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Review Article

FUSOBACTERIUM NECROPHORUM INFECTIONS: VIRULENCE FACTORS, PATHOGENIC MECHANISM AND CONTROL MEASURES

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ABSTRACT

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Fusobacterium necrophorum, a Gram-negative, non-spore-forming anaerobe, is a normal inhabitant of the alimentary tract of animals and humans. Two types of *F. necrophorum*, subspecies *necrophorum* (biotype A) and *funduliforme* (biotype B), have been recognized, which differ morphologically, biochemically, and biologically. The organism is an opportunistic pathogen that causes numerous necrotic conditions (necrobacillosis) such as bovine hepatic abscesses, ruminant foot abscesses and human oral infections. The pathogenic mechanism of *F. necrophorum* is complex and not well defined. Several toxins, such as leukotoxin, endotoxin, haemolysin, haemagglutinin and adhesin, have been implicated as virulence factors. Among these, leukotoxin and endotoxin are believed to be more important than other toxins in overcoming the host's defence mechanisms to establish the infection. *F. necrophorum* is encountered frequently in mixed infections and, therefore, synergisms between *F. necrophorum* and other pathogens may play an important role in infection. Several investigators have attempted to induce protective immunity against *F. necrophorum* using bacterins, toxoids, and other cytoplasmic components. Generally, none of the immunogens has afforded satisfactory protection against *Fusobacterium* infections. Because of the unavailability of suitable immunoprophylaxis, the control of *F. necrophorum* infection has depended mainly on the use of antimicrobial compounds.

Keywords: Bacteroidaceae, endotoxin, *Fusobacterium necrophorum*, immunity, leukotoxin, necrobacillosis

Abbreviations: CFU, colony-forming units; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; Eh, redox potential; ELISA, enzyme-linked immunosorbance assay; LPS, lipopolysaccharide; MPN, most-probable number; PMN, polymorphonuclear cells; rRNA, ribosomal ribonucleic acid; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; subsp., subspecies

INTRODUCTION

Fusobacterium necrophorum, of the Bacteroidaceae Family, is a Gram-negative, pleomorphically rod-shaped, non-spore-forming, and obligate anaerobic organism. It is frequently associated with necrotic lesions in many species of warm-blooded animals (Hofstad, 1986; Timoney *et al.*, 1988). The taxonomic description of *F. necrophorum* is confusing because of the number of synonyms used in the early literature. Previous publications have used as many as 20 different names for *F. necrophorum* (Moore *et al.*,

TABLE I
Differences between *Fusobacterium necrophorum* subsp. *necrophorum* and subsp. *funduliforme*

Criterion	Subsp. *necrophorum*	Subsp. *funduliforme*
Previous classification	Biovar/biotype A	Biovar/biotype B
General description		
Cell morphology	Pleomorphic filaments (2–100 μm)	Curved rods (1–10 μm)
Colony morphology	Smooth, opaque, and umbonate with irregular edges	Small, waxy, yellowish, raised, and sticky
Sedimentation in broth	Absent	Present
Major source	Bovine hepatic abscess, rumen	Human, rumen
Hydrophobicity	Present	Present
Biological activity		
Agglutination of chicken erythrocytes	+	–
Leukotoxin	+++	+/–
Platelet aggregation	+	–
Adherence to mammalian cells	+	–
Pathogenicity to mice	+++	+/–
Enhancement of infectivity with other bacteria	+	–
Biochemical properties		
Proteolytic activity	++	+
DNAase	+	–
Lipase	+/–	–
Phosphatase	+	–
DNA analysis		
G + C content (mol%)	28–32	27–33
DNA homology	53–76% relatedness to subsp. *funduliforme*	51–73% relatedness to subsp. *necrophorum*

1984). Based on cell morphology, haemagglutination properties, haemolytic activities, and virulence in mice, *F. necrophorum* was classified into A, B and C biovars/phases/biotypes (Langworth, 1977; Scanlan and Hathcock, 1983). Biovar C has been reclassified as a new species of *Fusobacterium* (*F. pseudonecrophorum*; Shinjo *et al.*, 1990). Biovars A and B have been designated as subsp. *necrophorum* and subsp. *funduliforme*, respectively (Shinjo *et al.*, 1991). The differences between the two subspecies in morphology, growth patterns, biological and biochemical characteristics, and DNA analysis are listed in Table I. Despite distinct differences between the two subspecies, many publications have not specified the subspecies (biovar/biotype) of *F.*

necrophorum being considered. Some *F. necrophorum* strains, isolated from ovine foot abscesses, were described as biovar AB because their biological characteristics were intermediate between those of subsp. *necrophorum* and subsp. *funduliforme* (Emery *et al.*, 1985). The taxonomic status of biovar AB is unclear. However, a phylogenetic analysis using the 16S rRNA gene sequence revealed a close relationship of biovar AB to subsp. *necrophorum* and subsp. *funduliforme* (Nicholson *et al.*, 1994).

F. necrophorum is a normal inhabitant of the alimentary tract of animals and humans (Langworth, 1977; Hofstad, 1986). The bacterium is frequently isolated from healthy hosts, although at a relatively low concentration. *F. necrophorum* concentrations in the bovine rumen were estimated to be 10^5–10^6 MPN/g of ruminal contents (Tan *et al.*, 1994b). Both subspecies of *F. necrophorum* have been isolated from bovine ruminal contents (Berg and Scanlan, 1982; Smith and Thornton, 1993a; Tan *et al.*, 1994c). Normal human faeces contained 10^8–10^{10} *Fusobacterium*/g, of which *F. necrophorum* was the predominant species (Ohtani, 1970). *F. necrophorum* is also a member of the normal flora of the human oral cavity and female genital tract (Gorbach and Bartlett, 1974).

F. necrophorum is commonly associated with various clinically significant anaerobic infections. However, the prevalence of *F. necrophorum* infection is still believed to be underestimated. Diagnosis often is not made because of difficulties with the anaerobic cultural procedures. *F. necrophorum* is the primary aetiological agent of bovine hepatic abscesses, from which it is frequently isolated in pure culture (Scanlan and Hathcock, 1983; Lechtenberg *et al.*, 1988). As an opportunistic pathogen, this organism, along with other bacteria, is associated with many disesases, such as foot abscesses in ruminants (Emery *et al.*, 1985) and human dental infections (Henry *et al.*, 1983; Uematsu and Hoshino, 1992).

In order to initiate an infection, bacteria must invade the host, overcome the host defence system, proliferate in tissue, and cause damage to the host. Several toxins (endotoxin, leukotoxin, and haemolysin) have been reported in *F. necrophorum* (Langworth, 1977; Emery *et al.*, 1986b; Kanoe, 1990; Tan *et al.*, 1992, 1994d). However, although over the years many efforts have been made to study *F. necrophorum* and its infections, the pathogenicity of this organism still remains unclear. The lack of knowledge on the determinants of virulence in *F. necrophorum* has hampered the development of an immunoprophylactic approach to the control of *Fusobacterium* infections. The characteristics of *F. necrophorum* and its role as an animal pathogen have been reviewed by Langworth (1977). The purpose of this paper is to summarize the involvement of *F. necrophorum* in various diseases and to provide an overview of the role of the virulence factors, possible pathogenic mechanisms, immunity and control of *Fusobacterium* infections. Information published since 1977 is emphasized.

FUSOBACTERIUM NECROPHORUM INFECTIONS

F. necrophorum has been known to be associated with numerous necrotic disease conditions, generally termed 'necrobacillosis' (Table II). Hepatic abscess in cattle is the most common *F. necrophorum* infection. Foot rot in cattle and sheep, calf diphtheria

TABLE II
Common diseases caused by *Fusobacterium necrophorum*

Disease	Host	Subsp./biovar	Bacteria commonly found in mixed infection
Hepatic abscesses	Cattle	*necrophorum funduliforme*	*Actinomyces pyogenes* and *Bacteriodes* spp.
Foot abscesses	Cattle, Sheep	*necrophorum funduliforme biovar* AB	*Actinomyces pyogenes* and *Dicholebacter nodosus Bacteriodes* spp.
Calf diphtheria	Calf	*necrophorum funduliforme*	*Actinomyces pyogenes*
Mastitis	Cattle	ND[a]	*Actinomyces pyogenes* and *Peptostreptococcus indolicus*
Oral/pharyngeal infections	Human	ND[a]	*Fusobacterium nucleatum* and others

[a]ND = not determined

and mastitis in cows are often caused by mixed infections of *F. necrophorum* and other pathogenic bacteria. In humans, *F. necrophorum* is a very common isolate associated with anaerobic infections.

Hepatic abscesses in cattle

Natural infection

Hepatic abscesses in grain-fed beef cattle are of great economic concern to the cattle industry. The prevalence of abscessed livers averages 18% to 32% in feedlot cattle (Brink *et al.*, 1990). The economic loss from condemnation of livers and reduced weight gain and feed efficiency associated with such abscesses is substantial (Brink *et al.*, 1990).

F. necrophorum is isolated in pure culture or mixed with other bacteria, such as *Actinomyces pyogenes* (Kanoe *et al.*, 1976; Lechtenberg *et al.*, 1988); *Staphylococcus* spp. (Kanoe *et al.*, 1976; Lechtenberg *et al.*, 1988); *Streptococcus* spp. (Lechtenberg *et al.*, 1988); and *Bacteroides* spp. (Kanoe *et al.*, 1976). Generally, subsp. *necrophorum* is isolated more frequently in pure culture than subsp. *funduliforme*, while the latter tends to be found more frequently in mixed infections (Berg and Scanlan, 1982; Lechtenberg *et al.*, 1988). *A. pyogenes* is the most common of the other bacteria isolated from hepatic abscesses.

Abscesses found in the liver at the time of slaughter or necropsy are often well

encapsulated, possessing a fibrotic wall measuring up to 1 cm in thickness (Lechtenberg *et al.*, 1988). Histologically, a typical abscess is pyogranulomatous, with a necrotic centre surrounded by zones of inflammatory tissue. Along with degenerated hepatocytes, bacteria can be observed within neutrophils, macrophages and giant cells. Generally, livers infected with subsp. *necrophorum* have more severe lesions than those infected with subsp. *funduliforme* (Lechtenberg *et al.*, 1988). Naturally occurring hepatic abscesses have been reported in sheep and goats (Tadayon *et al.*, 1980; Scanlan and Edwards, 1990).

Experimental infections

Hepatic abscesses have been studied in mice by inoculating *F. necrophorum* intraperitoneally (Abe *et al.*, 1976a; Conlon *et al.*, 1977; Garcia *et al.*, 1977), intravenously (Conlon *et al.*, 1977; Takeuchi *et al.*, 1984; Nakajima *et al.*, 1985), intraportally (Jensen *et al.*, 1954b; Shinjo *et al.*, 1981b), intrahepatically (Maestrone *et al.*, 1975), and subcutaneously (Maestrone *et al.*, 1975). Abe and colleagues (1976b) studied the pathological changes in the liver, spleen and lung of mice injected intraperitoneally with *F. necrophorum* of bovine hepatic abscess origin. The numbers of bacteria were higher in the liver than in the other organs. Moreover, abscesses were formed only in the liver, suggesting that liver may be a preferred site for *F. necrophorum* for some unknown nutritional or microenvironmental reason(s).

Experimental infections in cattle have been induced by infusing *F. necrophorum* into the portal vein (Scanlan and Berg, 1983; Nakajima *et al.*, 1986; Itabisashi *et al.*, 1987b; Lechtenberg and Nagaraja, 1991; Tan *et al.*, 1994a) or ruminal vein (Takeuchi *et al.*, 1984). Ultrasonography-guided intraportal inoculation has been employed as a noninvasive technique to induce liver abscesses in cattle (Nakajima *et al.*, 1986; Lechtenberg and Nagaraja, 1991; Tan *et al.*, 1994a). Another application of ultrasonography is in monitoring the onset and development of hepatic abscesses. However, ultrasonography cannot visualize the whole liver, so abscesses deep in the liver or on the visceral surface may not be discovered (Itabisashi *et al.*, 1987b; Lechtenberg and Nagaraja, 1991).

Foot rot and abscesses

Foot rot, also known as interdigital necrobacillosis, is an acute or chronic necrotizing infection of the interdigital and coronary skin and adjacent soft tissues and is the major cause of lameness in beef and dairy cattle. Foot rot in sheep is a mixed bacterial infection of the interdigital skin with *Dicholebacter (Bacteroides) nodosus*, a Gram-negative and obligatory anaerobic rod, as the primary causative agent (Stewart, 1989). Egerton and Roberts (1969) suggested that *F. necrophorum* plays an important role in the pathogenesis and that a synergistic infection of *D. nodosus* and *F. necrophorum* is the causal factor of foot rot in sheep. Emery and colleagues (1985) isolated *F. necrophorum* from foot lesions of cattle and sheep and from normal interdigital skin

of cattle. Most of the isolates from the foot lesions belonged to biovar AB. Other bacteria, such as *Prevotella (Bacteroides) melaninogenicus*, *Dicholebacter nodosus* and *Actinomyces pyogenes* may also be involved in the formation of the lesions (West, 1983; Timoney *et al.*, 1988). Early attempts at experimental induction of foot rot with *F. necrophorum* were unsuccessful (Langworth, 1977). However, Clark and colleagues (1985) induced foot abscesses by inoculating a pure culture of *F. necrophorum* subcutaneously into the interdigital spaces in cattle. In suppurative infections of the foot (abscesses), *F. necrophorum* is frequently associated with *A. pyogenes* (West, 1983, 1989).

Faecal excretion of *F. necrophorum* is believed to provide the primary source of infection in foot rot and foot abscesses. However, the presence of *F. necrophorum* in bovine and other animals' faeces is rare (Smith and Thornton, 1993a), suggesting that the organism is not normally excreted. Apparently, the disturbance of the normal gut flora induced by oral administration of certain antibacterial agents encouraged proliferation of *F. necrophorum* in the gut and subsequent faecal excretion (Smith and Thornton, 1993b).

Calf diphtheria

Calf diphtheria (necrotic laryngitis) is another disease associated with *F. necrophorum*. It is characterized by necrotic lesions in the larynx, oral cavity or pharynx and usually occurs in calves up to 2 years of age (Mackey, 1968). In severe cases the calf dies from subsequent aspiration pneumonia. In two surveys conducted by Jensen and co-workers, the prevalence of diphtheria was 6% in yearling feedlot cattle (Jensen *et al.*, 1976) and 1.4% in slaughtered beef cattle (Jensen *et al.*, 1981). In one outbreak of calf diphtheria, the mortality of calves below 3 months of age was 45% (Vanamayya and Charan, 1988). In a study involving bacteriological investigation of laryngeal swabs from animals with clinical laryngitis, *F. necrophorum* and *A. pyogenes* were isolated most frequently (Panciera *et al.*, 1989).

F. necrophorum is also a normal inhabitant of the respiratory tract of cattle (Jang and Hirsh, 1994). Because it does not penetrate a healthy mucosa, a breach in the mucosal barrier, possibly caused by viruses, allergens, irritants or other bacteria (Panciera *et al.*, 1989), is required for tissue invasion. Therefore, *F. necrophorum* is believed to be the secondary invader in calf diphtheria.

Mastitis

Bovine mastitis (summer mastitis) caused by *F. necrophorum* alone has been reported (McGillivery and Nicholls, 1984). However, in most cases, *A. pyogenes* and other bacteria are also involved (Shinjo, 1983). In one instance, the *F. necrophorum* involved in mastitis belonged to subsp. *funduliforme* (Shinjo, 1983). The organism has also been isolated from unaffected udders.

Watase (1983) observed that the prevalence of mastitis in heifers in Japan over a 6-

year period was 2.8%; *A. pyogenes* and *F. necrophorum* were the predominant isolates. Bacteriological investigations of summer mastitis in non-lactating cows and heifers have been conducted by several European researchers. *F. necrophorum* was isolated from 68% (Sorensen, 1978); 22% (Van den Bogaard and Hazen, 1987); 52% (Madsen *et al.*, 1990); and 14% (Jonsson *et al.*, 1991) of the cases. However, *A. pyogenes* and *Peptostreptococcus indolicus* were isolated more frequently than *F. necrophorum*, generally in mixed culture (21–100% of the cases).

Human infections

F. necrophorum is a normal inhabitant of the oral cavity, gastrointestinal tract and female genital tract of humans (Ohtani, 1970; Gorbach and Bartlett, 1974). The organism, being an opportunistic pathogen, causes bacteraemia by entering the blood from the oropharynx and the female genital tract (Gorbach and Bartlett, 1974). Eight species of *Fusobacterium* have been implicated in human infections. Among them, *F. nucleatum*, followed by *F. necrophorum*, were isolated most frequently from clinical specimens (Hofstad, 1986).

In a survey of 358 clinical cases, 24% of anaerobic bacteria were *F. necrophorum* (Finegold, 1977). Henry and colleagues (1983) reported 26 cases of *Fusobacterium* bacteraemia in a hospital over a 5-year period. The infections included oral and pharyngeal lesions, sinusitis, pelvic infection, aspiration pneumonitis and lung and hepatic abscesses. Death occurred in 3 of the 26 patients. *Fusobacterium* spp. were recovered from all blood samples, but species identification was not attempted.

Dental, oral and pharyngeal infections, such as periodontitis (Rams *et al.*, 1991; Uematsu and Hoshino, 1992), peritonsillar abscesses (Oleski *et al.*, 1976) and pharyngeal infection (Seidenfeld *et al.*, 1982; Henry *et al.*, 1983) are the most common diseases caused by *F. necrophorum* in humans. Pharyngotonsillar infection, a disease normally caused by viruses, is also associated with *F. necrophorum*. The disease is often complicated with multiple metastatic abscesses in the lung, liver and joints (Vogel and Boyer, 1980; Seidenfeld *et al.*, 1982; Kleinman and Flowers, 1984; Moreno *et al.*, 1989). In children, *F. necrophorum* causes various types of infections, including pharyngotonsillar infection (Vogel and Boyer, 1980; Brook *et al.*, 1984), meningitis (Islam and Shneerson, 1980; Pace-Balzan *et al.*, 1991), endocarditis (Stuart and Wren, 1992), pneumonia (Brook *et al.*, 1984), and septicaemia (Epstein *et al.*, 1992). *F. necrophorum* may also be involved in aspiration pneumonia, lung and hepatic abscesses, septicaemia, colitis, and other less common infections (Gorbach and Bartlett, 1974).

Generally, clinical reports of *F. necrophorum* infections in humans do not identify the subspecies involved. However, according to some early researchers (Langworth, 1977), human strains frequently belong to subsp. *funduliforme*. This observation is also supported by a recent study by Smith and Thornton (1993c). They reported that, of eight clinical isolates of *F. necrophorum*, seven belonged to subsp. *funduliforme*, based on the lack of enhancement of infectivity in mice when mixed with other bacteria. The seven strains were also less virulent and produced mild local lesions that rapidly healed when injected subcutaneously in mice (Smith and Thornton, 1993c).

VIRULENCE FACTORS

Pathogenic bacteria possess many strategies that allow them to escape host defence mechanisms and colonize the sites of infection. Several toxins have been implicated as virulence factors in the pathogenesis of *F. necrophorum* infections. They include endotoxin, leukotoxin, haemolysin, haemagglutinin, capsule, adhesins, platelet aggregation factor and certain extracellular enzymes. Endotoxin and leukotoxin have been investigated extensively.

Endotoxin

The outer membrane of *F. necrophorum*, as with other Gram-negative bacteria, contains endotoxic lipopolysaccharide (LPS). The chemical composition of *F. necrophorum* LPS has been analysed by several investigators (Inoue *et al.*, 1985; Okahashi *et al.*, 1988). The composition, which is variable and dependent on subspecies, strains, purity of LPS and analytical techniques, is 26% carbohydrate, 16–28% fatty acid or fatty acid ester, 4–34% lipid A, 6–8% protein, 0–2% 2-keto-3-deoxyoctonate, 7–12% hexosamine, 0.9–13% amino sugar, 0–12% heptose, 17–23% neutral sugar, 51% reducing sugar, 2–4% nitrogen, and 0.7–3% phosphorus.

The biological activity of *F. necrophorum* LPS has been studied by several investigators. The LPS is lethal to chicken embryos, mice and rabbits; causes localized and generalized Shwartzman reactions; and induces a biphasic pyrogenic response in rabbits (Langworth, 1977; Inoue *et al.*, 1985; Nakajima *et al.*, 1988). *F. necrophorum* LPS also causes erythema and oedema in rabbit skin (Berg and Scanlan, 1982), and hepatic degeneration and necrosis in mice (Nakajima *et al.*, 1985). The effects of LPS on the genesis of hepatic necrosis in rabbits have been investigated by Nakajima and colleagues (1987, 1988). Injection of a mixture of *F. necrophorum* and LPS into the bile duct produced hepatic necrosis, but inoculation of the bacterium or LPS separately caused only a slight lesion (Nakajima *et al.*, 1987). LPS from *F. necrophorum* or *E. coli* promoted the pathogenic effects of cytoplasmic or supernatant fractions of *F. necrophorum* in the formation of hepatic necrosis (Nakajima *et al.*, 1988).

The quantity and composition of LPS differ between subsp. *necrophorum* and subsp. *funduliforme* and, therefore, may be responsible for the difference in virulence (Table III). Inoue and colleagues (1985) reported a higher LPS content in subsp. *necrophorum* than in subsp. *funduliforme*. Lipid A, a primary toxophore of LPS responsible for the biological activity, is present in higher concentration in *F. necrophorum* subsp. *necrophorum* than in subsp. *funduliforme* (Table III). Also, the LPS of subsp. *necrophorum* was more potent in causing chick embryo mortality, haemorrhagic necrosis of solid tumours, a pyrogenic response, and a local Shwartzman reaction than that from subsp. *funduliforme*. However, Berg and Scanlan (1982) reported no differences between subsp. *necrophorum* and subsp. *funduliforme* in their ability to induce lethal shock in mice and skin lesions in rabbits. Therefore, further research is needed to relate the quantity and biological activity of LPS to the subspecies of *F. necrophorum*.

TABLE III
Chemical compositions and biological activities of lipopolysaccharides from *Fusobacterium necrophorum* subspecies[a]

Criterion	Subsp. *necrophorum*	Subsp. *funduliforme*
LPS (% of dry cell weight)	2.6	1.2
Composition (%)		
Lipid A	34.0	4.2
Protein	4.6	3.2
Hexose	21.5	66.2
Methylpentose	3.7	1.5
Amino sugar	12.9	0.9
Heptose	12.0	5.5
Keto-deoxy-octonate (KDO)	2.3	1.3
Total phosphorus	1.3	0.7
Biological activity		
LD_{50} for 10-day-old chick embryos (μg)	79.4	365.3
ED_{50} for haemorrhagic necrosis of solid tumours (μg)	0.8	42.6

[a]Adapted from Inoue *et al.* (1985)

Okahashi and colleagues (1988) studied the immunobiological characteristics of *F. necrophorum* LPS. The LPS induced mitogenesis of murine splenocytes in three different breeds of inbred mice. However, this effect might have been caused by contaminated mitogenic protein. Macrophages activated by LPS secrete various biologically active substances, including interleukin-1 (Okahashi *et al.*, 1988). *F. necrophorum* LPS is also a growth factor for oral *Bacteroides* (Price and McCallum, 1986). Enhancement of the growth of oral *Bacteroides* by *F. necrophorum* may occur in the subgingival flora in the gingival crevice, thus contributing to the infection of periodontium.

Leukotoxin

Roberts (1967) was the first to document a leukotoxic activity in *F. necrophorum* by observing that migration of leukocytes was inhibited and the cells were destroyed when *F. necrophorum* culture filtrate was injected intradermally into sheep, rabbits or guinea-pigs. Subsequent research has established that the leukotoxin is an important virulence factor in the pathogenesis of *F. necrophorum* infection. Coyle-Dennis and Lauerman (1979) reported that a leukotoxin-producing strain was more infective than a non-leukotoxin-producing strain in causing abscesses in mice. In another study (Emery *et*

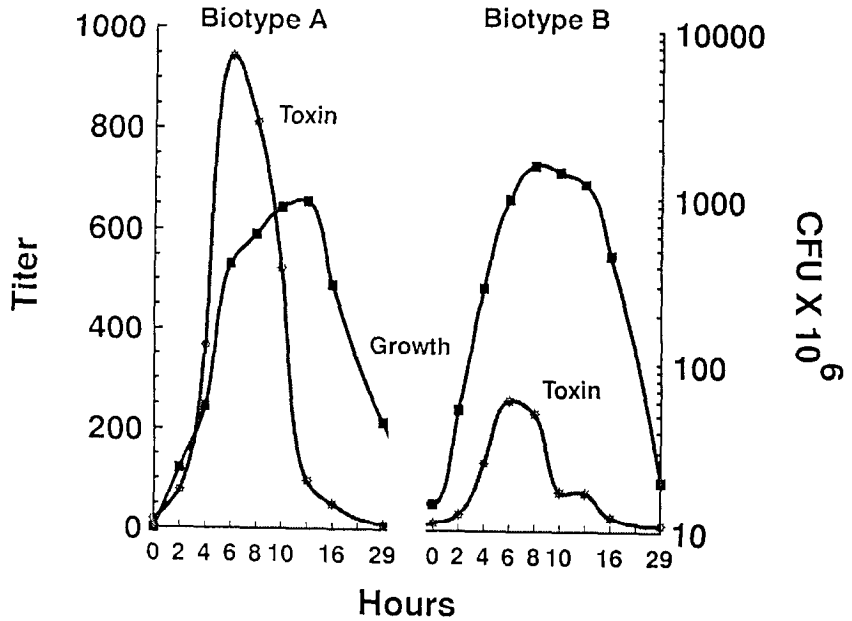

Figure 1. Leukotoxin production in relation to the growth phases of *Fusobacterium necrophorum* biotypes A and B. (From Tan *et al.*, 1992)

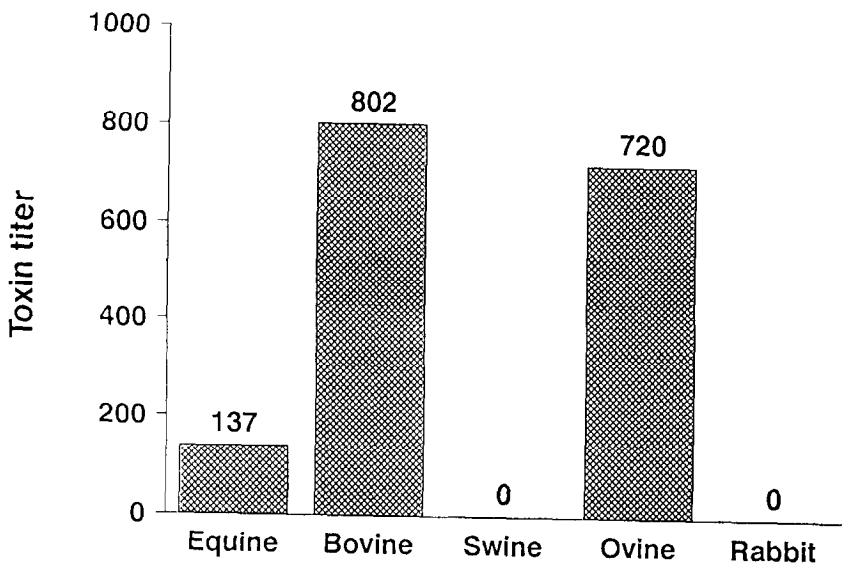

Figure 2. The leukotoxicity of *Fusobacterium necrophorum* against neutrophils isolated from various animal species. (From Tan *et al.*, 1994d)

al., 1986b), *F. necrophorum* strains with a high leukotoxin activity required fewer cells to induce infection and death in mice.

Several investigators have reported that subsp. *necrophorum* produces more leukotoxin than subsp. *funduliforme* (Scanlan *et al.*, 1982, 1986; Tan *et al.*, 1992,1994c,d). Tan and colleagues (1992) reported that subsp. *necrophorum* produced about 18 times more leukotoxin than subsp. *funduliforme*. The difference in toxin production may account for the difference in virulence between the two subspecies (Shinjo *et al.*, 1981a; Berg and Scanlan, 1982; Smith, 1992; Tan *et al.*, 1994c) and explain why subspecies *necrophorum* is encountered more frequently in bovine hepatic abscesses than subsp. *funduliforme* (Scanlan and Hathcock, 1983; Lechtenberg *et al.*, 1988).

In a recent study, Tan and colleagues (1994c) found that subsp. *necrophorum* strains isolated from the rumen were less leukotoxic (approximately 15-fold) than those isolated from bovine hepatic abscesses. The reason for the lack of or low leukotoxin activity in the ruminal isolates is unclear. However, it does indicate that considerable variation (none to low to high) occurs in the leukotoxicity of strains of subsp. *necrophorum*. Possibly, a gene(s) coding for leukotoxin in ruminal strains is expressed when they enter the liver, but not in the rumen. Additionally, the high leukotoxicity of the hepatic isolate may be an indication of selection, with non-leukotoxic or low-leukotoxic strains being phagocytosed in the liver.

Various factors (growth phase, composition of medium, culture pH, incubation temperature, redox potential, and iron concentration) affecting leukotoxin activity have been investigated (Tan *et al.*, 1992). In both subspecies of *F. necrophorum*, the maximal leukotoxin activity in the culture supernatant was observed during the late-log and early-stationary phases of growth (Figure 1). Brain–heart infusion broth, at a pH of 6.6–7.7 and redox potential between –230 and –280 mV, appeared to support maximal *F. necrophorum* growth and toxin production. *F. necrophorum* growth is stimulated by iron, but leukotoxin activity was not affected by iron at concentrations of <0.2 to 42 μmol/L. The specificity of *F. necrophorum* leukotoxin has been reported (Emery *et al.*, 1984; Tan *et al.*, 1994d). Leukocytes (PMN) from cattle and sheep were most susceptible, whereas leukocytes from rabbits and swine were not susceptible to the toxin (Figure 2). The resistance of swine neutrophils to leukotoxin may explain the failure to induce hepatic abscesses experimentally in swine (Jensen *et al.*, 1954b). It is not known whether the species specificity is due to differences in the structural determinants (receptors) between ruminant and non-ruminant neutrophils.

Attempts to characterize *F. necrophorum* leukotoxin have yielded conflicting data regarding its biochemical characteristics, such as molecular weight and stability to heat and enzymatic treatments (Fales *et al.*, 1977; Coyle-Dennis and Lauerman, 1978; Emery *et al.*, 1984; Kanoe *et al.*, 1986; Scanlan *et al.*, 1986). Again, these discrepancies may be attributable to differences in subspecies and strains, cultural conditions, and the techniques employed to detect and characterize the toxin. There is general agreement that *F. necrophorum* leukotoxin is a soluble, proteinaceous and heat-labile exotoxin with specificity for ruminant neutrophils (Coyle-Dennis and Lauerman, 1978; Emery *et al.*, 1984, 1985, 1986a; Kanoe *et al.*, 1986; Tan *et al.*, 1992, 1994d). Reducing agents like titanium(III) citrate, sodium sulphide and dithiothreitol markedly inhibit the leukotoxin (Tan *et al.*, 1994d). Presumably, reducing agents break disulphydral

bonds and, consequently, alter the toxin structure. Based on gel-filtration chromatography, the molecular weight of the native leukotoxin was $\geqslant$300 kDa (Emery *et al.*, 1984; Tan *et al.*, 1994d). Upon reduction and denaturation during sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the toxin dissociated into several components (Tan *et al.*, 1994d).

The instability of *F. necrophorum* leukotoxin has been a problem for its purification (Emery *et al.*, 1986a; Tan *et al.*, 1994d). Because *F. necrophorum* produces proteolytic enzyme(s) (Wallace and Brammall, 1985), proteolysis may be responsible, at least in part, for the loss of leukotoxicity. However, protease inhibitors specific for acid, thiol and serine proteases failed to preserve toxin activity (Tan *et al.*, 1994d), suggesting that some enzymes in the culture supernatant belong to other classes of proteases.

Haemolysin

The haemolytic activity of *F. necrophorum* has been reported in both the culture supernatant (Abe *et al.*, 1979; Kanoe *et al.*, 1984; Amoako *et al.*, 1994; Tan *et al.*, 1994c,d) and cell fraction (Tan *et al.*, 1994c,d). *F. necrophorum*, like many other pathogenic bacteria, requires iron as a growth factor (Tan *et al.*, 1992). Therefore, the haemolytic activity of *F. necrophorum* may aid in acquiring iron from the host. Another possible role of haemolysin may be to aid in creating an anaerobic environment for *F. necrophorum* growth, as lysis of erythrocytes will reduce oxygen transport to the site of infection.

Haemolytic activity has been demonstrated on erythrocytes from horses, humans, sheep, rabbits, cattle and chickens (Langworth, 1977; Amoako *et al.*, 1994). Abe and colleagues (1979) reported that erythrocytes from humans, dogs and rabbits were more susceptible to the partially purified haemolysin than those from cattle, sheep and goats. In contrast to these results, Emery and colleagues (1986b) reported that the haemolysin (culture supernatant) was more toxic to erythrocytes from cattle, horses and chickens than to those from sheep and least toxic to those from pigs and rabbits. Biovar AB produced more haemolysin than biovar B (subsp. *funduliforme*; Emery *et al.*, 1985). Tan and colleagues (1994c) observed that the haemolytic activity against sheep erythrocytes was similar in the supernatant and cellular fractions and between subsp. *necrophorum* and subsp. *funduliforme*. However, with horse erythrocytes, subsp. *necrophorum* was more haemolytic than subsp. *funduliforme* (Amoako *et al.*, 1994).

Based on thin-layer and gas–liquid chromatography, Abe and colleagues (1979) suggested that phospholipase A and lysophospholipase were the components responsible for the haemolytic activity of *F. necrophorum*. The toxin was inactivated by 56°C for 10 min. Kanoe (1990) was able to detect *F. necrophorum* haemolysin in abscessed bovine liver by using a fluorescence antibody. Haemolysin was purified by Kanoe and colleagues (1984) using diethylaminoethylcellulose chromatography and Sephadex G-200 gel filtration. In SDS-PAGE, the native toxin, which showed a single band in non-reducing and non-denaturing PAGE, disassociated into 54 kDa and 48 kDa molecular weight components. The purified toxin was heat labile and reversibly inactivated by oxygen.

Haemagglutinin

It has long been known that *F. necrophorum* subsp. *necrophorum* agglutinates erythrocytes from chickens and other animal species (Langworth, 1977). Shinjo and colleagues (1980) tested the ability of both subspecies of *F. necrophorum* to agglutinate erythrocytes from different animal species. Erythrocytes from cattle, pigs, dogs and rabbits were most susceptible. Subsp. *necrophorum* gave a higher agglutination titre than subsp. *funduliforme* when chicken or cattle erythrocytes were used. Nagai and colleagues (1984) purified the *F. necrophorum* haemagglutinin by gel filtration of trypsinized and sonicated bacterial cells. The haemagglutinin had a filamentous structure and frequently clustered as a macromolecule, as revealed by electron microscopy. It was heat labile and had a molecular weight of 19 kDa as determined by SDS-PAGE. This study did not ascertain whether the haemagglutinin was different from the bacterial fimbriae.

The occurrence of *F. necrophorum* haemagglutinin in subsp. *necrophorum* suggests that it may be important in pathogenesis. Shinjo and Kiyoyama (1986) reported that a non-haemagglutinating mutant strain of *F. necrophorum* did not kill mice, whereas a parent strain caused mice to die. Using an inhibition assay with an anti-agglutinin antiserum, the research group led by Kanoe reported that *F. necrophorum* haemagglutinin was responsible for the attachment of the organism to Vero cells (Kanoe *et al.*, 1985), bovine hepatocytes (Kanoe and Iwaki, 1986) and bovine ruminal cells (Kanoe and Iwaki, 1987); for aggregation of bovine platelets (Kanoe and Yamanaka, 1989); and for the formation of thrombi in the microcirculation (Kanoe *et al.*, 1989). Therefore, the haemaglutinin, in addition to aiding *F. necrophorum* to adhere to ruminal epithelial cells and hepatocytes, may also aid anaerobic microorganisms by promoting blood clotting.

Capsule

Bacterial capsules act as virulence factors by preventing complement-mediated serum killing the organism and by enhancing adherence of the pathogen to host cells (Brook, 1987). Brook and Walker (1986) revealed the presence of a mucopolysaccharide wall in *F. necrophorum* as visualized by electron microscopy. The importance of the capsule in *F. necrophorum* infection is not clear, but it appears to be related to the virulence of the bacteria. Two encapsulated *F. necrophorum* isolates induced subcutaneous abscesses in mice, whereas two non-capsulated strains did not (Brook and Walker, 1986). In another study (Emery, 1989), virulent *F. necrophorum* strains of biovar AB had mucoid capsules, whereas avirulent biovar B strains did not.

Adhesins

Attachment to a eukaryotic cell surface is the first major interaction between a pathogen and its host and an essential step for colonization by the bacterium. The

ability of pathogens to attach themselves to the mucosal surface of the host is mediated by fimbriae (pili), haemagglutinin, and a capsule (Finlay and Falkow, 1989).

Miyazato and colleagues (1978) demonstrated the presence of fimbriae in both subspecies of *F. necrophorum*. Each cell possessed a number of fimbriae, each up to 3 μm in length. Shinjo and Kiyoyama (1986) reported that *F. necrophorum* possessing fimbriae but no haemagglutinin did not cause severe infections in mice. These authors concluded that the mere presence of fimbriae was not sufficient to render the organism pathogenic. The stronger adherence ability of subsp. *necrophorum* than that of subsp. *funduliforme* may relate to the higher cell surface hydrophobicity in the former (Shinjo *et al.*, 1987, 1988). The receptor for bacterial adhesin is usually a specific carbohydrate residue on the eukaryotic cell surface (Finlay and Falkow, 1989). However, the adherence of *F. necrophorum* to ruminal cells was reduced by pretreatment of the cells with pepsin or trypsin (Kanoe and Iwaki, 1987), suggesting that the receptor contains a protein moiety.

Further research is needed to identify the exact mechanism of adherence of *F. necrophorum* to eukaryotic cells. Apparently, the organism has capsule, fimbriae, haemagglutinins and adhesins, and all of these alone or in combination could be the mediators of attachment. The recognition of the attachment mechanism could lead to an immunoprophylactic approach to preventing colonization and thus preventing *F. necrophorum* infections.

Platelet aggregation

Platelet aggregation relates to the virulence of bacteria, because of its involvement in thrombocytopenia, disseminated intravascular coagulation, fibrin deposition and other coagulative effects (Forrester *et al.*, 1985). The ability of *F. necrophorum* subsp. *necrophorum* but not subsp. *funduliforme* to aggregate human (Forrester *et al.*, 1985) and bovine platelets (Kanoe and Yamanaka, 1989) has been demonstrated. Cytosolic lactic dehydrogenase assay (Forrester *et al.*, 1985) and electron microscopic examination (Kanoe and Yamanaka, 1989) showed that platelet lysis did not occur during aggregation. Forrester and colleagues (1985) reported that the aggregation was a typical aggregation–degranulation reaction, and that the system did not appear to be ion-dependent or to involve complement. Kanoe and Yamanaka (1989) demonstrated that the haemagglutinin was associated with the platelet aggregation. The aggregation of bovine platelets was slower than that of human platelets (Forrester *et al.*, 1985; Kanoe and Yamanaka, 1989). The intravascular coagulation caused by platelet aggregation may contribute to the establishment of an anaerobic microenvironment, so enabling *F. necrophorum* to grow.

Proteolytic enzymes

Microbial proteolytic enzymes have been implicated as virulence factors (Lory and Tai, 1985). The enzymes promote invasion by the pathogen and damage host tissue by

breaking down physical barriers in the host, by non-specifically damaging leukocytes and immunoglobulins, and by providing amino acids and peptides for bacterial growth (Lory and Tai, 1985). Proteolytic activity by *F. necrophorum*, with collagen and gelatin as the substrate, has been demonstrated (Amoako *et al.*, 1993). There was no difference in the activity between the two subspecies. However, the use of a commercial kit of biochemical tests indicated that *F. necrophorum* did not hydrolyse gelatin (Scanlan *et al.*, 1992; Tan *et al.*, 1994c). The difference is possibly attributable to differences in the detection procedures used by the investigators.

The protease of *F. necrophorum* has been partially purified and characterized (Nakagaki *et al.*, 1991). It was heat labile, and its activity was inhibited by the protease inhibitor para-hydromercuribenzoate. Leukotoxin and haemolysin produced by *F. necrophorum* are themselves proteins and are inactivated by proteolytic enzymes (Coyle-Dennis and Lauerman, 1978; Tan *et al.*, 1994d). Accordingly, the synthesis and release of proteolytic enzymes may be highly regulated by the bacterium.

Other factors

Garcia and colleagues (1975) injected different preparations of *F. necrophorum* intradermally into the skin of guinea pigs and rabbits and observed that intact cells, sonicates and cell wall fractions all caused dermonecrosis and erythema. However, culture supernatant did not cause dermonecrosis. In other studies, dermonecrotic lesions were not induced by culture supernatant (Coyle-Dennis and Lauerman, 1978; Tan *et al.*, 1994d) or by sonicated cells (Tan *et al.*, 1994d). Recently, a dermonecrotic toxin was isolated from the cell wall preparation of *F. necrophorum* (Kanoe *et al.*, 1995). The toxin induced a haemorrhagic necrosis in the skins of guinea pigs and rabbits.

It has been documented that *F. necrophorum* produces DNAase and fibrinolysin (Hofstad, 1984; Moore *et al.*, 1984; Kanoe *et al.*, 1986; Tan *et al.*, 1994c). These enzymes are also potential virulence factors. More research is required to understand their role in the pathogenesis of *F. necrophorum* infection.

PATHOGENIC MECHANISM

Despite many research efforts, the pathogenic mechanisms of *F. necrophorum* infection, particularly the overall effect of virulence factors, the interaction between the bacterium and host, and any possible synergism between *F. necrophorum* and other pathogens, have not been well defined. The pathogenesis of *F. necrophorum* infection is clearly multifactorial and complex. *F. necrophorum* has several virulence factors, which probably act in concert to overcome host defences and allow growth in tissues. As an anaerobe, *F. necrophorum* faces oxygen toxicity in host tissue. Facultative organisms are known to play an important role in facilitating the growth of *F. necrophorum* by lowering oxygen tension and redox potential (Brook *et al.*, 1984; Hofstad, 1984). Of particular interest is the mode of entry of *F. necrophorum* from the rumen into the liver

and the possible mechanisms involved in establishing infection by an anaerobe in an organ that is highly oxygenated and well defended by macrophages.

Rumenitis–hepatic abscess complex

Because of the close correlation between the incidence of ruminal lesions and hepatic abscesses in cattle, the term 'rumenitis–liver abscess complex' was originally proposed by Jensen and colleagues (1954a). However, the precise pathogenic mechanism of bovine hepatic abscesses is still not clear. In the past several decades, feeding of high-grain diets has been a common practice to improve body weight gain and feed efficiency in feedlot finishing cattle. However, intensive grain feeding also increases the incidence of hepatic abscesses (Langworth, 1977; Scanlan and Hathcock, 1983).

The starch component of high-grain diets is rapidly fermented by ruminal microbes and the consequent accumulation of organic acids, particularly lactate, results in ruminal acidosis. The increase in ruminal lactate may also lead to increased numbers of *F. necrophorum*. Tan and colleagues (1994b) counted ruminal *F. necrophorum* using a selective medium and found that the bacterial concentration was higher in cattle fed a high-grain diet than in those fed a forage diet (Figure 3). Because *F. necrophorum* uses only lactate and not sugars as its energy source for growth, the increased bacterial concentration is probably due to increased lactate concentration. The acidic condition impairs the integrity of the rumen wall, and opportunistic organisms, principally *F. necrophorum*, colonize the wall and cause rumenitis (Kanoe *et al.*, 1978). The bacteria

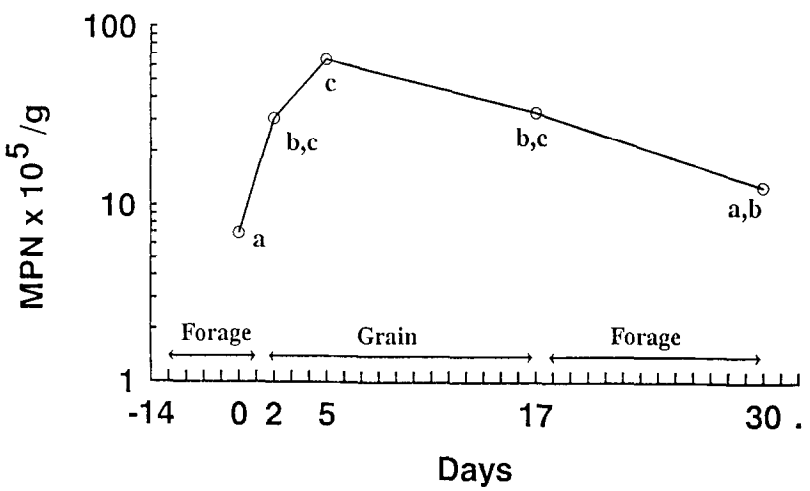

Figure 3. The effect of diet on the ruminal concentration of *Fusobacterium necrophorum*, as determined by the most-probable-number technique in a selective modified lactate medium. The data are means for six cattle, and different letters indicate a difference at $p < 0.05$ (From Tan *et al.*, 1994b)

then gain entry into the liver by the portal venous system. It is well documented that ruminal lesions are predisposing factors for hepatic abscesses (Scanlan and Hathcock, 1983).

There is no doubt that *F. necrophorum* toxins are critical in the establishment of infection in the liver. Leukotoxin and LPS may prevent phagocytosis by peripheral neutrophils and Kupffer cells. The release of mediators, as a consequence of destruction of phagocytes, has a detrimental effect on the liver parenchyma. Liver is a highly vascular organ with a blood redox potential around +126 to 442 mV (Meynell, 1963). In order to grow and establish an infection, *F. necrophorum* has to overcome the oxygen toxicity. Synergism with facultative bacteria, intravascular coagulation induced by LPS and platelet aggregation, formation of fibrin-encapsulated abscesses (Forrester *et al.*, 1985), and impairment of oxygen transport by damaging erythrocytes may all contribute to the establishment of an anaerobic microenvironment in liver tissue.

Interaction of toxins

The roles of *F. necrophorum* toxins and their modes of action are complex. Most of them seem to have multiple biological activities. All the major components of *F. necrophorum* cells have biological activity. Culture supernatant is leukotoxic and haemolytic (Tan *et al.*, 1994d), and it contributes towards the Shwartzman reaction (Nakajima *et al.*, 1988); cell wall contains endotoxic LPS and is pathogenic to mice (Nakajima *et al.*, 1985); cell lysate also contributes towards the Shwartzman reaction (Nakajima *et al.*, 1988) and is haemolytic (Tan *et al.*, 1994c,d), and pathogenic to mice (Garcia *et al.*, 1975); the cytoplasm also contains a haemagglutinin which is involved in attachment by the bacterium to host cells (Kanoe *et al.*, 1985, 1986; Kanoe and Iwaki, 1986) and platelet aggregation (Kanoe and Yamanaka, 1989).

The cytotoxic effect of LPS on mammalian cells has been studied (Nakajima *et al.*, 1985, 1988). The pathogenic properties of the endotoxin are believed to be mediated by activation of various cells, especially macrophages (Fenwick, 1990). It has been well documented that LPS has a direct effect on macrophages, including Kupffer cells, and neutrophils, and induces various biologically active substances that cause liver damage (Wilson, 1985; Van Bossuyt and Wisse, 1988). Nakajima and colleagues (1985) detected *F. necrophorum* LPS in the hepatic sinusoids in mice with hepatic abscesses. In addition, endotoxin has been found in periodontal tissues infected with *F. necrophorum* (Shapiro *et al.*, 1972). The detection of *F. necrophorum* endotoxin in damaged tissues suggests its involvement in pathogenesis.

Extracellular leukotoxin inhibits migration of leukocytes to the infected tissue. The mechanism of the action of *F. necrophorum* leukotoxin on leukocytes is not clear. Electron microscopy has revealed loss of microvilli and partial destruction of the murine macrophage membrane after treatment with *F. necrophorum* leukotoxin (Kanoe *et al.*, 1986). Monoclonal antibodies with a high binding affinity to the leukotoxic antigen of *F. necrophorum* partially neutralized the toxicity, suggesting that the association of leukotoxin with the target cell is essential for toxicity (Tan *et al.*, 1994e). It is not known whether *F. necrophorum* leukotoxin acts directly by causing cell

lysis or indirectly by altering the phospholipid composition and causing leakage of Ca^{2+}, as with *Pseudomonas aeruginosa* (Lory and Tai, 1985). *F. necrophorum* leukotoxin is also toxic to bovine ruminal cells, so it may be directly involved in the onset of rumenitis (Kanoe *et al.*, 1987).

F. necrophorum haemolysin is also leukotoxic. Kanoe and Iriki (1985) reported that purified haemolysin caused disruption and protoblastic extrusion of neutrophils from various animal species. The question has been raised whether *F. necrophorum* leukotoxic and haemolytic activities are controlled by the same effector molecule. However, Roberts (1967) reported that antihaemolysin antiserum did not neutralize leukotoxic activity. Kanoe and colleagues (1984, 1986) have separated the two toxins by purifying them from *F. necrophorum* culture supernatant. Tan and colleagues (1994c,d) found that haemolytic activity was present equally in the cellular and extracellular fractions, whereas leukotoxin activity was mainly found in the cell-free culture supernatant. Also, leukotoxic activity peaks in the late-log or early-stationary phase and then declines rapidly (Tan *et al.*, 1992), whereas haemolytic activity persists past the stationary phase of growth (Amoako *et al.*, 1994). Therefore, it seems more likely that the two toxins are two distinct molecules.

Synergism between bacteria

F. necrophorum is often isolated along with other facultative and anaerobic bacteria. In bovine hepatic abscesses, most mixed cultures contain *A. pyogenes* (Scanlan and Hathcock, 1983; Lechtenberg *et al.*, 1988). In foot rot and abscesses, *P. melaninogenicus*, *D. nodosus* and *A. pyogenes* are important (West, 1989; Timoney *et al.*, 1988). *F. necrophorum* and other *Fusobacterium* spp. are often found together in human oral infections (Gorbach and Bartlett, 1974; Henry *et al.*, 1983; Uematsu and Hoshino, 1992). The pathogenic synergy between *F. necrophorum* and other organisms possibly involves the following phenomena: facultative bacteria utilize oxygen and lower the redox potential to create an anaerobic environment for growth of *F. necrophorum*; the leukotoxin of *F. necrophorum* protects other pathogens from phagocytosis; and *F. necrophorum* and other organisms produce growth factors that stimulate bacterial growth (Price and McCallum, 1986). Oxygen is toxic to *F. necrophorum*, at least in part, because of its lack of superoxide dismutase (Gregory *et al.*, 1978). However, *F. necrophorum* was less susceptible to oxygen when cocultured with the facultative *Staphylococcus aureus* or *Escherichia coli*. The influence of redox potential (Eh) on the growth of *F. necrophorum* has been investigated (Tan *et al.*, 1992). In one study (Z.L. Tan; previously unpublished data), growth of *F. necrophorum* was inhibited in a medium with a high Eh (>−103 mV; Figure 4). Therefore, utilization of oxygen by facultative bacteria, leading to lowering of Eh, may be crucial for the establishment of *F. necrophorum*.

Studies have demonstrated that more hepatic abscesses were induced with *P. melaninogenicus*, *F. nucleatum* (Hill *et al.*, 1974), or *B. intermedius* (Price and McCallum, 1987) were injected with *F. necrophorum* into mice. In a similar study, Takeuchi and colleagues (1983) reported that *A. pyogenes* was a 'helper organism' for

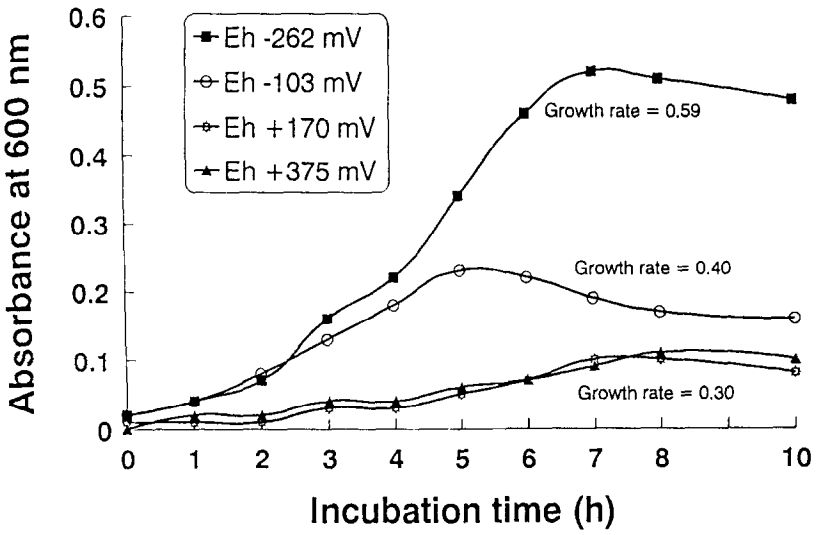

Figure 4. The influence of redox potential on the growth of *Fusobacterium necrophorum*. (From Tan *et al.*, 1992)

inducing hepatic abscesses in mice. Brook and Walker (1986) demonstrated a mutually beneficial relationship between *F. necrophorum* and other organisms, such as *Klebsiella pneumoniae* and *P. aeruginosa*. In a series of studies, Smith and co-workers (Smith *et al.*, 1989a, 1990, 1991a,b) demonstrated that *F. necrophorum* and *E. coli*, *A. pyogenes* or other bacteria acted synergistically in causing infection in mice. The minimum infective dose for *F. necrophorum* was reduced from $>10^6$ cells per mouse to <10 cells per mouse when other bacteria were involved.

IMMUNITY AND CONTROL OF INFECTION

Immunity

An ultimate goal of studying the virulence factors and pathogenesis of *F. necrophorum* is to develop a vaccine to control the infections it causes. Studies have demonstrated that serum antibodies against *F. necrophorum* were present in both healthy and infected animals and humans. This has caused doubts concerning the importance of anti-*F. necrophorum* immunity against infection. Antibody may be induced by the normal presence of *F. necrophorum*. Another concern is whether persistent exposure to *F. necrophorum* will lead to immunosuppression. Many investigators have attempted to induce protective immunity against *F. necrophorum* using bacterins, toxoids or other cellular components. Generally, none of these antigens afforded satisfactory protection in animals.

Laboratory animals have been used as models for studying the immune response to *F. necrophorum*. Roberts (1970) showed that formalinized *F. necrophorum* induced

humoral bacteriocidal antibodies that destroyed only half to two-thirds of a given inoculum either *in vitro* or in the tissues of a vaccinated rabbit, and the antibodies had no influence on the growth once the organism was established. He suggested that immunity against the exotoxin (leukotoxin) is necessary to render protection. Abe and colleagues (1976a) reported that repeated immunization (9–10 times) with formalinized *F. necrophorum* enhanced clearance of a challenge infection from the liver, lungs and spleen. In a similar study, Conlon and colleagues (1977) demonstrated that ethanol-killed *F. necrophorum* delayed death in mice after challenge. Based on the fact that *F. necrophorum* and *A. pyogenes* were often isolated together in infection, Cameron and Fuls (1977) immunized rabbits with bacterins of both organisms. However, the bacterins failed to protect the animals from experimental infection with both *F. necrophorum* and *A. pyogenes*. The vaccinated rabbits had high antitoxin antibodies against *A. pyogenes* but no antibodies against *F. necrophorum*. Garcia and McKay (1978) reported that weekly intraperitoneal injections of the cytoplasmic fraction of *F. necrophorum* afforded protection to mice against bacterial challenge. However, immune serum failed to protect mice from the infection in passive protection studies.

Smith and co-workers (1985, 1989b) and Smith and Wallace (1992) immunized mice with heat-killed *F. necrophorum* or live bacteria followed by treatment with metronidazole. Only slight or no protection was observed, although different means of immunization and challenges and various inocula sizes were tried. Smith and colleagues (1986) also reported that inactivated *F. necrophorum* failed to protect wallabies against *F. necrophorum* infection. Turner and colleagues (1986) reported a weak immune response to *F. necrophorum* in mice. Antibody titres were low in ELISA and almost nil in the passive haemagglutination test. Alexander and colleagues (1973) compared several preparations of *F. necrophorum* antigens and routes of injections for efficacy of immunizing cattle. Subcutaneous administration of alum-precipitated sonicate and cytoplasmic antigens was recommended. Garcia and colleagues (1974) reported that the incidence of hepatic abscess was reduced when cattle received cytoplasmic toxoid. A few years later, Garcia and McKay (1978) observed that the toxoid also induced protection in sheep. None of the sheep receiving three consecutive intraperitoneal injections of 20 mg cytoplasmic protein developed abscesses after challenge. In cattle with experimentally induced hepatic abscesses, the antibody titre increased approximately 1–2 weeks after challenge (Itabisashi *et al.*, 1987a; Takeuchi *et al.*, 1984). Takeuchi and colleagues (1984) further found that the immune response was against the culture supernatant (leukotoxin?) but not against LPS or the cytoplasmic fraction of *F. necrophorum*.

Immunity against the leukotoxin may be related to protection against *F. necrophorum* infection. Antibody to the leukotoxin was induced by injecting rabbits with semipurified leukotoxin (Emery *et al.*, 1986a). The specific antibody was able to prevent the cytolytic effect of the toxin on peripheral blood leukocytes. The antibody also recognized two subunits of toxin (14 and 13 kDa) of leukotoxin in immunoblots. However, antiserum against the two subunits failed to neutralize the native leukotoxin. Later, Emery and Vaughan (1986) immunized mice with LPS, culture supernatant, semipurified leukotoxin, and outer membrane protein, but only the semipurified toxin

afforded any significant protection against challenge. However, transfer of bovine or rabbit sera with high antileukotoxin antibody titres did not protect mice from *F. necrophorum* infection. Later, Emery and colleagues (1986b) reported that the same preparation of leukotoxin did not protect sheep challenged with *F. necrophorum*. Clark and colleages (1986) found that immunization of calves with culture supernatant, presumably containing leukotoxin, protected some animals from experimentally induced foot abscesses. Tan and colleagues (1994a) examined serum neutralizing antibody against *F. necrophorum* leukotoxin in feedlot cattle. Antibody titres were higher in cattle with abscessed livers than in those without. Also, a significant correlation was found between the severity of the abscesses and the antibody titre. They speculated that the presence of anti-leukotoxin antibody in non-abscessed cattle was due to other *F. necrophorum* infection, such as calf diphtheria or foot abscesses. In addition, the examination of livers at the slaughter plant was limited to superficial abscesses, so abscesses deep inside the liver could have been missed.

Control of infection

Because of the unavailability of a vaccine, control of *F. necrophorum* has depended mainly on the use of antimicrobial compounds. The antimicrobial sensitivity of *F. necrophorum* has been studied extensively (Berg and Scanlan, 1982; Seidenfeld *et al.*, 1982; Baba *et al.*, 1989; Duran *et al.*, 1990; Tan *et al.*, 1994c). In general, *F. necrophorum* is susceptible to the penicillins, tetracyclines and macrolides and resistant to aminoglycoside antibiotics. Werner and colleagues (1991) reported that *F. necrophorum* was also susceptible to cefpodoxime, an oral broad-spectrum antibiotic.

Several studies have examined the effect of antimicrobial agents as feed additives for the control of bovine hepatic abscesses. The commonly used antimicrobial feed additives to control liver abscesses include oxytetracycline, chlortetracycline and tylosin. In addition to improving weight gain and feed efficiency, tylosin significantly reduced the prevalence of hepatic abscesses in feedlot cattle (Potter *et al.*, 1985). Bacitracin (Haskins *et al.*, 1967) and ionophores such as monensin (Potter *et al.*, 1985; Beacom *et al.*, 1988; Tan *et al.*, 1994a,c) and lasalocid (Beacom *et al.*, 1988) have no effect on bovine hepatic abscesses. Recently, Rogers and colleagues (1995) reported that virginiamycin within a dose range of 16.5–19.3 mg/kg feed decreased the overall incidence of liver abscesses by about 38% compared to the control. In two separate studies, Berg and co-workers demonstrated that ethylenediamine dihydriodide could not prevent bovine foot rot experimentally induced with a high dose of *F. necrophorum* and *P. melaninogenicus* (Berg *et al.*, 1976) but did have efficacy against a challenge with a low dose of inoculum (Berg *et al.*, 1984).

PROSPECTS AND CONCLUSIONS

Much information has been developed over the past decades regarding the pathogenicity of *F. necrophorum* infection. *F. necrophorum* uses a variety of means to evade host

defensive mechanisms, to overcome oxygen toxicity, to colonize the host and to cause tissue damage. The virulence and pathogenesis of *F. necrophorum* are complex and, to some extent, unusual. Although many virulence factors have been identified, only a few, if any, have been characterized fully and many discrepancies exist regarding the properties of those toxins. Studies on *F. necrophorum* toxins are limited to their biological activity in animals and cell cultures. Their mode of action has not been determined at the molecular level.

One of the major problems in the control of *F. necrophorum* infection is the paucity of knowledge of the various virulence factors and their antigenic components responsible for stimulating protective immunity. The inability to purify *F. necrophorum* toxins by conventional chemical techniques has hindered the understanding of the protective immune response. For example, the effective cytoplasmic toxoid used in a field study (Garcia *et al.*, 1974) presumably contained several immunogenic components. An attempt has been made to use monoclonal antibodies to study the structure and function of leukotoxin (Tan *et al.*, 1994e). If monoclonal antibodies directed against different virulence factors are developed, it may be possible to define the protective antigens of *F. necrophorum*. Because *F. necrophorum* infection involves a series of events, complete protection probably requires immunity against more than one virulence factor. In other words, a successful candidate vaccine may consist of a major virulence factor plus a 'subunit' or 'helper' vaccine.

The technology of cloning and manipulating genes responsible for virulence factors is being used increasingly to understand the molecular nature of bacterial toxins and their involvement in the development of diseases (Lory and Tai, 1985; Finlay and Falkow, 1989). Molecular genetics will undoubtedly play an important role in our future efforts to understand the pathogenesis of *F. necrophorum*.

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