagocytosis and oxidative metabolism assays arises as a primary limitation since some PMN were more active than others. Other assays such as radioactive labeled bacteria for phagocytosis or myeloperoxidase production for bactericidal activity may prove to be more sensitive for evaluation of these PMN functions. In the PMN movement, phagocytosis and oxidative metabolism experiments, a finite number of bacteria (1×10^7) was used. A dose-response curve could of been performed on each bacteria to determine the optimal number of bacteria needed to induce the highest response by bovine PMN. Also the number of bacteria present at the site of injury compared to PMN is not known for this disease and therefore the variable (10:1 / bacteria:PMN) used from the literature must be taken as an arbitrary value. In the phagocytosis experiments, the time variable is a limitation as it was set for 30 minutes (incubation of bacteria and immune serum) and at 15 minutes (incubation of the bacteria and PMN). Obviously this is not the exact time used by the host *in vivo* and therefore the results must be examined as occurring over a specific unit of time. For the bactericidal experiments, a limitation is in the number of bactericidal mechanisms evaluated. Only one category (oxidative metabolism) was evaluated and analyzed on the PMN, but many different mechanisms for degrading bacteria are used by the PMN. An evaluation of these other mechanisms would either support or oppose the results already presented. During the immune serum experiments, heating the serum at

56°C for 30 minutes only destroys heat labile proteins (C3b, C3bi) (20) and thus some complement factors are still present along with other heat resistant proteins. Above are only a few of the limitations in the experimental protocols we employed to attempt to replicate the *in vivo* situation in AIP.

We are also confronted with limitations in interpreting the data that has been generated. The number of repetitions of each experiment forced us to analyze different groups of data with different statistical methodologies (non-parametric vs parametric).

Finally, by not being able to demonstrate the precise mechanism used by bacteria, such as alteration of PMN phagocytosis or the inability to induce oxidative metabolism, we have limitations on the conclusions that can be equivocally stated. Further research must be performed to be able to definitively conclude the processes by which these mechanisms operate. Overall our research has only addressed one or two areas of the complex inflammatory process involved in AIP. Thus, we can only limit our conclusions to the specific area that was investigated.

F. Conclusions

Based on the studies presented and the hypotheses tested, it can be concluded that:

1. *Porphyromonas levii*, *Fusobacterium necrophorum*, and *Prevotella intermedia* are all successful chemoattractants for bovine PMN *in vitro*.

2. *Porphyromonas levii* is not readily phagocytosed by bovine PMN *in vitro*.

3. High titre anti-*P. levii* bovine serum, and specifically IgG from this serum, enhances the ability of bovine PMN to phagocytose *P. levii in vitro*.

4. *Prevotella intermedia* failed to induce an oxidative metabolic response in bovine PMN *in vitro*.

5. *Porphyromonas levii* possesses a capsule *in vitro*.

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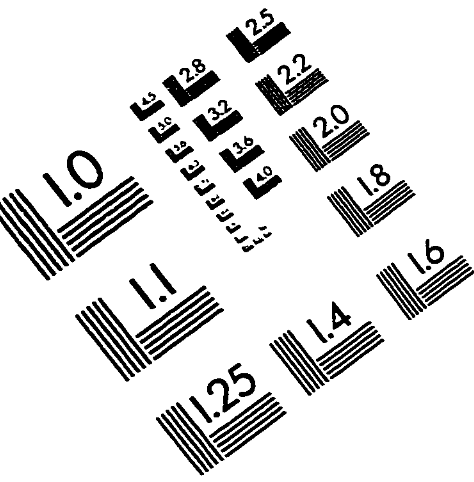
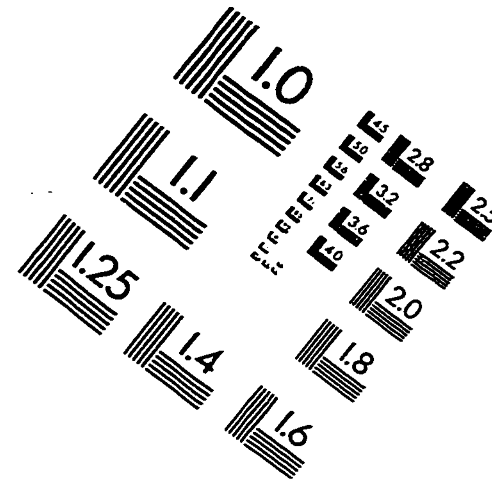
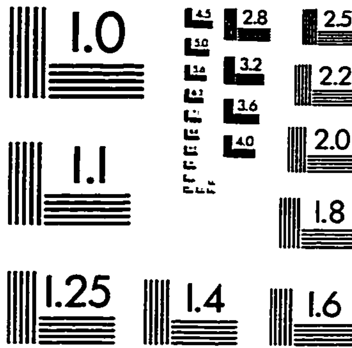
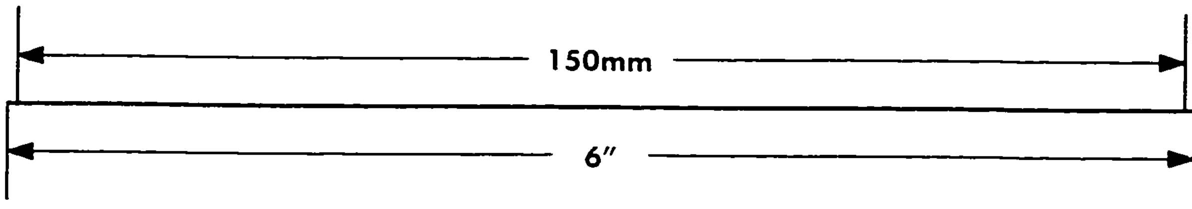
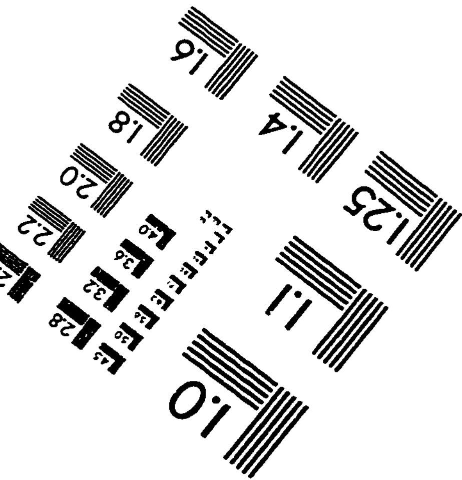
IMAGE EVALUATION TEST TARGET (QA-3)











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