THE UNIVERSITY OF CALGARY

Bovine Endothelial Cells Produce Tumour Necrosis Factor-α, Granulocyte Macrophage Colony Stimulating Factor and Interleukin 1ß in Response to *Porphyromonas levii* Lipopolysaccharide

by

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Abstract

Acute Interdigital Phlegmon or footrot is an important anaerobic bacterial infection affecting the cattle industry. The disease has a rapid onset with severe swelling and necrosis of the interdigital region leading to lameness, pain, and increasing inflammation. Damage to endothelial cells lining blood vessels of the limb may lead to edema and the release of pro-inflammatory mediators by these cells which contribute to inflammation. In footrot, this damage is thought to be caused by *Porphyromonas levii* and its LPS. The objectives of this study were to examine the cytopathic effects and cytokine responses of cultured bovine vascular endothelial cells exposed to *P. levii* LPS. Bovine endothelial cells responded to exposure with *P. levii* by producing GM-CSF, TNFα and IL-1ß. CPE occurred in response to exposure of endothelial cells with fractions of these bacteria over the same time period as cytokine production. LPS induced CPE and associated production of cytokines by endothelial cells may be important in the pathogenesis and immunity of footrot.

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Dedication

To: Suzanne, Dexter, Moyshi,
and the Sparrow and Nicol
Families.

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List of Abbreviations

ATCC	American Type Culture Collection
BBA	Brucella Blood Agar
BCIP	5 - bromo - 4 - chloro - 3 - indolyl phosphate
BSA	Bovine serum albumin
cDNA	complementary DNA
cMEM	complete Eagle's Minimum Essential Medium
CPE	cytopathic effect
cRPMI	complete Roswell Park Memorial Institute - 1640 Medium
ddH$_2$O	double distilled water
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eBHI	enriched Brain Heart Infusion
EDTA	ethylenediamine tetraacetic acid
eCMM	enriched Cooked Meat Medium
EJG	Bovine capillary endothelium, ATCC CRL - 8659
EtBr	ethidium bromide
FBS	fetal Bovine serum
GM - CSF	granulocyte, macrophage colony stimulating factor
HRP	horse radish peroxidase
IgG	immunoglobulin isotype G
IL - 1ß	interleukin one beta
kDa	kilodaltons
KDO	2 - keto - 3 - deoxyoctonate

KVLB	kanamycin, vancomycin laked blood
LB	Luria - Bertani
LPS	Lipopolysaccharide or Bacterial Endotoxin
MEM	Eagle's Minimum Essential Medium
MW	molecular weight
mRNA	messenger RNA
NBT	nitroblue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
P. levii	*Porphyromonas levii*
7.5 (Isolate)	*Porphyromonas levii*
PRAS	pre - reduced, anaerobic and sterile
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute - 1640 Medium
SDS	sodium dodecylsulphate
SSC	sodium chloride, sodium citrate buffer
TAE	Tris - acetate, EDTA buffer
TBS	TRIS buffered saline
TFB	transformation buffer
TNFα	tumour necrosis factor alpha
TRIS	tris(hydroxymethyl)aminomethane
VIDO	Veterinary Infectious Disease Organization
v/v	volume per volume
w/v	weight per volume

Introduction

Acute bovine footrot, is an anaerobic bacterial infection in cattle. Even though the bacteria thought to cause footrot are known to be anaerobic, little else is known about the disease. Research into the disease has been neglected in the past. This has often meant that insights into footrot have relied on the fact that most of the research in the field of anaerobic bacterial infections has pertained to infections of humans. Regardless, the knowledge gained in past research into anaerobic bacterial infections should reflect the nature of similar infections in animals, including those examined in cattle.

Historically, research into footrot has also been neglected because if successful diagnosis occurs early enough, treatment measures including the use of antibiotics, are most often successful (Morck, 1995, Morck, Olson, Louie, Koppe and Quinn, 1998). Those in the cattle industry have for some time, considered footrot to be of minor consequence, even though it is one of the most prevalent infections seen within the Canadian cattle industry (Morck, 1995). Recently, with the concern of antibiotic resistance becoming more of an issue within the scope of the disease, and the ever increasing interest into diseases affecting cattle, research looking at many aspects of footrot has been initiated (Morck, 1995, Feinman, 1998).

A comparable infection in sheep has been studied intensively, leading to more applicable control and treatment measures (Morck, Gard and Olson, 1994). Treatment of ovine footrot is largely controlled using a vaccine, antibiotic therapy and antiseptic footdips (Morck, 1995, Morck et al., 1994, Blood, Radostits, and Gay, 1994). Recent vaccines using a recombinant fimbrial antigen approach appear to be successful in preventing the onset of ovine footrot (Morck, 1995, Morck et al., 1994).

Footrot is a problematic and important anaerobic bacterial infection affecting the cattle industry as a whole (Morck, 1995, Jubb and Malmo, 1991). It is the second most frequently occurring infectious disease in feedlot cattle, and is also problematic for the dairy industry, pastured cattle, and breeding programs (Morck, 1995, Jubb and Malmo, 1991). The disease is common in most countries, and is probably responsible for the majority of lameness seen in cattle world wide (Blood, Radostits, and Gay, 1994, Kruse, Weiskopf and Agger, 1988). The disease can affect cattle of all ages, including young calves, but the disease is much more common in heavier adults (Blood et al., 1994, Kruse et al., 1988). Footrot may be observed in any of the four legs, but the majority of cases is seen in the hind legs (Kruse et al., 1988). There seems to be a complex correlation between many factors, including animal management practices and environmental factors, that predispose animals to the disease (Johnson, Dommert and Kiger, 1969). The bovine foot has anatomic features that may contribute to its susceptibility to infection by footrot - causing bacteria (Johnson et al., 1969). For example, the interdigital space of the hoof is an area of unprotected epidermis that is consistently exposed to many factors that predispose infection (Johnson et al., 1969). Footrot usually occurs when pen or pasture conditions are moist and unsanitary. In feedlot cattle, pen cleaning, which occurs in spring and autumn, may create such conditions. In Southern Alberta, chinooks are often a good indication that new cases of the disease will occur, however footrot is not necessarily a seasonal disease, and may affect cattle year around (Morck, 1995, Blood et al., 1994). Stony ground, lanes filled with sharp gravel and pasturing on coarse stubble are some of the abrasive conditions which also seem to predispose cattle to footrot (Blood et al., 1994). It is suggested that footrot is due to traumatic abrasions occurring in the interdigital hoof region followed by secondary penetration of bacteria (Blood et al., 1994, Kruse et al., 1988). Abrasions are also more likely to occur when the skin is swollen and soft due to continual wetting, implicating the wetness under-foot again, as a major factor in disease incidence (Blood et al., 1994).

Regardless of when the disease occurs, or how it is initiated, footrot has major economic implications for the cattle industry, including feedlot producers and the purebred livestock industry (Morck, 1995, Morck et al., 1998, Kruse et al., 1988). Economic losses are often substantial, primarily due to lost production in commodities such as milk or in terms of animal growth, during the course of the illness and treatment (Morck, 1995, Kruse et al., 1988). Animals commonly go off their feed and lose weight, and normal breeding is interrupted (Kruse et al., 1988). Footrot is of greatest economic importance world wide in dairy cattle, because of the intensive conditions under which they are kept, increasing the incidence of the disease (Blood et al., 1994). In rare and severe cases, the animal may have to be euthanised if treatment is not successful, or if the animal suffers from severe joint involvement, further adding to the economic loss due to the disease (Blood et al., 1994). The cost of treatment and the suffering experienced by the cattle further complicates the problems caused by footrot (Morck, 1995, Kruse et al., 1988).

Virtually nothing is known of the specific pathogenic mechanisms of footrot (Morck, 1995, Blood et al., 1994). The disease is diagnosed primarily on the basis of viewing the abnormal stance and gait exhibited by affected animals, which results from the lameness caused by symmetrical and bilateral lesions in the lower limbs (Mgasa, 1987). The disease has a rapid onset with severe swelling and necrosis of the interdigital region of the hoof leading to lameness, pain and increasing inflammation (Morck, 1995, Egerton, Yong and Riffken, 1989, Blood et al., 1994). Early treatment of footrot is considered essential, and broad-spectrum injectable antibiotics have proven to be successful treatment options in the past (Kruse et al., 1988). However, if left unchecked or if treatment fails, the swelling and lameness may spread proximally in the limb, with sequelae such as: septic arthritis, fibrosis of ligaments and tendons, and in severe cases, permanent lameness (Morck, 1995). A few cattle develop such severe joint and bone involvement, that surgery may be required, or the animals have to be euthanised (Johnson, Dommert and Kiger, 1969).

Some of the pathological hallmarks of footrot are inflammation with edema and a interdigital lesion, often emitting a purulent discharge (Morck, 1995). Edema may be caused by increased blood pressure, lowered colloidal osmotic pressure, lymphatic damage and/or damage to vasculature. Certain gram - negative bacteria are known to directly damage endothelial cells primarily through the action of the outer membrane surface molecule, lipopolysaccharide (LPS) (Endo, Shibazaki, Nakamura and Takada, 1997). No investigations have evaluated the effects of LPS from the anaerobic bacterial footrot isolates on bovine endothelial cells, in footrot. Interaction of bacteria and endothelial cells may lead to the production and release of pro-inflammatory mediators by endothelial cells, leading to inflammatory events that either contribute to the resolution of infection, or exaggerate the pathology of the diseased area (Gallin, Goldstein and Snyderman, 1988). With the void in knowledge and literature pertaining to footrot, we have initiated investigations into the interactions of the anaerobic bacteria isolated from footrot lesions in cattle, with bovine endothelial cells *in vitro*, to evaluate potential roles of these factors in the immunopathophysiology of this infection. With this research, we hope to gain further knowledge of the pathogenesis and immune response triggered by the causative agent. This information, will be a significant advancement in the understanding of footrot and will aid to provide basic information for designing efficacious therapies and prevention strategies for the disease.

Literature Review

Anaerobic Bacteria

Despite the extensive use of antibiotics and vaccination programs, infectious diseases, particularly bacterial diseases, continue to be a leading cause of morbidity and mortality worldwide (Finlay and Cossart, 1997). Anaerobes are important in various mixed infections, and numerically, they constitute a major portion of the normal microbial flora found in most mammalian species (Aldridge, 1995). In mixed infections, anaerobes may outnumber aerobic bacteria at the site of infection (Aldridge, 1995). For many years, the contribution of these anaerobic bacteria to the pathogenesis of these infections was considered to be insignificant (Rotstein, 1993). However, new evidence supporting the role of these anaerobes, in mixed infections, has come about by improved isolation and culture techniques, and with studying antimicrobial susceptibility profiles of the anaerobic bacteria in question (Rotstein, 1993). Anaerobic bacteria are also involved in infections caused by a single species of bacteria (Aldridge, 1995).

Normal host flora (bacteria), colonize certain mucosal surfaces to form a protective layer of organisms that prevents attachment and invasion of more pathogenic organisms; this protective layer or biofilm produces substances that act as bacteriocins to other organisms (Aldrige, 1995). However, when this protection is interrupted by tissue damage, either through ischemia or other trauma, the microflora may invade normally asceptic tissues in various body sites and potentially become pathogenic (Aldridge, 1995). Many of the anaerobic bacteria isolated from infections at various locations in the body, are normal flora of the affected host (Aldridge, 1995, Paster, Dewhirst, Olsen and Fraser, 1994). The precise identity of the pathogen(s) involved in anaerobic infections may remain unknown due to the expense and complexity of isolation and identification to the species level (Moncla, Braham, Rabe and Hillier, 1991, Durmaz, Jousimies-Somer and Finegold, 1995). Despite their clinical relevance, very little is known about the pathogenic mechanisms in anaerobic bacterial infections (Botta, Arzese, Minisini and Trani, 1994).

Examples of anaerobic infections include post-trauma abscesses, some cases of pneumonia, necrotizing skin infections and sepsis, which often results in death due to shock (Aldridge, 1995). Abscesses caused by anaerobes may occur at any and all anatomical sites imaginable (Aldrige, 1995). Clinical clues suggestive of anaerobic infection include: the presence of a foul smelling exudate, tissue necrosis with abscess formation, and the failure of the infection to respond to antibiotics (Aldridge, 1995). On the basis of culture and isolation of mixed infections, the causative agents are most often anaerobic, gram - negative, non spore-forming bacilli (Aldridge, 1995). These include *Bacteroides fragilis,* non-fragilis *Bacteroides, Prevotella sp., Porphyromonas sp.* and *Fusobacterium sp.* (Aldridge, 1995, Paster et al., 1994, Garcia, Becker, Brooks, Berg and Finegold, 1992, Scanlan, Berg and Campbell, 1986, Gibson, Tzianabos and Onderdonk, 1996, Frazier and Yeager, 1996). Several potential mechanisms have been proposed for the involvement of anaerobic bacteria in the pathogenicity of polymicrobial infections (Rotstein, 1993, Marra and Isberg, 1996). These include the ability of anaerobes to impair host defenses, thereby allowing their co-pathogens to exert their intrinsic virulence; the provision of nutrients by one bacterial species to enhance growth of its bacterial partners; the capacity of anaerobes to alter the local microenvironment, thereby rendering it more conducive to bacterial survival and proliferation; and the transfer of virulence factors to other microorganisms involved in the mixed infections (Rotstein, 1993). Within the anaerobic, gram - negative, non spore forming bacilli are the black - pigmented anaerobic rods which include a number of gram - negative bacterial species isolated from humans and animals (Grenier, Labbe, Mouton and Meyrand, 1994). Although some species have yet to be classified, recent taxonomic studies recognize two genera: *Porphyromonas* for the asaccharolytic group and *Prevotella* for the saccharolytic group (Grenier et al., 1994, Summanen, 1995). Most of these pathogenic anaerobic bacteria possess an extensive array of mechanisms to invade and infect host tissue, and to escape host defense mechanisms (Aldridge, 1995, Grenier et al., 1994). It is the *Porphyromonas* group of anaerobes which are of interest for this project.

Pathogenic Mechanisms of Anaerobic Bacteria

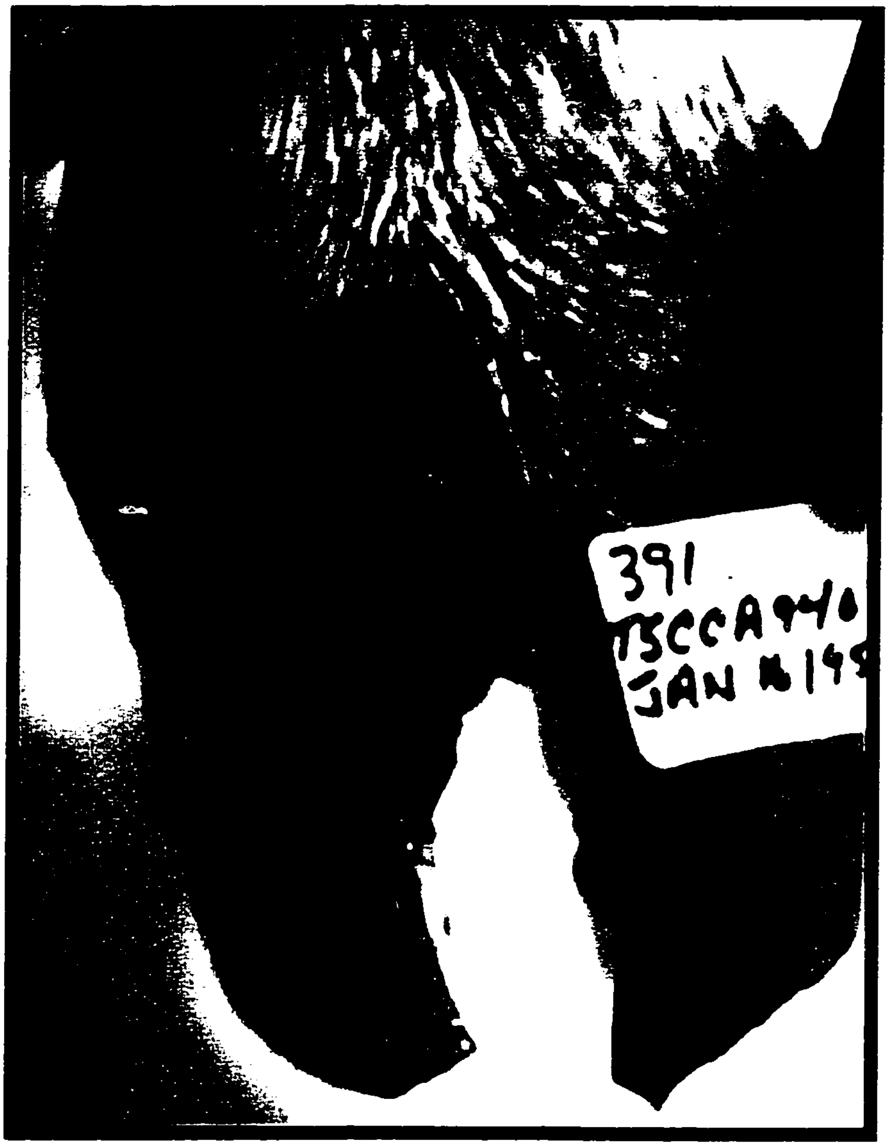
Without aiming to be in any way exhaustive in terms of the many areas of research in this field, a review examining some of the more prominent issues with regard to selected aspects of virulence factors in anaerobes seems prudent. With the gap in literature and knowledge about the specific pathogenic mechanisms of individual anaerobes, it seems only rational to postulate that anaerobes may contain any of the following virulence factors, aiding in their infectivity. Capsules are a structural component of many anaerobes, and can aid in preventing phagocytosis, opsonization and promote abscess formation (Botta et al., 1994). The fimbriae of anaerobes are known to play an important role in adhesion, an initial event in the bacteria - host interaction that may be necessary but not sufficient for the development of an infectious process in humans or animals (Botta et al., 1994). Fimbriae and capsules have been implicated in hemagglutination activity, which may be a central mechanism in diseases caused by anaerobic bacteria, through prevention of bacterial clearance, and a resultant reduction in oxygen concentration in the affected tissues (Botta et al., 1994). Endotoxin (Lipopolysaccharide/LPS) which is associated with gram - negative anaerobic pathogens, is pyrogenic and has toxic effects on host cells (Botta et al., 1994). LPS can also stimulate host immune responses, sometimes leading to inappropriate consequences including inflammation (Botta et al., 1994). Enzymes of tissue destruction such as collagenase, hyaluronidase, elastase and a host of others, play roles in invasion and dissemination, and are many times implicated in anaerobic infections (Botta et al., 1994). *Porphyromonas* strains, have been shown to possess trypsin - like, phospholipase C, and chymotrypsin - like activities, which may represent additional virulence determinants for these pathogens (Grenier et al., 1994). Immunoglobulin proteases produced and released by anaerobes may allow these pathogens to evade host immunity (Botta et al., 1994). For instance in some *Porphyromonas* strains, enzymes have been found that were able to digest IgG, IgM and IgA (Botta et al., 1994). Many anaerobes produce enzymes such as ß - lactamases, which provide the pathogens resistance to ß - lactam antibiotics (Botta et al., 1994). Many anaerobes also show resistance to non - ß - lactam antibiotics (e.g. macrolides, aminoglycosides) for which the mechanisms of resistance are still not completely understood (Aldridge, 1993). Metabolic end products including short chain fatty acids can be released by the pathogens, reducing the pH of the surrounding area (Aldridge, 1995). This can diminish phagocytic activity and limit the effectiveness of pH dependent ß - lactam antibiotics (Aldridge, 1995). Recently it was found that the short chain fatty acid, succinic acid, was responsible for an inhibitory effect on neutrophil/phagocytic function (Rotstein, 1993). It was found that succinic acid mediated this effect by shuttling protons from the extracellular to cytoplasmic space of the neutrophils, thereby causing intracellular acidification and resultant cell dysfunction (Rotstein, 1993). Cellular dysfunction may limit chemotactic activities, the release of toxic metabolic/oxidative bactericidal bursts via lost lysosome function, and the release of immuno - regulatory substances may be negatively affected (Botta et al., 1994). Another potential mechanism by which anaerobes impair leukocyte function and predispose to abscess formation is the direct promotion of fibrin deposition at the site of infection (Rotstein, 1993). Interactions between anaerobes and macrophages have been shown to induce cell associated procoagulant activity, which appears to impair bacterial clearance (Rotstein, 1993). Infections caused by anaerobes often lead to extensive tissue destruction (Botta et al., 1994). The affected tissue becomes hypoxic and/or anoxic (Botta et al., 1994). This impairs the function of phagocytic cells and therefore protection from the infecting organisms as well (Botta et al., 1994). The production of specific leukotoxins by anaerobes has been described (Botta et al., 1994). This loss and destruction of leukocyte function appears to be important in anaerobic virulence (Botta et al., 1994). Similar virulence factors likely operate in most anaerobic infections, including those affecting animals, such as bovine footrot. It has only been in the past few decades that clinicians and researchers have acknowledged the importance of anaerobes as pathogens in potentially harmful and fatal diseases such as footrot (Aldridge, 1995, Morck, 1995).

Pathogenesis of Footrot

The primary pathological features of footrot include the following: 1) edema (abnormal fluid accumulation) of subcutaneous tissues which is one of the cardinal signs of inflammation and can also be caused because of direct damage to vascular endothelium; 2) inflammation with diffuse infiltration of the affected area by specific types of white blood cells (neutrophils); 3) Clinical pain, warmth and redness and 4) the presence of a necrotic interdigital lesion (Morck, 1995, Egerton et al., 1989, Blood et al., 1994). The typical lesion occurs within the skin of the interdigital cleft and takes the form of a fissure with swollen, protruding edges which may extend along the length of the cleft or be confined to the anterior part or that part between the bulbs of the heal (Blood et al., 1994). The presence and infiltration of neutrophils with the finding of low serum immunoglobulin levels specific for the footrot pathogen, suggests a possible role for cellular immunity in combating this infection (Morck, 1995). Overall it is suggested that the footrot pathogen affects the functioning of the blood vessel linings in the affected area, and that neutrophils probably act to clear the bacteria from affected tissue (Morck, 1995).

Histological studies of biopsy specimens confirm that endothelial damage occurs with footrot (Morck, 1995). How the footrot pathogen causes endothelial damage is still not understood. Dogma states that two main possibilities for this edema in footrot, exist (Gallin, Goldstein and Snyderman, 1988). Each of these possibilities involves bacterial endotoxin or lipopolysaccharide (LPS) directly or indirectly. The first is that endotoxin acts directly to damage endothelial cells promoting fluid movement into adjacent tissue (Gallin, et al., 1988). The second involves intermediary cells such as tissue resident leukocytes or circulating leukocytes recruited into the area of infection because of the interaction of the endothelial cells and the LPS (Gallin, et al., 1988). These leukocytes can

Figure 1: A photograph of the gross lesions typical of footrot. The interdigital area shows substantial swelling, redness and a purulent discharge.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lead to endothelium damage as an indirect result of the presence of LPS (Gallin et al., 1988). The mechanisms by which LPS from gram - negative pathogens, including anaerobes, induce endothelial cell injury and pathology, may be crucial in footrot pathogenesis.

How the bacteria enter and invade the interdigital region is not exactly understood. One of the most probable modes is by direct inoculation via trauma, or by dermal translocation (Aldridge, 1995, Paster et al., 1994). Other possibilities include systemic seeding from other sites of the body where perhaps the pathogen resides as normal flora (Aldridge, 1995, Botta et al., 1994). Cultures of biopsy specimens often are negative for any bacteria, which may reflect the limitations and difficulty in transporting and culturing anaerobes from clinical specimens (Engelkirk, Duben - Engelkirk, Dowell, 1992, Gerhardt, Murray, Costilow, Nester, Wood, Krieg, Phillips, 1981). Furthermore, it's been shown that because anaerobes often release highly acidic products (short chain fatty acids) into their environment, the low pH often limits or slows down their growth, to the extent that they are not detected in routine culture (Aldridge, 1995). However, in approximately 50% of the cases, cultures are positive for the presence of anaerobes. Anaerobes are considered significant as pathogens in infections in most sites of the body, especially in highly vascular tissues, where the amounts of oxygen would limit growth under normal, healthy conditions (Aldridge, 1995, Botta et al., 1994). Consequently, culturing anaerobic bacteria from sites of infection, supplies significant evidence that they play an important role in pathogenesis.

Up until recently, it was thought that the three most probable anaerobes involved in bovine footrot are *Dichelobacter nodosus* (ovine footrot), *Fusobacterium necrophorum* and/or *Bacteroides melaninogenicus* (Morck, 1995, Egerton et al., 1989, Blood et al., 1994, Laing and Egerton, 1978, Berg and Loan, 1975). The taxonomy of the "Bacteroides" has undergone significant changes in the past few years (Paster, Dewhirst, Olsen and Fraser, 1994). Species of this bacterial group were once loosely defined as obligately anaerobic, gram - negative, nonsporulating, pleomorphic rods (Paster et al., 1994). Attempts to better classify this bacterial group have been made on the basis of physiologic characteristics, electrophoretic patterns of dehydrogenases, cellular fatty acid and sugar composition, lipid analysis, serology and bacteriophage typing (Paster et al., 1994). Newer methodologies have also been employed to classify these organisms including nucleic acid analyses and 16s rRNA sequencing (Jouisimies - Somer, 1995). On the basis of these studies, it has been proposed that the genus *Bacteroides* be divided into three genera (Paster et al., 1994). The three divisions include the *Bacteroides*, *Prevotella* and *Porphyromonas* (Paster et al., 1994, Jousimies - Somer, 1995). *Porphyromonas* generally consists of asaccharolytic, black pigmented species including *Porphyromonas levii* and *Porphyromonas macacae*, both of animal origin and once known as *Bacteroides melaninogenicus* (Paster et. al, 1994, Jousimies - Somer, 1995). The pigmented anaerobic gram - negative rods are often encountered in clinical specimens but are also found as part of the indigenous flora on various mucosal surfaces (Jousimies - Somer, 1995).

Footrot biopsy specimen cultures, have isolated a novel species of anaerobic, pigmented, gram - negative rod. Continual isolation of these dominant pigmented, gram - negative isolates above all others, warranted further investigation. Upon selective and differential culturing techniques, one dominant isolate was recovered but not clearly identified. To date, standard bacteriological techniques have only been developed for detecting a limited number of anaerobes to any great success, so further techniques needed to be employed including gas-liquid chromatography (GLC) and complex sugar utilization tests (Moncla et al., 1991). The most recent and reliable GLC analysis from the University of California Davis identified the footrot isolate as *Porphyromonas levii.* Other sources give support for this identification as well. *P. levii* is stated as one of the only known black pigmented isolates, from animal sources outside of humans (Paster et al., 1994). In fact, ATCC isolated its stocks of this organism from the rumen of cattle (ATCC 29147) (Paster et al., 1994). This classification, tends to support the idea that most anaerobic pathogens reside as normal flora of the host and provides a clue as to where and how the pathogen may come into contact with the host (Aldridge, 1995, Blood et al., 1994). Also in favor of this identification, it's been shown by our Laboratory, that inoculating healthy cattle with this isolate, via direct inoclulation after abrasion of the interdigital hoof region, causes footrot in cattle (Morck, 1995).

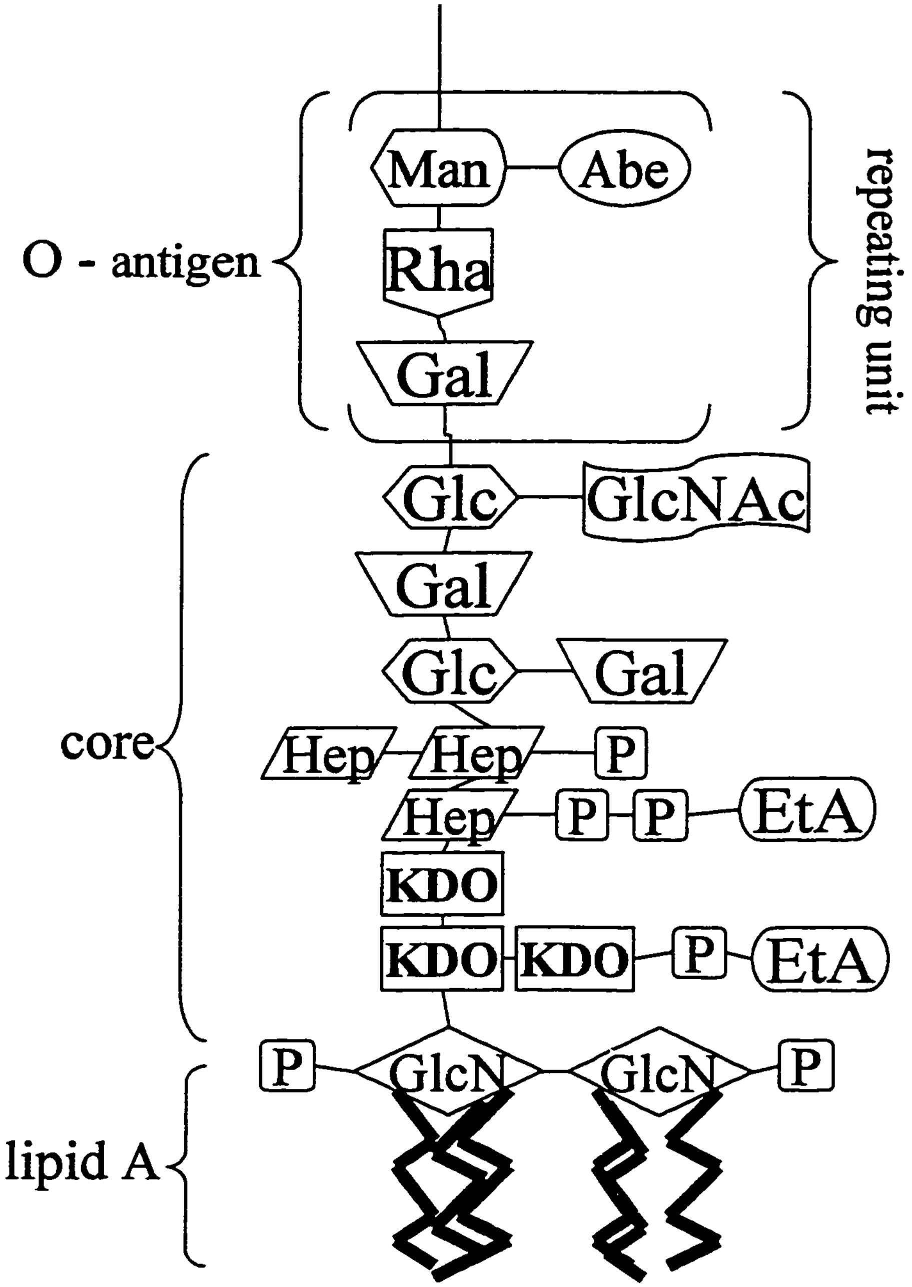
Porphyromonas levii

P. levii is a pleomorphic gram - negative rod, and an obligate anaerobe. Its complex growth requirements often make it very difficult to culture in any great amount. To overcome this limitation, it's often cultured in Cooked Meat Broth, supplemented with hemin and vitamin K, and maintained in a constant anaerobic environment (Engelkirk et al., 1992, Atlas, 1993). Growth on kanamycin/vancomycin laked blood agar (KVLB) gives the characteristic black pigmentation, which fluoresces brick red when exposed to UV light (Engelkirk et al., 1992). Dental research has enabled one to speculate on possible virulence factors possessed by *P. levii*, when considering what is known about other *Porphyromonas sp.*. These factors include: LPS (endotoxin), exotoxins - leukotoxins, encapsulation, fimbriae for attachment, proteolytic enzymes, outer membrane proteins and antibiotic resistance (Aldridge, 1995, Botta et al., 1994). These virulence factors, in any combination may play roles in the pathogenesis of footrot, but for this study, only *P. levii* endotoxin and its effects on bovine endothelial cells will be examined. At this time, no significant research has been carried out studying *P. levii* or its LPS. Research into other *Porphyromonas sp.* has shown common features found within most LPS molecules isolated from gram - negative bacteria, anaerobes or aerobes (Paster et al., 1994, Botta et al., 1994). With this, we can assume that closely related *Porphyromonas sp.* have LPS molecules which contain the characteristic moieties of other species endotoxins, and may only differ in the nature of certain components of the molecule such as the O - side chain.

Bacterial Endotoxin

In gram - negative bacteria, lipopolysaccharides (LPS or Endotoxin) exist as structural components in the outer membrane of the cell envelope (Helander, 1985). Lipopolysaccharides appear to be built according to a common structural principle in all bacteria possessing the molecule (Helander, 1985). LPS molecules consist of a lipid moiety called lipid A, which is covalently linked to a heteropolysaccharide chain (Helander, 1985). The latter consists of two substructures, the core oligosaccharide (proximal to lipid A) and the O - specific chain consisting of repeating oligosaccharide units (Helander, 1985). The lipid A moiety is the hydrophobic part of this large molecule and contains six saturated fatty acid chains that are linked to two glucosamine residues (Stryer, 1990). The core oligosaccharide region of the LPS consists of ten sugar units, followed by the O - side chain, which is made up of many repeating tetrasaccharide units (Stryer, 1990). These two regions contain several sugars that are rarely present elsewhere in nature: 2 - keto - 3 deoxyoctonate (KDO, rarely found elsewhere in nature), an eight carbon sugar; heptose, a seven carbon sugar; and L - rhamnose and abequose, six carbon sugars with -CH_3 instead of -CH_2OH at C-6 (Stryer, 1990). The LPS molecule is negatively charged because several of its sugars are phosphorylated (Stryer, 1990). In most gram - negative bacteria, KDO connects the lipid A and the core oligosaccharide (Kumada, Haishima, Kondo, Umemoto, and Hisatsune, 1993). Hence, KDO plays an important role as a constituent of the inner core region for molecular assembly of LPS (Kumada et al., 1993). This type of structure confers on LPS an amphiphilic character, since these molecules contain both a hydrophobic part (lipid A) and a hydrophilic part (the heteropolysaccharide chain) (Helander, 1985). The LPS molecules are embedded in the outer leaflet of the gram - negative outer membrane through their lipid A component, leaving the polysaccharide fraction protruding outside the membrane (Helander, 1985). Lipopolysaccharides are associated with other outer membrane components through hydrophobic and ionic interactions, whereas they do not appear to be covalently interlinked or linked to cell envelopes (Helander, 1985). Accordingly, LPS molecules are extractable from the bacteria

Figure 2: General representation of the structure of lipopolysaccharides. Present is the basal unit, lipid A. Next to lipid A is the core subunit, containing six (i.e. Galactose, Glucose) and eight carbon sugars (i.e. KDO, heptulose). Connected to the core and extending outwards is the O - antigen region (smooth phenotype only), consisting of repeating oligosaccharide units. (modified from Wolfe, 1993, Figure 8-36)


Man
Abe
Rha
Gal
O - antigen
repeating unit
Glc
GlcNAc
Gal
Glc
Gal
Hep
Hep
P
Hep
P
P
EtA
KDO
KDO
KDO
P
EtA
core
P
GlcN
GlcN
P
lipid A

by relatively mild techniques which do not involve cleavage of covalent bonds (Helander, 1985, Eidhin and Mouton, 1993, Davies, Ali, Parton, Coote, Gibbs and Freer, 1991).

LPS affects myriads of cellular and physiologic functions in animals; such functions are collectively referred to as the endotoxic response (LeGrand, 1990). Many of the body's defense systems (coagulation, inflammation/immune responses, and acute - phase reactions) are triggered by LPS (LeGrand, 1990). LPS is generally viewed by the veterinary profession as one of the major toxic and pathogenic components of gram - negative bacteria (LeGrand, 1990). Endotoxin in the outer membrane of gram - negative bacteria is a highly unusual molecule consisting of three basic components (Stryer, 1990). Lipid A the fraction with most of the endotoxic activity, is an essential structural membrane component; gram - negative bacteria lacking lipid A are not viable (LeGrand, 1990). The inner Lipid A (hydrophobic) component is the "toxic component of the molecule" and is pyrogenic. Next to Lipid A, are the core oligosaccharides with ten sugar units followed by the O - side chain. The O - side chain composition varies among different bacteria (Rietschel et al., 1992). LPS can be lacking the O - side chain altogether (Rough phenotype), or if present (Smooth phenotype), can be made up of 19-40 repeating units consisting of up to 8 different sugars (Rietschel et al., 1992). The O - side chain is also a major immunogenic component of the molecule (Rietschel et al., 1992). Resistance to infection can be conferred by antibodies specific for the O - side chain (Rietschel et al., 1992). The nature of LPS toxicity is still not completely understood; however the connection between LPS and disease has become more apparent in recent times (Rietschel et al., 1992).

Inflammation

The inflammatory process is vital to the survival of all complex organisms, and its functions play a profound role in health and disease (Gallin et al., 1992). Inflammation is normally a localized protective response which serves to destroy, dilute, or wall - off both the injurious agent and the injured tissue. It is characterized in the acute form by the classical signs of pain (dolor), heat (calor), redness (rubor), swelling (tumor), and loss of function (functio laesa) (Gallin et al., 1992). Acute inflammation is characterized during the early stages by the presence of polymorphonuclear neutrophil leukocytes (PMN) which migrate to the inflammatory site (Perretti, Solito and Parente, 1992). Microscopically it involves a complex series of events, including: dilation of arterioles, capillaries, and venules, with increased permeability and blood flow; exudation of fluids, including plasma proteins; and leukocytic migration into the inflammatory focus (Gallin et al., 1992). Diseases characterized by inflammation are an important cause of morbidity and mortality in humans and animals. Commonly, inflammation occurs as a defensive response to invasion of the host by foreign material, frequently microbial in nature (Gallin et al., 1992). Responses to mechanical trauma, toxins, and neoplasia also may result in inflammatory reactions. The accumulation and subsequent activation of leukocytes are central events in the pathogenesis of virtually all forms of inflammation (Gallin et al., 1992). Deficiencies of inflammation lead to a compromised host. Excessive inflammation, either secondary to abnormal recognition of host tissue as foreign, or abnormal turn - off of an otherwise normal inflammatory process, leads to inflammatory diseases. Information concerning the mechanisms whereby inflammatory cells accumulate in tissues, as well as the mechanisms whereby such cells are stimulated to successfully control infection or damage tissues, should provide better insights into the pathogenesis of diseases and may also provide clues for developing more rational forms of therapy (Gallin et al., 1992).

Regardless of etiology, most forms of acute and chronic inflammation are amplified as well as propagated as a result of the recruitment of humoral and cellular components of the immune system (Gallin et al., 1992). The material to be eliminated is recognized as being "foreign" by specific or non - specific means. Non-specific forms of recognition (i.e., recognition of denatured proteins or endotoxins) can be mediated directly by phagocytes (Gallin et al., 1992). Recognition of an antigen generally leads to activation of an amplification system, initiating production of pro-inflammatory substances. Pro-inflammatory cytokines, some of the substances released, are proteinaceous mediators produced by inflammatory cells (Moldawer, 1994). The function of pro-inflammatory cytokines is to communicate to somatic tissues the presence of an inflammatory stimulus (Moldawer, 1994). Unlike mediators derived from the classical endocrine system, cytokines are paracrine agents, meaning that they act locally in a variety of tissues where they are produced (Moldawer, 1994). Pro-inflammatory mediators alter blood flow, increase vascular permeability, augment adherence of circulating leukocytes to vascular endothelium, promote migration of leukocytes into tissues and stimulate leukocytes to destroy the inciting agent (Gallin et al., 1992). The actual destruction of antigens by immune mechanisms is mediated by phagocytic cells. Such cells may migrate freely or may exist at fixed tissue sites as components of the mononuclear phagocyte system (Gallin et al., 1992). Destruction of antigens outside of the mononuclear phagocyte system generally takes place in tissue spaces and is mediated by polymorphonuclear leukocytes (neutrophils) or monocytes, which are recruited from circulating blood. The development of clinically apparent inflammation indicates that the immune system has encountered either an unusually large amount of antigen, an antigen in an unusual location, or an antigen that was difficult to digest (Gallin et al., 1992). Inflammation and tissue injury on an immune basis characterize a wide variety of diseases. Inflammatory processes not only play a central role in mediating immune host defense and wound healing but also participate in the pathogenesis of many diseases (Gallin et al., 1992).

Endothelial Cells

Given the complex structural and functional properties of endothelial cells, it appears appropriate to consider the vascular endothelium as a systemically disseminated organ (Augustin, Kozian and Johnson, 1994, Garlanda and Dejana, 1997). Endothelial cells line the insides of all blood vessels, forming a structurally and functionally heterogeneous population of cells (Gallin et al., 1988, Augustin et al., 1994). Not only do they form the structural basis of blood vessels and provide an antithrombogenic surface, but they also contribute to numerous metabolic functions including coagulation and thrombolysis, control of vasotonus and antigen presentation, as well as basement membrane growth factor synthesis (Augustin et al., 1994). Due to their location and contact with the flowing blood stream, endothelial cells interact with, as well as modulate, the activities of the various biologic systems in blood, particularly circulating leukocytes (Gallin et al., 1988). Endothelial cell adhesion molecules regulate the trafficking of circulating cells and provide an internal map that regulates body compartmentation (Augustin et al., 1994). *In vivo*, endothelial cells are attached to the blood vessel wall by their interaction with the underlying basement membrane which also forms a secondary barrier to the passage of fluid and formed elements into the extravascular compartment (Gallin et al., 1988). Endothelial cell basement membranes contain collagen, glycosaminoglycans, elastin, microfibrils, laminin and some fibronectin and thrombospondin (Gallin et al., 1988). The underlying extracellular matrix forms a complex meshwork consisting of type IV collagen and laminin associated with heparin sulfates with large hydration shells that may form anionic channels (Partridge, Jeffrey and Malik, 1993). These channels may influence permeability of the extracellular matrix to molecules of different charges and sizes (Partridge et al., 1993). Due to the fact that the extracellular matrix serves as a substratum for cell attachment, it also influences endothelial cell shape and cytoskeletal conformation (Partridge et al., 1993).

In tissue culture, endothelial cells are relatively slow - growing and may retain the ability to organize into capillary - like networks when provided with the appropriate substrate (Beilke, 1989). Endothelial cells in culture form a confluent monolayer with intercellular junctions that contribute to the barrier function of the monolayer (Partridge et al., 1993). Endothelial cells are dependent on serum - derived growth factors and attachment factors for successful cultivation (Beilke, 1989). Recent refinements in tissue culture reagents, such as optimized media and purified growth factors, have helped to overcome previous obstacles in obtaining the appropriate growth conditions for study of the role of these cells in infection (Beilke, 1989).

Vascular Endothelium in Infection and Inflammation

A) LPS induced Damage

The first of the two major roles that LPS may play in footrot involves the hypothesis that endotoxin from the footrot pathogen may directly damage endothelial cells (Rietschel et al., 1992, Breider, Kumar and Corstvet, 1990, Sharma, Olchowy and Breider, 1992, Arditi, Zhou, Huang, Luckett, Marra and Kim, 1994, Ishii, Shuyi and Kitamura, 1995, Kang and Williams, 1991). As noted in one study, a single infusion of *Escherichia coli* LPS into sheep resulted in structural evidence of pulmonary endothelial injury, increases in both prostacyclin and prostaglandin E_2 in lung lymph, and an increase in pulmonary microvascular permeability (Meyrick, Hoover, Jones, Berry, and Brigham, 1989). *In vitro,* the action of the *E. coli* LPS on endothelial cells not only resulted in increased production of prostanoids, but also resulted in the cells changing shape, retracting, pyknosis, a lost barrier function and eventually cell lysis (Meyrick, Hoover, Jones, Berry, and Brigham, 1989). LPS molecules from *E. coli* and *Salmonella typhosa* have both been shown to be directly toxic to bovine pulmonary arterial endothelial cells (Paulsen, Mosier, Clinkenbeard and Confer, 1989). *In vitro* bovine pulmonary endothelial monolayers were treated with *Pasteurella haemolytica* LPS (Breider et al., 1990, Paulsen et al., 1989). Postinoculation, the LPS appeared to have produced severe damage to the endothelial cells, indicated by extensive cellular detachment, and morphologic changes characterized by cell contraction, cytoplasmic blebbing, and loss of monolayer confluency (Breider et al., 1990, Paulsen et al., 1989). Subsequently the cells became round and detached (Paulsen et al., 1989, Ryan, Vann and Nolop, 1989). Other studies have shown that gram - negative bacterial endotoxin can directly damage pulmonary endothelial cells *in vitro* in quantities of as little as 1 µg, per milliliter (Paulsen et al., 1989). *In vivo*, it has been shown that intravenous inoculation of LPS in sheep incites acute pulmonary inflammation and endothelial cell injury (Breider et al., 1990). A similar process may occur in cattle subcutaneous tissues, as a result of anaerobic gram - negative infection. Endotoxin exposure of endothelial cells can cause release of toxic oxidative mediators such as hydrogen peroxide, by the endothelial cells affected (Gallin et al., 1988). These are released to kill bacteria, but may result in toxic effects on endothelial cells themselves as well (Gallin et al., 1988, Ryan et al., 1989). These cytotoxic effects may play a major role in the clinical signs seen in footrot.

In response to bacterial LPS, endothelial cells are converted to an activated phenotype expressing both pro-inflammatory and procoagulant properties that include the induction of leukocyte adhesion molecules and tissue factor expression (Noel, Sato, Mendez, Johnson and Pohlman, 1995). It has been shown that LPS - induced endothelial cell injury or activation, is serum dependent and that a soluble form of the CD14 receptor in serum as well as LPS binding protein (LBP) are required for LPS - induced vascular endothelial cell responses, including cytotoxicity (Arditi et al., 1994, Ishii et al., 1995, Townsend and Scheld, 1995, Yang, Khemlani, Dean, Carter, Slauson and Bochsler, 1994, Noel et al., 1995, Rietschel et al., 1992). These responses seen in endothelial cells are thought to occur via glycosylphosphatidylinositol anchored to the plasma membrane of endothelial cells (Noel et al., 1995). The interaction between the LPS/CD14 complex and glycosylphosphatidylinositol induces rapid phosphorylation of tyrosine residues in mitogen - activated protein kinases (Noel et al., 1995). Activation of a distinct 38 - kDa protein kinase of the mitogen - activated protein kinase family leads to further phosphorylation events in a protein kinase cascade that is ultimately thought to result in the changes seen within endothelium when LPS is present, such as the cytotoxicity and the expression of pro-inflammatory mediators (Noel et al., 1995).

B) LPS induced Pro-inflammatory Responses

LPS molecules are known to induce a variety of inflammatory responses (Endo et al., 1997). Of these, increased capillary permeability and dilation of fine blood vessels are major features of inflammation and help to provide a suitable local environment for effective immune reactions and the activities of immune cells (Endo et al., 1997). However, excessive inflammation is harmful and sometimes lethal (Endo et al., 1997). During inflammation, resident cells, circulating leukocytes, and platelets, as well as the endothelium lining the vessels in the infected area, become both the targets and producers of cytokines (Gallin et al., 1992, Houston, Carson and Esmon, 1997). The endothelium cannot be regarded as an inert surface; it is under the composite influence of circulating immune effector cells and foreign antigens (LPS), and is consequently subject to a number of morphologic and functional alterations (Beilke, 1989).

The vascular endothelium is a critical target for endotoxin and many cytokines are released during gram - negative infection, which may play important roles in vascular injury (Arditi et al., 1994). If the characteristics of vascular endothelium are altered by infection, a localized cellular inflammatory process may ensue (Beilke, 1989). Indirectly, LPS from footrot pathogens may trigger an inappropriate immune response leading to further endothelial cell and surrounding tissue damage as seen in footrot (Endo et al., 1997, Fratti, Ghannoum, Edwards and Filler, 1996). Indirectly, bound or released LPS from the pathogen, may stimulate a variety of responses in endothelial cells related to pro-inflammatory immunity. It's been shown that endothelial cells found in various parts of the body, have the capacity to produce immunoregulatory substances and pro-inflammatory cytokines in response to exposure with gram - negative bacterial endotoxin (Feder, Todaro and Laskin, 1993, Houston et al., 1997, Meyrick et al., 1989, Noel et al., 1995, Beilke et al., 1989). LPS activated endothelial cells have been shown to release interleukin - 1, interleukin - 6, interleukin - 8, monocyte chemotactic protein - 1, and platelet activating factor which are substances involved in the recruitment and activation of leukocytes (Noel et al., 1995, Townsend et al., 1995, Kang et al., 1991, Tissot Van Patot et al., 1996, Huber, Kunkel, Todd and Weiss, 1991, Smart and Casale, 1993, Hoch, Schraufstatter and Cochrane, 1996, Sticherling, Hetzel, Shroder and Christophers, 1993, Baggioline, Loetscher and Moser, 1995). Studies have shown that endothelial cells express class 2 immune response genes and the interleukin-1 (IL-1) gene to a greater degree during gram - negative bacterial infection and thus may be capable of amplifying the lymphocytic proliferative process (Beilke, 1989). Other studies have implicated roles for the cytokines TNFα (Tumor Necrosis Factor Alpha), and GM - CSF (Granulocyte Macrophage Colony Stimulating Factor) produced by LPS activated endothelial cells, in the immune response as well (Shapira et al., 1996, Sordillo et al., 1992, Smart and Castle, 1993, Tissot Van Patot et al., 1996). The release of these inflammatory substances can eventually lead to an excessive stimulation of host immunity, particularly neutrophils and macrophages (Shapira, Soskolne, Houri, Barak, Halabi and Stabholz, 1996, Ishii et al., 1995, Sharma et al., 1992). Excessive immunological responses result in these cells called into the site of infection to release active oxygen species and other mediators that result in the cytopathology of endothelial cells (Janssen, Houten, Borm and Mossman, 1993, Mandell, 1995). Active oxygen species for example, are potentially harmful to cells because they interact with and modify a spectrum of biomolecules (Janssen et al., 1993). Some biochemical effects of active oxygen species resulting in progressive endothelial cell damage include lipid peroxidation, oxidative modification of proteins and DNA alterations (Janssen et al., 1993). Thus, endothelial cell activation may promote leukocyte infiltration and microvascular thrombosis contributing to the pathogenesis of anaerobic bacterial infections including bovine footrot (Noel et al., 1995)

C) Neutrophil - Endothelial Interactions

Cell - Cell interactions represent an important aspect on inflammation (Tissot Van Patot, MacKenzie, Tucker and Voelkel, 1996). Activated cells such as monocytes, neutrophils and endothelial cells release proinflammatory cytokines, lipid mediators and oxygen radicals, causing adhesion of cells to endothelium and subsequent migration through the intima (Tissot Van Patot et al., 1996). Acute inflammatory reactions are usually characterized by PMN margination in postcapillary venules and emigration across the vascular endothelium (Wiedermann, Schratzberger and Kahler, 1994). Polymorphonuclear leukocytes adhere to the surfaces of endothelial cells *in vivo* and must traverse across the endothelial cell barrier before reaching the extravascular space (Gallin et al., 1988, Tissot Van Patot et al., 1996). *In vitro*, neutrophils adhere to monolayers of endothelial cells to a much greater degree than to either smooth muscle cells or fibroblasts (Gallin et al., 1988). Neutrophils move about on the surface of endothelial cells and migrate through the cell layer via intercellular junctions (Gallin et al., 1988). Several recent studies all corroborate that neutrophils and other leukocytes bind to endothelium via specific receptors (Beilke, 1989). Adhesion of neutrophils to endothelial cells has been shown to increase after exposing endothelial cells to pro-inflammatory mediators such as IL-1 and TNFα and LPS (Gallin et al., 1988, Wiedermann et al., 1994). These mediators appear to induce synthesis of an endothelial cell - surface protein(s) that promotes neutrophil adherence by a mechanism involving the CDw18 complex present on neutrophils (Gallin et al., 1988). The cytokine induced phenotype of endothelial cells during inflammation has been characterized most extensively in the last few years (Augustin et al., 1994). Inducible endothelial cells adhesion molecules, such as E - selectin, P - selectin, VCAM - 1 (vascular cell adhesion molecule 1), ELAM - 1 (endothelial leukocyte adhesion molecule 1), GMP - 140 (granule membrane protein 140), PADGEM (platelet - activation - dependent granule - external membrane protein), and ICAM - 1 (intercellular adhesion molecule 1), are expressed on the cell surface following cytokine exposure, enabling them to direct circulating cells to sites of inflammation (Augustin et al., 1994, Gallin et al., 1992). Mice made deficient in ICAM - 1 by targeted gene disruption are resistant to a lethal dose of LPS, which mediates the majority of adverse effects observed with endotoxemia (Augustin et al., 1994).

Near sites of inflammation neutrophils undergo a series of morphologic changes associated with adhesion to the endothelium (Abramson and Wheeler, 1993). Assuming a spherical shape and rolling along the blood vessel walls, these include cessation of movement and flattening, membrane ruffling, and modulation of membrane receptors (Abramson et al., 1993). The PMN then migrate through endothelial cell junctions apparently without disrupting the electrical resistance of the endothelial monolayer, and travel to the actual inflammatory site by amoeboid movements still under the influence of chemoattractants (Abramson et al., 1993, Wiedermann et al., 1994, Russman, Ruckdeschel and Heesemann, 1996). The importance of the CD11/CD18 molecules in neutrophil adherence to endothelial cells has been well documented (Gallin et al., 1992). Each of the three heterodimeric leukocyte surface molecules (CD11a/CD18, CD11b/CD18, CD11c/CD18) contains a common ß chain (CD18) and a unique α chain (CD11a, CD11b, CD11c) (Gallin et al., 1992). *In vitro* studies using unstimulated neutrophils indicate that these leukocytes bind to ICAM - 1 on the surface of endothelium by CD11a/CD18 (lymphocyte function associated antigen - 1 [LFA - 1]) - dependent mechanisms (Gallin et al., 1992). Stimulation of neutrophils with chemotactic factors enhances neutrophil binding to ICAM - 1 by a CD11b/CD18 (MAC - 1) - dependent processes (Gallin et al., 1992). Chemotactic factors also stimulate adherent neutrophils to produce H_2O_2, and this adhesion - dependent respiratory burst is mediated by CD11b/CD18 (Gallin et al., 1992).

Transendothelial migration of neutrophils in response to a chemotactic gradient also involves CD11a/CD18, CD11b/CD18, and ICAM - 1 molecules (Gallin et al., 1992, Wiedermann et al., 1994). However, all the molecules involved in leukocyte extravasation are not known (Gallin et al, 1992). Significant migration of neutrophils across endothelial cells can also occur in the absence of a chemotactic gradient, by exposure of the endothelial cells to cytokines or LPS (Gallin et al., 1992). This endothelium induced trans - endothelial neutrophil migration also appears to be dependent on the CD11a/CD18, CD11b/CD18, and ICAM - 1 molecules (Gallin et al., 1992). It was recently suggested that once tight contact with the endothelium is established, the neutrophils are attracted to components of the endothelial intercellular junctions or the subendothelial matrix (Wiedermann et al., 1994). Consequently, it is possible that a loosening of intercellular junctions is induced by some unknown mediator to allow PMNs to be attracted, and thus migrate through endothelial monolayers via intercellular junctions (Wiedermann et al., 1994).

Inflammatory Cytokines

A) Overview

The generation of a protective response in the setting of infectious challenge is a complex and dynamic process that involves the coordinated expression of both proinflammatory and antiinflammatory cytokines (Standiford and Huffnagle, 1997). Cytokines are a class of soluble proteins that mediate signals throughout the immune system as well as between immune effector cells and other cell populations (Ye and Young, 1997). Proinflammatory cytokines, including IL-1ß, TNFα and GM - CSF, are mediators produced by a variety of cells including vascular endothelium and inflammatory cells (Moldawer, 1994, Feder et al., 1993). The function of proinflammatory cytokines is to communicate to somatic tissues the presence of an inflammatory stimulus such as LPS (Moldawer, 1994, Mackay, 1997). The large number and variety of cytokines that are produced by the body, is indicative of the importance of these molecules, and suggests that diversity in the chemokine system is important for such events as regulating leukocyte movement through tissues (Mackay, 1997). This class of proteins includes interleukins, interferons, and chemokines (Ye et al., 1997). Unlike mediators derived from the classical endocrine system, cytokines are paracrine agents, meaning that they act locally in a variety of tissues where they are produced (Moldawer, 1994). For example, the reticuloendothelial system contains inflammatory cells, capable of secreting proinflammatory cytokines, including interleukin - 1 and TNFα (Moldawer, 1994). In bacterial infection, the elaboration of activating and/or chemotactic cytokines is necessary to generate sufficient leukocyte recruitment/activation required for eradication of the particular pathogen (Standiford et al., 1997). Cytokines can modulate innate or acquired immune responses by either directly or indirectly mediating leukocyte recruitment or by activating leukocytes that have accumulated at specific sites of microbial invasion (Standiford et al., 1997). Cytokines can also influence microbial clearance by serving as afferent signals that regulate the expression of other effector molecules (Standiford et al., 1997). The list of cytokines involved in these processes is long and their relative importance varies depending on the type of infection (Standiford et al., 1997).

The production of cytokines at various tissue sites depends, in part, on the proximity of the site to the injurious stimulus. Trends have been observed suggesting that cytokine concentrations increase with the magnitude of injury (Moldawer, 1994). It has been shown that the expression of cytokines is tightly controlled in the producing cells, and one of the most important regulatory steps in this control is via gene transcription (Ye et al., 1997, Dinarello, 1996). The transcription of most cytokine genes is silent until a producing cell is activated by extracellular stimuli such as LPS (Ye et al., 1997).

B) Interleukin - 1ß and Tumor Necrosis Factor α

Two inflammatory cytokines, IL-1ß and TNFα appear to play predominant roles in the normal inflammatory response (Moldawer, 1994). Although these cytokines have become synonymous with various clinical pathologies, they exert useful effects in the normal physiologic inflammatory response (Moldawer, 1994). The cytokines IL-1ß and TNFα affect nearly every tissue and organ system (Gallin et al., 1992, Armstrong, Gardiner, Kirk, Halliday and Rowlands, 1997). These cytokines are the prototype of the proinflammatory cytokines; IL-1ß and TNFα induce the expression of a variety of genes and the synthesis of proteins which, in turn, induce acute and chronic inflammatory changes (Gallin et. al, 1992). IL-1ß and TNFα are often implicated as key mediators of the biological responses to bacterial LPS, infection and inflammatory stimulants (Gallin et. al, 1992).

The functions of IL-1ß and TNFα overlap to a great degree except for the fact that unlike TNFα, IL-1ß can activate T lymphocytes and is involved in stem cell activation (Gallin et al., 1992). These cytokines increase neutrophil margination and activate the antimicrobial activity of monocytes, macrophages, neutrophils and eosinophils, leading to induction or enhancement of microbicidal activity, protease release, respiratory burst, adhesion molecules, chemokines and/or other cytokines (Moldawer, 1994, Standiford et al., 1997, Perretti et al., 1992, Dinarello, 1996). Both cytokines induce a non specific acute - phase response, characterized by fever and anorexia (Moldawer, 1994). These cytokines also increase skeletal muscle degradation to yield amino acids for gluconeogenic precursors and substrates for increased visceral protein synthesis (Moldawer, 1994). At the wound site, these cytokines increase vascular proliferation, osteoclastic activity, and collagen synthesis (Moldawer, 1994, Standiford et al., 1997). These responses, induced by the appropriate level and duration of cytokine production, constitute a beneficial response to clinical insult and are involved in the remodeling of wound tissue (Moldawer, 1994). However, when excessive acute concentrations or chronic long - term increases of these cytokines occur, these benefits are often outweighed by adverse physiological effects (Moldawer, 1994).

C) IL-1ß

IL-1ß is a proinflammatory cytokine that is defined as a "primary cytokine" based upon the prediction that its release, as an isolated event, would be sufficient to induce inflammation (Kupper and Groves, 1995). There is a dramatic increase in IL-1ß production by a variety of cells in response to infection, microbial toxins, inflammatory agents, products of activated lymphocytes, complement and clotting factors (Gallin et al., 1992). Recent studies of the functions of IL -1ß have implicated this cytokine as a mediator of gene expression in a number of cells it effects, including endothelial cells during an inflammatory response (Dinarello, 1996) Increased expression of various genes by IL-1ß include: genes for cytokines including TNFα and CM - CSF, cytokine receptors, pro-inflammatory mediators (inducible nitric oxide synthase), hepatic acute phase reactants, growth factors, clotting factors (fibrinogen), tissue remodeling, neuropeptides, lipid synthesis, oncogenes, adhesion molecules (ICAM - 1, ELAM - 1, VCAM - 1), receptors, extracellular matrix components (laminin), and many others (Dinarello, 1996). Decreased expression of genes by IL-1ß include: housekeeping genes, receptors, cytokines, extracellular matrix proteins, and many others (Dinarello, 1996). It then becomes even more apparent that the biological effects of IL-1ß are broad and extensive, and that the outcome of its induction may result in a variety of responses in receptor cells, with the aim of combating infection and other cellular disfunctions (Dinarello et al., 1996). It also becomes apparent that an inappropriate induction of IL-1ß release and function, may result in inappropriate responses in receptor cells, and thus damage to these cells may ensue (Dinarello, 1996). For example, the generation of nitric oxide in disease appears to be a fundamental event (Dinarello, 1996). Several studies have shown that IL-1ß induces nitric oxide synthesis in a variety of cells, including endothelial cells, *in vivo* and *in vitro* (Dinarello, 1996). With the purpose of producing nitric oxide to combat infection, it can be seen that over - stimulation of nitric oxide synthesis by these cells, would eventually have detrimental effects on the cells themselves, leading to their own destruction and death (Dinarello, 1996). Other studies, further implicate IL-1ß in receptor mediated cell death because it has been shown that this cytokine induced apoptosis or programmed cell death, in cells for which it was in contact with (Zychlinsky and Sansonetti, 1997, Wendt, Polunovsky, Peterson, Bitterman and Ingbar, 1994). In a variety of bacterial infections, IL-1ß has been implicated as a eukaryotic host cell programmed cell death mediator, and it again becomes evident that inappropriate expression of IL-1ß could have detrimental effects on cells (i.e. endothelial cells) within its localized area of expression (Zychlinsky et al., 1997).

D) TNFα

TNFα is one of the major cytokines produced immediately following both antigen specific and non - specific stimulation, hence the designation of TNFα as an early response cytokine (Standiford et al., 1997). TNFα, a product of stimulated monocytes and macrophages, is also produced by lymphocytes, endothelial cells and keratinocytes (Gallin et al., 1992). The proinflammatory properties of TNFα are absolutely required for the development of effective innate and acquired immunity (Standiford et al., 1997).

Cytokines exist both as free secreted and cell - associated forms. For example, TNFα exists as a high molecular weight, cell associated membrane form in inflammatory cells (Moldawer, 1994). This form of TNFα acts by direct cell to cell contact (Moldawer, 1994). The dichotomous nature of this cytokine also helps to explain why systemic concentrations of circulating TNFα may not be reflective of the degree of local TNFα activity (Moldawer, 1994)

Recently it has been shown that TNFα may increase vascular endothelium permeability through alteration of the extracellular matrix via the production of a 96 - kDA gelatinase (Partridge et al., 1993). The induced metalloproteinase was purified *in vitro* presumptively from TNFα exposed bovine endothelial cells, and found to cleave fibronectin, laminin, collagen and gelatin (Partridge et al., 1993). Further evidence that TNFα influences bovine vascular endothelial barrier function *in vitro* comes from the finding that TNFα induces endothelial cell F - actin depolymerization, new actin synthesis and barrier dysfunction (Goldblum, Ding and Campbell - Washington, 1993). It has also been shown that bovine endothelial barrier dysfunction and p42 oxidation is influenced by TNFα induced expression of nitric oxide (Ferro, Gertzberg, Selden, Neumann and Johnson, 1996). One study indicated that a mechanism through which TNFα alters bovine endothelial cell barrier function involves a reduction in intracellular adenosine 3', 5' - cyclic monophosphate (cAMP) content due in part to increased cyclic nucleotide phosphodiesterase activities (Koga, Morris, Ogawa, Liao, Bilezikian, Chen, Thompson, Ashikaga, Brett, Stern and Pinsky, 1995). Inducers such as TNFα , like IL-1ß, cause a large percentage of endothelial cells to undergo apoptosis, even in the absence of protein synthesis (Wendt et al., 1994). Endothelial cells do not have a propensity to undergo apoptosis spontaneously, since only a small percentage of cells undergo apoptosis when they are cultured in the absence of serum (Wendt et al., 1994). Endothelial cell apoptosis induced by TNFα, probably is a complex process involving intracellular components in addition to extracellular inducers (Wendt et al., 1994)

E) Granulocyte - Macrophage Colony Stimulating Factor

Another mediator shown to be released by endothelial cells in response to infection and endotoxin is Granulocyte - Macrophage Colony Stimulating Factor (GM - CSF) (Gallin, et al., 1988). Colony - stimulating factors are cytokines produced by a variety of immune cells that are required for the proliferation and maturation of hematopoietic stem cells (Standiford et al., 1997). In addition, these factors have been shown to regulate the cell activities of mature leukocyte populations (Standiford et al., 1997). Hence, Colony-stimulating factors are a group of cytokines that control the production, proliferation and differentiation of leukocytes (Abramson et al., 1993).

The production of granulocytes and mononuclear phagocytes from bone marrow progenitors is tightly regulated in the adult to maintain a relatively constant circulating level of effector cells in the peripheral blood (Gallin et al., 1992). There is no theoretical limit to the expansion of myelopoiesis, and in disease states there may be more than a 10 - fold increase in circulating granulocytes (Gallin et al., 1992). The humoral regulators of myelopoiesis has been defined by *in vitro* culture systems (Gallin et al., 1992). Of these regulators, GM - CSF was analyzed, and it was found that the purified substance was a glycoprotein with a major species of approximately 22,000 molecular weight and was found to be identical to an earlier molecule described as "neutrophil - migration inhibitory factor" (Gallin et al., 1992).

GM - CSF can be produced by a wide spectrum of cell types, including fibroblasts, epithelial, endothelial, smooth muscle cells, lymphocytes, monocytes/macrophages, mast cells, granulocytes and some carcinoma cells (Xing, Braciak, Ohkawara, Sallenave, Foley, Sime, Jordana, Graham and Gauldie, 1996). Few studies have assessed the role of GM-CSF in antimicrobial host defense (Standiford et al., 1997). It has been shown that GM - CSF stimulates the proliferation and maturation of both PMN and macrophages, with the activating properties of the cytokine being primarily directed toward mature cell populations (Standiford et al., 1997). *In vitro* it has been shown that GM - CSF stimulates the production of neutrophil, monocyte/macrophage, and eosinophil colonies in semisolid gel culture (Gallin et al., 1992). Therefore it has been shown that GM - CSF is a eosinophiloprotein, as well as a stimulator of neutrophils and monocytes (Gallin et al., 1992). GM - CSF receptors have been identified on the above cells, as well as on endothelial cells, indicating further functional properties of this mediator outside of its myelopoiesis properties (Gallin et al., 1992). GM - CSF has a multitude of actions on mature neutrophils, and other leukocytes, which results in increased functional capacity

(Gallin et al., 1992). GM - CSF increases the life span of neutrophils *in vivo* and *in vitro*, which increases their metabolic activity, and enhances the ability of neutrophils to phagocytose and kill microorganisms (Gallin et al., 1992, Sordillo et al., 1992). GM - CSF profoundly affects the movement of neutrophils by inhibiting random migration and by increasing chemotactic responses to some stimuli (Gallin et al., 1992). GM - CSF is known to prime neutrophils, resulting in the neutrophils becoming more responsive to chemotractants (Abramson et al., 1993, Sordillo et al., 1992). However, GM - CSF also functions in decreasing neutrophil and macrophage migratory activities after arriving into an area of inflammation (Abramson et al., 1993, Sordillo et al., 1992). These findings have been confirmed *in vitro* by demonstrating that bovine GM - CSF has similar and direct actions on neutrophils (Sordillo et al., 1992). GM - CSF also causes increased expression of neutrophil adhesion proteins and induction of IL - 1 synthesis (Gallin et al., 1992). An important action of GM - CSF on neutrophils is cellular priming for enhanced oxidative metabolism (Gallin et al., 1992, Sordillo et al., 1992, Tao, Dougherty, Johnson and Pickett, 1993). This action of GM - CSF has been confirmed using bovine GM - CSF by monitoring the production and activity of superoxide as well as ß - glucosaminidase by bovine neutrophils *in vitro* (Tao et al., 1993). GM - CSF also promotes migration and/or proliferation of structural cells including fibroblasts, endothelial, and smooth muscle cells, perhaps playing a role in tissue repair as well (Xing et al., 1996).

These findings clearly demonstrate that GM -CSF is more than just a hematopoietic cytokine, and may play a pivotal role in the multiple pathological processes underlying numerous illnesses, including bacterial infections (Xing et al., 1996, Tao et al., 1993). It also becomes apparent that some of the functions of GM - CSF overlap with those cytokines previously discussed, and may also act in conjunction with IL-1ß and TNFα, contributing to the inflammatory processes aimed at combating bacterial infections including bovine footrot.

Neutrophils

A) Beneficial Effects of...

The outcome of the interaction between certain micro-organisms and neutrophils determines health or disease (Abramson et al., 1993). Once the barriers of the skin and mucous membranes have been breached, the host's health depends on PMN and other host resistance factors to combat invading microorganisms that can cause infection (Abramson et al., 1993). As soon as microbes invade the tissues, circulating PMN are activated, adhere to activated endothelial cells, and move through the endothelial barrier to the site of the infection (Abramson et al., 1993). While PMN migration occurs, the microbes are opsonized; that is, the microbial surface is coated with antibody and complement factors for recognition by PMN (Abramson et al., 1993, Marks, Todd and Ward, 1989). PMN have receptors specifically designed to bind to the Fc fragment of the immunoglobulin molecules present on the surface of the opsonized bacteria and other receptors designed to bind the activated complement factors (Abramson et al., 1993). The complement factors and the antibody molecules are ligands that promote attachment of the microbe to the cell enhancing otherwise inefficient microbe - phagocyte interactions (Abramson et al., 1993). Uptake of unopsonized *Staphylococcus aureus* by neutrophils is much higher on an endothelial cell surface than on plastic, suggesting that endothelial cells support neutrophils in killing bacteria, although the mechanism is not understood (Gallin et al., 1988). A possible serum factor other than complement or antibody that functions as an opsonin are the lipopolysaccharide binding proteins (LBP) (Abramson et al., 1993). LBP is an acute - phase reactant that binds bacterial LPS (Abramson et al., 1993). LBP can bind to the surface of gram - negative bacilli and strongly enhances attachment of these particles to the cell membrane of phagocytic cells (Abramson et al., 1993). This binding leads to enhanced phagocytosis (Abramson et al., 1993). After this receptor - mediated attachment, PMN engulf the microbes and ingestion takes place (Abramson et al., 1993). Once a microbe is phagocytosed by the PMN, it is usually rapidly killed and digested (Abramson et al., 1993).

Phagocytosis is the process that leads to ingestion of the bacterium (Abramson et al., 1993). It involves both attachment to receptors and subsequent engulfment (Abramson et al., 1993). Neutrophils take up microorganisms into phagosomes that fuse with secretory granules (lysosomes) to form phagolysosomes (Abramson et al., 1993). Killing and digestion of microorganisms take place within these phagolysosomes (Abramson et al., 1993). PMN are able to kill microorganisms by two distinct mechanisms (Abramson et al., 1993). One antimicrobial system is oxygen dependent, while the other can kill bacteria in the absence of oxygen (Abramson et al., 1993, Mandell, 1995). The oxygen - dependent antimicrobial mechanisms are set in motion when PMN undergo a 'respiratory burst' (Abramson et al., 1993). NADPH oxidase in the phagolysosome membrane is activated and reduces O_2 to superoxide ($^{\cdot}O_2^-$) (Abramson et al., 1993). O_2 reduction is the first step in a series of reactions that produce toxic oxygen species such as H_2O_2 (hydrogen peroxide), superoxide, and hypochlorous acid (Abramson et al., 1993, Mandell, 1995). These species are powerful oxidants, and the oxidation of bacterial surface and cytoplasmic components by these oxygen species results in the killing of the microorganism within the phagolysosome (Abramson et al., 1993). PMN cytoplasmic granules contain additional antimicrobial agents that are released into phagolysosomes and do not require the production of oxidants for activity (Abramson et al., 1993, Mandell, 1995). These agents include proteases, other hydrolytic enzymes such as phospholipases, glycosidases and lysozymes and other proteins and peptides that disrupt microbial functions or structural components (Abramson et al., 1993). Thus non - oxygen dependent killing mechanisms include an array of biologically active molecules that kill the invading microorganism. It is assumed that most microorganisms are digested after being killed, and that bacterial components are rapidly degraded by the numerous granule - associated degradation enzymes (Abramson et al., 1993).

B) Detrimental Effects of...

Recently, the detrimental potential of neutrophils has been increasingly realized and has become a part of the analysis of the pathophysiology and treatment of a variety of common diseases (Abramson et al., 1993). It should be noted that non - neutrophil related injury could incite an inflammatory response which could then recruit neutrophils as a secondary phenomenon (Abramson et al., 1993). Several ways exist in which neutrophils might induce tissue injury (Abramson et al., 1993). The major possibilities include production and release of toxic oxygen metabolites, granular components, or products of arachidonic acid metabolism (Abramson et al., 1993). Neutrophils contain a number of components that can cause endothelial cell injury or detachment *in vitro* (Gallin et al., 1988). Activation of neutrophils on endothelial cell monolayers causes endothelial cell detachment that is due to release of elastase from neutrophils (Gallin et al., 1988). PMN leukocytes contain heparinase, which can digest heparin sulfate in the basement membrane; this degradation is facilitated by serine proteases (Gallin et al., 1988). Neutrophils also release hydrogen peroxide and oxygen radicals, which can cause endothelial cell lysis (Gallin et al., 1988). Thus, when the neutrophil is exposed to one or various stimuli, a process occurs which is similar to the reaction following ingestion of bacteria, except that the reaction appears to be directed extracellularly into surrounding tissues rather than internally into phagocytic vacuoles (Abramson et al., 1993).

Polymorphonuclear neutrophils have been shown to be a major contributor to tissue injury and organ failure in various forms of ischemia and shock (Barroso - Aranda, Zweifach, Mathison and Schmid - Schonbein, 1995). Studies have implicated neutrophils as important mediators of vascular damage in the context of endotoxemia and as participants in the release of pro-inflammatory cytokines such as IL-1ß, TNFα and GM - CSF (Klein, Ison, Peakman, Levin, Hammerschmidt, Frosh and Heyderman, 1996). In support of this, the severity of infection appears to correlate directly with the degree of host inflammatory cell activation (Klein et al., 1996). It has become increasingly evident that along with the above mentioned cytokines, neutrophils play a significant role in the pathogenesis of bacterial infections. In response to infection, neutrophils are intended to engulf invading pathogens via phagocytosis, whereby the microbes are rapidly ingested and killed (Abramson et al., 1993). When studying bacterial meningitis and the role of LPS in disrupting the blood brain barrier composed mostly of endothelial cells, it has been found that large amounts of cytokines are produced and released into the CSF (cerebral spinal fluid) including IL-1ß and TNF-α (Townsend and Scheld, 1995). Furthermore one of the hallmarks of bacterial meningitis is the finding of high concentrations of leukocytes in CSF, which is ordinarily devoid of cells (Townsend et al., 1995). Thus the release of the pro-inflammatory mediators by the blood brain barrier after exposure to LPS, results in the movement of monocytes into the area, leading to further inflammation and tissue damage (Townsend et al., 1995). Monocytes and neutrophils inflict endothelial injury in such cases through the release of proteases, glycanases, and reactive oxygen metabolites; a process that is consequent on their activation and adhesion to the endothelium (Klein et al., 1996). Oxidative damage by neutrophils and tissue breakdown products cause endothelial cell lysis, vascular leakage, disseminated intravascular coagulation, and other sequelae of septicemia (Beilke, 1989). Neutrophils are usually the predominant inflammatory cell initially recruited to sites of inflammation via the release of cytokines (Smart and Castle, 1993). It appears that after endothelial activation by endotoxin, pro-inflammatory mediators are released to recruit neutrophils and other leukocytes, leading to detrimental consequences. With this, a link between LPS, endothelial cells, cytokines and neutrophils is becoming evident, leading to an explanation into the severity of many bacterial infections including bovine footrot.

Summary

To conclude this introduction and review, with the scope and complexity of this field, and based on the background material provided, a short summary dealing with the role of endothelial cells, *P. levii* LPS, cytokines and neutrophils in bovine footrot is needed to be able to focus on the objectives of the proposed research. In terms of the immunopathogenesis of acute footrot, it is proposed that with a *P. levii* infection, LPS stimulated endothelial cells will produce and release pro-inflammatory mediators (IL-1ß, TNFα, GM-CSF) which will result in the attraction and proliferation of circulating leukocytes, including neutrophils, into the area. The early inciting events governing leukocyte - endothelial cell binding during infection have not been fully identified but certainly represent a critical step in the ensuing inflammatory process seen in footrot (Beilke, 1989). PMNs arrive at the area of infection and bind to endothelial cells, via receptor mediated events. Once in the infected area, and/or bound to the endothelial cells, the neutrophils carry out their functions as professional phagocytes, and with the release of oxidative mediators and proteins/enzymes, attempt to rid the area of the *P. levii* pathogens. However, as mentioned previously, this may end up having detrimental effects on the endothelium and tissues in proximity to the neutrophils. As the neutrophils carry out their functions including the release of cytokines, endothelial cells continue to release cytokines and all together this contributes to an inappropriate amplification of the immune response, which in turn amplifies the damage to infected tissues (Gallin, et al., 1992). The initial endothelial produced cascade of mediators and cellular responses is meant to limit and localize the size and amount of the infection and inflammation (LeGrand, 1990). Nevertheless, the amplifying effects of the continuous cytokine release and neutrophil infiltration, actually contributes to the initiation of a deeper seated, more serious pathological lesion. In summary, it is hoped that with this brief and limited overview of footrot and the possible pathoimmunological events involved in the disease, that the research following, will aid in uncovering the properties of *P. levii*, that result in the hallmarks of footrot seen *in vivo*.

Research Objectives

The methods and materials proposed for this study are designed to complete two main objectives. The first is to examine the cytopathic effect (CPE) of *Porphyromonas levii* LPS on bovine endothelial cells *in vitro*. The second is to examine the specific pro-inflammatory cytokine responses of bovine endothelial cells to *Porphyromonas levii* LPS *in vitro*.

Methods and Materials

1. Bacteria

1.1 Growth

The clinical Bovine footrot isolate 7.5, presumptively identified as *Porphyromonas levii (P. levii),* was used exclusively in these investigations. *P. levii* was initially isolated from interdigital biopsy samples taken from animals suffering from footrot by Dr. DW Morck. *P. levii* was stored in -85°C (Cryo - Fridge, Baxter Scientific) in culture swabs (Difco, Detroit, MI) or biobeeds (Microbank™, Pro Lab Diagnostics, ON, Canada) until needed. Growth medium was either obtained commercially or made, depending on the proposed use of the bacterial cultures grown. All media were pre - reduced, anaerobic and sterile (PRAS) before used to culture *P. levii.* User prepared media were made as per manufacturer's instructions. The specific use of media by composition depended on what the bacteria were needed for at that particular time in the research and will be described in later sections. All *P. levii* cultures were grown under strict anaerobic conditions (5% H_2: 5% CO_2: 90% N_2) (Bactron II Anaerobic Chamber, Sheldon Manufacturing Inc., Portland, OR). Kanamycin, vancomycin, laked blood agar plates (KVLB, Dalynn Laboratory Products, Calgary, AB), enriched Brain Heart Infusion broth and agar (eBHI, Difco), supplemented with kanamycin and vancomycin (Dalynn) and enriched Cooked Meat Broth (eCMM, BBL®, Becton Dickinson, Cockeysville, MD) were the media used in these investigations. Enrichment supplements included hemin used at 10 µg/ml (w/v) (Sigma Chemical Co., St. Louis, MO.), and vitamin K (Sigma) used at 1 µg/ml (w/v). 3.25 ml/L (v/v) of a 0.025% resazurin (Sigma) solution was added to prepared media as an indicator of anaerobic conditions. All *P. levii* cultures were routinely monitored and checked for purity either by gram staining described below, or by growth on non-selective agar such as Brucella Blood Agar (BBA, Dalynn).

1.2 *P. levii* Culture Purity

A) Gram Staining

Gram staining was carried out as described by Ross (1996). From broth or plate cultures, thin smears of *P. levii* were prepared in tap distilled water on microscope slides and allowed to air dry. The cells were then fixed to the slide using methanol (BDH Chemicals, Toronto, ON) for 1 minute, or passed through a flame for a short time to heat fix the cells to the slide. The cells were primarily stained using a 1% (w/v) crystal violet (Difco) solution for 1 minute and rinsed with distilled water. Lugol's iodine solution (Difco) was then added for 1 minute, the slides were rinsed with distilled water, and then destained using 95% ethanol (BDH) until crystal violet no longer rinsed from the smear. The cell smear was then counter-stained using a 1% (w/v) safranin solution (Difco) or a 1% (w/v) basic fuchsin (BDH) solution. The cell smear was then examined using light microscopy under oil immersion at 1000X magnification (Zeiss, West Germany). Purity of the culture was verified by examining the typical cell morphology of *P. levii.*

B) Plate Cultures

From cultures *P. levii* (plate or broth) loop samples of the cultures were taken and subcultured to non-selective BBA (Dalynn). Purity of the original culture was verified by viewing the single colony morphology of the sub-culture. Black pigment production by *P. levii* was also indicative of culture purity (Engelkirk, Duben-Engelkirk, Dowel, 1992). All cultures were grown under anaerobic conditions as described previously.

1.3 Lipopolysaccharide Isolation and Quantification

A) Lipopolysaccharide Purification

Lipopolysaccharide was isolated by the hot aqueous phenol method of Westhphal and Jan (1965). *P. levii* was grown using eCCM, eBHI broth and eBHI plates with purity of all cultures confirmed as described previously. Broth cultures (approximately 7 L) were grown until turbid, approximately 4-6 days. Enriched CMM cultures were then filtered through Whatman #2 filter papers (Whatman, England) to get rid of any residual meat particles. From this point on, all broth cultures were pooled and centrifuged at 3000 x g.

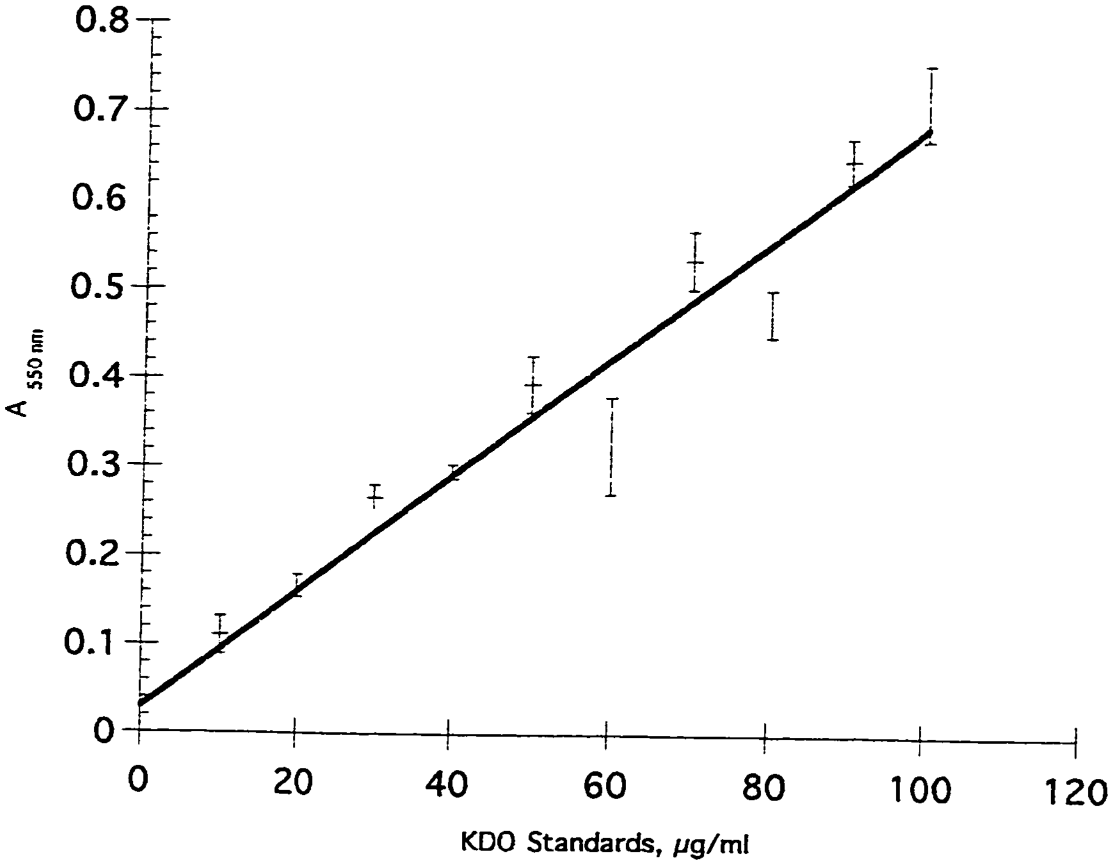
for 30 minutes (Model HN-S Centrifuge, Int. Equip. Comp. (I.E.C.), Needham Heights, Mass). Culture supernatants were saved for studies described below. Plate cultures were flooded with sterile Phosphate Buffered Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4), scraped, and pooled into centrifuge tubes. These preparations were then centrifuged at 3000 x g. for 10 minutes. Cell pellets of *P. levii* were then freeze dried for approximately 18 hours (Virtis Comp. Inc., Gardiner, NY). Ten grams of freeze dried *P. levii* was then resuspended in 300 ml of deionized glass distilled water (ddH_20) and 300 ml of 90% phenol (BDH) in water (w/v) and heated to 68°C for 15 minutes with agitation. The mixture was cooled in ice to 10°C and then centrifuged for 30 minutes at 3000 x g. (Model HN-S) to yield separate aqueous and phenol phases. The different phases where separated (approx. 300 ml each) and then brought back up to 68°C and extracted again by mixing each of the above phases with the reverse phase as described above. This procedure was repeated a third time and all aqueous phases where then pooled and dialyzed in running tap water for 2 days using a 3500 MW cut off dialysis membrane (Spectra/Por® Membrane, Spectrum®, Houston, TX). The same dialysis sample was then dialyzed for 2 days in running tap distilled water using the same membrane. The resultant dialyzate was concentrated down to 20 ml by using freeze drying techniques for approximately 4 days. The 20 ml solution was then brought to a magnesium chloride (BDH) concentration of 100 mM and then treated with DNase I (Sigma) and RNase B (Sigma), each at 0.1 mg/ml. The solution was incubated with agitation (Gyrotory Shaker M-G2, New Brunswick Scientific, Edison, NJ) at 37°C for 8 hours. The solution was then treated with a general protease (Pronase, Sigma) at a concentration of 0.1 mg/ml and incubated for 14 hours at 37°C with agitation (Gyrotory Shaker). The enzyme treated suspension was then dialyzed using a 50,000 MW cut off dialysis tubing (Spectra/Por®) overnight in running distilled water. The dialyzate was then ultracentrifuged at 105,000 x g. overnight (Sorvall T875 Ultracentrifuge, 33K). The resultant pellets were resuspended and pooled using deionized glass distilled water (ddH_20) and ultracentrifuged as above for

4 hours. The resultant purified LPS was dissolved and aliquoted using ddH_20 and frozen at -85°C.

B) LPS Quantification

The method of Osborne (1963), was used to determine the 2 - keto - 3 - deoxyoctonate (KDO) concentration of the purified LPS sample which served as a measure of the resultant LPS concentration. This procedure was carried out in triplicate for reliability of the results obtained. *P. levii* eCCM culture supernatant, *P. levii* purified LPS and ddH_20 were tested in parallel with KDO (Sigma) standards. KDO (0.25 ml) standards were made up in the range from 10-100 μg/ml (v/v) in 0.05 M sulfuric acid (BDH) in sealable microfuge tubes. The *P. levii* samples as well as the ddH_20 samples were made up to 0.25 ml in 0.05 M sulfuric acid by adding 50 μl of each sample to twist cap microfuge tubes and topped up to 0.25 ml (test samples). The samples where heat hydrolyzed in the sealed microfuge tubes at 100°C for 30 minutes. The samples were then allowed to cool to room temperature. Periodic acid was prepared by dissolving 0.267 g of Na - metaperiodate (Sigma) in 50 ml of 0.0625 M sulfuric acid (Sigma). To all of the above samples, 0.25 ml of periodic acid was added. The tubes were sealed and warmed to 55°C for 20 minutes. A 2% (w/v) solution of sodium arsenite (Fisher Scientific, Fair Lawn, NJ) was prepared by dissolving 1 g of sodium arsenite in 50 ml of 0.5 M hydrochloric acid (Fisher). To all samples 0.5 ml of the sodium arsenite solution was added, and then the samples were vortexed (Fisherbrand Vortex Genie 2) thoroughly. Thiobarbituric acid (Sigma) was prepared (0.3% w/v) by dissolving 0.15 g in 50 ml of ddH_20 and filter sterilized through a 0.22 μm filter (Sigma) under vacuum. To all the samples, 2.0 ml of the thiobarbituric acid solution was added, and then the samples were rapidly heated and maintained at 100°C for 20 minutes. Each sample was then extracted with 5 ml of KDO extraction buffer. KDO extraction buffer consists of 5 ml concentrated hydrochloric acid (10.36 M, Fisher) mixed with 95 ml n - butanol (Fisher) (v/v). The samples were then centrifuged at 3000 x g. for 10 minutes (Model HN-S) and the top layers of the separated mixtures were taken for

Figure 3: Standard curve for the 2-keto-3-deoxyoctonate assay using KDO standard solutions (μg/ml). (n=3) (R=0.97586)


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$A_{550\ nm}$
0
20
40
60
80
100
120
KDO Standards, µg/ml

immediate A_{550} readings (Unicam SP1800 UV Spectrophotometer). Readings of the *P. levii* LPS, eCMM culture supernatant and the ddH_20 were then compared to the standard curve created by the mean values of the A_{550} readings of the KDO standards.

2. Tissue Culture

2.1 Bovine Endothelial Cells

A) Bovine Capillary Endothelium

Bovine capillary endothelium (EJG) was obtained from the American Type Culture Collection (ATCC). The cell line used in this study is classified as EJG, ATCC CRL - 8659 and was obtained and maintained at ATCC without knowing the exact passage number. Therefore, for the purposes of this study the passage number is indicative of when the cell line was received and first cultured in our lab. Thus, in all studies, cells from passages numbering 1 to 4 were used, and this was possible because the cell line was easily cultured and frozen down. EJG, when confluent demonstrates a fibroblast like appearance, and appears as long, parallel arranged cells with tight proximity.

B) Growth and Maintenance

The EJG cells were cultured immediately upon receipt from ATCC. EJG cells were maintained and grown in Eagle's Minimum Essential Medium (MEM) with Earle's salts and sodium bicarbonate without L - glutamine (Sigma). A 200 mM solution of L-glutamine (Sigma) was added in the volume of 10 ml/L, to the MEM at the time of use. EJG growth medium was also supplemented with 10% (v/v) heat inactivated complement, fetal bovine serum (FBS, Sigma), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 0.08 mg/ml tylosin solution (Sigma). This medium formulation is called complete or cMEM. All media components were sterile and tested to be LPS free by the manufacturer. EJG cells were grown in a humid, 5% CO_2 incubator at 37°C (Nuaire™ US Autoflow, Plymouth, MN) in a variety of tissue culture treated culture vessels, specific for each experiment. The EJG cells were given fresh cMEM every two to three days until they became confluent, at which time it was necessary to either use the cells experimentally, freeze the cells down or passage them. All work with the EJG cultures was carried out in a laminar flow hood (Canadian Cabinets Company Ltd. (CCC), Ottawa, ON) to maintain culture sterility.

C) EJG Passaging

EJG cells were passaged in two distinct ways. Firstly, EJG cells were scraped passaged from one flask to the other at a maximum ratio of 1 to 4. Confluent monolayers were dislodged from the culture vessels using sterile cells scrapers (Falcon® 3086 Cell Scraper, Becton-Dickson, Lincoln Park, NJ). The cell suspension was then broken up by repeatedly passing the cells in and out of a sterile pipette. The suspension was then transferred to new culture vessels and cMEM was added in appropriate volumes. The cells were then maintained under the appropriate incubation conditions. Secondly, confluent EJG cultures were passaged using Trypsin - Ethylenediamine Tetraacetic Acid (EDTA). Confluent monolayers of EJG cells were aspirated of their cMEM and rinsed with sterile PBS without calcium and magnesium (Sigma). A cation - free solution containing 0.5 g porcine trypsin and 0.2 g of EDTA · 4 Na per litre (Sigma) was added at the appropriate volume per culture vessel size. EJG cell detachment from the culture vessel was monitored until the monolayer had been significantly disrupted. The trypsin reaction was then stopped with the addition of cMEM and then the appropriate dilutions of the trypsinized cell solution were made to a maximum passage ratio of 1 to 4 or a seeding cell count of 1×10^5 cells/ml. Cell numbers were counted using a hemacytometer under standard techniques (Freshney, 1987).

D) Cell Freezing and Storage

EJG cells were cultured and passaged as described above. Cells at passage number 2 were frozen down to ensure experiments to follow would be carried out utilizing cells within the 1-4 passage limit. EJG cells grown to confluence in 75 cm^2 tissue culture treated flasks (Falcon 3111 Vented Cap) were aspirated of their media. The cells were then rinsed with cation free PBS as previously described. The cells were trypsinised as previously described using a 7 ml volume of the trypsin - EDTA solution. The trypsin reaction was stopped using cMEM and the cells were then pelleted down at 30 x g. (I.E.C. Centra-7R, Refrigerated Centrifuge), at a temperature of 4°C in sterile 50 ml (Falcon, Blue Max) centrifuge tubes. After centrifugation, the supernatant was aspirated off the cell pellet, which was then resuspended in serum free cMEM supplemented with 7% sterile, tissue culture grade dimethyl sulfoxide (DMSO, Sigma). Immediately after resuspending the cell pellet, the cells were aliquoted in 1 ml volumes into sterile cryovials (2 ml, Sigma). Resuspension of the centrifuged cell pellet was carried out so that the volume of the resultant suspension would equal that of 1 ml suspension per 75 cm^2 flask initially trypsinised. The cells were then slowly frozen down by keeping them in -85°C (Cryo - Fridge) overnight (16-18 hours), and then quickly transferring them to liquid nitrogen the next day. All frozen cultures were kept in the liquid phase of liquid nitrogen to ensure viability of the cells. To bring the cells out of freezing, the 1 ml aliquots were removed from the liquid nitrogen and quickly thawed in a 37°C water bath. The thawed EJG suspension was then added to 10 ml of cMEM in 25 cm^2 tissue culture treated flasks (Falcon 3108 Vented Cap), and maintained as mentioned previously.

2.2 Hybridoma Tissue Culture

A) Cell Clones and Origin

With the aid of Dr. L. Babiuk from the Veterinary Infectious Disease Organization (VIDO) in Saskatoon, hybridomas (specific clones) producing monoclonal antibodies specific for bovine TNFα and bovine GM - CSF were obtained. The hybridomas were cultured and sufficient numbers of each clone were injected into mice following specific ascites fluid production procedures as outlined in Harlow and Lane, (1988). These procedures are described below, with the outcome of producing sufficient amounts of each monoclonal antibody, used to carry out cytokine protein detection procedures after EJG cell exposure to *P. levii* LPS and crude bacterial culture supernatant. As well, these monoclonal antibodies were used to detect TNFα and GM - CSF in immunohistology studies described below. Work at VIDO was carried out to isotype the monoclonal antibodies, aiding in protein detection procedures. The immunoglobulin isotype of the TNFα monoclonal antibody is IgG_1 and the isotype of the GM - CSF antibody is IgG.

B) Growth, Maintenance and Freezing

The hybridomas cultures were grown immediately upon receipt from VIDO, with the initial aid of a feeder cell layer, which was set up as described below. Hybridoma cells were maintained and grown in Roswell Park Memorial Institute - 1640 medium (RPMI - 1640) containing sodium bicarbonate without L - glutamine (Sigma). A 200 mM solution of L - glutamine (Sigma) was added in the volume of 10 ml/L, to the RPMI - 1640 at the time of use. Hybridomas growth medium was also supplemented with 15% (v/v) heat inactivated complement, fetal bovine serum (FBS, Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 0.08 mg/ml tylosin solution (Sigma). This medium formulation is thus called complete or cRPMI. All media components were sterile and tested to be LPS free by the manufacturer. Hybridomas cultures were grown in a humid, 5% CO2 incubator at 37°C (Nuaire™) in 25 cm^2 or 75 cm^2 tissue culture flasks (Falcon), depending on the size of culture required (ascites fluid production or freezing down). Hybridomas cultures were refreshed every two days with fresh cRPMI and passaged easily from flask to flask due to the fact that these cultures grow in suspension and do not attach to the tissue culture treated flasks. Hybridomas cultures were frozen down in a similar manner as described previously except without the use of Trypsin - EDTA, and with the use of FBS free cRPMI instead of cMEM. Hybridomas cultures used for freezing were grown in 75 cm^2 flasks (Falcon) to cell numbers approaching 10^8 cells/ml in a 20 ml culture per flask. One 75 cm^2 (20 ml) flask of hybridomas would also be frozen in a 1 ml volume as described for EJG cells. Thawing of hybridomas cultures was also carried out in a similar fashion as the EJG cells with the exception that cRPMI was used instead of cMEM, and a feeder layer of cells was needed to ensure retrieval of viable hybridomas cells out of freezing. All work with the hybridomas cultures was carried out in a laminar flow hood to maintain culture sterility (CCC, Ottawa, ON).

C) Macrophage Feeder Layer Preparation

Feeder layers served the purpose of providing growth factors for hybridomas cells to ensure viability of newly started hybridomas cultures. Two mice (Balb/c or Swiss Webster) were euthanized by cervical dislocation as per standard operating procedures (Animal Resource Center, University of Calgary). The mice were then dipped in 70% ethanol (BDH, Toronto, ON) and placed on a dissection board. The skin of the bottom left abdominal quadrant was incised to reveal the peritoneal cavity. Using a sterile 5 ml syringe and an 20 gauge needle, 5 ml of cRPMI was injected into the left side of the peritoneal cavity of each mouse. Leaving the needle in the peritoneal cavity, the cRPMI was repeatedly withdrawn and re-injected into the mice (approx. 5 times). After the final re - injection, as much of the 5 ml cRPMI was removed from the peritoneal cavity as possible. The needle was then removed and the cRPMI containing the feeder cells was put into a sterile 50 ml centrifuge tube (Falcon, Blue Max). The volume was then brought up to 50 ml in total for the two mice using cRPMI. The feeder cells would then be aliquoted in 10 ml volumes into 5, 25 cm^2 tissue culture flasks (Falcon). The feeder cells were allowed to attach to the flasks for 2 days in a 5% CO_2 incubator (Nuaire™), before hybridomas cultures out of freezing were added.

D) Ascites Fluid Production

Balb/c mice of at least 6 months of age were used in these procedures. At least 1 day before starting ascites fluid production, 0.3 ml of Freund's Incomplete Adjuvant (Difco) would be injected intraperitoneally into each mouse. This served to "prime" the mice for ascites fluid production. A suspension of 0.25 ml of 10^6 hybridomas cells per ml (specific clones) would then be injected intraperitoneally into each mouse. The mice were then inspected daily for 1 to 2 weeks for the appearance of fluid production within the peritoneum. When acceptable limits of the amount of fluid contained within each mouse were reached, the mice were euthanized by cervical dislocation as per standard operating procedures (Animal Resource Center, University of Calgary). The fluid was removed from the peritoneal cavity and centrifuged at 500 x g. for 5 minutes (I.E.C. Model HN-S). The bottom layer of the resultant two layer suspension was then saved being the ascites fluid. The cell pellet was discarded.

3. EJG Exposure Studies

3.1 Exposure For Cytokine Protein Expression and Cytopathic Effect

A) *P. levii* eCMM Culture Supernatant Exposure

As described previously, *P. levii* was grown in eCMM and the culture supernatant was saved after centrifuging the bacterial cells. This culture supernatant was stored in -85°C until used. Confluent monolayers of EJG cells grown in 25 cm^2 tissue culture flasks (Falcon) were exposed to 2 ml of a 1:100 dilution of crude *P. levii* culture supernatant in cMEM for 60, 120, 180 and 240 minutes. The exposure medium was sterilized by passing it through a 0.22 μm filter (Sigma) before exposure of the EJG cells. The exposed cells were viewed for CPE (cytopathic effect) as described below. After exposure, the endothelial cells were aspirated of their media which was immediately put into -85°C (Cryo - Fridge) storage for cytokine expression analysis described below. The exposed monolayers were washed once with cMEM and then frozen at -85°C to allow for cell detachment from the culture flask. The cells were then thawed, collected and stored at -85°C for cytokine analysis as described below.

B) Purified *P. levii* LPS Exposure

Confluent monolayers of EJG cells grown in 25 cm^2 tissue culture flasks were exposed to 2 ml of 25 μg/ml of purified *P. levii* LPS in cMEM for 2, 15, 30, 45, 60, 120 and 240 minutes. The quantity of LPS used was determined from the results of the KDO assay described earlier, and the LPS/cMEM mixture was filter sterilized (0.22 μm, Sigma) before EJG monolayer exposure. Over the timed intervals the EJG cells monolayers were observed for CPE. As described above, exposure supernatants and cell fractions were saved for cytokine detection, described below.

3.2 Exposure For Cytokine mRNA Expression

A) *P. levii* eCMM Culture Supernatant Exposure

All procedures herein were carried out using aseptic technique and a laminar flow hood (CCC). A 50:50 mixture of crude *P. levii* culture supernatant and cMEM was prepared (exposure medium). The mixture was filter sterilized through a 0.22 μm filter (Sigma). Ten 75 cm^2 flasks (Falcon) of confluent EJG cells at passage number 4 were prepared. To 2, only 20 ml of cMEM was added for non-exposed controls. To the other 8, 20 ml of the exposure medium was added. All flasks were immediately put into 5% CO_2, 37°C (Nuaire™) incubation except for two exposure flasks used as time 0 samples. The other 6 exposure flasks were incubated for 0, 30, 60, 90, 120 and 150 minutes. After the individual incubation times were complete, each flask was immediately prepared for total RNA isolation procedures as described below. The non - exposed control flasks were incubated for 60 and 360 minutes and then prepared for RNA isolation as well.

B) *E.coli* LPS Exposure

All procedures herein were carried out using aseptic technique and a laminar flow hood (CCC). Sterile lipopolysaccharide from *E. coli* 0111:B4 (Smooth Strain, Sigma, Product Number L 4391) was mixed with cMEM to a final concentration of 5 μg/ml. Eight 75 cm^2 flasks (Falcon) of confluent EJG cells at passage number 4 were prepared. To two of these flasks was added 20 ml of cMEM to act as time 0 and 150 minute non - exposed cell controls. To the other 6, 20 ml of the *E. coli* LPS exposure medium was added, and then the flasks were put into 5% CO_2, 37°C (Nuaire™), for incubation times including 0, 30, 60, 90, 120 and 150 minutes. The time 0 exposed and non-exposed flasks were immediately prepared for RNA isolation as described below, as were all other flasks after incubation times were complete.

C) Purified *P. levii* LPS Exposure

All procedures herein were carried out using aseptic technique and a laminar flow hood (CCC). This procedure was used to back up the results seen with the *E. coli* LPS exposure experiments, due to the limited quantity of pure *P. levii* LPS, so as to confirm the optimal time of cytokine production caused by LPS from *P. levii*. LPS purified from *P. levii* was mixed with cMEM to a final approximate concentration of 5 μg/ml as dictated by the KDO assay performed earlier. This mixture was then filter sterilized through a 0.22 μm filter (Sigma Bio-Science). Fourteen confluent 75 cm^2 flasks (Falcon) of EJG cells were prepared. To two of these flasks, only cMEM was added as non-exposed controls. Two flasks were used as time 0 controls after being exposed briefly to 20 ml of the *P. levii* LPS exposure medium. To 10 of these flasks, 20 ml of the *P. levii* LPS exposure medium was added and then these flasks were incubated at 5% CO_2, 37°C (Nuaire™) at incubations times of 30, 60, 90, 120 and 150 minutes (2 flasks for each time). The non - exposed control flasks were incubated for 150 minutes. After all incubations, flasks were immediately prepared for total RNA isolation as described below.

D) Removal of RNase Contamination

These procedures were carried out for all reagents and equipment that were used for RNA isolation or any procedure where RNA analysis was being carried out. All reagents and equipment after being treated for RNase contamination were kept exclusively for RNA experiments. Some reagents such as tris(hydroxymethyl)aminomethane (TRIS), were bought RNase free, and used exclusively for RNA experiments. For the case of any equipment or reagents that were autoclavable, these were allowed to soak in 0.1% diethylpyrocarbonate (DEPC, Sigma) treated ddH_20 (v/v) or were treated directly with 0.1% DEPC (v/v), and then autoclaved. The exception to this were TRIS buffered reagents, which were made in RNase free glassware and autoclaved, but not treated directly with 0.1% DEPC. Reagents non-autoclavable would be treated with 0.1% DEPC and then filter sterilized through 0.22 μm filters where appropriate. Equipment that was non-autoclavable which included plastic and glass vessels, general equipment, work surfaces and pipettes, would be treated with RNaseZAP™ (Sigma, Product Number R-2020) as per manufacturer's instructions, and then rinsed with sterile 0.1% treated ddH_20. RNaseZAP™ is a cleaning agent for the complete removal of RNase contamination.

E) RNA Isolation

RNA was isolated by two methods in these experiments. Following the protocol in Sambrook, Fritsch and Maniatis, (1989), the first method is the rapid isolation of total RNA from mammalian cells. Working with 75 cm^2 EJG monolayers (exposed or not), the following procedure was carried out per flask of cells. First, all medium was aspirated from the flask, which was then rinsed twice with 14 ml of ice-cold PBS lacking calcium and magnesium (Sigma). Next, 4 ml of 10 mM EDTA (pH 8.0), 0.5% sodium dodecylsulphate (SDS) was added to lyse the cells, which were then scraped from the tissue culture flask using a cell scraper (Falcon) and placed in a centrifuge tube (40 ml). The flasks where then rinsed with 4 ml of 0.1 M sodium acetate (pH 5.2) and 4 ml of 10 mM EDTA (pH 8.0). The rinse would then be transferred to the tube containing the cell lysate. To the cell lysate, 8 ml of phenol (equilibrated with water, Sigma), was added, and the contents of the tube were shaken for 2 minutes at room temperature. The suspension was then centrifuged at 3000 x g. for 10 minutes at 4°C (Sorvall Superspeed RC2-B, Newton, Connecticut). The upper aqueous phase was then transferred to a fresh tube containing 880 µl of ice-cold 1M Tris - Cl (pH 8.0) and 360 µl of ice-cold 5 M NaCl. Two volumes of ice-cold ethanol (BDH) were added, the tube was mixed by hand, and then stored on ice for 30 minutes. The RNA was collected by centrifugation at 3000 x g. (Sorvall RC2 - B) for 10 minutes at 4°C. The ethanol was removed and allowed to drain away from the RNA pellet. The RNA was then dissolved in 400 µl of ice - cold TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) and transferred to a microfuge tube (2 ml). To this was added 8 µl of cold 5 M NaCl and 1 ml of ice-cold ethanol. The RNA was collect by centrifugation at 12,000 x g. for 5 minutes at 4°C (Baxter, Canlab, Biofuge A). The ethanol was then removed and drained, and the RNA pellet was redissolved in RNA dilution buffer as described below.

Secondly, RNA was isolated with the aid of TRI REAGENT™ (Sigma) as per manufacturer's instructions. This is a quick and convenient reagent containing guanidine thiocyanate and phenol for the use in the isolation of total cell RNA. Briefly, for one monolayer of EJG cells in a 75 cm^2 flask (Falcon), the cells would be lysed by adding 7.5 ml of Tri Reagent™. The cell lysate was incubated at room temperature for 5 minutes followed by the addition of 1.5 ml of chloroform (BDH). The lysate was shaken for 15 seconds and allowed to stand for 10 minutes at room temperature. This solution would then be divided equally into 10 microfuge tubes and centrifuged at 12,000 x g. for 15 minutes at 4°C (Biofuge A). For each of the ten tubes, the top aqueous phase would be transferred to a fresh tube, with the addition of 375 μl of isopropanol and mixing. The tubes were allowed to stand at room temperature for 7 minutes and then micro - centrifuged for 10 minutes at 4°C at 12,000 x g.. The supernatant was removed, and the RNA pellet in each tube was washed with 750 μl of 75% ethanol by vortexing (Fisher). The tubes were micro - centrifuged at 7,500 x g. for 5 minutes at 4°C, after which the ethanol was removed and drained. The RNA pellets were dissolved and pooled in RNA dilution buffer (described below) and stored at -85°C (Cryo - Fridge) until further analysis.

F) RNA Quantification

RNA (and DNA) was quantified with the aid of Nucleic Acid Quicksticks (Clontech Laboratories Inc., Palo Alto, CA). This kit was designed for the quantification of DNA, RNA or oligonucleotides. Quantification was performed using 1 μl of each RNA sample and was determined by comparing the relative color intensities on the Quicksticks with standards provided on a reference chart. Briefly, 1 μl of each RNA sample was blotted onto a Quickstick and them immersed in 1 ml of Dye Solution provided. The stick was immersed and rinsed in 1 ml of ddH_2O for 3 seconds and then washed in 1 ml of Wash

Solution provided for 1 minute. The stick was then allowed to air dry and the resulting color intensity was compared with the reference chart.

4. Examination of Cytopathic Effect (CPE)

4.1 Visual Examination of CPE

Characteristics of CPE that were looked for included rounding up and detachment of individual cells from the monolayer, destruction of the cell monolayer, the appearance of refractile individual cells, cytoplasmic vacuolation of cells, granulation of cells, swollen cells, formation of syncytia, and generally an overall appearance of cellular degeneration at higher magnifications as described by Paulsen et al., 1989. Photographs of healthy and exposed monolayers were taken with the aid of an inverted light microscope equipped with photographic equipment.(Axiovert 25, Carl Zeiss Inc., West Germany, Canon EOS Rebel X, Canon Inc., Taiwan).

4.2 Transwell Experiments

A) EJG Transwell Cultures

The goal of using the Transwell (Transwell-COL, Costar, Cambridge, MA) system, was to mimic the barrier function of endothelium *in vitro* . Unexposed cell controls were run in parallel to all Transwell experiments to aid in confirmation of results. Growing confluent EJG monolayers on microporous membranes (Transwell), and measuring confluence via electrical resistance was carried out as described below. Confluent monolayers of EJG cells at passage number 3, were passaged as described previously. The trypsinised cells were then centrifuged at 30 x g. (I.E.C. Centra - 7R). The resultant cell pellet from one confluent 75 cm^2 flask (Falcon) of EJG, was then resuspended in 20 ml of cMEM. A 1 : 9 dilution of this suspension was made in a buffered solution of 0.1% Trypan Blue (Sigma) for cell enumeration and cell viability analysis as outlined in Freshney, (1987). The cell concentration was then adjusted to 8 x 10^5 cells/ml in cMEM. For each Transwell microporous membrane (6.5 mm diameter insert, 3.0 μm pore size, Costar) set up in 24 well tissue culture plates (Costar), 200 μl of the 8 x 10^5

Figure 4: Side representation of a Transwell (Costar) tissue culture insert. *a*: electrical resistance measuring electrode; *b*: upper chamber; *c*: monolayer of cells growing on the semi-permeable membrane; *d*: lower chamber and basolateral surface of membrane. (courtesy of D. Teoh, University of Calgary)

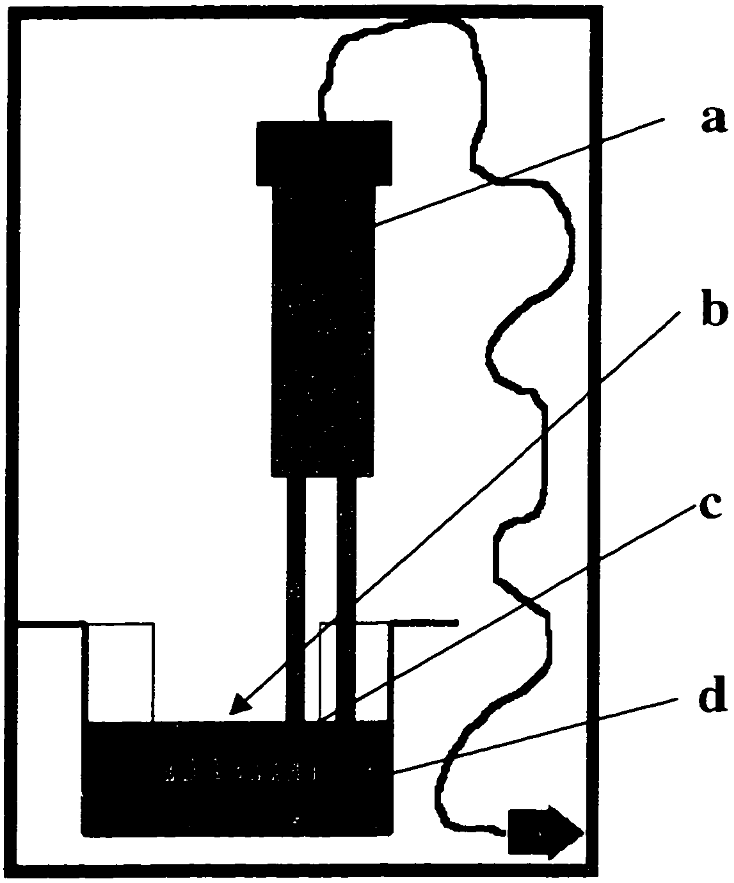
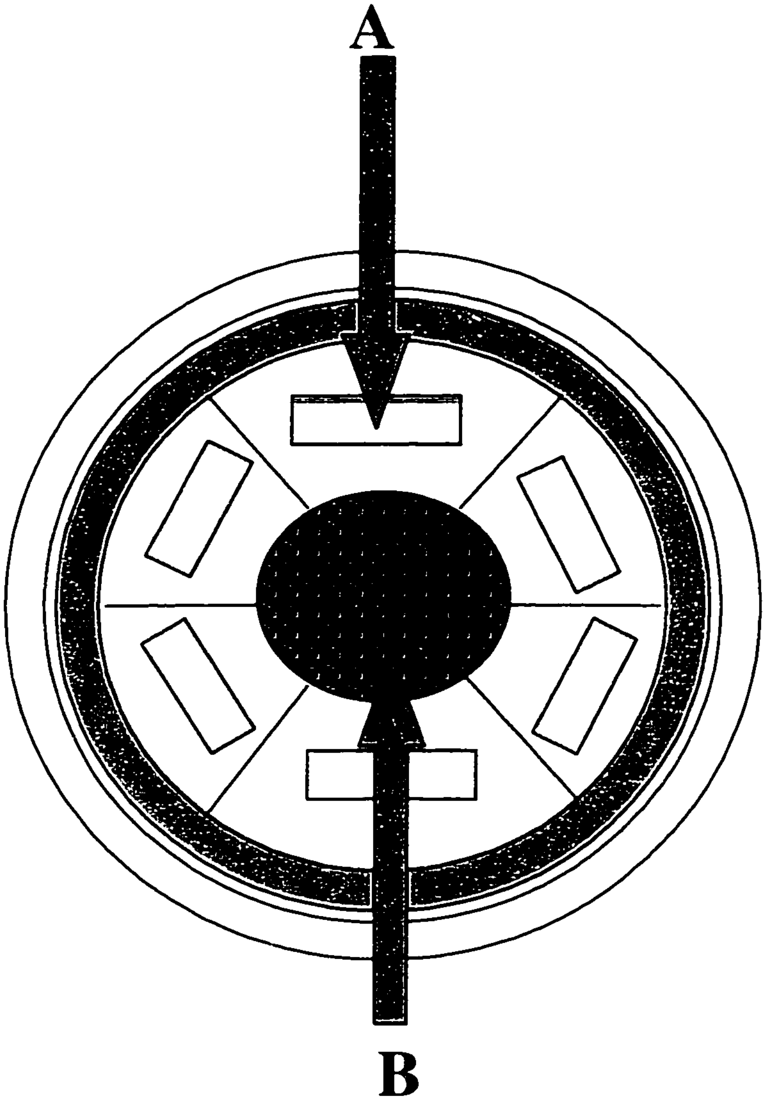

a
b
c
d

Figure 5: Top representation of a Transwell (Costar) tissue culture insert. *A*. Entrance into the lower compartment or basolateral surface. *B*. Upper chamber and cell growth on semi-permeable membrane.


A
B

cells/ml suspension was added to the upper compartment of the Transwell insert. To the lower compartment (basolateral surface) of the insert, 500 µl of cMEM was added. The plates were then be incubated at 37°C in 5% CO_2 (Nuaire™) and refreshed every two days with cMEM until confluence was reached as measured by electrical resistance across the membrane as described below.

B) Electrical Resistance and Confluence Analysis

After the Transwell membranes were seeded with EJG cultures, it was necessary to measure the electrical resistance across the cells and the Transwell - COL (Costar) microporous membrane. On alternate days, different Transwell cultures were measured for electrical resistance daily using a sterile electrode and a resistance meter (Endohm[12], EVOM™, EVOM™ Electrode Set, World Precision Instruments, Sarasota, FL). Once the cells reached confluence, at a resistance measurement of approximately 200 ohms, they were also visually inspected for confluence under 400 X magnification (Axiovert 25, Carl Zeiss Inc., West Germany), to ensure the cell monolayer was indeed confluent over the whole Transwell membrane. Cultures were then ready for the biotin, LPS exposure, leakage assay as described below.

C) LPS Exposure, Biotin Leakage Studies

ImmunoPure® Biotinylated Horseradish Peroxidase (B-HRP) (Pierce, Rockford, IL) a detectable substrate was added along with either *E. coli* 0111:B4 (Smooth Strain, Sigma, Product Number L 4391) LPS or purified *P. levii* LPS in the top chambers of healthy EJG monolayers growing on Transwell membranes (Costar). These sterile test exposure mediums consisted of 1:2500 dilution of 1 mg/ml biotinylated - HRP and 12.5 µg/ml of purified LPS in cMEM. Other medium containing just the 1:2500 (0.4 µg/ml) dilution of B-HRP in cMEM (control exposure media) was made and added for the purpose of non - LPS exposed controls. Two hundred microlitres (200 µl) of control and exposure medium were added to the top chambers of 24 confluent EJG - Transwell cultures. To the basolateral surface was added 500 µl of cMEM as indicated previously. Of the 24

Figure 6: Electrical resistance profile of EJG cells growing on Transwell (Costar) membranes. Electrical resistance was used as an indicator of EJG monolayer confluencey.

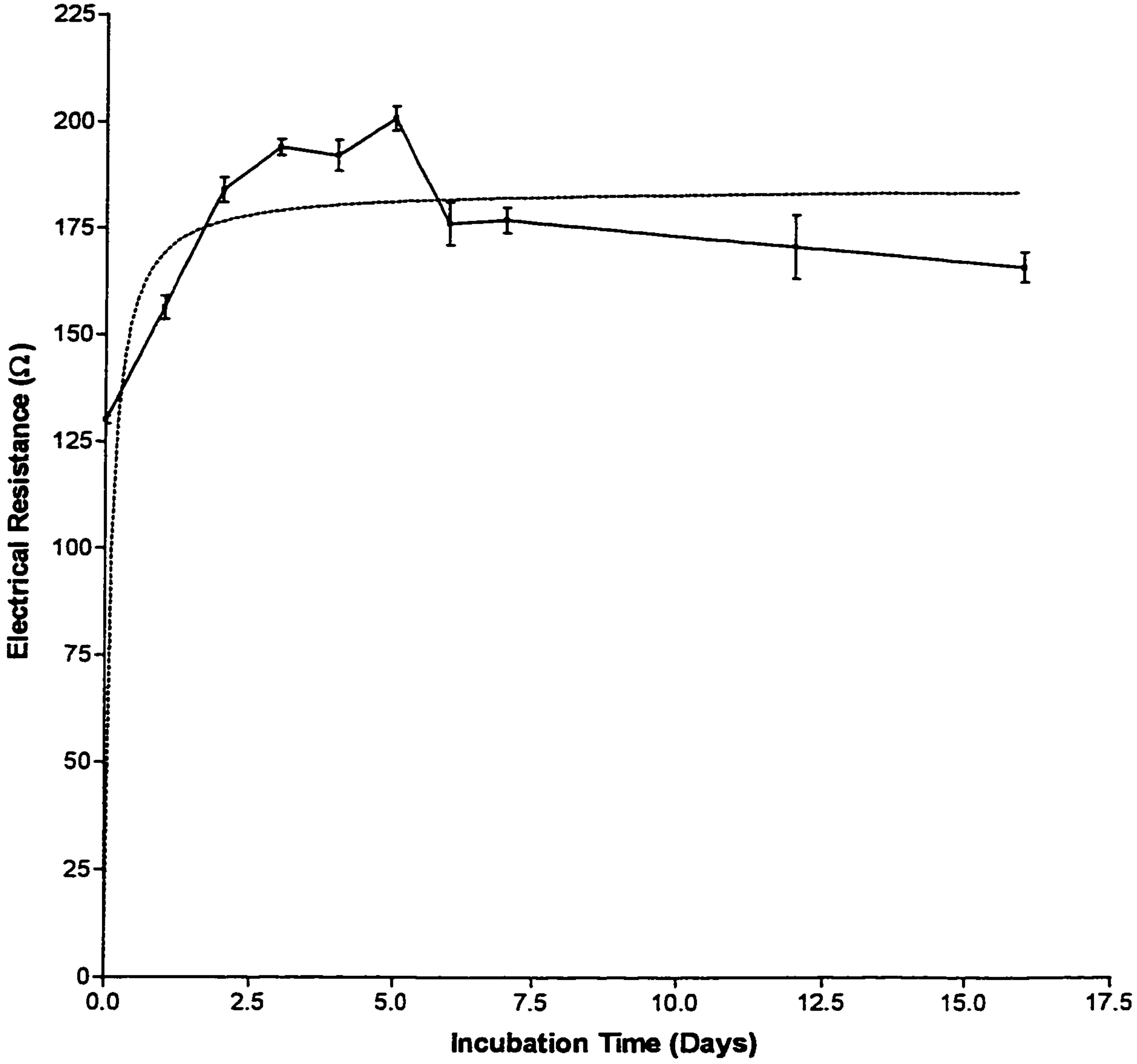
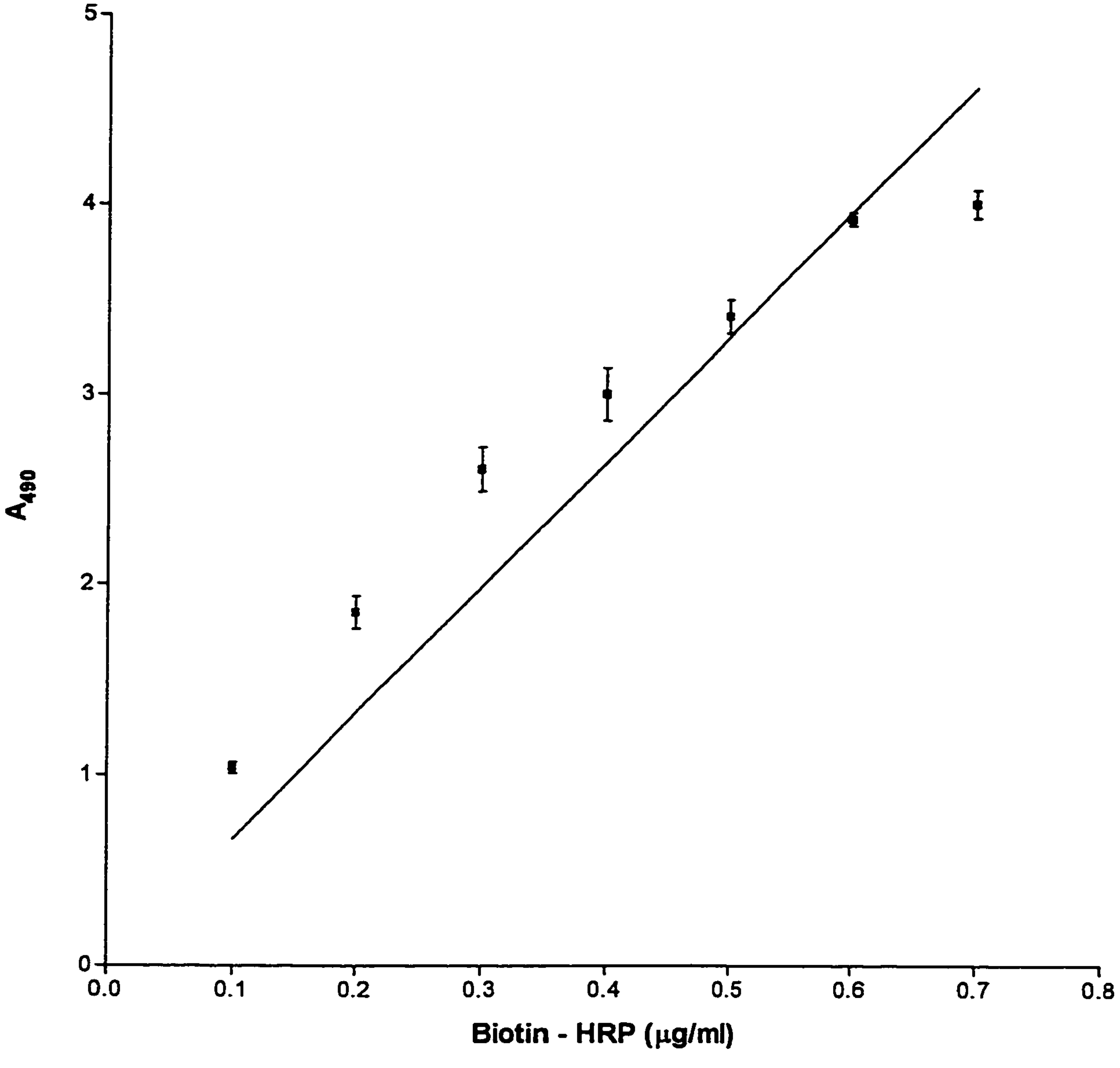

225
200
175
150
125
100
75
50
25
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Electrical Resistance (Ω)
Incubation Time (Days)

Figure 7: Standard curve for the EJG, LPS Exposure, Biotin Leakage studies using standard Biotin - HRP solutions (μg/ml). (n=16) (R=0.978449)



5
4
3
2
1
0
A_{490}
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Biotin - HRP (µg/ml)

Transwell cultures, twelve confluent cultures were exposed to test exposure medium from times 0 to 2 hours and 30 minutes. At the end of each individual incubation period, the 500 µl of cMEM on the basolateral surface was retrieved (every 15 minutes), for quantification of biotin leakage across the EJG monolayers and the Transwell membranes. Control (12 non - LPS exposed) Transwell cultures were set up and examined in parallel to the 12 exposed cultures. Experiments using *P. levii* LPS or *E. coli* 0111:B4 (Smooth Strain, Sigma, Product Number L 4391) LPS were run separately, with the experimental design for each run being identical. During these trials, the 24 Transwell cultures were incubated at 37°C, 5% CO_2, (Nuaire™) as per usual tissue culture protocols.

D) Quantification of Biotin Leakage

Leakage across the monolayer was measured over time after exposure to *P. levii* LPS, thus indicating leakiness or loss of monolayer confluence. The 500 µl of basolateral cMEM taken from the LPS exposure, biotin leakage studies, was analyzed for the amount of Biotin-HRP that passed across the EJG - Transwell (Costar) cultures for both exposed and control samples, as mentioned previously. Reacti - Bind™ Streptavidin Coated Pre-Blocked Polystyrene 96-well plates were obtained for this purpose (Pierce, Rockford, IL). For quantification of the amount of Biotin - HRP that leaked across the membranes, standards of this reagent were tested in parallel with the test samples. Standards included those ranging from 0.1 µg/ml up to 1 µg/ml of biotinylated - HRP diluted in cMEM. Blank samples tested consisted of just cMEM. Briefly, as per manufacturer's instructions, 200 µl of a 1 : 100 µl dilution (in cMEM) of all test and control samples were added to the Reacti - Bind™ plates in triplicate, and allowed to incubate for 2 hours at room temperature. Each well was rinsed 6 x with 200 µl of wash buffer (Tris-buffered saline, 0.1% BSA, 0.05% Tween 20). Color development followed with the addition of 200 µl per well of o-phenylenediamine dihydrochloride (OPD, 0.5 mg/ml) dissolved in 0.5 M phosphate citrate buffer pH 5.0, with 40 µl per 100 ml buffer of fresh hydrogen peroxide (30%) (Sigma). The reaction was allowed to take place for 30 minutes at 37°C. The color reaction was stopped by adding 50 µl of 2.5 M H_2SO_4. The plates were then read at 490 nm using a plate reader ($\text{Thermo}_{\text{max}}$, Microplate Reader, Molecular Devices, Menlo Park, CA) Non - parametric analysis was performed within groups of the same incubation time, using a two-tailed P value Mann-Whitney test.

5. Cytokine Protein Detection

5.1 Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS - PAGE) was carried out utilizing a Mini - Protein™II Dual Slab Cell vertical electrophoresis unit (Bio-Rad Laboratories, Richmond, CA). The method of Laemmli, (1970) was used to cast discontinuous gels consisting of a 4% stacking gel and a 12% resolving gel. Gels were cast using either a 8 well comb or more commonly a 1 well preparative comb with a reference slot for molecular weight standards. Cell and supernatant exposure samples, from the LPS and crude supernatant exposure trials (4 classes of sample) were mixed 50 : 50 with SDS reducing buffer (with 0.05% Bromophenol Blue) and boiled for 10 minutes. Test gels were run with all samples to determine the maximum amount of each sample that could be loaded without overloading the gel when visualized by staining methods described below. One hundred microlitres (100 µl) of the 50 : 50 exposure/sample buffer samples for each time trial described previously were added to the single well using gel loading pipette tips and an adjustable volume pipette. Non-exposed samples were also run in a similar fashion. Pre-reduced low range molecular weight color markers (Sigma) were run with each gel and loaded in a similar fashion at a volume of 5 µl per reference well. Markers consist of mixture of 5 - 8 proteins with each protein conjugated to a different dye, thereby providing a visual monitor of protein migration during electrophoresis and/or a visual check of transfer efficiency of protein to nitrocellulose membranes as described below. The molecular weight markers were necessary to estimate the molecular weight of specimen proteins on Western blots by comparing Rf values. All gels were run using a high voltage power supply (Bio-Rad, Power PAC 3000) using a voltage of 150 volts for

15 minutes, and then a constant voltage of 200 for approximately 20 minutes, on ice to reduce smiling effect. Gels were immediately prepared for Western blotting except for those described earlier, used for direct gel staining.

5.2 Gel Staining

Silver staining (Bio-Rad's Silver Stain Kit) was used exclusively in these investigations as per manufacturer's instructions. Gels were placed in a 40 : 10 : 50 mixture of methanol:acetic acid:water for 30 minutes at room temperature. A washing solution of a 10 : 5 : 85 mixture of ethanol:acetic acid:water was used to rinse the gel twice (15 minutes each). The washed gels were then allowed to incubate for 5 minutes in Bio-Rad oxidizing reagent. The gels were then washed with ddH_20 multiple times until no visual evidence of oxidizer remained on the gels. Bio-Rad silver stain reagent was then added and incubated for 20 minutes followed by a 1 minute rinse in ddH_20. Bio-Rad developer solution was then added for 30 seconds, removed, and added again for 5 minutes. A 5 % (v/v) aqueous acetic acid solution was then added to stop the development of the gel. Gels were then photographed and/or stored in ddH_20.

5.3 Western Blotting

The methods outlined in Towbin, Staehelin and Gordon, (1979), were used for these investigations. Immediately after electrophoresis, SDS-PAGE gels (of each exposure method and time period) were allowed to equilibrate in cool (4°C) transfer buffer (25 mM Tris, 193 mM glycine, 20% HPLC methanol (v/v), pH 8.3, Bio-Rad) for 15 minutes. At the same time a nitrocellulose membrane (Bio-Rad, 0.20 μm pore size) was cut to match the size of the gel and allowed to equilibrate in cool transfer buffer for 15 minutes. Using the Mini Trans-blot™ apparatus (Bio-Rad Laboratories, Richmond, CA), a transfer stack/sandwich was assembled with the nitrocellulose and SDS-PAGE gel stacked next to each other for electrophoretic transfer. Transfer took place at 4°C using fresh transfer buffer and a constant voltage (FB 135, Fisher Scientific) of 20 volts for 16 hours. Transfer of proteins was confirmed by visually inspecting the nitrocellulose sheets for transfer of the color molecular weight markers (Sigma) run as reference on the SDS-PAGE gel. The molecular weight markers were then cut from the nitrocellulose, which was also cut into strips to allow for concurrent investigation of cytokine production for each exposure sample prepared earlier. The cut strips were then placed in blocking solution (10 mM Tris, 0.9% (w/v) NaCl pH 7.4, 0.05% Tween 20, 0.02 g/ml skim milk powder) and incubated for 1 hour at 37°C on a rocking platform (Gyrotory Shaker M-G2). The strips were then washed 3 times (15 min.) in TBS/Tween (10 mM Tris, 0.9% (w/v) NaCl pH 7.4, 0.05% Tween 20) on a rocking platform at 37°C. The strips were then used for cytokine protein detection as described below.

A) Western Blot Development

Ascites fluids containing monoclonal antibodies specific for Bovine TNFα and Bovine GM-CSF were produced as described earlier. For each cytokine detection/development a non-exposed control blot strip was exposed to the primary antibody in parallel with each exposure test strip and carried through the development procedure. The following procedure follows those outlined in Towbin et al., (1979) and were used for detecting both TNFα and GM-CSF in an exact manner. Ascites fluid was diluted 1 : 100 in TBS/Tween and incubated with the blot strips for 16 hours at 4°C. As for controls, strips of nitrocellulose blots for each antigen were not exposed to the primary antibody to test for endogenous alkaline phosphatase activity and cross reactivity of the secondary antibody. The blot strips were then washed 3 times in TBS/Tween (10 min. each) on a rocking platform (Gyrotory Shaker M-G2). The membrane strips were then incubated with a 1 : 30,000 preparation (in TBS/Tween) of sheep derived anti - mouse IgG (whole molecule) alkaline phosphatase conjugated secondary antibody (Sigma Bio-Chemicals Product No. A-5324). This incubation was carried out for 2 hours at 37°C on a rocking platform. The blot strips were again washed 3 times in TBS/Tween (10 min. each) on a rocking platform. Ten (10 mg) of nitroblue tetrazolium (NBT) was dissolved in 100 ml of 0.2 M Tris-HCL, 4 mM $MgCl_2$, pH 8.8, and to this was added 1 ml of 5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in DMSO (Sigma). This development solution was then immediately added to the test strips and development was allowed to take place at room temperature, on a rocking platform for exactly 10 minutes. The development reaction was then stopped by pouring off the developing solution and rinsing the test strips with cold ddH_20. The test strips were then photographed and stored in ddH_20 at 4°C.

6. Cytokine mRNA Detection

6.1 Plasmid Vectors

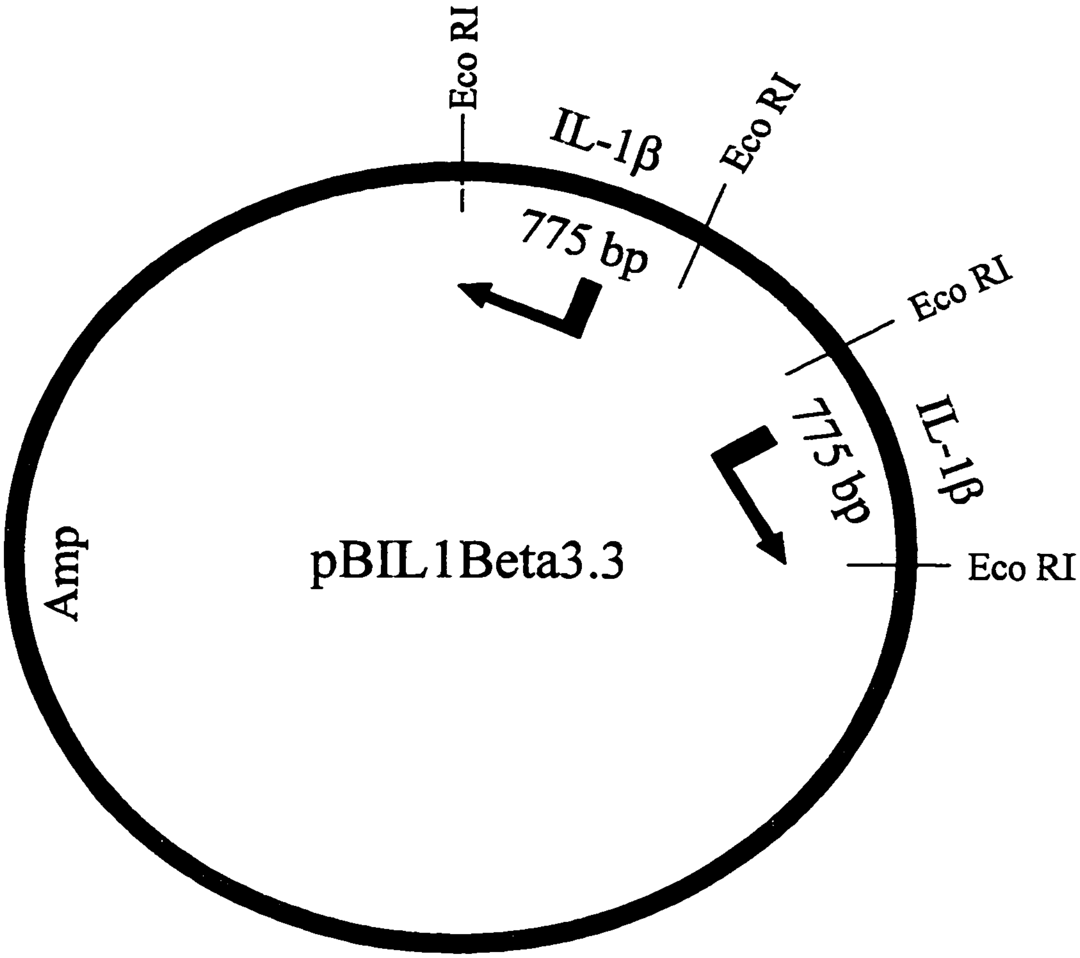
A) Type and Origin

With the aid of Dr. L. Babiuk from the Veterinary Infectious Disease Organization (VIDO) in Saskatoon, we received a plasmid containing the cDNA sequence of bovine IL-1ß. The pBIL1Beta 3.3 (pUC based) plasmid has Eco RI linked Bovine IL-1ß ligated into 2 restriction enzyme sites in opposite orientations. The plasmid also confirms bacteria carrying the plasmid with ampicillin resistance, making it possible to select transformants, as described below. The goal was to transform the plasmid into an acceptable strain of bacteria, grow out the bacteria to large cell numbers, and then isolate the plasmid back out of the bacterium. From there, IL-1ß cDNA sequences were restriction digested from isolated plasmids for probe production and thusly, hybridization experiments, all described below.

B) Transformation

The following procedures followed those outlined in Sambrook et al., (1989). Transformation buffer (TFB), containing 1 M 2-[N-morpholino]ethanesulfonic acid (MES) was prepared (Sambrook et al., 1989). A single colony of *E. coli* strain DH5-α from an 18 hour incubation was transferred into 100 ml of LB (Luria - Bertani) broth. This culture was grown with vigorous shaking for 3.5 hours at 37°C (Incubator Model 1915, VWR Scientific, Mississauga, ON) until a cell density of 10^7 cells/ml was reached. The culture

Figure 8: Representation of the plasmid vector (pBIL1Beta3.3) containing the sequence for bovine IL-1ß cDNA. IL-1ß cDNA was inserted twice into the plasmid, with each insert flanked by Eco RI restriction sites.


Eco RI
IL-1β
Eco RI
775 bp
Eco RI
775 bp
IL-1β
Eco RI
Amp
pBIL1Beta3.3

was transferred to 2 sterile 50 ml ice-cold polypropylene tubes (Falcon) and cooled on ice for 10 minutes. The cells were pelleted by centrifugation at 2000 x g. for 10 minutes at 4°C (Sorvall RC2-B). The LB media was allowed to drain away from the cell pellets and then each pellet was resuspended in 10 ml of TFB, on ice. The DH5-α cells were pelleted again by the same method and drained. Each cell pellet was then resuspended in 2 ml of ice-cold TFB and then pooled. At this point the cells were competent, and the majority of the competent cells were then frozen down for future transformations (Sambrook et al., 1989). On ice, 200 μl of competent cells were added to a sterile microfuge tube. Plasmid DNA (pBIL1Beta 3.3) was added at no more than 50 ng in a volume of 10 μl. The contents of the tube were mixed gently and stored on ice for 30 minutes. The tube was then incubated at 42°C for 90 seconds (water bath), and then returned to ice for 2 minutes. To the tube, 800 μl of SOC (pre litre, 20 ml 1M glucose, 20 g bacto-tryptone, 5 g yeast extract, 0.5 g NaCl, 10 ml of 250 mM KCl, 5 ml of $MgCl_2$) medium was added, and then incubated at 37°C for 45 minutes (Sambrook et al., 1989). Ten plates of LB medium containing 50 μg/ml ampicillin (LB-amp) (Sigma) were then spread with 100 μl each of the transformation mixture. Two plates of competent cells without DNA were plated as controls. The plates were then allowed to air dry and then incubated for 16 hours at 37°C (Model 1915, VWR). Transformant colonies were then frozen down for future use or used directly for plasmid preparation procedures described below (Sambrook et al., 1989).

C) Plasmid Preparation

A 16 hour broth culture of pBIL1Beta 3.3 transformants grown in LB - amp broth at 37°C was prepared. The PlasmidPURE™ DNA mini-prep kit was used which is a complete plasmid purification system for the quick isolation of plasmid DNA (Sigma). The kit is based on a modified alkaline lysis procedure (Sambrook et al., 1989). The procedure was carried out as per manufacturer's instructions. Briefly, 1.5 ml of the LB - amp culture was microcentrifuged at 7,500 x g. for 1 minute (Biofuge A, Baxter, McGaw Park, IL).

The cells were resuspended in 250 µl of resuspension solution A. Two hundred and fifty microlitres of lysis solution B (contains SDS) was then added and the solution was mixed gently until the suspension cleared. The suspension was neutralized and centrifuged for 5 minutes at 12,000 x g.. The supernatant was then transferred into a PlasmidPure™ spin filter and centrifuged for 1 minute at 12,000 x g.. The filter was then washed three times with intermittent centrifugation using 500 µl of wash solution D. The plasmid DNA was then eluted from the filter into a microfuge tube by using elution buffer E (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and centrifugation at 12,000 x g. for 1 minute. The purified plasmid DNA was then stored at -20°C or directly analyzed by agarose electrophoresis or restriction enzyme digestion as described below.

D) Restriction Enzyme Digestion

The following procedures followed those outlined in Sambrook et al., (1989). Briefly, from the PlasmidPURE™ DNA mini - prep kit (Sigma), 6.7 µl (~1 µg) of pBIL1Beta 3.3 plasmid was mixed with 11.3 µl of sterile ddH_20. To this was added 2 µl of 10x restriction enzyme buffer (Palette Buffer™ Black, Sigma, 1 x = 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml bovine serum albumin (BSA), 1.0 mM 2-mercaptoethanol) with mixing by tapping the tube. Two units or 1 µl of Eco RI (Sigma, Product Number R-2881) were added to the mixture with gentle mixing to follow. The solution was incubated at 37°C (Model 1915, VWR) for 6 hours and then the enzymatic digest was stopped with the addition of 1 µl of 0.5 M EDTA. The digest was then frozen at -20°C or prepared for direct analysis using agarose electrophoresis as described below. Agarose gels were used to view the success of the restriction analysis as well as to isolate the cDNA sequences out of the restriction enzyme reaction.

E) Agarose Electrophoresis

The following procedures followed those outlined in Sambrook et al., (1989). Briefly, gels were cast using horizontal gel apparatus (Bio-Rad Sub-cell® GT, Hoefer HE 33B Minnie Horizontal Agarose Unit). Using Agarose LE (low electroendosmosis, Boehringer Mannheim), 1.2% (w/v) agarose gels were prepared in 1x TAE buffer (≥88°C) (0.04 M Tris-acetate, 0.001 M EDTA) and solidified (36°C) with sample wells, in the horizontal gel units. The use of 1.2% gels was appropriate for an efficient range of separation of DNA molecules in the range of 0.4 - 6.0 kilobases. Samples of DNA (described below) were mixed with gel - loading buffer (0.25% Bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and loaded into the sample wells with the gel submerged in 1x TAE buffer. Often 3 μl of a DNA ladder (phage ϕX174 Hae III Digest, Sigma, Product Number D-0672) was run with the samples, which had fragments ranging from 1, 353 bp - 72 bp. Gels were run at 5 V/cm (80-100 volts) (Labconco 1000 Volt Power Supply, Buchler Instruments, Saddle Brook, NJ), until the xylene cyanol had run approximately 3/4 of the way down the gel. Gels were then stained for 45 minutes in 0.5 μg/ml Ethidium Bromide (EtBr, Bio-Rad), and then destained in ddH_20 for 20 minutes. The gels were then either photographed or cut depending on what procedure they were being used for, as described below.

6.2 cDNA Probe Production

A) Probe Purification

Agarose gels were prepared as mentioned previously (1.2%). Samples of the pBIL1Beta 3.3 plasmid digests (40 μl per lane) were mixed with gel loading buffer and loaded onto the gels. The gels were run at 80 volts for 1 and 1/2 hours and then stained with EtBr (0.5 μg/ml) (Sambrook et al., 1989). Gels were visualized and photographed (Image Store 7500, Video Copy Processor P67UA, White/UV Transilluminator, Diamond Scan, Ultra - Violet Products (UVP) Inc., San Gabriel, CA) to confirm digestion when compared to non-digested plasmid samples. The size of the IL-1ß cDNA (~775 bp) sequences was confirmed with comparison to the DNA standards run with each gel (Sigma). Using an ultra-violet trans-illuminator (Chromato - VUE® Transilluminator, UVP Inc., San Gabriel, CA (312 nm), the cDNA sequences within the agarose were cut and trimmed from the gel. To remove the EtBr stained IL-1ß cDNA sequences from the agarose, and to destain the DNA, the protocol for eliminating ethidium bromide from the IL-1ß cDNA sequences and for purifying the sequences using GenElute™ Minus EtBr Spin Columns was used (Supelco, Bellefonte, PA). Briefly, as per manufacturer's instructions, the agarose gel slices containing the cDNA were applied directly to the spin columns. The spin columns containing the cDNA/agarose were then placed in sterile centrifuge tubes and microcentrifuged for 10 minutes at 12,000 x g. (Biofuge A, Baxter, McGaw Park, IL). The cDNA was collected at the bottom of the tubes, free of EtBr and now ready for labeling reactions described below.

B) Probe Labeling

Labeling of the bovine cDNA IL-1ß sequences isolated as above was carried out using the DIG DNA Labeling Kit, which utilized random primed DNA - labeling with digoxigenin (DIG) - dUTP (Boehringer Mannheim) (Feinberg and Vogelstein, 1983). This method was based on the hybridization of random oligonucleotides to the denatured IL-1ß DNA templates (Feinberg et al., 1983). The complementary DNA strand was synthesized by Klenow enzyme which used the 3' OH termini of the random oligonucleotides as primers and a mixture of deoxyribonucleotides containing DIG - 11 - dUTP, alkali - labile for elongation (Feinberg et al., 1983). This resulted in incorporation of DIG into the newly synthesized DNA (Feinberg et al., 1983). Briefly, as per manufacturer's instructions, for each labeling reaction, 15 µl (approx. 10 ng of DNA) of the purified IL-1ß cDNA sequences from the GenElute™ preparations described above, was denatured by heating for 10 minutes in a boiling water bath then quickly chilled on ice. Then 20 ml of labeling mixture (hexanucleotides, dNTP (DIG-UTP) mix, ddH_20 and Klenow enzyme), was added to the denatured cDNA sequences, and incubated for 20 hours at 37°C (Model 1915, VWR). The labeling reaction was then stopped with 2.0 µl of 0.2 M EDTA pH 8.0. For the purposes of these investigations, further probe purification was unnecessary because unincorporated nucleotides do not adhere to blotting membranes, they are washed away by stringency washes during detection procedures described below. This was then followed by quantification of the amount of probe produced as described below.

C) Quantification of Probe

These procedures were carried out utilizing DIG Quantification Teststrips (Boehringer Mannheim, Product Number 1669958) and DIG Control Teststrips (Boehringer Mannheim, Product Number 1669966) for the fast quantification of digoxigenin - labeled DNA probes in comparison to standardized digoxigenin - labeled control DNA. An accurate quantification of the amount of DIG - labeled DNA was important for optimal and reproducible results in membrane hybridization techniques as described below. Briefly, as per manufacturer's instructions, a series of dilutions (1:3.3 to 1:330) of a 1:40 diluted DIG - labeled IL-1ß probe mixture was applied to the marked squares on the DIG quantification teststrips. DIG control teststrips were already loaded with 5 defined dilutions (300, 100, 30, 10 and 3 pg) of a control DNA and were used as standards. The teststrips were then subjected to immunological detection with anti - digoxigenin - alkaline phosphatase and the color substrates NBT/BCIP (DIG Nucleic Acid Detection Kit, Boehringer Mannheim, Product Number 1175041). The amount of labeled IL-1ß probe was then determined by comparing the signal intensities of the spots on the quantification teststrips with the control teststrips. Comparison was done visually as well as with the aid of photo - densitometric analysis of spot intensity (Ultra-Violet Products (UVP) Inc., San Gabriel, CA).

6.3 Detection of Cytokine mRNA

A) Formaldehyde/Agarose Gel Electrophoresis

The following procedures followed those outlined in Sambrook et al., (1989) for the electrophoresis of RNA through gels containing formaldehyde. All equipment and solutions were treated for RNase contamination as mentioned previously. Briefly, 1.5% agarose (Agarose LE, Boehringer Mannheim) was prepared and allowed to cool to 60°C. To this (depending on the size of gel) was added the appropriate volumes of 12.3 M formaldehyde, pH>4 (BDH), deionized formamide (Sigma), and 5x formaldehyde gel running buffer (0.1 M MOPS [3-{N-morpholino}propanesulfonic acid] pH 7.0, 40 mM sodium acetate, 5 mM EDTA pH 8.0, Sigma). The gel was poured and allowed to set for 30 minutes at room temperature (Bio-Rad Sub-cell® GT, Hoefer HE-33B). While the gel was cooling RNA samples, isolated as mentioned previously, were prepared. Four and one half microlitres of each RNA sample (approx 75 ng/µl) in RNA dilution buffer (DEPC-treated ddH_20, 20X SSC buffer [3 M NaCl, 300 mM Sodium Citrate, pH 7.0], 12.3 M fromaldehyde, 5:3:2 mixture), from isolation procedures mentioned previously were mixed with 2.0 µl of 5x formaldehyde gel - running buffer, 3.5 µl of formaldehyde (12.3 M, BDH), and 10 µl of formamide (Sigma) in sterile microfuge tubes (1 per sample). The samples were then mixed and incubated for 15 minutes in a 65°C water bath and then chilled on ice. To each RNA sample 2 µl of formaldehyde gel loading buffer was added (50% glycerol (v/v) (BDH), 1 mM EDTA pH 8.0, 0.25% Bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v)). The gel was pre-run in 1x formaldehyde gel - running buffer (5x diluted in ddH_20) for 5 minutes at 5 V/cm before loading the 20 µl RNA samples and 20 µl of 20 ng/µl RNA molecular weight marker (RNA molecular weight marker 1, digoxigenin-labeled (0.3 - 7.4 kb), Boehringer Mannheim). The gel was run submerged in 1x formaldehyde gel running buffer at 4 V/cm until the bromophenol blue had migrated approximately 6 - 8 cm depending on the size of the gel. The gels were then stained for 45 minutes in 0.5 µg/ml EtBr (Bio-Rad) and photographed with the aid of UV-transillumination (UVP). The gels were then used to transfer the RNA to nylon membranes as described below.

B) Northern Blotting

The following procedures followed those outlined in Sambrook et al., (1989), for the alkaline transfer of denatured RNA to nylon membranes by capillary transfer. All equipment and solutions were treated for RNase contamination as mentioned previously. Charged nylon membranes retain RNA in alkaline solutions irreversibly, so there was no need to bake the membrane or to expose it to ultraviolet irradiation, to fix the RNA before hybridization procedures as described below. Briefly, RNA - gels were trimmed and rinsed in ddH_20 to remove the formaldehyde. Gels were then soaked for 20 minutes in 0.05 N NaOH and then for 45 minutes in 20x SSC buffer to improve transfer efficiency. A capillary transfer stack was prepared, which included (from bottom to top, all pre-soaked in transfer buffer) a solid support, over hanging Whatman 3MM paper (Whatman, England), RNA gel, nylon membrane, 2x Whatman 3MM paper, paper towels (8 cm thick), glass plate, and a 500 g weight, all in a glass reservoir dish. All stacking materials were cut to be close to the size of the gel and nylon membrane, except for the over hanging Whatman 3MM paper which was used to provide a moving stream of buffer (75 mN NaOH) from the reservoir dish to the transfer system. Transfer was allowed to take place for 6 hours. After transfer was complete, the nylon membranes were rinsed in 2x SSC (20x diluted in ddH_20), 0.1% SDS and allowed to dry at room temperature. From here the nylon membranes were prepared for hybridization and detection as described below.

C) Dot Blotting

All equipment and solutions were treated for RNase contamination as mentioned previously. Briefly, pieces of nylon membranes (Boehringer Mannheim) were wet in ddH_20 and then soaked in 20x SSC buffer for 1 hour at room temperature. Isolated exposure RNA samples (75 ng/μl) were suspended in RNA dilution buffer (DEPC-treated ddH_20, 20x SSC buffer, 12.3 M fromaldehyde, 5:3:2 mixture) at a dilution of 1 : 5 (15 ng/μl), and incubated for 15 minutes in a 68°C water bath. Standards of RNA ranging from 3 - 300 pg/μl were prepared as well. To the nylon membranes, 1 μl of each sample and standard were applied at marked locations at regular intervals. The nylon membranes were then allowed to dry completely at room temperature. The RNA was then fixed to the membranes by UV crosslinking (312 nm) for 5 minutes (Chromato - VUE®, UVP, San Gabriel, CA). From here, the nylon membranes were prepared for hybridization and detection as described below.

D) Hybridization

The following procedures were carried out as outlined in The DIG System User's Guide for Filter Hybridization (Beohringer Mannheim, 1995). All equipment and solutions were treated for RNase contamination as mentioned previously. Briefly, blots were placed and sealed in hybridization bags (Tubular Roll Stock, Kapak® Corp. Minneapolis, Minn) containing 20 ml of northern prehybridization buffer (7% SDS, 50mM sodium phosphate buffer pH 7.0, 50% formamide [Sigma], 2% blocking reagent [Boehringer Mannheim], 50 µg/ml total yeast RNA [Boehringer Mannheim], 5x SSC buffer [20x diluted in ddH_20], 0.1% lauroylsarcosine [Sigma]), per 100 cm^2 of nylon membrane. The blots were then prehybridized for at least 1 hour at 50°C. The probe (IL-1ß, 12.5 ng/ml, determined previously with the DIG Quantification Teststrips [Boehringer Mannheim]), was heat denatured in a boiling water bath for 10 minutes, diluted in northern prehybridization buffer. The blots were then drained and the probe containing buffer was added (20 ml) to the same bag and sealed. Hybridization was allowed to take place overnight (18 hours) at 50°C. At the end of hybridization, the membranes were washed 3 times, 5 minutes each, in 2 x wash solution (2x SSC [20x diluted in ddH_20], 0.1% SDS) at room temperature. The membranes were then washed twice, 15 minutes per wash in 0.1 x wash solution (0.1x SSC [20x diluted in ddH_20], 0.1% SDS) at 68°C. For control hybridizations, DIG - labeled actin RNA probes (Beohringer Mannheim) were used in hybridization procedures as described above. DIG actin probes were used in quantities of 100 ng/ml to examine reporter gene mRNA production in the exposed/non-exposed EJG cells. From here, membranes were used in detection procedures as outlined below.

E) Detection

The following procedures were carried out as outlined in The DIG System User's Guide for Filter Hybridization (Beohringer Mannheim, 1995) using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Product Number 1175041). All equipment and solutions were treated for RNase contamination as mentioned previously. Membranes were allowed to equilibrate in Buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 1 minute in sealed bags (Kapak®). In fresh bags, the membranes were blocked with gentle agitation (Gyrotory Shaker M-G2) for 30 minutes in Buffer 2 (1% (w/v) blocking reagent [Boehringer Mannheim], in Buffer 1). During blocking, Anti - DIG - Alkaline Phosphatase (Boehringer Mannheim), was diluted 1:5000 in Buffer 2 to a working concentration of 150 mU/ml (antibody solution). After blocking, Buffer 2 was decanted, and the membranes were incubated in the antibody solution (20 ml) for 30 minutes with agitation at room temperature. The membranes were then transferred to new bags and washed twice (15 minutes each) in 20 ml of Buffer 1. The membranes were then allowed to equilibrate in 20 ml of Buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 2 minutes at room temperature. A color substrate solution was made by mixing 45 μl of NBT solution (75 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethlyformamide) with 35 μl X-Phosphate solution (50 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100% dimethylformamide) in 10 ml of Buffer 3 (Boehringer Mannheim). To each membrane 10 ml of color substrate solution was added in a sealed plastic bag, without any air bubbles present. Color development took place in the dark and checked periodically until the desired spots or bands were detected. The color reaction was stopped by washing the membrane in 50 ml of Buffer 1 for 5 minutes. The blots were then photographed, and prepared for quantification procedures as described below.

F) Quantification

Intensities of RNA samples were compared over time with each other, as well as with the RNA standards applied to the dot blots as mentioned previously. RNA samples were semi - quantified by visually inspecting the intensities of test dots (spots) and comparing them to the intensities of the RNA standards.

7. Immunohistology

7.1 Tissue Preparation

A) Origin and Histology of Tissues and Cells

Tissue biopsy samples taken from animals suffering from footrot were fixed, processed and sectioned for the procedures to follow. Tissues were taken from cattle either experimentally or clinically affected with footrot. Tissues were either embedded in paraffin or Immuno-Histochemistry Embedding Medium (Immuno-Bed™ Kit, Polysciences, Inc., Warrington, PA) Sections of the embedded tissues were mounted on slides treated with 2% silane (3-aminopropyltriethoxysilane, Sigma), to prevent the tissue sections from sliding off the slides during staining procedures described below.

Bovine capillary endothelium (EJG) cells were passaged as mentioned previously and 500 µl of 10^5 cells/ml were seeded to tissue culture treated, sterile Lab - Tek Chamber slides (8 wells), with covers (Nalge Nunc International, Naperville, IL). The cells were grown to confluence (37°C, 5% CO_2) determined visually with microscopy, and then exposed to 12.5 µg/ml (in cMEM) of purified *P. levii* LPS for two hours. Unexposed cells were grown in cMEM, in parallel as controls. Exposed and unexposed cells were then fixed in 5 % acetic acid (BDH) in methanol (BDH) for 10 minutes, and then stained as described below.

B) Tissue Staining

Tissue sections embedded in paraffin or Immuno-Bed™ were treated in the same way, except for those in paraffin, which were first deparaffinnized using three, three minute washes in Americlear (Stephens Scientific, Riverdale, NJ). From here, all slides including those of EJG cells, were re - hydrated with 3 minute consecutive washes in 100%, 100% and 95% ethanol. The slides were then rinsed in ddH_20 for 3 minutes and then rinsed in 0.15 M Tris buffered saline (TBS), pH 7.5 for 3 minutes. The tissues were then blocked for endogenous alkaline phosphatase activity by submerging them in 1.0 mM tetramisole (Sigma) in TBS for 60 minutes. Further blocking was then carried out by submerging the slides in 10% sheep serum, 0.2% Bovine serum albumin (Sigma) in TBS for 1 hour at room temperature. At this point, neat tissue culture supernatant from hybridomas cultured previously, producing anti - bovine TNFα or anti - bovine GM - CSF (primary antibody sources) or just cRPMI (exposed EJG control), were added, enough to cover the whole sections, and incubated overnight (18 hours) at 4°C. After overnight incubation, slides were washed four times (3 minutes each) in TBS. From here a 1:100 dilution of sheep derived anti - mouse IgG alkaline phosphatase conjugated secondary antibody (Sigma Bio-Chemicals Product No. A-5324) in 0.2% BSA, TBS, was added to the tissue sections which were incubated at room temperature for 30 minutes. The slides were then washed four times as before with TBS. Color development followed by the addition of 0.2 ml of SIGMA FAST™ Fast Red TR/Napthol AS-MX (Fast Red TR 1.0 mg/ml, Napthol AS - MX 0.4 mg/ml, Levamisol 0.15 mg/ml in 0.1 M Tris Buffer) prepared from tablets (Sigma), to each section for exactly 10 minutes. Color development was then stopped by gently rinsing the samples with ddH_20 for 3 minutes. Counterstaining followed by submerging the slides in Gill's Hemotoxylin (Fisher Scientific) for 1 minute and then saturated lithium carbonate for 1 minute with intermittent and final rinses in ddH_20. The slides were then allowed to air dry, and mounted with coverslips using aqueous mounting media (Aqua Poly/Mount, Polysciences Inc., Warrington, PA) and allowed to set overnight before light microscope examination.

C) Slide Examination and Photography

Slides were examined by light microscopy at various magnifications and under oil immersion at 1000 X magnification (Nikon Labophot - 2, Japan). Photographs were taken (Nikon FX - 35DX, Japan) after viewing evidence of detecting the cytokines in question. Mounted, stained slides were stored for future reference as were negative control slides.

Results

1. Growth, Purity and LPS of *Porphyromonas levii*

A) *P. levii* Culture Purity

P. levii was grown in broth cultures (eCMM) and on plates (KVLB, eBHI, BBA) under strict anaerobic conditions. From these cultures, whole cells were used for LPS purification and culture supernatants were used in exposure studies. Culture purity was essential to ensure the reliability of results obtained. Figure 9A shows the results of gram staining *P. levii* and Figure 9B, the growth of *P. levii* on PRAS KVLB agar. Both techniques were utilized for ensuring purity of cultures grown for and used in experiments described earlier. Figure 9A shows the characteristic appearance of *P. levii,* after gram staining. Note the characteristic and identifiable cell morphology including the pleomorphic size and shape of the cells, the random arrangement of cells and the gram - negative staining reaction. Figure 9B shows the growth of *P. levii* on KVLB after 4 days of incubation. Note the characteristic and identifiable colony morphology of *P. levii* , being circular, smooth, raised, black pigmented, and opaque. All *P. levii* cultures used in experiments and preparative procedures were pure as determined by gram staining, growth on KVLB and growth on non - selective BBA (not shown).

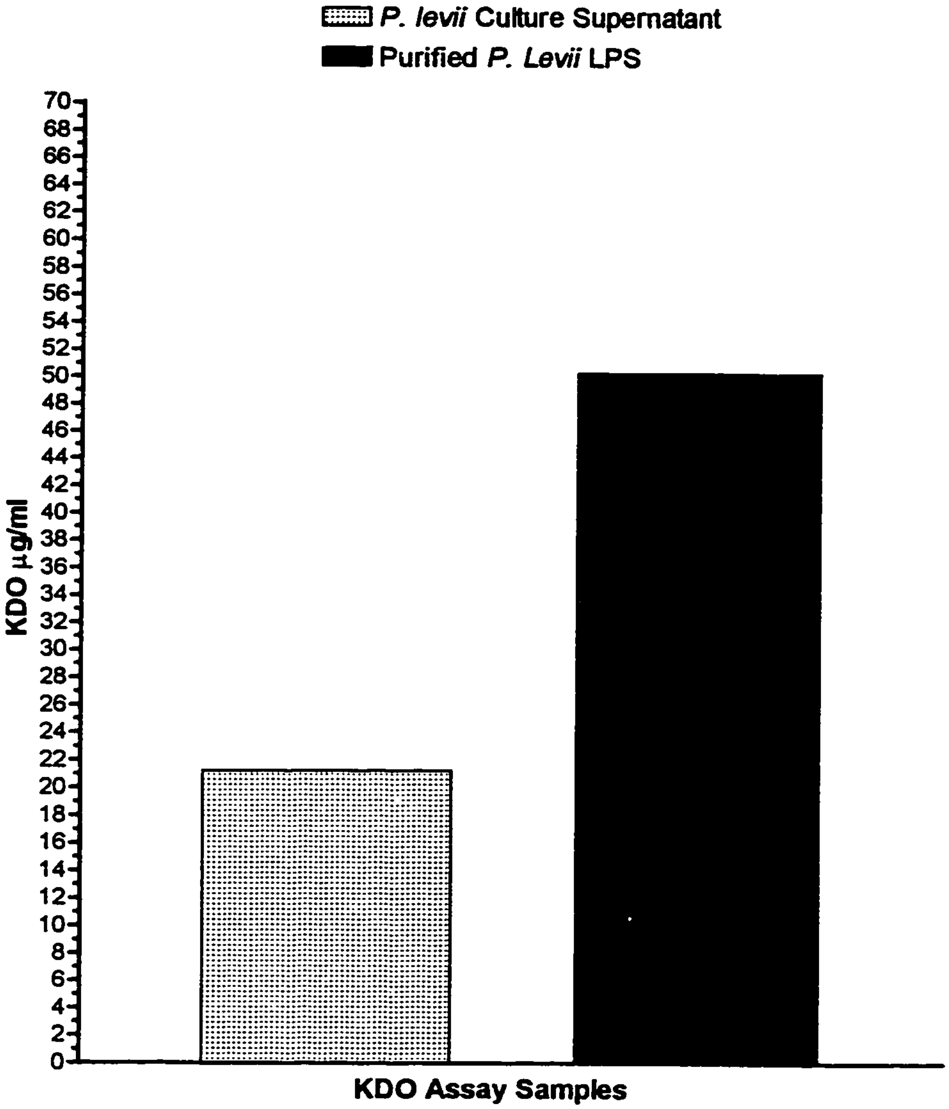
B) *P. levii* Culture Supernatant and Purified LPS Quantification

Porphyromonas levii culture supernatants and purified LPS extract were assayed for KDO, allowing for quantification of LPS used in experiments described earlier. The quantities of KDO measured in each sample, represented by the mean amount value calculated from the KDO standard curve (Figure 3) of each sample (n=3) are shown in Figure 10. From 10 g of freeze dried *P. levii* whole cells, 50 μg/ml of KDO was quantified in the purified LPS extract. This was also used to represent the LPS concentration in the purified LPS extract as being 50 μg/ml. KDO/LPS was found to be

Figure 9: A) Gram stained smear of *Porphyromonas levii* from a pure culture grown anaerobically showing characteristic cell morphology (1000X). B) Pure culture of *Porphyromonas levii* grown anaerobically on KVLB agar after 4 days incubation, showing typical colony morphology, including black pigmentation.

A

Figure 10: Bar graph showing the results of KDO assays performed on *P. levii* crude culture supernatant samples (22 µg/ml KDO/LPS) and purified *P. levii* LPS samples (50 µg/ml KDO/LPS)(n=3).


P. levii Culture Supernatant
Purified P. Levii LPS
KDO µg/ml
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
52
54
56
58
60
62
64
66
68
70
KDO Assay Samples

present in *P. levii* culture supernatants in amounts reaching 22 µg/ml. Controls (ddH_2O) indicated KDO levels were undetectable.

2. Bovine Endothelial (EJG) Cytopathic Effect Experiments

A) Exposures for the Appearance of Cytopathic Effect

All EJG cells were grown to confluence and were morphologically normal before exposure studies were carried out. Figure 11 shows a healthy EJG monolayer at 400X magnification. Note in Figure 11 the fibroblast like appearance of the EJG cells and the long, parallel arranged cells in tight proximity, indicative of a healthy monolayer of EJG. Healthy monolayers similar in appearance to that seen in Figure 11 were exposed to 25 µg/ml of *P. levii* LPS and diluted *P. levii* culture supernatant for up to 240 minutes. Signs of CPE became apparent in the LPS exposed cells starting at t = 120 minutes and in the culture supernatant exposed cells at t = 180 minutes. Figure 12A shows the LPS exposed EJG cells after 240 minutes of exposure shown at 400X magnification, and Figure 12B shows the culture supernatant exposed cells after 240 minutes of exposure seen at 400X magnification. Note the characteristics of CPE seen in Figures 12A and 12B including rounding up of cells, destruction of the monolayer with refractile individual cells, vacuolation of cells, granulation of cells, swollen cells and generally an overall appearance of cellular degeneration.

B) Transwell/Biotin Leakage Experiments

Figure 13 represents the typical cell morphology (400X) of EJG cells viewed after confluence was reached on Transwell membranes (Costar). This monolayer also measured an electrical resistance of 200 ohms and was thus ready for LPS exposure and biotin leakage studies. The results of the biotin leakage studies are seen in Figure 14. ELISA absorbence values of basolateral Transwell (Costar) test samples were compared to the biotin standard curve (Figure 7), and amounts of biotin leakage across the cells/membranes were determined (µg/ml). Biotin - HRP was measured from the basolateral surface in

Figure 11: Normal, healthy EJG monolayer at confluence (400X). (incubation t=0 to t=150).

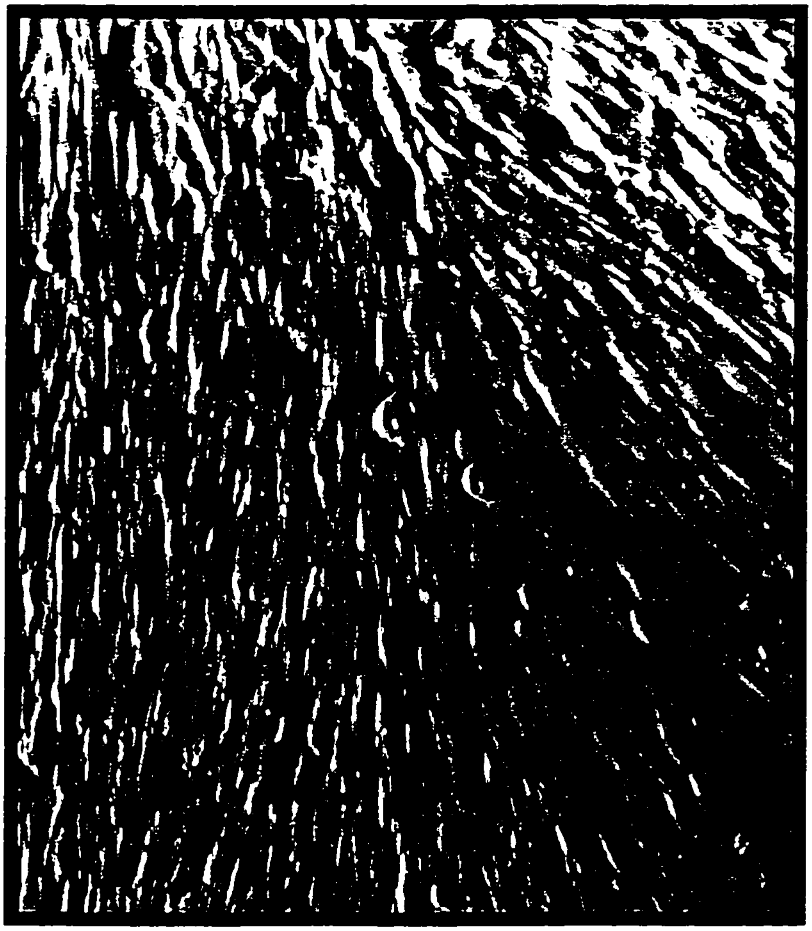
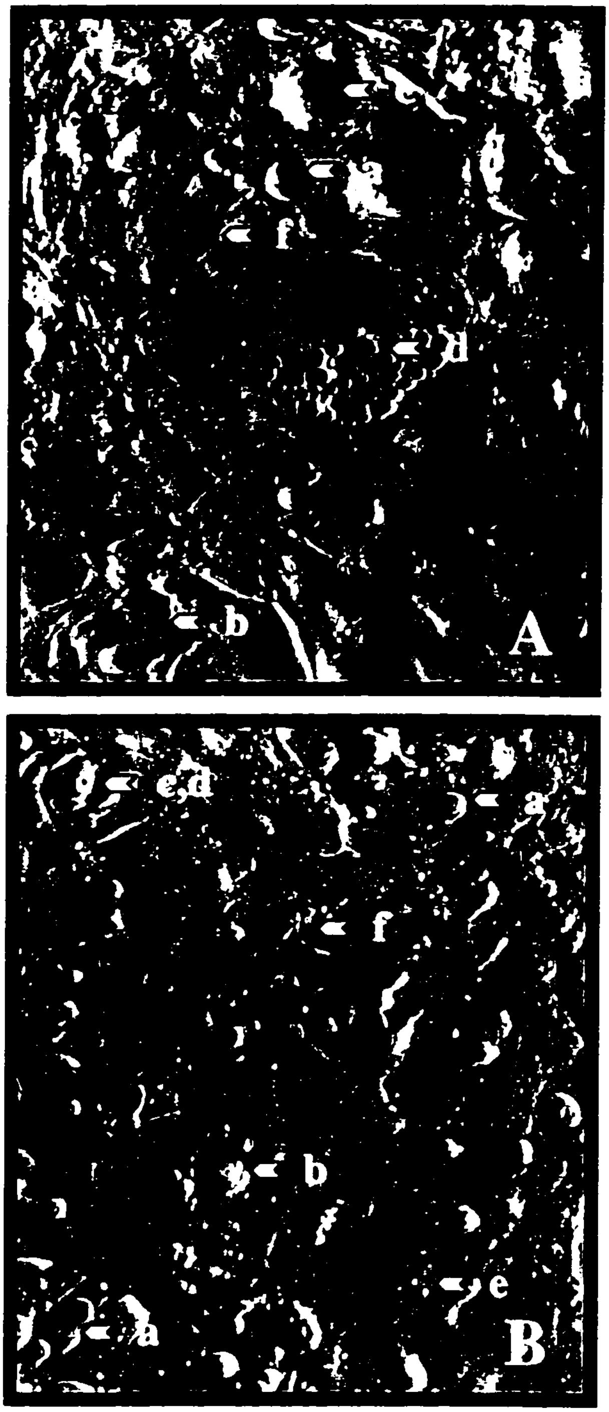


Figure 12: *A*. EJG monolayer after 240 minutes of exposure to purified *P. levii* LPS (400X). *B*. EJG monolayer after 240 minutes of exposure to *P. levii* culture supernatant (400X). Characteristics of CPE seen in Figures 11A and 11B (arrows): **a** - rounding up of cells, **b** - destruction of the monolayer, **c** - refractile individual cells, **d** - vacuolation of cells, **e** - granulation of cells and **f** - swollen cells.


e
a
f
d
b
A
e,d
a
f
b
e
a
B

quantities between 0 and 0.1 µg/ml for all samples. Data (µg/ml) was then normalized and the means graphed so that biotin leakage across LPS exposed and non-exposed EJG confluent Transwells (Costar) (n=21), could be compared at each time interval of incubation. For *E. coli* 0111:B4 LPS exposed Transwell (Costar) cultures, biotin leakage was statistically significant when compared to controls at the same time interval for times: 0, 15, 30, 45, 60, 105, 120 and 150 minutes. For Purified *P. levii* LPS exposed Transwell (Costar) cultures, biotin leakage was statistically significant when compared to controls at the same time interval for times: 15, 30, 105, 120, and 135 minutes. Significance indicated that LPS disrupted EJG confluence (CPE) at these incubation times, allowing more biotin to cross the LPS exposed monolayers when compared to corresponding non-exposed controls. Non - parametric analysis was performed within groups of the same time interval using a two-tailed P value Mann-Whitney Test.

3. Cytokine Protein Detection

Figure 15 is a representation of a silver stained SDS - PAGE gel loaded with samples (lanes B and C) after EJG cells were exposed to purified *P. levii* LPS. Lane B shows proteins stained from EJG cell fractions after LPS exposure and Lane C shows proteins stained from EJG crude culture supernatants after LPS exposure. Both samples (lanes B and C) were prepared after 240 minutes of incubation. Other incubation times (described previously) were run on SDS - PAGE gels as well, after EJG cell monolayers were exposed to *P. levii* LPS, showing similar staining profiles for both EJG cell and crude culture supernatant samples (data not shown). EJG cells exposed to *P. levii* crude culture supernatant gave similar results as did the non - exposed controls over similar incubation times (data not shown). No clear detectable difference in silver staining protein profiles was seen for any of the samples that were run, despite differences in staining intensities. Lane A shows silver stained molecular weight protein markers with masses in kilodaltons (kDa).

Figure 13: Photomicrograph of a Confluent EJG monolayer growing on a Transwell (Costar) membrane measuring an electrical resistance of 200 ohms (400X).

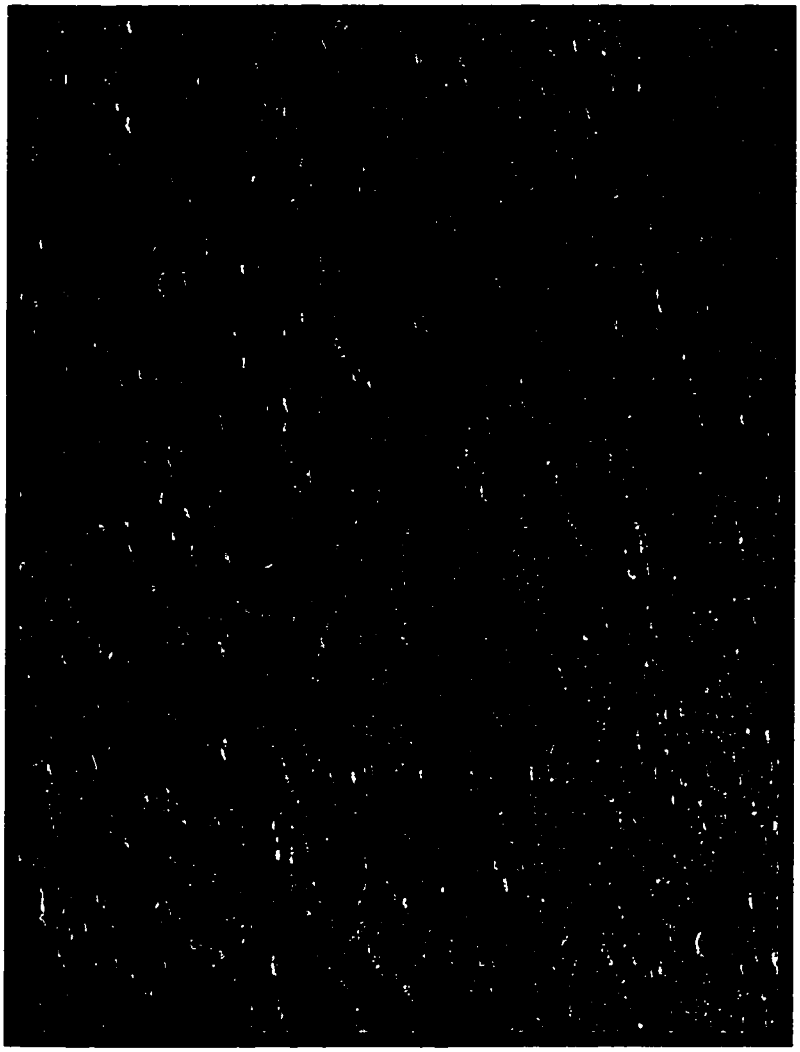
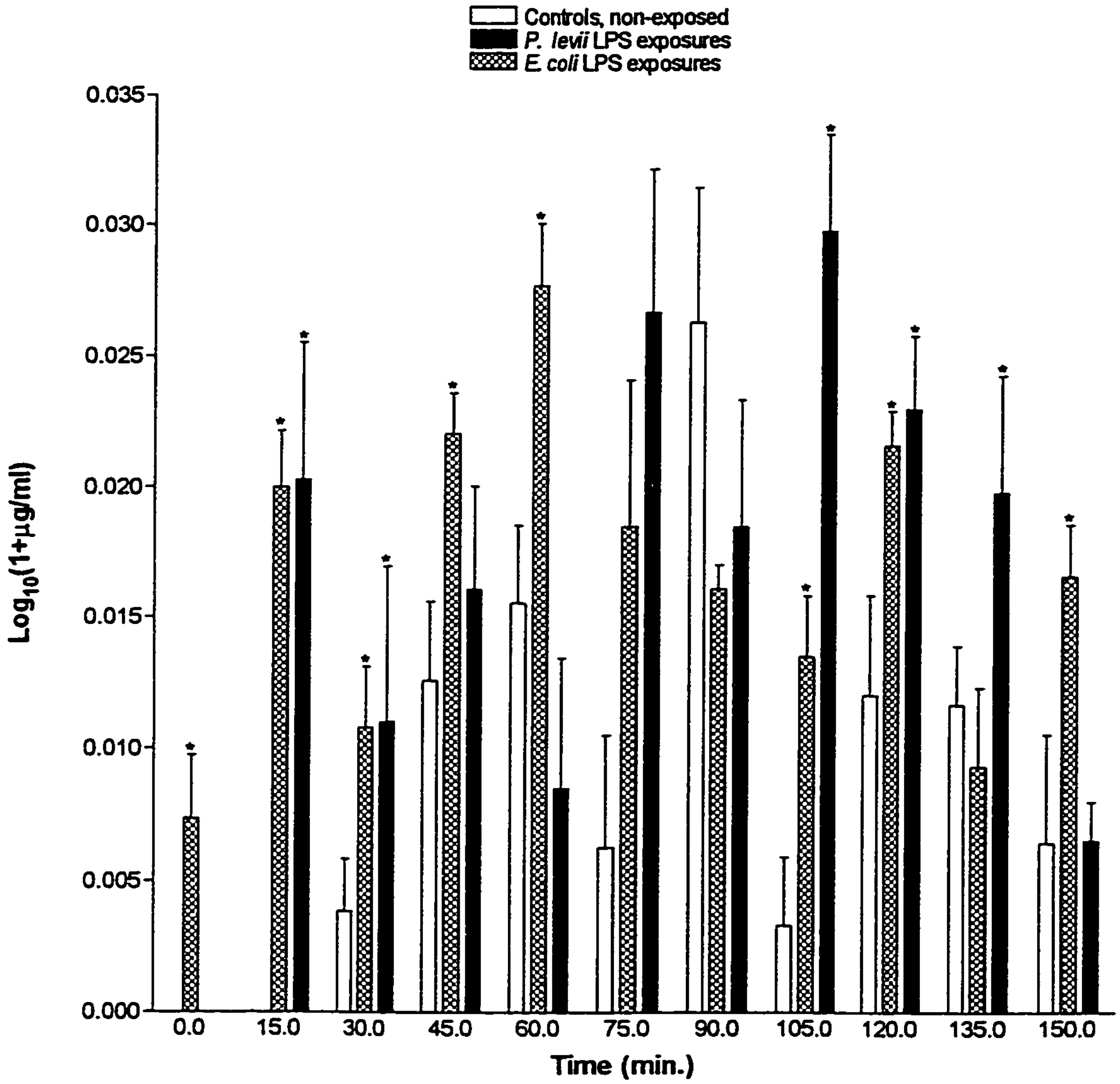


Figure 14: Bar graph showing the results of the Transwell/Biotin leakage studies representing the comparison of the mean $\mathrm{Log}_{10}(1+\mu g/ml)$ values of biotin - HRP recovered from the basolateral surface of Transwell cultures within each incubation time. All test and control groups had n values of 21 (±SEM), (* = significantly different $P<0.05$).


Controls, non-exposed
P. levii LPS exposures
E. coli LPS exposures
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Log$_{10}$(1+μg/ml)
0.0
15.0
30.0
45.0
60.0
75.0
90.0
105.0
120.0
135.0
150.0
Time (min.)
*

A) Western Blotting and Detection of TNFα and GM-CSF

Figure 16 demonstrates that EJG cells produced TNFα and GM - CSF after monolayers were exposed to purified *P. levii* LPS at 60 minutes, 120 minutes, 180 minutes and 240 minutes (Figures 16A and 16B). Bands appeared similar at all time periods for both TNFα (14A) and GM - CSF (14B). Exposure of monolayers to *P. levii* crude culture supernatant gave similar results (data not shown) at the same time intervals as did the LPS exposures. Band intensities were greater for EJG cell - associated fractions of exposed EJG cells, than for EJG crude culture supernatant associated fractions. By comparing Rf values of molecular weight markers run in parallel with each samples (not shown), the molecular weight of the major band seen in each blot was estimated. In Figure 16A, the major band (TNFα) had a molecular weight of approximately 36 kDa. In Figure 16B, the major band (GM - CSF) had a molecular weight of approximately 35 kDa. Lane "a" in both Figures A and B, represents non - exposed control samples for which negligible amounts of either cytokine were detectable.

4. IL-1ß mRNA Detection

A) Probe Preparation

Figure 17 represents a 1.2% agarose gel stained with EtBr, showing the various stages of IL-1ß cDNA sequence purification, for DIG - labeled probe purification procedures. Molecular weight markers shown in lane "a" confirmed the size of the purified IL-1ß cDNA to be approximately 775 base pairs (lane d). Lane "b" shows the results of isolating the pBIL1Beta 3.3 plasmid out of transformed *E. coli* strain DH5-α. Note the appearance of multiple bands in lane "b" due to the different confirmations of pBIL1Beta 3.3 (i.e. supercoiled and relaxed). Lane "c" shows the results of a successful Eco RI

Figure 15: SDS - PAGE/Silver stain analysis of EJG culture fractions after *P. levii* LPS exposure. *Lane A* shows silver stained molecular weight protein markers with masses in kilodaltons (kDa). *Lane B* shows proteins stained from EJG cell fractions after LPS exposure, *Lane C* shows proteins stained from EJG crude culture supernatants after LPS exposure and *Lane D* shows proteins stained from EJG cells, not exposed to LPS or crude culture supernatant (healthy EJG cells).

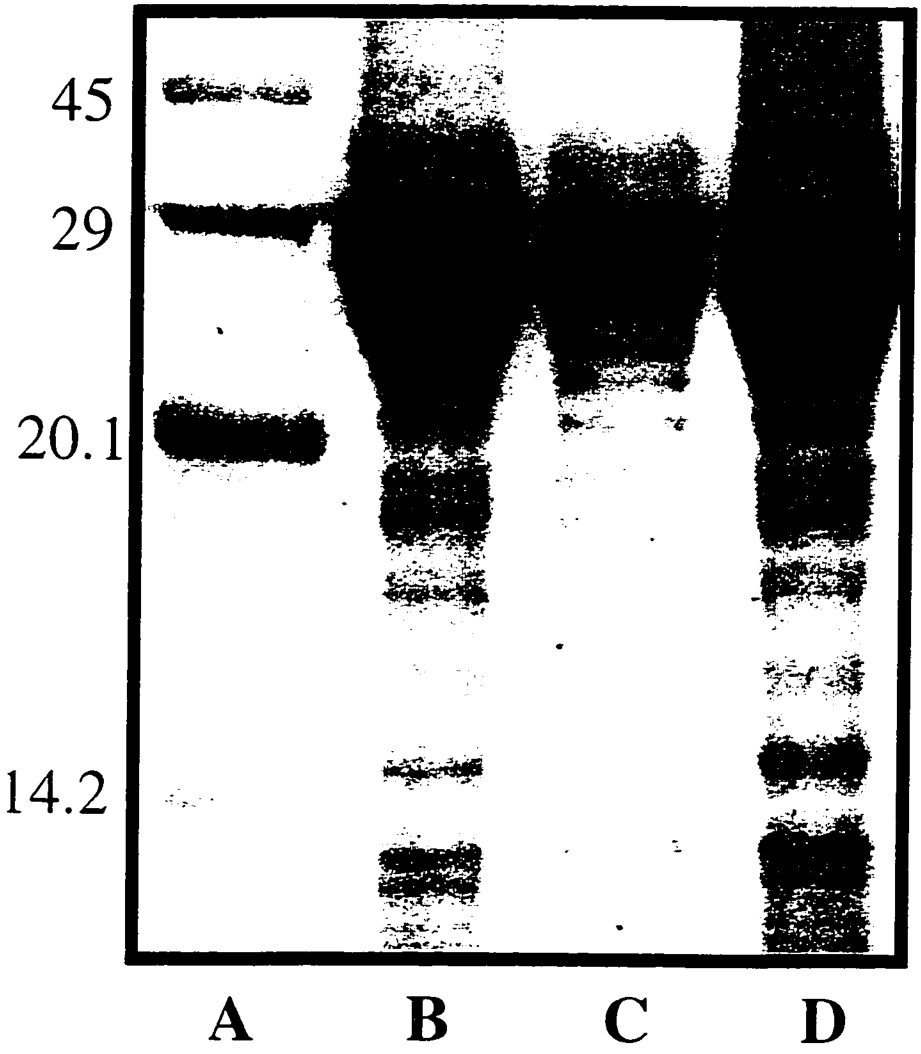

45
29
20.1
14.2
A
B
C
D

Figure 16: *A*. Western blot showing the presence of bovine endothelial cell (EJG) TNFα. *B*. Western blot showing the presence of bovine endothelial cell (EJG) GM-CSF. *Lane a*. negative control; *Lane b*: 60-minute LPS exposure; *Lane c*: 120-minute exposure; *Lane d*: 180-minute exposure; *Lane e*: 240-minute exposure.

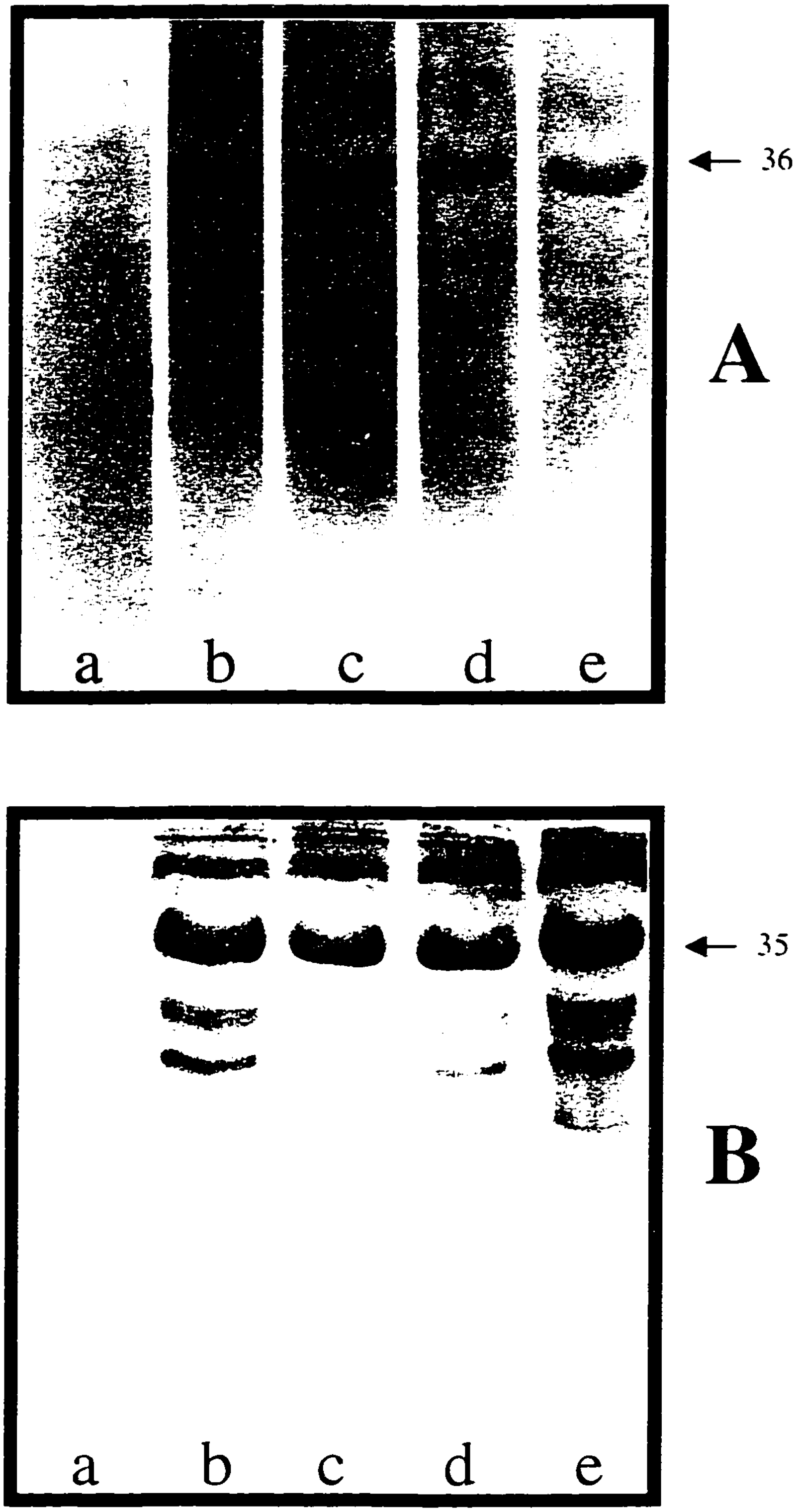
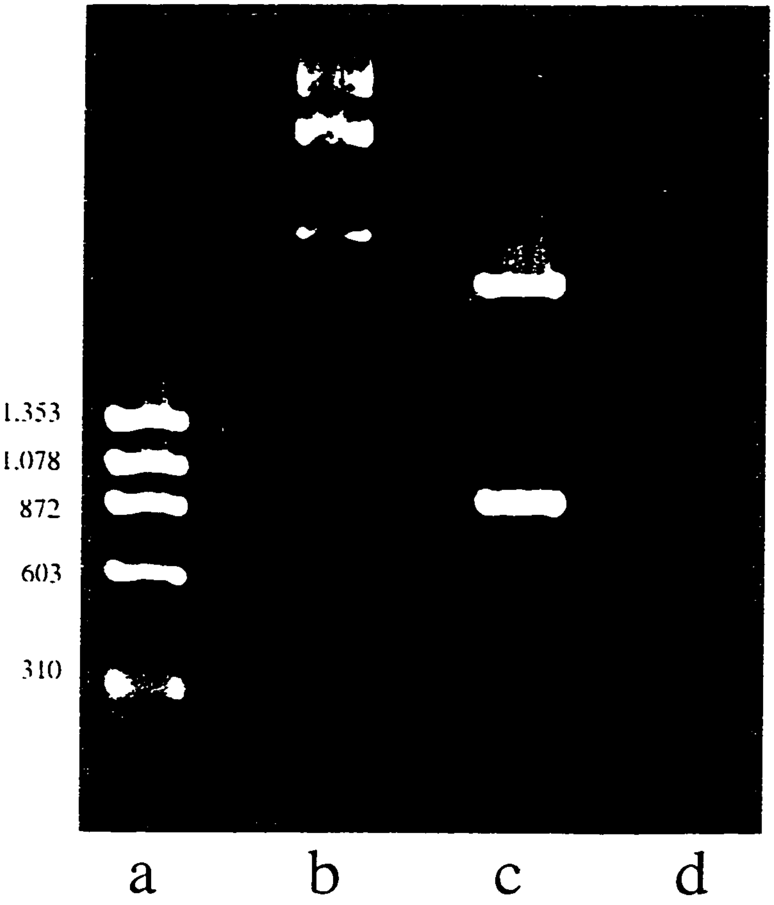

36
A
a
b
c
d
e
35
B
a
b
c
d
e

Figure 17: Agarose gel illuminated by ultra violet light showing the various stages of IL-1ß cDNA sequence purification. *Lane a*: DNA molecular weight markers (bp); *Lane b*: pBIL1Beta 3.3 plasmid; *Lane c*: digested pBIL1Beta 3.3; *Lane d*: purified IL-1ß cDNA.


1.353
1.078
872
603
310
a
b
c
d

digest of pBILlBeta 3.3. The two bands appearing in lane "c" represent the nicked (digested) plasmid (large molecular weight), and the IL-1ß cDNA sequence.

B) Quantification of DIG - Labeled IL-1ß cDNA Probes

Figure 18 shows the results of utilizing DIG Quantification Teststrips (Boehringer Mannheim) and DIG Control Teststrips (Boehringer Mannheim) for quantification of digoxigenin - labeled IL-1ß cDNA probes in comparison to standardized digoxigenin - labeled control DNA. Dilutions of a pre-diluted (1/40) IL-1ß probe labeling reactions are seen in lane A (n=3). Lane B represents DIG Control Teststrips (Boehringer Mannheim) with the amounts of DIG control DNA indicated (pg/μl) (n=3). Measuring the spot intensities of the DIG Control Teststrips (Boehringer Mannheim) allowed for a standard curve of spot intensity compared to DIG DNA concentrations to be constructed as seen in Figure 19. Measuring the intensities of the IL-1ß DIG-probe spots on the test strips (Lane A) allowed for quantification of the DIG labeled probe produced, when compared to the standard curve (Figure 19). For example, the amount of probe produced was measured from the 1:100 dilution spot (Figure 18, Lane A) and determined to be 2.84×10^5 pg/μl.

C) Total EJG Cell RNA Quantification

Before RNA gel electrophoresis and Northern Blotting experiments were performed, RNA from each exposure sample was quantified, to ensure approximately equal amounts of RNA were loaded into each sample well. In Figure 20A, the reference chart for comparison quantification is shown (Clontech). Figure 20B represents Nucleic Acid Quicksticks (Clontech) loaded with purified EJG RNA from exposure studies. Figure 20-B1 represents unexposed EJG RNA. Figure 20-B2 represents *P. levii* LPS exposed EJG RNA from time 0. Figure 20-B3 represents *P. levii* LPS exposed EJG RNA from time 45 minutes. Figure 20-B4 represents *P. levii* LPS exposed EJG RNA from time 2 hours and 30 minutes. As demonstrated in Figure 20B, staining intensities from all quantification samples appeared similar (all data not shown), and when compared to the

Figure 18: *A*. DIG Quantification Teststrips (Boehringer Mannheim) and *B*. DIG Control Teststrips (Boehringer Mannheim) for quantification of digoxigenin - labeled IL-1ß cDNA probes. *Lane A* shows dilutions of a IL-1ß probe labeling reaction. *Lane B* shows control teststrips with amounts of DIG - DNA in pg/µl.

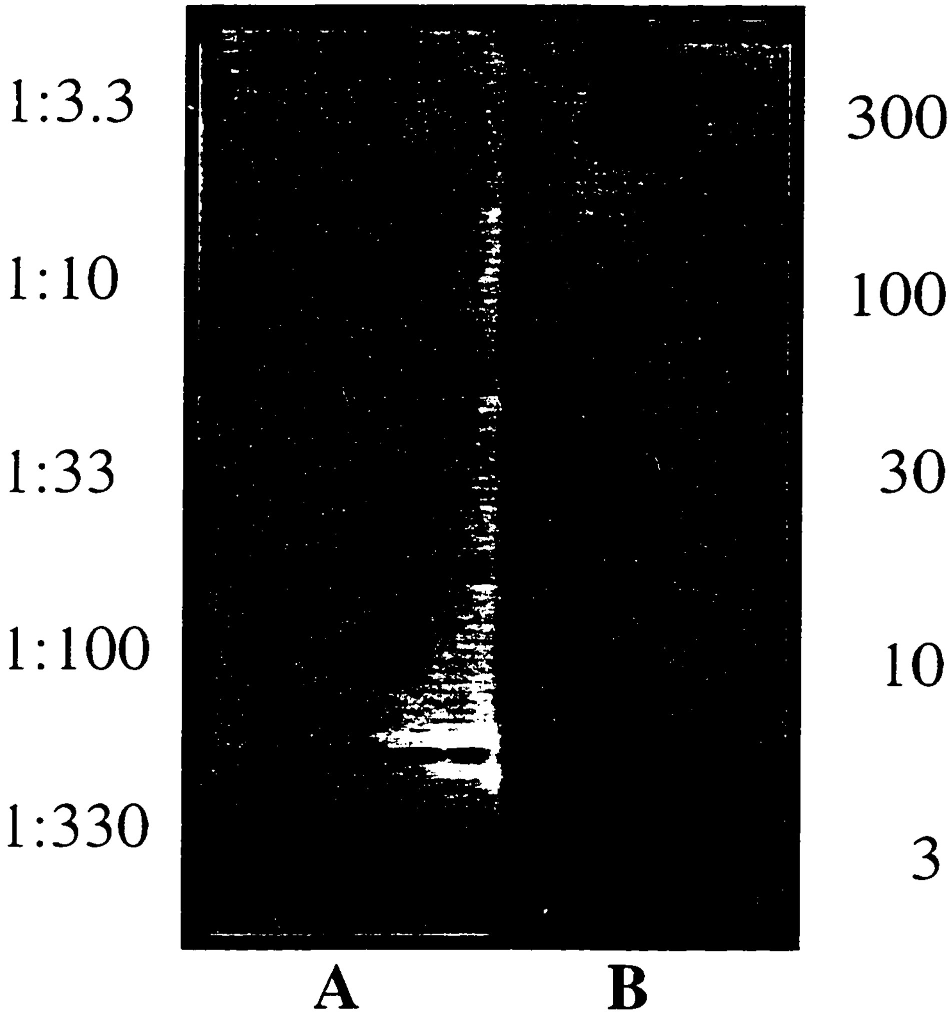

1:3.3
1:10
1:33
1:100
1:330
300
100
30
10
3
A
B

Figure 19: Standard curve for the DIG Control Teststrips (Boehringer Mannheim) for quantification of digoxigenin - labeled IL-1ß cDNA probes in comparison to standardized digoxigenin - labeled control DNA. Intensity values were converted from percent values and plotted as 1-(%/100). Control amounts of DIG labeled DNA (3, 10, 30, 100, 300 pg/μl) had intensities plotted for generation of the standard curve (R=0.91979).

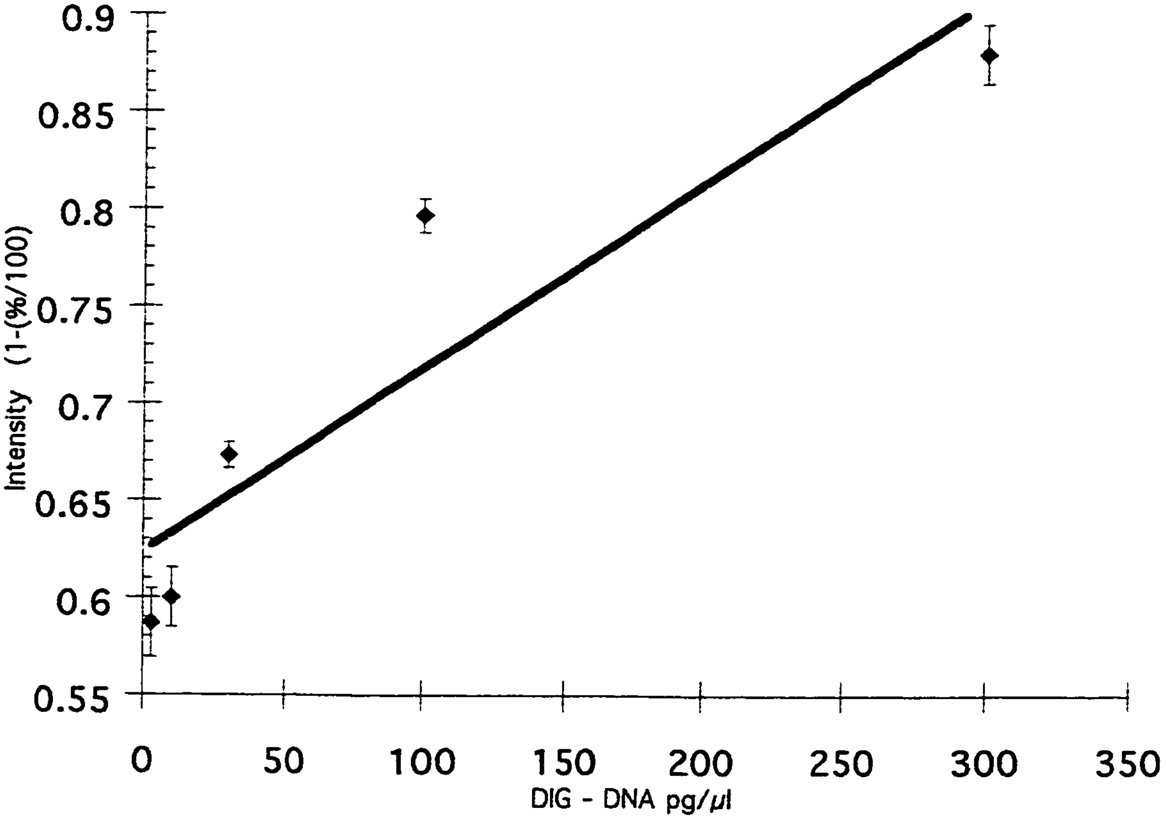
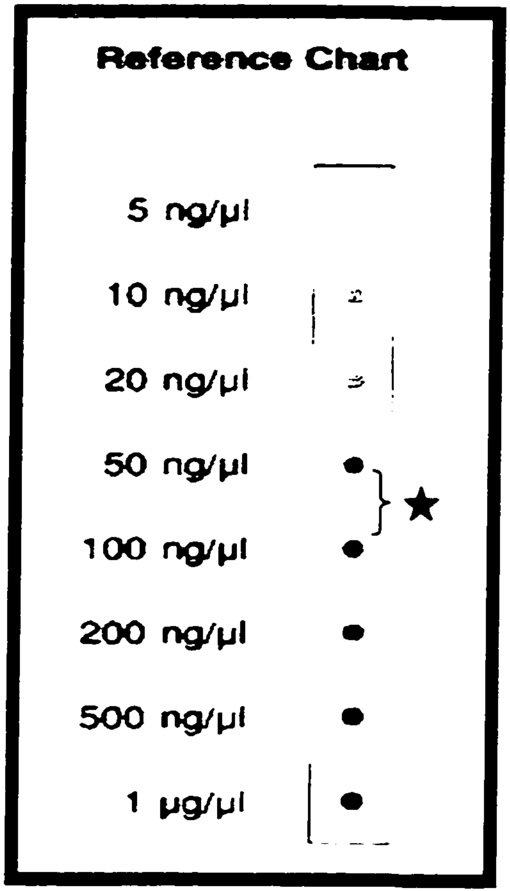
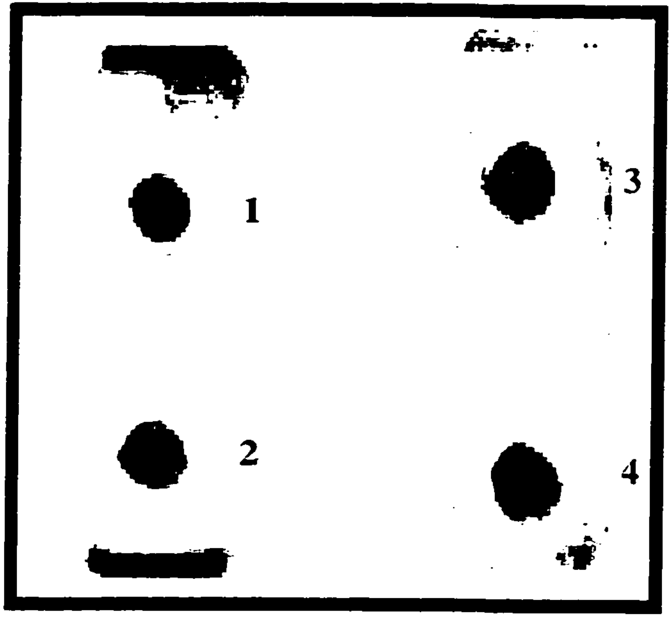

0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
Intensity (1-(%/100)
0
50
100
150
200
250
300
350
DIG - DNA pg/µl

Figure 20: *A*. Reference chart (Clontech) for the quantification of RNA isolated from EJG cells. *B*. Quantification test spots of RNA isolated from EJG cells which were compared to the reference chart *(A)*. *B1*: unexposed EJG RNA; *B2*: t=0 min., *P. levii* LPS exposed EJG cells; *B3*: t=45 min. *P. levii* LPS exposed EJG cells; *B4*: t=150 min. *P. levii* LPS exposed EJG cells. *A*. ★ indicates approximate test spot intensity correlation's for all quantified samples (n=3) (~75 ng/µl RNA).


Reference Chart
5 ng/µl
10 ng/µl
20 ng/µl
50 ng/µl
100 ng/µl
200 ng/µl
500 ng/µl
1 µg/µl
★

A


1
2
3
4

B

reference chart it was determined that approximately 75 ng/μl (Figure 20 ★) of RNA was purified from all exposed and non - exposed EJG samples. It was found that the yield of RNA isolated utilizing TRI REAGENT™ (Sigma) was superior when compared to the method outlined in Sambrook et al., 1989, for the rapid isolation of total RNA from mammalian cells.

D) RNA Formaldehyde Agarose Gel Electrophoresis

Denaturing RNA gels were set up and run before Northern Blotting to nylon membranes. Figure 21A shows EtBr stained RNA bands (major and minor) from purified EJG total RNA samples, where cell monolayers were exposed to *P. levii* LPS over time. Figure 21B shows EtBr stained RNA bands from purified EJG total RNA samples where cell monolayers were exposed to *E. coli* 0111:B4 LPS (Sigma) over time. Each lane (a-f) was loaded with 15 ng/μl of purified RNA. For both gels, bands in each lane appeared to have similar intensities when viewed using ultra violet light. Figure 21 A+B, lanes a to f, represent 30 minute interval RNA samples from t=0 to t=150 min. respectively. Lane g represents RNA molecular weight markers. Gels (not shown) containing non-exposed EJG cell RNA appeared very similar to those in Figure 21. After Northern Blotting, no RNA remained in either Gel A or Gel B when viewed using ultra violet light.

E) EJG IL-1ß Northern Blot Analysis

RNA gels similar to those seen in Figure 21 were blotted to nylon membranes and either probed for actin mRNA (Figure 21A) or IL-1ß mRNA (Figure 21B) using DIG - labeled DNA probes. Figure 21A shows a successful hybridization of DIG-actin probe to actin mRNA, with the major hybridization product appearing just above the 1000 base pair DIG - labeled RNA molecular weight marker (lane a). This size corresponds to that expected for mammalian actin mRNA. It is also seen in Figure 21A, that actin mRNA production from *P. levii* LPS exposed EJG cells is down regulated from times t=0 to t=150 minutes (lanes b-g respectively). Results were similar for *E. coli* 0111:B4 LPS exposed

Figure 21: *A*. RNA gel with *P. levii* exposed EJG RNA. *B*. RNA gel with *E. coli* O111:B4 exposed EJG RNA. *Lane a*: t=0; *Lane b*: t=30; *Lane c*: t=60; *Lane d*: t=90; *Lane e*: t=120; *Lane f*: t=150; *Lane g*: RNA molecular weight markers (t=minutes).

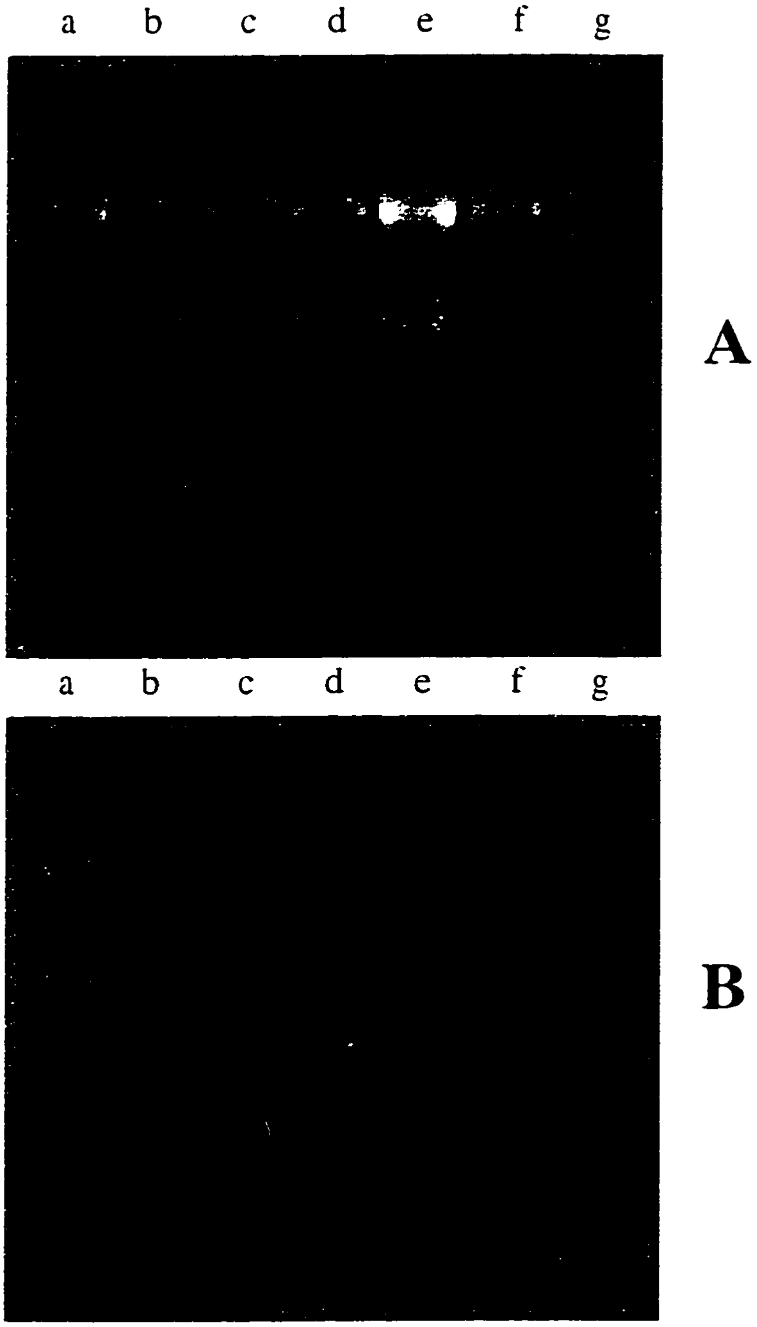
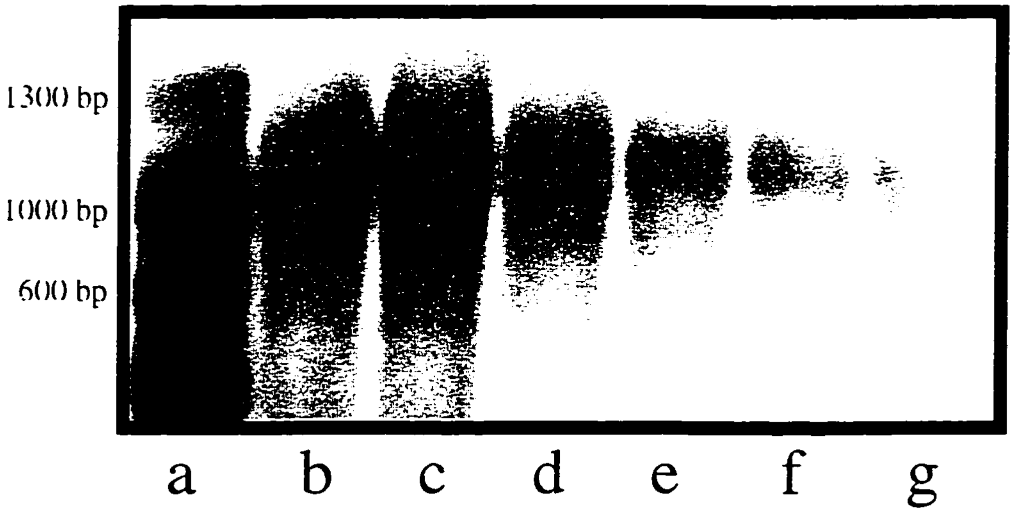
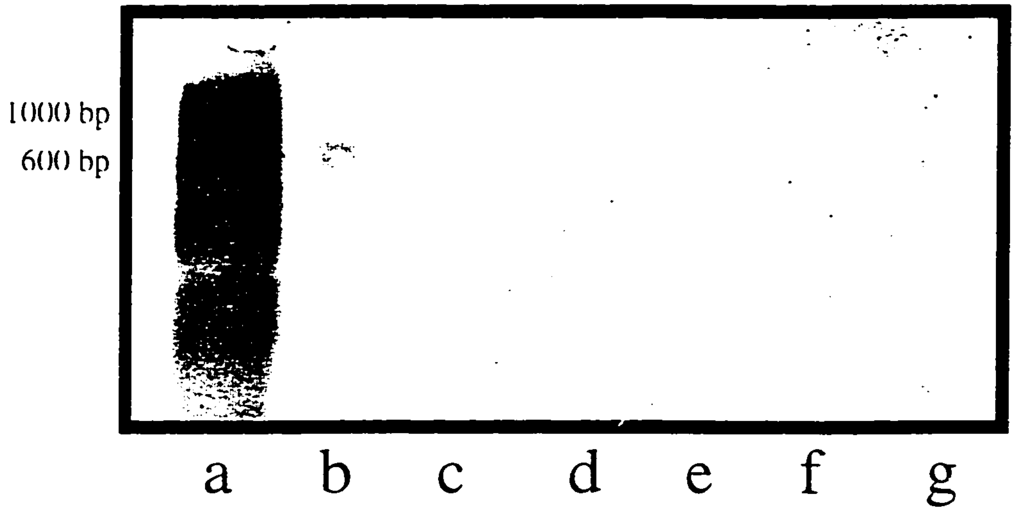

a b c d e f g
A
a b c d e f g
B

Figure 22: *A*. Northern Blot showing the hybridization products for *P. levii* LPS exposed EJG cell actin mRNA over time. *B*. Northern Blot showing the hybridization products for *P. levii* LPS exposed EJG cell IL-1ß mRNA over time. *Lane a*: DIG-Labeled RNA molecular weight markers; *Lane b*: t=0 products; *Lane c*: t=30 min. products; *Lane d*: t=30 min. products: *Lane e*: t=60 min. products: *Lane e*: t=90 min. products; *Lane f*: t=120 min. products; *Lane g*: t=150 min. products.


1300 bp
1000 bp
600 bp
a
b
c
d
e
f
g

A


1000 bp
600 bp
a
b
c
d
e
f
g

B

samples (not shown). Actin mRNA synthesis did not change in unexposed control EJG cells (not shown).

Figure 21B shows a successful hybridization of DIG-IL-1ß probe to EJG IL-1ß mRNA, with the major hybridization product appearing in-between the 600 and 1000 base pair DIG - labeled RNA molecular weight marker (lane a). This size range would correspond to that expected for IL-1ß RNA (~775 bp). It is also seen in Figure 21B that IL-1ß mRNA synthesis is upregulated from t=0 to t=60 minutes (lanes b-d) and then remains unchanged or decreased from t=60 minutes to t=150 minutes (lanes d-g). Results were similar for *E. coli* 0111:B4 LPS exposed samples (not shown). IL-1ß mRNA was not detectable in unexposed EJG cells (not shown).

F) EJG IL-1ß Dot Blot Analysis

With the goal of semi-quantifying IL-1ß mRNA production from LPS exposed EJG cells over time, the results are shown in Figure 23. DIG - Labeled RNA controls are seen in Figure 23, Lanes A and C. DIG - Detection products were seen for control RNA amounts of 300 pg/µl (1), 100 pg/µl (2) and 30 pg/µl (3), but not for 10 or 3 pg/µl. IL-1ß hybridization products were seen for all times (t=30-t=150 minutes, a-e) except for t = 0 and unexposed cell controls (not indicated on Figure 23). 15 ng/µl of RNA was added to each dot for each time sample in Figure 23, Lanes B and D (n=5). For this quantity of total *P. levii* LPS exposed EJG cell RNA, for times 30 to 120 minutes, quantities of IL-1ß mRNA would be below 30 pg/µl and above 10 pg/µl. For time 150 minutes, IL-1ß mRNA appeared to be present in quantities above 30 pg/µl and below 100 pg/µl. Results appeared similar for RNA isolated from *E. coli* 0111:B4 LPS (sigma) (data not shown).

5. TNFα and GM-CSF Immunohistological Staining *In Vitro* and *In Vivo*.

A) *P. levii* Exposed EJG Cells

Figure 24 shows control EJG samples stained for TNFα and evaluated for non-specific staining reactions. In Figure 24A, it is seen that no red precipitate (Fast Red TR,

Figure 23: Dot Blot representing the quantification of *P. levii* exposed EJG cell IL-1ß mRNA. Lanes *A* and *C* contain DIG - labeled RNA controls in the amounts of *1*: 300 pg/µl; *2*: 100 pg/µl; *3*: 30 pg/µl. Lanes *B* and *D* contain hybridization products for EJG IL-1ß mRNA seen in *a*: t=30 min.; *b*: t=60 min.; *c*: t=90 min.; *d*: t=120min; *e*: t=150 min.. ★ indicates the locations where t=0 min. controls were applied.

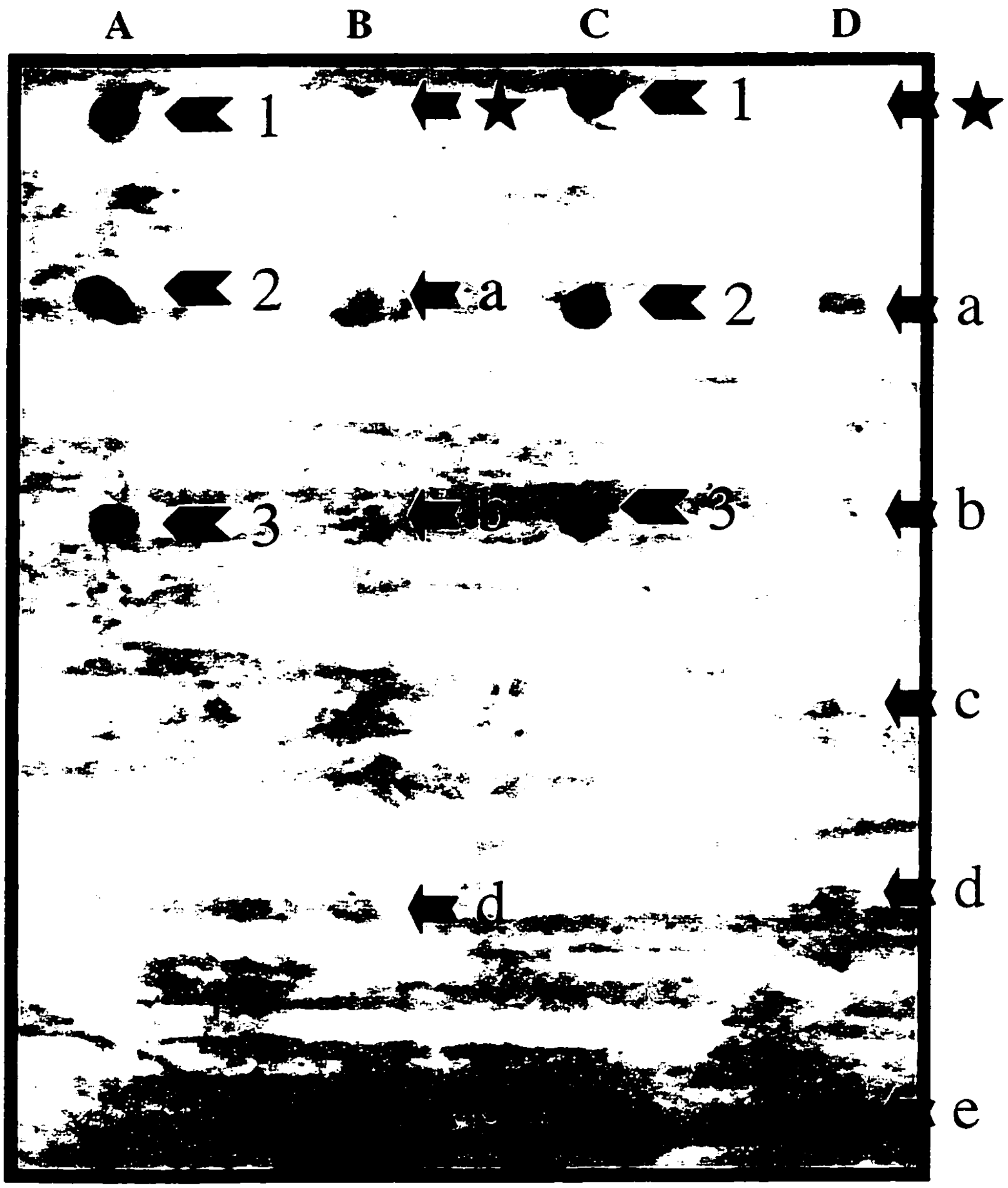
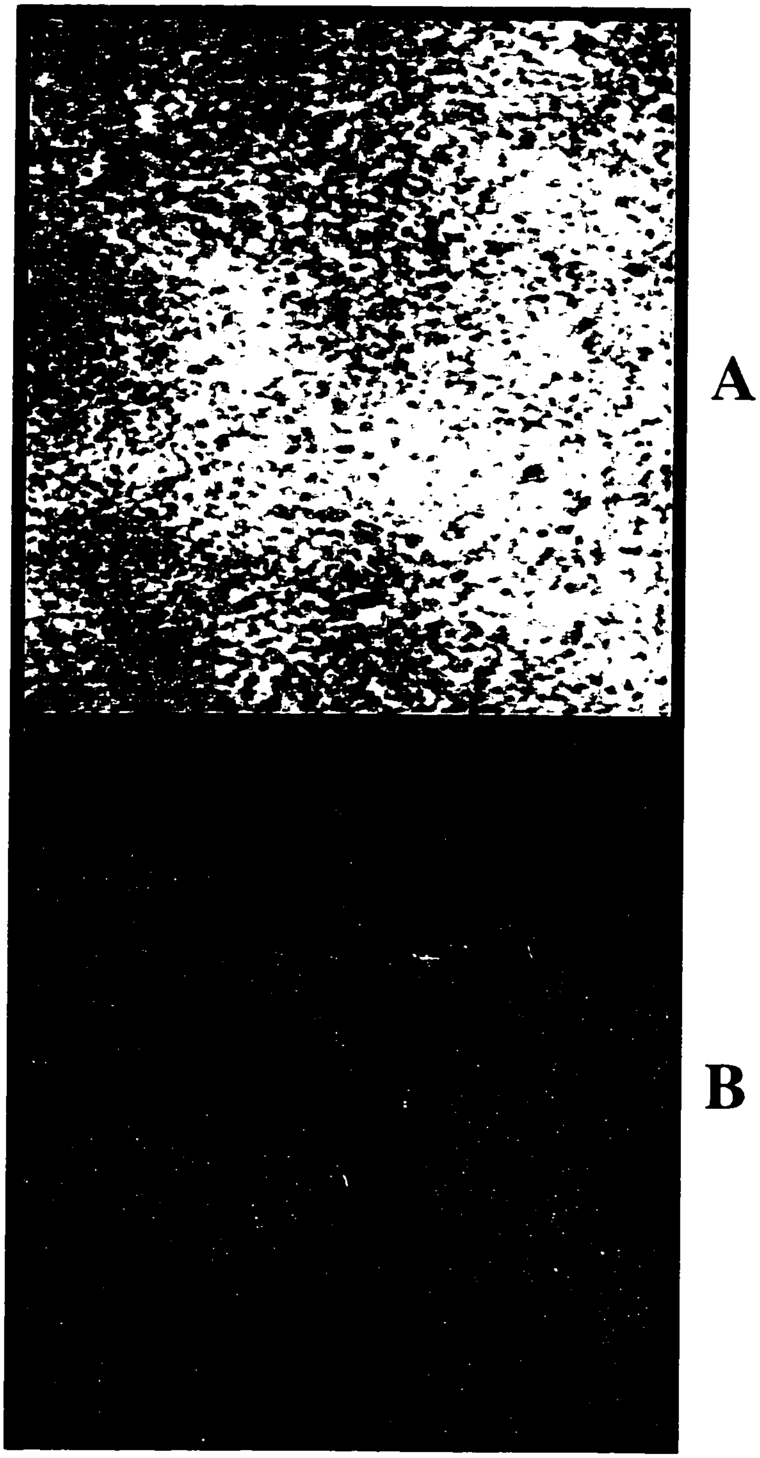

A
B
C
D
1
★
1
★
2
a
2
a
3
b
3
b
c
d
d
e

Figure 24: *A*. Non LPS exposed EJG cells stained for TNFα. *B*. *P. levii* LPS Exposed EJG cells stained with 2° antibody only (control).


A
B

Sigma) and therefore no TNFα was detectable in EJG cells not exposed to *P. levii* LPS. In Figure 24B, LPS exposed EJG cells were not incubated with primary antibody (anti - TNFα monoclonal), but were incubated with secondary antibody (anti - mouse alk-phos. conjugate). As shown in Figure 24B, no red precipitate was seen and thus no non-specific binding by the conjugate antibody would be expected in further staining procedures.

Figure 25 shows the presence of TNFα in *P. levii* LPS exposed EJG cells incubated for 2 hours. The presence of Fast Red TR precipitate indicated the presence of TNFα, produced by the EJG cells in response to *P. levii* LPS exposure. Staining for GM-CSF was inconclusive (data not shown).

B) Footrot Biopsy Samples

Tissue biopsy samples were taken from animals suffering from footrot, and stained for the presence of GM-CSF and TNFα. Both cytokines were found to be present in the stained biopsy samples as seen in Figure 26. Figure 26A and 26B, shows that GM-CSF is produced in tissues taken from the interdigital region of cattle suffering from footrot. Figure 26C and 26D, shows that TNFα is produced in tissues taken from the interdigital region of cattle suffering from footrot. The presence of a red precipitate indicated positive staining for each cytokine protein and that these cytokines are produced in this disease. Controls using no primary antibody were carried out, and came out negative for any non-specific staining results (not shown).

Figure 25: Photomicrographs of EJG cells staining positive (red) for TNFα after *P. levii* LPS exposure. *A*: 100X; *B*: 400X; *C*: 1000X.

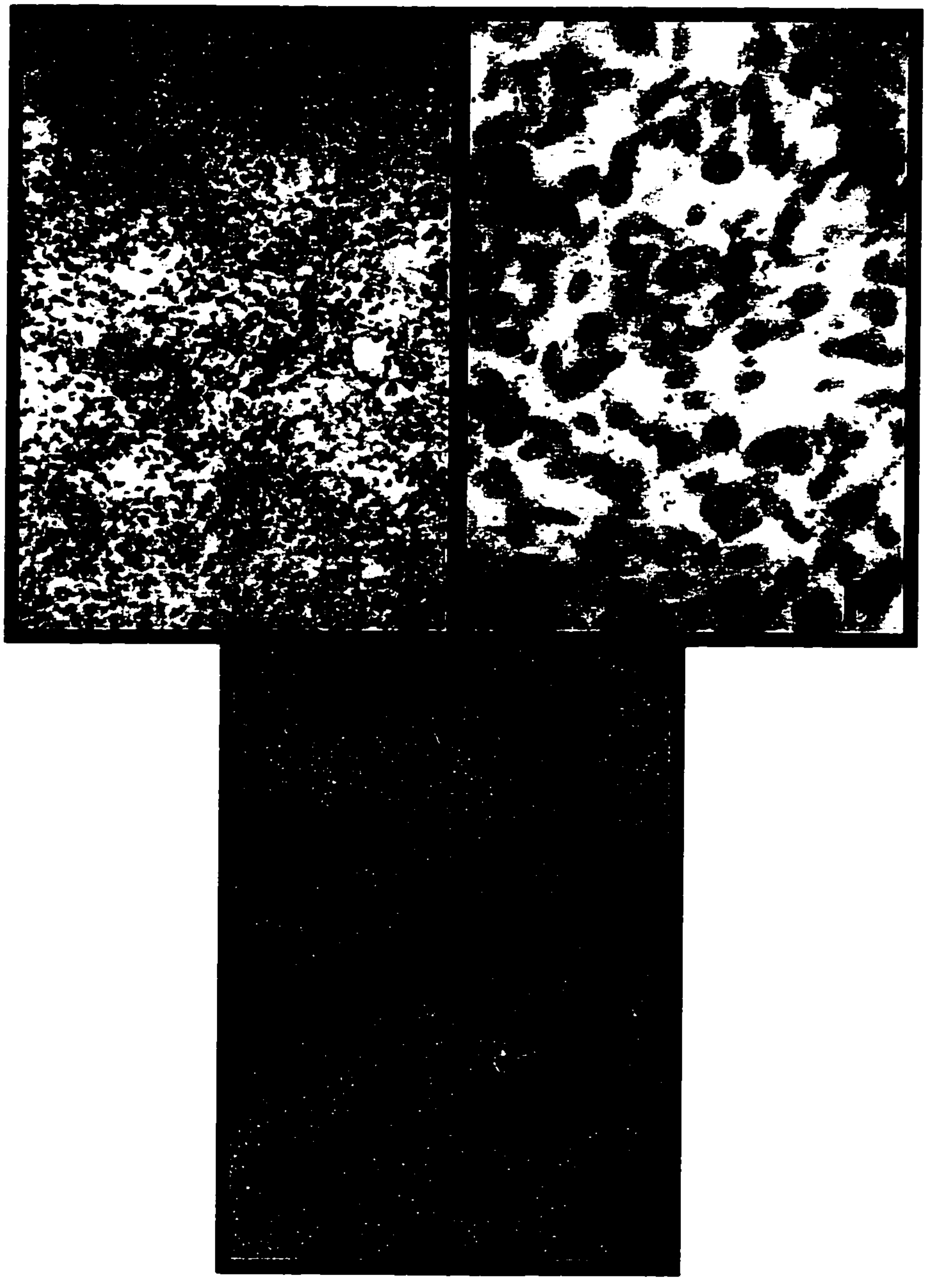
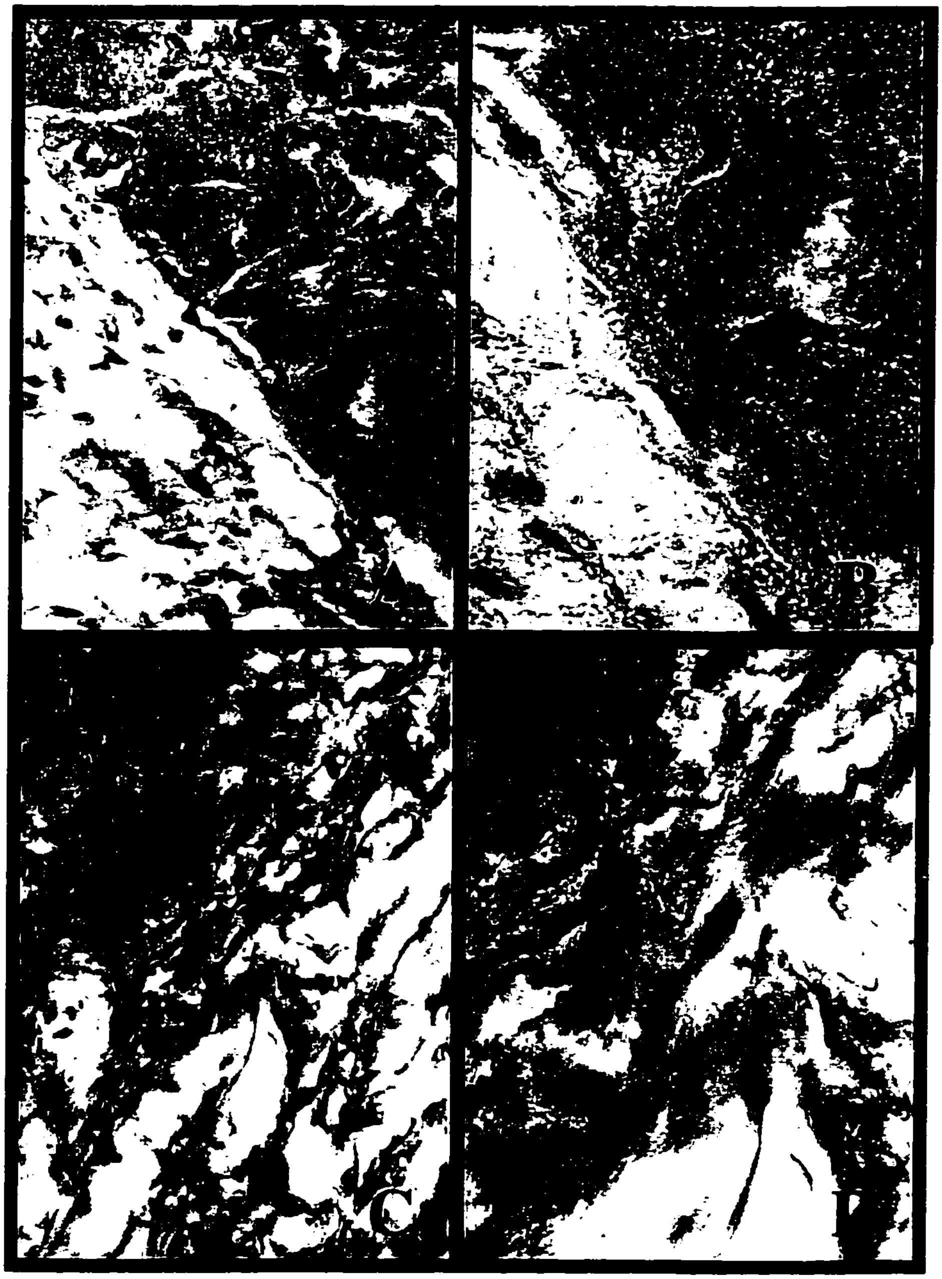


Figure 26: Photomicrographs of tissue biopsy sample taken from the interdigital region of cattle suffering from footrot and stained for the presence of GM-CSF and TNFα. A.: Positive stain for GM-CSF (400X); B: Positive stain for GM-CSF (1000X); C: Positive stain for TNFα (400X); Positive stain for TNFα (1000X). Presence of red precipitate indicates positive staining.


A
B
C
D

Discussion

Porphyromonas levii is commonly isolated from animals suffering from acute footrot as cited in Morck, 1995 (Morck et al., 1998). The exact role of this obligate anaerobe in the pathogenesis of footrot is still unknown. The pathological hallmarks of footrot including edema and inflammation, lead investigators to postulate that *P. levii* may interact with bovine endothelial cells at the site of infection. It was thought that this interaction, may influence the resulting immunopathogenesis of this infection. For this reason, the objectives of this study were designed to examine some of the possible roles *P. levii* and its LPS may play in damaging bovine endothelial cells and in stimulating them to act as immunocontrolling and immunomodulating cells *in vitro*. From the results of this study, it was theorized that the information gained may be descriptive in what occurs immunopathologically in footrot *in vivo*.

P. levii and its LPS interact with bovine endothelial cells (EJG), damaging them, as shown by the CPE and Transwell (Costar) experiments. The evidence shown by the results of this study, indicate that *P. levii* and LPS from this bacterium damage endothelial cells *in vitro* and may contribute to the pathology seen in footrot. These investigations also suggest that endothelial cells may act as immunocontrolling and immunomodulating cells in response to the bacteria that cause footrot (Sparrow et al., 1997). *P. levii* and its LPS interact with and activate EJG cells to release pro-inflammatory cytokines (TNFα, GM-CSF and IL-1ß) *in vitro* (Sparrow et al, 1997). The results presented, suggest that endothelial cells are in part involved in the production of pro-inflammatory compounds as well as the recruitment and activation of neutrophils into the area of the footrot infection *in vivo* (Sparrow et al., 1997).

1. Cytopathic Effect Investigations.

A) *P. levii* and its LPS

Ideal growth conditions were established for *Porphyromonas levii* . This allowed for obtaining enough pure cultures of *P. levii* to utilize in EJG exposure studies. Sufficient growth of *P. levii* allowed for the successful isolation of its LPS, at concentrations above that found in *P. levii* culture supernatants (50 μg/ml vs. 22 μg/ml, Figure 10). Both preparations gave sufficient concentrations of LPS to utilize in EJG exposure studies, where it has been shown that as little as 1 μg/ml of LPS is needed to induce its effects (Paulsen et al., 1989). Purifying the *P. levii* LPS, allowed for the investigation of only the effects of its LPS on EJG cells rather than some other unknown virulence factor present.

The quantification of LPS by the KDO assay, indicated that *P. levii* likely possesses LPS of the smooth phenotype (Stryer, 1990). Evidence for this comes from the fact that KDO is not only found in the core of LPS molecules, but usually makes up a considerable amount of those sugars found in the O - antigen region of LPS molecules (Kumada et al., 1993, Stryer, 1990). However, the exact nature of the LPS molecule of *P. levii* cannot be confirmed by these results, without further examination of its electrophoretic profile using SDS-PAGE electrophoresis. Priority for the use of the purified LPS was given to exposure studies, and thus the nature of the *P. levii* LPS was not verified in these investigations.

B) Examination of CPE

The involvement of *P. levii* culture supernatants and purified LPS in damaging bovine endothelial cells was evaluated. Both the isolated *P. levii* LPS and *P. levii* crude culture supernatant were shown to contain LPS at different concentrations from KDO assays. Using light microscopy, it was shown that both *P. levii* LPS and centrifuged *P. levii* culture supernatant induced CPE in EJG cells over the same incubation period, however, the pure fraction of LPS resulted in the appearance of CPE more quickly. Using light microscopy, it was shown that the characteristic signs of LPS induced CPE were present in the exposed EJG cell monolayers (Breider et al., 1990, Meyrick et al., 1989, Paulsen et al., 1989). CPE was present in the form of, rounded cells, refractile cells, disrupted cell monolayer, vacuolated cells, granulated cells, swollen cells, syncytia formation, and the general appearance of cellular degeneration (Figure 12). These results were sufficient to show that *P. levii* and its LPS damaged bovine endothelial cells. Intracellular investigations of CPE, such as the condensation of chromatin and the degeneration of the nuclear membrane, using transmission electron microscopy, would have been indicative of the cytotoxic effects that *P. levii* LPS has on EJG cells as well (Meyrick et al., 1989). This procedure was not included within these investigations.

The findings that *P. levii* LPS damages bovine endothelial cells *in vitro* may relate to the hallmarks of footrot seen *in vivo*. *In vivo*, it has been shown that LPS induces endothelial damage as seen by structural evidence of endothelial injury and microvascular permeability (Meyrick et al., 1989). It is postulated that in footrot, *P. levii* LPS damages the endothelium of the microvasculature surrounding the infection, contributing to edema. LPS exposure of endothelial cells can cause a release of toxic oxidative mediators such as hydrogen peroxide, by endothelial cells affected by infection (Gallin et al., 1992). In footrot, these mediators may be released by endothelial cells, to kill the infecting *P. levii*, aiding in clearing the infection. Alternatively, these mediators may also result in toxic effects on the surrounding cells themselves, contributing to the pathology and clinical signs of footrot.

C) Transwell (Costar) CPE Experiments

In footrot, edema is one of the major clinical signs associated with the infection (Egerton et al., 1989). As noted previously, it has been shown that LPS increases microvascular permeability by damaging the endothelial lining of vasculature (Gallin et al, 1992). The goal of the Transwell (Costar) investigations was to mimic the cause of this edema *in vitro*. It was postulated that *P. levii* LPS, when added to confluent EJG monolayers, would damage the EJG cells and cause degeneration of the integrity of the cell monolayer. This endothelial cell damage and monolayer leakiness was then measured by detecting the passage of Biotin-HRP (Pierce) across the membrane over known incubation times, as seen in Figure 14. At most individual incubation times, both *P. levii* LPS and *E. coli* 0111:B4 (positive control) LPS caused significantly more leakage of the Biotin-HRP across the monolayer/membrane, when compared to non-exposed EJG monolayer controls. This appeared to be caused by LPS induced damage of the EJG cell monolayer. Thusly, it can be said that LPS and more specifically *P. levii* LPS can disrupt endothelial cell function and result in increased permeability *in vitro* . Relating back to footrot, it is assumed that LPS from *P. levii* contributes to the edema seen surrounding the infection by disrupting the endothelial linings of surrounding blood vessels in a similar fashion.

It was hoped that within the Transwell experiments, a clearer pattern of functional disruption would have been seen from time 0 to time 150 minutes. As seen in Figure 14, no clear evidence of increased endothelial damage/leakiness can be seen over time. Trying another bovine endothelial cell line may have improved upon this. Also, due to the inconsistent measurements seen over time, the sensitivity of this assay may be questionable. Choosing a more established detection/substrate protocol may have helped to clarify and improve upon the results shown in Figure 14.

2. EJG Pro-inflammatory Cytokine Investigations.

A) The Effect of *P. levii* on EJG GM-CSF Production

LPS molecules are known to induce a variety of inflammatory responses (Endo et al., 1997). It's been shown that endothelial cells, have the capacity to produce immunoregulatory substances, including GM-CSF in response to exposure with LPS (Houston et al., 1997). It was proposed that *P. levii* LPS and culture supernatant containing LPS could induce EJG cells to produce GM-CSF in response to this interaction. Consistent with previous findings and as seen in Figure 14B, GM-CSF was successfully detected/produced by EJG cells incubated with *P. levii* LPS containing fractions over time, using Western Blotting techniques. The observation that GM-CSF was not seen in EJG cells exposed to *P. levii* LPS, immunostained for GM-CSF, supports the idea that this cytokine is secreted from cells (Moldawer, 1994). The fact that GM-CSF, detected using

Western Blotting techniques was found in the cell associated EJG fractions may represent the detection of GM-CSF, not yet secreted by the EJG cells into the surrounding medium. The exact amounts of GM-CSF could not be measured by these investigations. Finding more sensitive assays for the detection of bovine cytokines, including GM-CSF would aid to elucidate these results.

The production of GM-CSF by endothelial cells gives insights into what may be occurring in footrot. It is postulated that GM-CSF produced by endothelial cells and secreted within the microvasculature surrounding an infection by *P. levii*, may play several roles in resolving the infection. GM-CSF is known to prime neutrophils, increasing their functional capacity, their ability to phagocytose bacteria and increasing their oxidative killing functions (Gallin et al,., 1992, Abramson et al., 1993). GM-CSF acts as a chemotractant for neutrophils and other leukocytes, calling these cells into the area of infection and then aids in keeping them there to resolve the infection (Xing et al., 1996). In footrot, these consequences of GM-CSF production by endothelial cells would promote resolution of the *P. levii* infection by host immune responses. GM-CSF may also promote tissue repair after the footrot infection is resolved, by recruiting the actions of non-immune cells such as fibroblasts to partake in such activities (Xing et al., 1996). A downside of the upregulation of host cellular immune responses via GM-CSF production in footrot, may be an excessive, inappropriate inflammatory response, further contributing to the pathology of the disease (Shapira et al, 1996, Sharma et al., 1992).

B) The Effect of *P. levii* on EJG TNFα Production

In vivo the vascular endothelium is a critical target for endotoxin and many cytokines are released during gram - negative infections, which may play important roles in resolving the infection, or in vascular injury (Arditi et al., 1994). The pro-inflammatory properties of TNFα are absolutely required for the development of effective innate and acquired immunity (Standiford et al., 1997). Using Western Blotting Techniques, these investigations examined if *P. levii* LPS or crude culture supernatants (containing LPS) could induce EJG cells to produce TNFα. As seen in Figure 14A, *P. levii* LPS can induce TNFα production by bovine endothelial cells (EJG), starting at 30 minutes of exposure and lasting for up to 240 minutes. Cytokines exist both as free secreted and cell - associated forms (Moldawer, 1994) In these investigations, TNFα was more noticeably found in the cell-associated EJG fractions. The findings mentioned above were supported by the results seen in Figure 25. EJG cells exposed to *P. levii* LPS immunostained positive for TNFα, which appeared to be associated and bound to the surface of the EJG cells. Consequently, it can be said that bovine TNFα is produced by endothelial cells in the cell - associated form in the presence of *P. levii* LPS. In Footrot, it is assumed that *P. levii* and its LPS would induce endothelial cells of the surrounding vasculature to produce TNFα in response to infection. This production of TNFα may increase vascular endothelium permeability via gelatinase production, F-actin depolymerisation, induced expression of nitric oxide and a reduction in intracellular adenosine 3',5'-cyclic monophosphate (Goldblum et al., 1993, Ferro et al., 1996, Koga et al., 1995, Partridge et al., 1993). TNFα may also play an active role in promoting an inflammatory response, aimed at resolving the footrot infection.

An improvement on this application would be to find a more sensitive means of measuring the exact amount of TNFα produced by EJG cells over time. As seen in Figure 14A, no discernible difference in EJG TNFα production could be detected over time. Perhaps using a quantifiable ELISA would clarify these results, or with the production of a

TNFα nucleic acid probe, the production of TNFα mRNA over time would aid in refining these results.

C) The Effect of *P. levii* on EJG IL-1ß Production

IL-1ß, shown to be produced by endothelial cells *in vitro*, is a pro-inflammatory cytokine that is defined as a “primary cytokine” based upon the prediction that its release, as an isolated event, would be sufficient to induce inflammation (Kupper et al., 1995). In these investigations the production of IL-1ß was investigated after exposing EJG cells to purified LPS from *P. levii* and *E. coli* 0111:B4 (positive control). Figures 22B and 23 show that EJG cells produced IL-1ß mRNA at detectable levels, at 30 minutes of incubation, when compared to negative controls. From Figure 22B, it appeared that IL-1ß mRNA production peaked at 1 hour and then remained constant or decreased slightly, up to 2.5 hours. This suggested that LPS can induce EJG cells to produce IL-1ß mRNA over extended exposure times. Attempting to quantify the production of IL-1ß mRNA over time, Dot blots of RNA from each incubation time, were carried out in parallel with quantified RNA standards. The results seen in Figure 23, confirmed that IL-1ß mRNA is produced by EJG cells when challenged with LPS. However, a more precise quantification of IL-1ß mRNA production over time needs to be established. Dot blots indicated that IL-1ß mRNA production started at 30 minutes of incubation, remained relatively constant, and then peaked at 2.5 hours. These results conflicted with those seen in figure 22B. The dot blots were successful in semi-quantifying the amounts of IL-1ß mRNA produced. IL-1ß mRNA levels never reached levels above 100 pg/µl or below 10 pg/µl. Within the results seen, it was clear that *P. levii* LPS induced IL-1ß gene transcription in EJG cells. This did not appear to be a consequence of increased total gene expression by EJG cells as a whole. It has been shown that the production of IL-1ß results in decreased expression of genes in cells exposed to this cytokine (Dinarello, 1996). Figure 22A shows the results of probing for actin mRNA, using the same RNA samples used for IL-1ß mRNA detection. Not only was it seen that actin mRNA synthesis was not increased over time with *P. levii* LPS exposure, but consistent with the literature, actin mRNA was down regulated from t=0 to t=2.5 hours. IL-1ß has been shown to upregulate gene expression in endothelial cells as well, including genes known to encode for pro-inflammatory mediators such as TNFα and GM-CSF (Dinarello, 1996). It is postulated that in footrot, the production of IL-1ß results in an inflammatory response, and upregulates the production of other mediators by endothelial cells of the surrounding vasculature. Through the combined actions of these pro-inflammatory mediators, a potent inflammatory response in the host results, aiding in resolving the infection seen in footrot.

The procedures performed here were successful in establishing that IL-1ß mRNA is produced by EJG cells exposed to *P. levii* LPS. Improvements upon the results seen in Figures 22 and 23 may be accomplished by using RNA probes instead of DNA probes, optimizing the stringency wash steps, optimizing probe concentrations used, optimizing hybridization and developing times etc. (Miltenburg et al., 1995).

D) Consequences of TNFα and IL-1ß Production in Footrot

The functions of TNFα and IL-1ß overlap to a great degree except for the fact that unlike TNFα, IL-1ß can activate T lymphocytes and is involved in stem cell activation (Gallin et al., 1992) As seen *in vitro*, bovine endothelial cells produce both of these cytokines in response to *P. levii* LPS (Figures 16A, 22B and 23). The production of both of these cytokines associated with *P. levii* infection in footrot would promote leukocyte infiltration into the area of infection, leading to induction and/or enhancement of microbicidal activity, contributing to the resolution of the infection (Perretti et al., 1992). In the infected interdigital area, these cytokines may increase vascular proliferation and collagen synthesis, promoting healing of the infected area (Moldawer, 1994, Standiford et al., 1997). It has been shown that both of these cytokines can induce apoptosis in cells they come into contact with, and perhaps in footrot, these cytokines may aid in regulating the viability of cells within the area of infection (Zychlinsky et al, 1997). In footrot, these responses, induced by the appropriate level and duration of IL-1ß and TNFα production by endothelial cells and other immunoregulatory cells, would constitute a beneficial response to *P. levii* infection, and aid in the remodeling of wounded tissues (Moldawer, 1994). However, it also becomes apparent that in cases of footrot, an inappropriate or an exaggerated induction of IL-1ß and/or TNFα release and function, may result in inappropriate responses in receptor cells (Dinarello, 1996). The outcome of this response, may contribute to the pathology already present in footrot, due to *P. levii* infection.

E) TNFα and GM-CSF Production Associated with Footrot *In Vivo*

With the production of GM-CSF, TNFα and IL-1ß by EJG cells confirmed *in vitro*, further studies looked for the presence of GM-CSF and TNFα *in vivo*. As seen in Figure 26, biopsy samples taken from animals suffering from footrot stained positive for GM-CSF and TNFα. The consequences due to the production of these pro-inflammatory mediators in response to *P. levii* infection in footrot were described previously. It can be restated that the production of these cytokines in footrot indicates cells surrounding the septic foci, respond to infection by producing these cytokines, given the hallmark pathological features seen in footrot. The role that endothelial cells played in producing these cytokines is unclear, due to the dispersion of the cytokines seen in Figure 26. Detailed histological examination of the sections seen in Figure 26, may identify the cells surrounding those areas stained positive for either cytokine.

From these investigations, it can be assumed that endothelial cells near the area that these biopsy samples (Figure 26) were taken, were actively involved in mounting an immune response against the footrot pathogen(s) including *P. levii*. The production of

GM-CSF and TNFα by endothelial cells *in vivo*, would contribute to the resolution of infection via inflammatory responses to their presence. If the response or production of these cytokines was unsuitable in footrot, the results could be detrimental to the course and outcome of the infection.

Conclusions

1. *P. levii* causes CPE in bovine endothelial cells (EJG).
2. *P. levii* LPS alters the integrity of EJG cell monolayers.
3. Cytopathic effects occurred in response to exposure of EJG cells with *P. levii* fractions (LPS) over the same time period as cytokine production.
4. EJG cells responded to exposure with *P. levii* and its LPS by producing IL-1ß, TNFα and GM-CSF.
5. TNFα and GM-CSF are produced in response to infection seen in footrot, *in vivo*.
6. Bovine Endothelial cells may act as immunocontrolling and immunomodulating cells in response to the footrot causing bacteria (*P. levii*).
7. LPS - induced CPE and associated production of cytokines by EJG cells, may give important insights into the pathogenesis, immunity and resolution of this infection.
8. A fundamental understanding of the interactions of specific cytokines, endothelial cells and phagocytic leukocytes will aid investigators in predetermining and possibly altering the clinical outcome of footrot in cattle.

References

Abraham, D., Ince, T., Muir, H., Olsen, I. (1989) Fibroblast Matrix and Surface Components that Mediate Cell - to - Cell Interaction With Lymphocytes. *J Invest Dermatol.* **93,** 335-340.

Abramson, J.S., Wheeler, J.G., editors. (1993) "The Neutrophil." New York, IRL Press, pp. 15, 46, 68, 88, 156-157.

Aldridge, K.E. (1995) The Occurrence, Virulence, and Antimicrobial Resistance of Anaerobes in Polymicrobial Infections. *Am J of Surg.* **169,** 2S-7S.

Arditi, M., Zhou, J., Huang, S.H., Luckett, P.M., Marra, M.N., Kim, K.S. (1994) Bactericidal/Permeability-Increasing Protein Protects Vascular Endothelial Cells from Lipopolysaccharide-Induced Activation and Injury. *Infection and Immunity.* **62,** 3930-3936.

Armstrong, A.M., Gardiner, K.R., Kirk, S.J., Halliday, M.I., Rowlands, B.J. (1997) Tumor Necrosis Factor and Inflammatory Bowel Disease. *British J of Surg.* **84,** 1051-1058.

Atlas, M.A. (1993) "Handbook of Microbiological Media." Boca Raton, CRC Press, pp. 151, 63, 262-263, 465.

Augustin, H.G., Kozian, D.H., Johnson, R.C. (1994) Differentiation of Endothelial Cells: Analysis of the Constitutive and Activated Endothelial Cell Phenotypes. *BioEssays.* **16,** 901-906.

Baggioline, M., Loetscher, P., Moser, B. (1995) Interleukin - 8 and the Chemokine Family. *Int J Immunopharmac.* **17,** 103-108.

Bannerman, D.D., Fitzpatrick, M.J., Anderson, D.Y., Bhattacharjee, A.K., Novitsky, T.J., Hasday, J.D., Cross, A.S., Goldblum, S.E. (1998) Endotoxin - Neutralizing Protein Protects Against Endotoxin - Induced Endothelial Barrier Dysfunction. *Infection and Immunity.* **66,** 1400-1407.

Barroso-Aranda, J., Zweifach, B.W., Mathison, J.C., Schmid-Schonbein, G.W. (1995) Neutrophil Activation, Tumor Necrosis Factor, and Survival After Endotoxic and Hemorrhagic Shock. *J of Card Pharma.* **25,** S23-S29.

Beilke, M.A. (1989) Vascular Endothelium in Immunology and Infectious Disease. *Rev of Inf Dis.* **2,** 273-283.

Berg, J.N., Loan, R.W. (1975) *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* as Etiologic Agents of Foot Rot in Cattle. *Am J Vet Res.* **36,** 1115-1122.

Blood, D.C., Radostits, O.M., Gay, C.C. (1994) "Veterinary Medicine, A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses. 8th ed." London, Bailliere Tindal, pp. 662-669, 867-870.

Blowey, R. (1992) Diseases of the Bovine Digit, Parts 1 and 2. *In Practice.* **5,** 85-124.

Botta, G.A., Arzese, A., Minisini, R., Trani, G. (1994) Role of Structural and Extracellular Virulence Factors in Gram-Negative Anaerobic Bacteria. *Clin Inf Dis.* **18,** S260-S264.

Breider, M.A., Kumar, S., Corstvet, R.E. (1990) Bovine Pulmonary Endothelial Cell Damage Mediated by *Pasteurella haemolytica* Pathogenic Factors. *Infection and Immunity.* **58,** 1671-1677.

Brook, I., Frazier, E.H., Yeager, J.K. (1996) Microbiology of Infected Atopic Dermatitis. *Int J of Dermatol.* **35,** 791-793.

Corder, R., Carrier, M., Khan, N., Klemm, P., Vane, J.R. (1995) Cytokine Regulation of Endothelin-1 Release from Bovine Aortic Endothelial Cells. *J of Card Pharma.* **26,** S56-58.

Darveau, R.P., Belmont, C.M., Reife, R.A., Lamont, R.J. (1998) Local Chemokine Paralysis, a Novel Pathogenic Mechanism for *Porphyromonas gingivalis. Infection and Immunity.* **66,** 1660-1665.

Davies, R.L., Ali, Q., Parton, R., Coote, J.G., Gibbs, A., Freer, J.H. (1991) Optimal Conditions for the Analysis Of *Pasteurella haemolytica* Lipopolysaccharide by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis. *FEMS Microbiology Letters.* **90,** 23-28.

Dinarello, C.A. (1996) Biologic Basis for Interleukin - 1 in Disease. *Blood.* **87,** 2095-2147.

Durmaz, B., Jousimies-Somer, H.R., Finegold, S.M. (1995) Enzymatic Profiles of *Prevotella, Porphyromonas,* and *Bacteroides* Species Obtained with the API ZYM System and Rosco Diagnostic Tablets. *Clin Infect Dis.* **20,** S192-194.

Egerton, J.R., Yong, W.K., Riffken, G.G. (1989) "Footrot and Foot Abscesses of Ruminants." Boca Raton, CRC Press, pp. 47-56.

Eidhin, D.N., Mouton, C. (1993) A rapid method for preparation of rough and smooth lipopolysaccharide from *Bacteroides, Porphyromonas* and Prevotella. *FEMS Microbiology Letters.* **110,** 133-138.

Endo, Y., Shibazaki, M., Nakamura, M., Takada, H. (1997) Contrasting Effects of Lipopolysaccharides (Endotoxins) from Oral Black - Pigmented Bacteria and Enterobacteriaceae on Platelets, a Major Source of Serotonin, and on Histamine - Forming Enzyme in Mice. *J of Inf Dis.* **175,** 1404-1412.

Engelkirk, P.G., Duben-Engelkirk, J., Dowell, V.R. (1992) "Principles and Practice of Clinical Anaerobic Bacteriology." Belmont, Star Publishing Company.

Ellis, J.A., Godson, D. Campos, M., Sileghem, M., Babiuk, L.A. (1993) Capture Immunoassay for Ruminant Tumor Necrosis Factor-α: Comparison with Bioassay. *Vet Immuno and Immunopath.* **35,** 289-300.

Engler-Blum, G., Meier, M., Frank, J., Muller, G.A. (1993) Reduction of Background Problems in Nonradioactive Northern and Southern Blot Analyses Enables Higher Sensitivity Than 32P-Based Hybridizations. *Analyt Biochem.* **210,** 235-244.

Farrell, R.E. (1993) "RNA Methodologies, A Laboratory Guide For Isolation and Characterization." San Diego, Academic Press, INC. 113-202.

Feder, L.S., Todaro, J.A., Laskin, D.L. (1993) Characterization of interleukin-1 and interleukin-6 production by hepatic endothelial cells and macrophages. *J of Leukocyte Biol.* **53,** 126-132.

Feinberg, A.P., Vogelstein, B. A Technique For Radiolabeling DNA Restriction Endonuclease Fragments to High Specific Activity. *Analytical Biochemistry.* **132,** 6-13.

Feinman, S.E. (1998) Antibiotics in Animal Feed - Drug Resistance Revisited. *ASM News.* **64,** 24-30.

Ferro, T.J., Gertzberg, N., Selden, L., Neumann, P., Johnson, A. (1997) Endothelial Barrier Dysfunction and p42 Oxidation Induced by TNF-alpha are Mediated by Nitric Oxide. *A J of Phys.* **272,** L979-988.

Finlay, B.B., Cossart, P. (1997) Exploitation of Mammalian Host Cell Functions by Bacterial Pathogens. *Science.* **276,** 718-725.

Fratti, R.A., Ghannoum, M.A., Edwards, J.E., Filler, S.G. (1996) Gamma Interferon Protects Endothelial Cells from Damage by *Candida albicans* by Inhibiting Endothelial Cell Phagocytosis. *Infection and Immunity.* **64,** 4714-4718.

Freshney, R. I. (1987) "Culture of Animal Cells, A Manual of Basic Technique 2 ed." New York, Alan R. Liss, Inc., pp. 127-134, 275, 334.

Gallin, J.I., Goldstein, I.M., Snyderman, R., editors. (1988) "Inflammation, Basic Principles and Clinical Correlates." New York, Raven Press, pp. 169-253.

Gallin, J.I., Goldstein, I.M., Snyderman, R., editors. (1992) "Inflammation, Basic Principles and Clinical Correlates. Second Edition" New York, Raven Press, pp. 169-253.

Garcia, M.M., Becker, S.A.W.E., Brooks, B.W., Berg, J.N., Finegold, S.M. (1992) Ultrastructure and Molecular Characterization of *Fusobacterium necrophorum* Biovars. *Can J Vet Res.* **56,** 318-325.

Garlanda, C., Dejana, E. (1997) Heterogeneity of Endothelial Cells: Specific Markers. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **17,** 1193-1202.

Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N. R., Phillips, G.B. (1981) "Manual of Methods for General Bacteriology." Washington, American Society for Microbiology, pp. 337-338.

Gibson III, F.C., Tzianabos, A.O., Onderdonk, A.B. (1996) The Capsular Polysaccharide Complex of *Bacteroides fragilis* Induces Cytokine Production from Human and Murine Phagocytic Cells. *Infection and Immunity.* **64,** 1065-1069.

Goldblum, S.E., Ding, X., Campbell-Washingtion, J. (1993) TNF-α Induces Endothelial Cell F-actin Depolymerization, New Actin Synthesis, and Barrier Dysfunction. *A J of Phys.* **264,** C894-905.

Grenier, D., Labbe, S., Mouton, C., Meyrand, D. (1994) Hydrolytic enzymes and Lectin-Binding Activity of Black-Pigmented Anaerobic Rods. *Microbiology.* **140,** 873-878.

Harlow, E., Lane, D. (1988) "Antibodies, A Laboratory Manual." New York, Cold Spring Harbor Laboratory Press.

Helander, I.M., author. Dawes, E.A., Korhonen, T.K., Makela, P.H., editors. (1985) "Enterobacterial Surface Antigens, Methods for Molecular Characterisation. CH. 17. Isolation and Electrophoretic Analysis of Bacterial Lipopolysaccharides." New York, Elsevier Science Publishers, pp. 263-274.

Hoch, R.C., Schraufstatter, I.U., Cochrane, C.G. (1996) In Vivo, in Vitro and Molecular Aspects of Interleukin-8 and the Interleukin-8 Receptors. *J Lab Clin Med.* **128,** 134-145.

Houston, D.S., Carson, C.W., Esmon, C.T. (1997) Endothelial Cells and Extracellular Calmodulin Inhibit Monocyte Tumor Necrosis Factor Release and Augment Neutrophil Elastase Release. *J of Biol Chem.* **272,** 11778-11785.

Huber, A.R., Kunkel, S. L., Todd, E.F., Weiss, S.J. (1991) Regulation of Transendothelial Neutrophil Migration by Endogenous Interleukin-8. *Science Reports* **254,** 99-102.

Ishii, Y., Shuyi, W., Kitamura, S. (1995) Soluble CD14 in Serum Mediates LPS-Induced Increase in Permeability of Bovine Pulmonary Arterial Endothelial Cell Monolayers In Vitro. *Life Sciences.* **56,** 2263-2272.

Janssen, Y.M.W., Van Houten, B., Borm, P.J.A., Mossman, B.T. (1993) Biology of Disease: Cell and Tissue Responses to Oxidative Damage. *Laboratory Investigation.* **69,** 261-268

Johnson, D.W., Dommert, A.R., Kiger, D.G. (1969) Clinical Investigation of Infectious Foot Rot of Cattle. *JAVMA.* **155,** 1886-1891.

Jousimies-Somer, H.R. (1995) Update on the Taxonomy and the Clinical and Laboratory Characteristics of Pigmented Anaerobic Gram-Negative Rods. *Clin Inf Dis.* **20,** S187-191.

Jubb, T.F., Malmo, J. (1991) Lesions Causing Lameness Requiring Veterinary Treatment in Pasture-Fed Dairy Cows in East Gippsland. *Aust Vet J.* **68,** 21-24

Kang, Y.H., Williams, R. (1991) Endotoxin-Induced Endothelial Injury and Subendothelial Accumulation of Fibronectin in Rat Aorta. *The Anatomical Record.* **229,** 86-102.

Klein, N.J., Ison, C.A., Peakman, M., Levin, M., Hammerschmidt, S., Frosch, M., Heyderman, R.S. (1996) The Influence of Capsulation and Lipooligosaccharide Structure on Neutrophil Adhesion Molecule Expression and Endothelial Injury by *Neisseria meningitidis. J of Inf Dis.* **173,** 172-179.

Koga, S., Morris, S., Ogawa, S., Liao, H., Bilezikian, J.P., Chen, G., Thompson W.J., Ashikaga, T., Brett, J., Stern, D.M., Pinsky, D.J. (1995) TNF Modulates Endothelial Properties by Decreasing cAMP. *A J of Phys.* **268,** C1104-1113.

Kruse, G.O.W., Weiskopf, S., Agger, N. (1988) Therapeutic Use of Terramycin*/LA Against Footrot in Cattle. *As published in Proceedings (vol. II) of the 15th World Buiatrics Congress, Palma de Mallorca, Spain.* **Oct.,** 1111-1116.

Kumada, H., Haishima, Y., Kondo, S., Umemoto, T., Hisatsune, K. (1993) Occurrence of 2-Keto-3-Deoxyoctonate (KDO) and KDO Phosphate in Lipopolysaccharides of *Bacteroides* Species. *Current Microbiology.* **26,** 239-244.

Kupper, T.S., Groves, R.W. (1995) The Interleukin - 1 Axis and Cutaneous Inflammation. *J of Invest Derma.* **105,** 62S-66S.

Laemmli, U.K. (1970) Cleavage of Structural Proteins During The Assembly of the Head of Bacteriophage T4. *Nature.* **227,** 680-685.

Laing. E.A., Egerton, J.R. (1978) The Occurrence, Prevalence and Transmission of *Bacteroides nodosus* Infection in Cattle. *Res in Vet Science.* **24,** 300-304.

LeGrand, E.K. (1990) Endotoxin as an Alarm Signal of Bacterial Invasion: Current Evidence and Implications. *JAVMA.* **197,** 454-456.

Mackay, R.C. (1997) Chemokine: What Chemokine is That? *Current Biol.* **7,** R384-386.

Mandell, G.L. (1995) Cytokines, Phagocytes and Pentoxifylline. *J of Card Pharma.* **25,** S20-22.

Marks, R.M., Todd, R.F., Ward, P.A. (1989) Rapid Induction of Neutrophil-Endothelial Adhesion by Endothelial Complement Fixation. *Nature.* **339,** 314-317.

Marra, A., Isberg, R.R. (1996) Bacterial Pathogenesis: Common Entry Mechanisms. *Current Biology.* **6,** 1084-1086.

Meyrick, B., Hoover, R., Jones, M.R., Berry, L. C., Brigham, K.L. (1989) In Vitro Effects of Endotoxin on Bovine and Sheep Lung Microvascular and Pulmonary Artery Endothelial Cells. *J of Cell Physio.* **138,** 165-174.

Mgasa, M.N. (1987) Bovine Pododermatitis Aseptica Diffusa (Laminitis) Aetiology, Pathogenesis, Treatment and Control. *Vet Res Com.* **11,** 235-241.

Moldawer, L.L. (1994) Biology of Proinflammatory Cytokines and their Antagonists. *Crit Care Med.* **22,** S3-7

Moncla, B.J., Braham, P., Rabe, L.K., Hillier, S.L. (1991) Rapid Presumptive Identification of Black-Pigmented Gram-Negative Anaerobic Bacteria by Using 4-Methylumbelliferone Derivatives. *J of Clin Micro.* **29,** 1955-1958.

Morck, D.W. (1995) Footrot - New Info On An Old Problem. *Alberta Beef* **3,** 49-54.

Morck, D.W., Gard, M.S., Olson, M.E. (1994) Experimental Evaluation of a Commercial Footrot Vaccine Against Native Canadian Strains of *Dichelobacter nodosus. Can J Vet Res.* **58,** 122-126.

Morck, D.W., Merrill, J.K., Gard, M.S., Olson, M.E., Nation, P.N. (1997) Treatment of Experimentally Induced Pneumonic Pasteurellosis of Young Calves with Tilmicosin. *Can J Vet Res.* **61,** 187-192.

Morck, D.W., Olson, M.E., Louie, T.J., Koppe, A., Quinn, B. (1998) Comparison of Ceftiofur Sodium and Oxytetracycline for Treatment of Acute Interdigital Phlegmon (foot rot) in Feedlot Cattle. *Am J Vet Res.* **212,** 254-257.

Morello, J.P., Plamondon, J., Meyrick, B., Hoover, R., O'Connor-McCourt, M.D. (1995) Transforming Growth Factor-ß Receptor Expression on Endothelial Cells: Heterogeneity of Type III Receptor Expression. *J of Cell Phys.* **165,** 201-211.

Noel, R.F., Sato, T.T., Mendez, S., Johnson, M.C., Pohlman, T.H. (1995) Activation of Human Endothelial Cells by Viable or Heat-Killed Gram-Negative Bacteria Requires Soluble CD14. *Infection and Immunity.* **63,** 4046-4053.

Osborne, M.J. (1963) Studies on the Gram Negative Cell Wall. 1. Evidence for the Role of 2-Keto-3-Deoxyoctonate in the Lipopolysaccharide of *Salmonella typhimurium. Proc Natl Acad Sci USA.* **50,** 499-506.

Partridge, C.A., Jeffrey, J.J., Malik, A.B. (1993) A 96-kDA Gelatinase Induced by TNF-α Contributes to Increased Microvascular Endothelial Permeability. *A J of Phys.* **265,** L438-447.

Paster, B.J., Dewhirst, D.E., Olsen, I., Fraser, F.J. (1994) Phylogeny of *Bacteroides, Prevotella,* and *Porphyromonas* spp. and Related Bacteria. *J of Bact.* **176,** 725-732.

Paulsen, B.P., Mosier, D.A., Clinkenbeard, K.D., Confer, A.W. (1989) Direct effects of *Pasteurella haemolytica* lipopolysaccharide on bovine pulmonary endothelial cells in vitro. *Am J Vet Res.* **50,** 1633-1637.

Perretti, M., Solito, E., Parente, L. (1992) Evidence that Endogenous Interleukin - 1 is Involved in Leukocyte Migration in Acute Experimental Inflammation in Rats and Mice. *Agents Actions.* **35,** 71-78.

Rietschel, E.T., Brade, H. (1992) Bacterial Endotoxins. *Scientific American* . **Aug.,** 54-61.

Ross, H. (1996) "Laboratory Manual, Microbiology 343, Department of Biological Sciences." Calgary, Alberta, The University of Calgary, Appendix 5.

Rotstein, O.D. (1993) Interactions Between Leukocytes and Anaerobic Bacteria in Polymicrobial Surgical Infections. *Clin Inf Dis.* **16,** S190-194.

Russmann, H., Ruckdeschel, K., Heesemann, J. (1996) Translocation of *Yersinia enterocolitica* Through an Endothelial Monolayer by Polymorphonuclear Leukocytes. *Infection and Immunity.* **64,** 1016-1019.

Ryan, U.S., Vann, J.M., Nolop, K.B. (1989) Endothelial Cells and Bacterial Infection. *Chest.* **95,** 174S-175S.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) "Molecular Cloning, A Laboratory Manual 2 ed." New York, Cold Spring Harbor Laboratory Press, pp. 1.11-1.14, 1.21-1.32, 1.74-1.105, 6.3-6.49, 7.3-7.84, 11.3-11.17.

Scanlan, C.M., Berg, J.N., Campbell, F.F. (1986) Biochemical Characterization of Leukotoxins of Three Bovine Strains of *Fusobacterium necrophorum. Am J Vet Res.* **47,** 1422-1425.

Shapira, L., Soskolne, W.A., Houri, Y., Barak, V., Halabi, A., Stabholz, A. (1996) Protection against Endotoxic Shock and Lipopolysaccharide-Induced Local Inflammation by Tetracycline: Correlation with Inhibition of Cytokine Secretion. *Am Soc for Micro.* **64,** 825-828.

Sharma, S.A., Olchowy, T.W.J., Breider, M.A. (1992) Alveolar Macrophage and Neutrophil Interactions in *Pasteurella haemolytica* Induced Endothelial Cell Injury. *J of Infect Dis.* **165,** 651-657.

Smart, S.J., Casale, T.B. (1993) Interleukin-8-induced Transcellular Neutrophil Migration Is Facilitated by Endothelial and Pulmonary Epithelial Cells. *Am J Respir Cell Mol Biol.* **9,** 489-495.

Sordillo, L.M., Afseth, G., Davies, G., Babiuk, L.A. (1992) Effects of Recombinant Granulocyte-macrophage Colony-stimulating Factor on Bovine Peripheral Blood and Mammary Gland Neutrophil Function *in vitro. Can J Vet Res.* **56,** 16-21.

Sparrow, D.G., Gard, M.S., Buret, A., Ceri, H., Olson, M., Morck, D.W. (1997) Expression of Tumor Necrosis Factor α and Granulocyte-Macrophage Colony-Stimulating Factor by Bovine Endothelial Cells In Vitro. *Clin Inf Dis.* **25,** S178-179.

Standiford, T.J., Huffnagle, G.B. (1997) Cytokines in Host Defense Against Pneumonia. *J of Invest Med.* **45,** 335-345.

Sticherling, M., Hetzel, F., Schroder, J.M., Christophers, E. (1993) Time and Stimulus Dependent Secretion of NAP-1/IL-8 by Human Fibroblasts and Endothelial Cells. *J Invest Dermatol.* **101,** 573-576.

Stryer, L. (1990) "Biochemistry, 3rd. ed." New York, W.H. Freeman and Company, pp. 782-783.

Summanen, P. (1995) Microbiology Terminology Update: Clinically Significant Anaerobic Gram-Positive and Gram-Negative Bacteria (Excluding Spirochetes). *Clin Infect Dis.* **21,** 273-276.

Tao, W., Dougherty, R., Johnston, P., Pickett, W. (1993) Recombinant Bovine GM-CSF Primes Superoxide Production but Not Degranulation Induced by Recombinant Bovine Interleukin-1ß in Bovine Neutrophils. *J of Leuk Biol.* **53,** 679-684.

Tissot Van Patot, M.C., MacKenzie, S., Tucker, A., Voelkel, N.F. (1996) Endotoxin-Induced Adhesion of Red Blood Cells to Pulmonary Artery Endothelial Cells. *A J of Phys.* **270,** L28-36.

Townsend, G.C., Scheld, W.M. (1995) Microbe-Endothelium Interactions in Blood-Brain Barrier Permeability during Bacterial Meningitis. *ASM News.* **61,** 294-298.

Towbin, H., Staehelin, T., Gordon, J. (1979) Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. *Proceedings of the National Academy of Sciences of the United States of America.* **76,** 4350-4354.

Trayhurn, P., Duncan, J.S., Nestor, A., Thoman, M.E.A., Rayner, D.V. (1994) Chemiluminescent Detection of mRNAs on Northern Blots with Digoxigenin End-Labeled Oligonucleotides. *Analyt Biochem.* **222,** 224-230.

Van Miltenburg, R., Ruger, B., Grunewald-Janho, S., Leons, M., Shroder, C. (1995) "The DIG System User's Guide for Filter Hybridization." Mannheim, Germany, Boehringer Mannheim GmbH Biochemica, ch.2, ch. 5, ch.8, ch.9.

Wendt, CH., Polunovsky, V.A., Peterson, M. S., Bitterman, P. B., Ingbar, D.H. (1994) Alveolar Epithelial Cells Regulate the Induction of Endothelial Cell Apoptosis. *A J of Phys.* **267,** C893-900.

Westphal, O., Jann, K. (1965) Bacterial Lipopolysaccharides, Extraction with Phenol-Water and Further Applications of the Procedure. *Meth of Carb Chem.* **5,** 83-91.

Wiedermann, C.J., Schratzberger, P. Kahler, C.M. (1994) Migration of Neutrophils Across Endothelial Monolayers Is Stimulated by Treatment of the Monolayers with ß-Endorphin. *Brain, Behavior and Immun.* **8,** 270-277.

Wolfe, S.L. (1993) "Molecular and Cellular Biology." Belmont, CA., page 306, Figure 8-36.

Xing, Z., Braciak, T., Ohkawara, Y., Sallenave, J.M., Foley, R., Sime, P.J., Jordana, M., Graham, F.L., Gauldie, J. (1996) Gene Transfer for Cytokine Functional Studies in the Lung: The Multifunctional Role of GM-CSF in Pulmonary Inflammation. *J of Leuk Biol.* **59,** 481-488.

Yang, Z., Khemlani, L.S., Dean, D.F., Carter, C.D., Slauson, D.O., Bochsler, P.N. (1994) Serum Components Enhance Bacterial Lipopolysaccharide-Induced Tissue Factor Expression and Tumor Necrosis Factor-α Secretion by Bovine Alveolar Macrophages In Vitro. *J of Leuk Biol.* **55,** 483-488.

Ye, J., Young, H.A. (1997) Negative Regulation of Cytokine Gene Transcription. *FASEB Journal.* **11,** 825-833.

Yoo, Maheswaran, Lin, Townsend and Ames, (1995) Induction of Inflammatory cytokines in Bovine Alveolar Macrophages following stimulus with Pasteurella haemolytica Lipopolysaccharide. *Infection and Immunity.* **63,** 381-388.

Zychlinsky, A., Sansonetti, P. (1997) Apoptosis in Bacterial Pathogenesis. *J Clin Invest.* **100,** 493-496.

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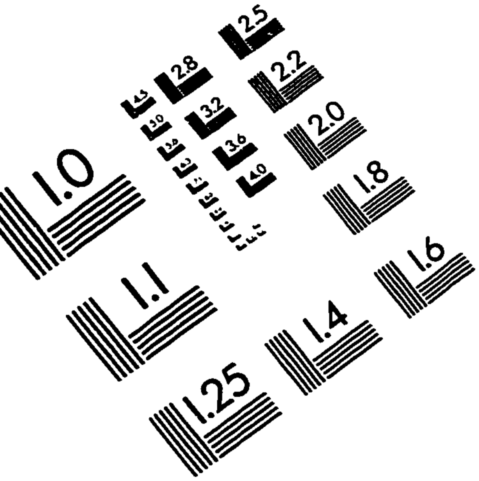
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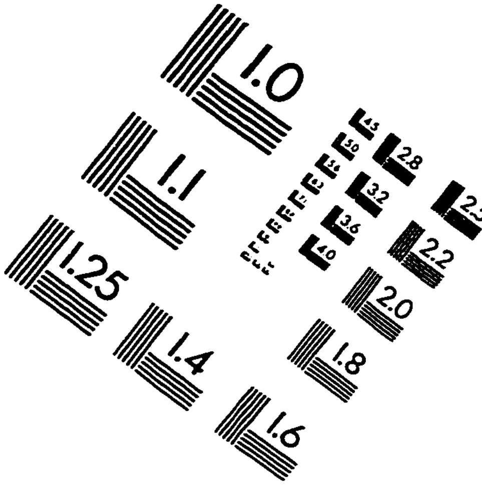
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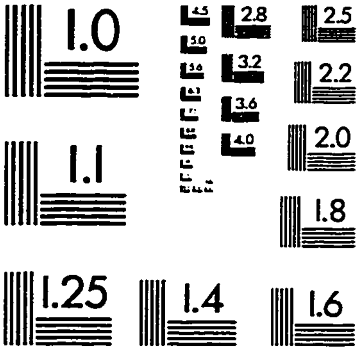
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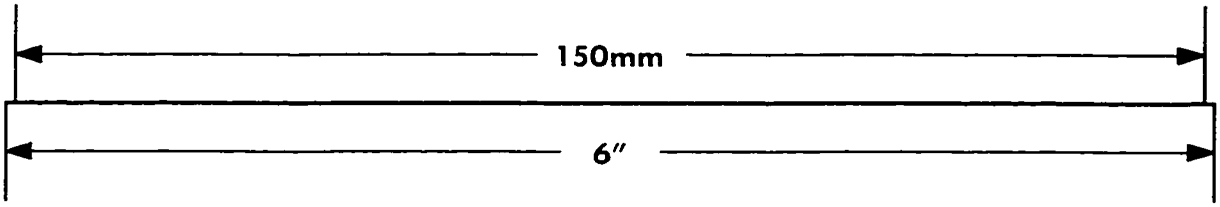

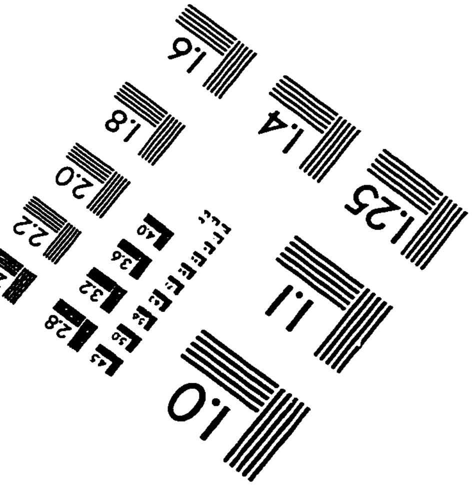
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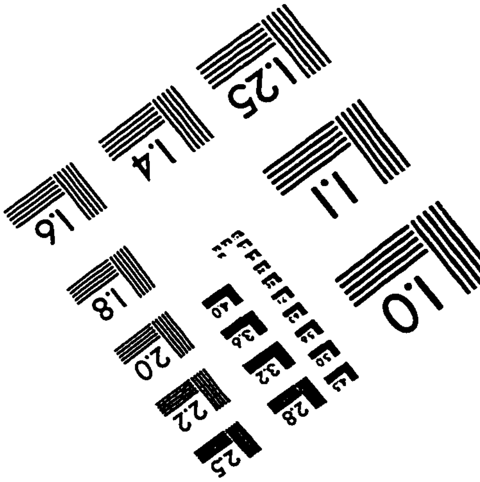
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