MICROAEROPHILIC GRAM-NEGATIVE RODS IN PERIODONTAL DISEASE

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This thesis is the original work of the author.

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Summary

A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species are capnophilic Gram-negative rods that have been isolated frequently from dental plaque. They have been studied to varying extents with regard to the role that they may play in the aetiology of human periodontal disease. A.actinomycetemcomitans has been the most extensively studied of the three bacteria and has been implicated in the aetiology of periodontitis, particularly localized juvenile periodontitis. Capnocytophaga species have been implicated in some forms of periodontitis, though the evidence is far from complete and more studies are needed to verify their role. However, few studies have addressed the aetiological role of H.aphrophilus in periodontitis.

In the present study a number of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains freshly isolated from subgingival plaque samples, together with type strains of each bacterium, were examined for their ability to produce the following virulence factors. First the ability of each organism to haemagglutinate human erythrocytes was measured. Secondly, their ability to

produce a leukotoxin capable of killing HL60 cells and human PMNLs was determined by using two assay systems, the trypan blue exclusion and luminol-dependent chemiluminescence inhibition assays respectively. Thirdly, the ability of human PMNLs to phagocytose strains of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species was tested by using a luminol-dependent chemiluminescence assay. Fourthly, the ability of these groups of bacteria to interfere with the proliferation of cultured human skin fibroblasts was examined by using a bio-assay to measure the metabolic activity of cultured fibroblasts. Finally the ability of the organisms to cleave human IgA by production of an IgA protease was investigated by using SDS-PAGE and immunoblotting techniques.

The results obtained in the current study indicated that all three groups of bacteria studied are capable of producing virulence factors that may cause ranging degrees of damage to host tissues. The results of this study also indicated that strains of H.aphrophilus are capable of producing virulence factors, though not to the same extent as the other two tested bacteria. To verify the role of H.aphrophilus in periodontitis more studies are clearly

required. These should include careful examination of the epidemiology.

PREFACE

Some parts of this thesis have been presented at a scientific meeting and published as an abstract as follows:

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ABBREVIATIONS

ABB:	Anaerobic blood Broth
ATCC:	American Type Culture Collection
NCTC:	National Collection of Type Cultures
CBA:	Colombia Blood Agar
PBS:	Phosphate buffered saline
SEM:	Standard error of mean
TSBV:	Trypticase Soy Serum Bacitracin Vancomycin agar
TSBVF:	Trypticase Soy Serum Bacitracin Vancomycin Fluoride agar
PMNLs	Polymorphonuclear leukocytes
TGB	Thioglycollate Broth
EMEM	Eagle's Minimum Essential Medium
TSA	Trypticase Soy Agar
PYG	Bacto-peptone, Trypticase peptone, Yeast extract and glucose medium
MTT	Methylthiazole diphenyl tetrazolium bromide
BHIB	Brain Heart Infusion Broth
TSB	Trypticase Soy Broth
PMA	Phorbol 12-myristate-13-acetate
DMSO	Dimethyl sulphoxide

Chapter 1

Introduction and review of the literature

1 Introduction and review of literature

1.1 Introduction

Periodontal diseases may be thought of as a group of pathological conditions that affect individual or multiple sites of the periodontium. The search for aetiological agents of destructive periodontal disease has been progressing for over 100 years (Socransky and Haffajee, 1992). Although periodontal disease (gingivitis and periodontitis) has been recognised for centuries, it was only relatively recently that the aetiology of periodontal disease has been related clearly and scientifically to microbial agents. Over the years different potential periodontal pathogens have been singled out as causative agents of periodontal disease, though the progress in these studies has been slow and sometimes uncertain because of the special characteristics which make these studies difficult. Some of these problems include the large number of bacterial species present in subgingival plaque as it is estimated that about 300-400 species are capable of colonizing the mouth and can be cultured from periodontal sites; some bacteria are difficult or

some times impossible to culture and identify; some pathogens are present in small numbers in the periodontium of healthy individuals and this makes it difficult to study their role in causing periodontal disease. Furthermore, only a few species (about 10-30) may cause the disease and the search and identification of this small group, has occupied workers in this field for the past 100 years (Haffajee and Socransky, 1994). A detailed account of the difficulties experienced in research in this field are discussed by Socransky and Haffajee (1987).

In this review of literature the aetiology of periodontal disease, suspected periodontopathogens and their role in causing the disease, including virulence factors, will be presented and discussed.

1.2 The periodontium

The supporting structures of the teeth are known collectively as the periodontium (from the Greek word "peri" meaning around and "dontos" a tooth). The periodontium consists of gingiva, periodontal ligament, cementum and alveolar bone. The functions of periodontium include the attachment of the teeth in the correct position to the alveolar bone, and the

resolution of the forces generated by mastication, speech and deglutition (Lindh and Karring, 1989).

The normal periodontium is characterised clinically by its pale pink colour, firm consistency, shallow sulcus with an intact junctional epithelium, and lack of bleeding on probing (Ainamo and Löe, 1966; Wennström 1988).

1.2.1 Definition and classification of periodontal disease

The term periodontal disease, if used in the general sense, includes all diseases that affect the periodontium (Kinane and Davies, 1990). Gingivitis and periodontitis are inflammatory diseases of the periodontium which are associated with the presence of dental plaque. Gingivitis is the most common form of periodontal disease and is manifested clinically by a change of colour and appearance (swelling and redness) with bleeding after probing (Taichman and Lindhe, 1989). Periodontitis is a disease of the deeper tissues of the periodontium including bone, the periodontal ligament and cementum.

In the World Workshop in Clinical Periodontics (1989) the different forms of periodontitis were classified as follows:

I Adult periodontitis

II Early-onset periodontitis

1- Prepubertal periodontitis (localized and generalized)

2- Juvenile periodontitis (localized and generalized)

3- Rapidly progressive periodontitis

III Periodontitis associated with systemic disease.

IV Necrotizing ulcerative periodontitis.

V Refractory periodontitis.

It should be noted that an overlap exists between different types.

Adult periodontitis is the most common form of periodontal disease and it is characterised by an age of onset of 35 years or older. The presence of microbial deposits is in proportion to the amount of periodontal destruction seen. The disease is not confined to any particular teeth (Page *et al.*, 1983).

Prepubertal periodontitis is seen early in life around the time of eruption of the primary teeth. In the generalized form all primary teeth became affected, and is characterised by inflammation and rapid destruction

of the alveolar bone. In localized periodontitis little or no inflammation is present around the affected teeth, and the condition is rare. It has been suggested that there is a genetic component to this condition. (Page and Schroeder 1982).

The onset of juvenile periodontitis occurs at puberty. Lesions are confined predominantly to the first permanent molars and/or incisors (Baer, 1971).

The onset of rapidly progressive periodontitis is between puberty and about the age of 35 years. In this form of disease the lesions are generalized and affect most of the teeth without any consistent pattern (Page and Schroeder, 1982).

Necrotizing ulcerative periodontitis is characterised by the formation of ulcers in the papillae. The lesions may be confined to a few papillae or affect all available sites. The acute form of disease is accompanied by pain, bleeding and halitosis (Nisengard, Newman and Zambon, 1994).

1.2.2 Dental plaque

Dental plaque is a layer of dense non-calcified bacterial mass attached firmly to the tooth surface. Bacteria in dental plaque adhere to one another or to

the tooth surface either directly or with a matrix of carbohydrates and proteins (inter-microbial matrix). The inter-microbial matrix forms from salivary proteins and extracellular products of bacterial metabolism. After the exposure of a clean tooth surface to saliva, an acellular and homogenous pellicle called the acquired pellicle is formed (predominantly glycoproteins from saliva). Bacteria attach to the pellicle and to the tooth surface. Dental plaque matures by a complex process involving the colonization and proliferation of bacteria which produces bacterial metabolites. The plaque becomes visible to the naked eye and increases in thickness to an optimal extent if not removed. The early predominant microflora is mainly Gram-positive bacteria (mainly Streptoccus species) though a small number of Gram-negative organisms are also present. After the initial colonization by Gram-positive bacteria, the number of Gram-negative organisms increases.

The changes in dental plaque are accompanied by gradual changes in the microorganisms present and the early Gram-positive colonizers are gradually displaced by more filamentous, Gram-negative microbiota (Listgarten, 1988). During three weeks of plaque accumulation, there

is an increase of Gram-positive rods, especially A.israelii and of Fusobacterium, Veillonella and Treponema species. Further at this stage bacterial species associated with periodontitis lesions can be isolated with sites with gingivitis. Numbers of bacteria such as E.corrodens, Fusobacterium and Capnocytophaga species are also elevated in sites with gingivitis. Plaque is very resistant to removal by the washing action of saliva or the physiologic movement of soft tissues. Calculus is a hard mineral substance strongly adherent to tooth structure which results from calcification of dental plaque (Listgarten and Levin, 1981; Pawlac and Hoag, 1984; Nyvad and Kilian, 1987; Marsh, 1993).

1.2.3 Subgingival plaque

Plaque that forms below the gingival margin is associated with the accumulation of supragingival plaque, which influence the establishment and relative proportion of subgingival plaque organisms. Supragingival plaque accumulation and maturation is accompanied by changes in the gingival tissues adjacent to the plaque layer. These changes include inflammation and erythema. The swelling causes enlargement which

increases the capacity of the subgingival area for bacterial colonization. This may result in an environment suitable for the growth of anaerobic microflora. Bacteria in this space are protected from the usual cleansing mechanisms; at the same time the crevicular fluid flow increases as does the pocket epithelial cell turnover. The end result is an altered ecological environment which is quite separate from the supragingival domain. The presence of the crevicular fluid, desquamated epithelial cells and bacterial end-products subsequently influence the establishment and relative proportions of subgingival microorganisms. The colonization of the subgingival area by microorganisms then follows, and as indicated above, this environment allows the fastidious strictly anaerobic (mainly Gram-negative and many spirochetes and other motile bacteria) bacteria to become predominate. Possibly because of local environmental changes, and host defence mechanisms, certain microorganims increase or decrease up to a stage when they can elicit disease (Page, 1986; Listgarten, 1988; Sanz and Newman, 1994). Studies by light and electron microscopy of extracted human teeth and their adjacent tissues have provided information about the internal structure of subgingival

plaque; these studies reported the separation of plaque into tooth associated, epithelium associated and connective tissue associated (Sanz and Newman, 1994). The microorganisms associated with the tooth are mainly Gram-positive filamentous bacteria, Gram-positive cocci and rods are also present. Gram-negative cocci and rods can be found also. The epithelium associated microflora is composed of mainly Gram-negative rods and cocci as well as large numbers of flagellated bacteria and spirochetes. The connective tissue microflora has been shown to be invaded by subgingival bacteria in all forms of periodontitis.

1.2.4 Aetiology of periodontal disease

The role of bacteria as causative agents in periodontal disease has been only 15 years ago acknowledged and the evidence implicating bacteria has been reviewed by Socransky and Haffajee (1994). Initially the evidence of bacterial involvement in periodontal disease was provided by early experiments in which oral hygiene measures were withdrawn from healthy individuals. This resulted in the accumulation of dental plaque and the development of gingivitis within 21 days. When oral hygiene measures were re-instituted the health of the

gingival tissues returned to normal. These simple experiments led to the conclusion that dental plaque was the cause of gingivitis (Löe, Theilade and Jensen, 1965; Theilade *et al.*, 1966). Since then there have been large numbers of studies presenting evidence indicating the causative role of one 'pathogen' or another, and in the quest for the aetiologic agents of periodontitis, two different theories have developed. The first is the "the non-specific theory" which suggests that periodontal disease is caused by the combined biologic effect of all the microorganisms present in plaque rather than the presence of specific plaque microorganisms. The second theory, "the specific theory" suggests that periodontitis is caused by the presence of a specific small number of bacterial species in dental plaque. The initial phase of the periodontal research for the specific theory was prominent upto 1930s. A middle stage was when the non-specific theory dominated. Finally there was a return to the concept of specificity in the nineteen seventies (Tonetti, 1993; Socransky and Haffajee, 1994).

Evidence for a primary role for bacteria in periodontal disease

There are a number of studies indicating that treatment of certain forms of periodontal disease by antimicrobial agents results in clinical improvement. For example Christersson and Zambon (1993) studied the possible elimination of Actinobacillus actinomycetemcomitans (A.actinomycetemcomitans) from 35 subgingival sites of 6 subjects with localized juvenile periodontitis, and reported that systemic tetracycline can be used to monitor A.actinomycetemcomitans in localized juvenile periodontitis. Antibiotics have been shown to be beneficial in the treatments of other forms of periodontal disease; Dibart *et al.*, (1993) reported that adjunctive systemic antibiotics used in 31 subjects with adult periodontitis, resulted in an improved periodontal condition as evident from an increase in the periodontal attachment level and a reduction in the numbers of certain periodontal pathogens for example fewer sites were colonized by detectable numbers of P.gingivalis after treatment. van Winkelhoff, Tijhof and de Graaff, 1992) reported that mechanical treatment combined with antibiotics

(metronidazole and amoxicillin) were effective in suppressing subgingival A.actinomycetemcomitans in patients with severe periodontitis. Furthermore, a number of studies in the literature indicate that antimicrobial therapy may be effective in controlling the extent and rate of disease progression in patients with refractory adult periodontitis (Gorden *et al.*, (1993). It should be observed though that even although the use of antimicrobial agents is successful in the treatment of patients in the diseased groups, a number of subjects did not respond positively probably due to differences in the aetiological agents. Additional evidence for the involvement of bacteria in periodontal disease is provided by a number of studies. In these studies the ability of certain suspected periodontopathogens to trigger the host immune response as indicated by an elevated serum antibody titre specific to certain subgingival organisms was reported (Ebersole *et al.*, 1982; Gunsolly *et al.*, 1990). Studies in experimental animals also provided additional evidence for implicating some oral bacterial species in the aetiology of periodontal disease (Kornman, Holt and Robertson, 1981; Kornman *et al.*, 1981; Holt *et al.*, 1988).

The search for specific aetiologic agents of periodontal disease is long-standing and as indicated above it is a complex problem that is hampered by many difficulties linked to clinical diagnosis and laboratory methodology (Socransky and Haffajee, 1987; Socransky and Haffjee, 1990).

Researchers have used Koch's postulates to help identify suspected periodontal pathogens. However, these are generally unsuitable criteria for chronic infections caused by a mixture of bacterial species and therefore, workers in the field have extended Koch's postulates to include the following:

1 Association with disease. This requires the detection of the suspected periodontopathogen more frequently and in higher numbers in disease sites than in healthy sites.

2 Treatment elimination. The basis of elimination studies is that the treatment given to a diseased subject results in the reduction or elimination of the pathogen and at the same time halts the disease progression.

3 Induction of host immune response by periodontal pathogens or their components.

4 The ability to cause disease in laboratory animals.

5 The detection of virulence factors. The ability of certain species to produce virulence factors has been used to support the role that they may have in causing the disease (Tonetti, 1993; Socransky and Haffajee, 1994). Based on the evidence obtained using the above criteria, several periodontopathogens have been implicated in some forms of destructive periodontal disease. However, it is likely that in due course some of these species will be removed from the list and quite possible that others will be added (Socransky and Haffajee, 1992). Based on the criteria described above a number of organisms have been implicated in the aetiology of periodontitis. These studies have implicated the following organisms in adult forms of periodontitis: Porphyromonas gingivalis; Prevotella intermedia; Eikenella corrodens; Campylobacter (Wolinella) rectus; Eubacterium species; Selenomonas species; Bacteroides forsythus and spirochetes. A summary of suspected periodontopathogens and the supporting references are shown in Table 1.1. As mentioned above the evidence supporting each of these bacteria varies and some have been studied more frequently than others. All mentioned organisms implicated in periodontitis are reviewed by Haffajee

Table 1.1 Summary of some studies suggesting an etiologic role in periodontitis for the listed organisms

Species	Association (Reference)	Elimination (Reference)	Host response (Reference)	Virulence factors (Reference)	Animal experiments (Reference)
P.intermedia	Walker and Gordon (1990)	Walker and Gordon (1990)	Zafiropoulos *et al.*, (1992)	Greneir, Mayrand and McBride (1989)	van Steenbergen *et al.*, (1982)
F. nucleatum	Haffajee *et al.*, (1988)	Kulkarni *et al.*, (1991)	Haffajee *et al.*, (1988)	Tuttle *et al.*, (1992)	Baumgartner, Falkler and Beckerman (1992)
B.forsythus	Haffajee *et al.*, (1988)	Haffajee, Dzink and Socransky , (1988)	Taubman *et al.*, (1992)	Tanner *et al.*, (1985)	
C.rectus	Haffajee *et al.*, (1988)	Haffajee, Dzink and Socransky, (1988)	Ebersole *et al.*, (1987)	Gillespie *et al.*, (1992)	
E.corrodens	Wolff, Aeppli and Pihlstrom (1993)	Tanner *et al.*, (1987)	Ebersole *et al.*, (1987)	Meghji *et al.*, (1992)	Behling *et al.*, (1981)
Selenomonas species	Moore *et al.*, (1991); Moore *et al.*, (1985)				

Table 1.1 Continued

Species	Association (Reference)	Elimination (Reference)	Host response (Reference)	Virulence factors (Reference)	Animal experiments (Reference)
					Behling *et al.*, (1981)
Eubacterium species	Moore *et al.*, (1991)		Gunsolly et al., (1990)	Persson *et al.*, (1990)	
spirochetes	Moore *et al.*, (1991)	Loesche *et al.*, (1992)	Lai *et al.*, (1986)	Schenkein and Berry (1991)	Miky, Maltha and van Campen (1990)

and Socransky (1994). Other bacteria have been implicated in juvenile form of periodontitis, these include A.actinomycetemcomitans, Eikenella corrodens, Prevotella intermedia, and Capnocytophaga species. E.corrodens is_ a Gram-negative small rod that has been associated in some studies with A.actinomycetemcomitans in localized periodontitis (Mandel, Ebersole and Socransky, 1987). There are a number of Gram-negative rods, capnophilic bacteria that can be isolated from the dental plaque samples frequently though the evidence implicating some of these bacteria varies, as some were studied more extensively than others. For example A.actinomycetemcomitans has been the most extensively studied periodontopathogen and the evidence supporting its aetiologic role in periodontitis is overwhelming especially in localized juvenile periodontitis. Although Capnocytophaga species have been implicated as aetiologic agents in periodontitis in some early studies (Slots, 1976; Mashimo *et al.*, 1983), the role that they may play in the aetiology of periodontitis is not clear. There is only little information regarding H.aphrophilus and its aetiologic role in periodontitis and it was not studied as the other two mentioned capnophilic bacteria and

clearly more studies are needed to verify the role that H.aphrophilus may play in the aetiology of periodontitis. Therefore the present study was undertaken to study the latter group of Gram-negative capnophilic bacteria to verify the role that they may have in periodontitis.

In the following sections, microbiological and clinical "historical" reviews about the 3 organisms will be presented, after which will follow a full detailed section dealing with the pathogenicity of periodontopathogenic bacteria and virulence factors produced by suspected pathogens especially those which are the subject of this study.

1.3 H.aphrophilus, A.actinomycetemcomitans and Capnocytophaga species (Historical review)

1.3.1 H.aphrophilus

Haemophilus aphrophilus was first isolated and described in 1940 by Khairat, who isolated the organism from blood cultures of a patient with endocarditis and suggested the species name "aphrophilus" because it required carbon dioxide for isolation (Khairat, 1940). In later years H.aphrophilus was isolated from various

infections and sites (see review by King and Tatum 1962). In 1978 Bieger, Brewer and Washington (1978) reviewed 90 cases of infections due to H.aphrophilus, the most common being endocarditis and brain abscess.

Morphological characteristics

Khairat (1940), described colonies of H.aphrophilus after 24 hours growth on blood agar (37°C; 5% CO_2 in air) as circular, entire edged, convex, small, with a smooth glistening surface, homogeneous in structure with a butyrous consistency that could be easily emulsified. After incubation for up to 3 days, colonies were slightly larger, yellowish in colour, and the growth was confluent; olive-green discoloration of the blood agar observed was without clear haemolysis. Broth cultures were described as very granular with colonies sticking to the side of the glass container. Other colonial morphological variants were also described. For example similar descriptions were reported by Toschach and Bain (1958) and by King and Tatum (1962). Tempro and Slots (1986) described the colonies of H.aphrophilus isolates grown on the selective medium TSBVF as circular, small (0.5 to 1mm in diameter), and convex.

Morphologically the organism was described as Gram-negative, coccoid to coccobacillary in shape, measuring about (1.5-2 by 0.4-0.5μm), non-capsulated, non-spore-forming and non-motile. After several passes in the laboratory, cells showed a tendency to become more definitely rod-shaped.

Metabolic and growth requirements

H.aphrophilus grows best in an atmosphere of air and 5% CO_2. Strictly anaerobic conditions potentially depressed growth while a heavy inoculum was essential for aerobic growth. It grew well at 37° C but not at 22° C (Khairat, 1940). When first isolated by Khairat (Khairat, 1940), H.aphrophilus was haemin (X factor) dependent. Nicotinamide adenine dinucleotide (NAD)(V factor) was not required for growth and neither factor was required for anaerobic growth. Tempro and Slots (1986), reported that neither X or V factor was required. Later King and Tatum (1962) reported that H.aphrophilus was able to grow in an atmosphere of CO_2 (since a candle jar was used the authors did not determine the exact percentage of CO_2), without the addition of either factor; this was later supported by Kilian and Schiott (1975). Growth on MacConkey agar, glucose or glycerol agar was

negative according to Khairat (Khairat, 1940). However, King and Tatum (1962) reported that some H.aphrophilus strains showed visible growth on MacConkey agar after 3 days incubation but there was no growth on Salmonella, Shigella, Citrate or Cetrimide agar. H.aphrophilus is commonly isolated on the selective medium for Haemophilus species which contains X and V factors and bacitracin or other suitable antimicrobial agents to suppress the growth of other bacteria (Kilian, Prachyabrued and Theilade, 1976). However the recognition of H.aphrophilus on general media for Haemophilus species is difficult because of the similarity in colonial and morphological characteristics between a number of the different species. Later a selective medium for the isolation of H.aphrophilus was formulated based on susceptibility studies and the ability of the organism to resist sodium fluoride. This medium was composed of Tryptic Soy Agar, heat inactivated horse serum, bacitracin, vancomycin and sodium fluoride and it was designated TSBVF (Tempro and Sots, 1986). The TSBVF medium yielded a three fold recovery of oral H.aphrophilus than other medium routinely used for the isolation of Haemophilus

species (for example Chocolate agar with 75µg/ml bacitracin).

Biochemical characteristics

There are only a few studies in which the biochemical characteristics of H.aphrophilus have been addressed since the early reports by Khairat (1940) and King and Tatum (1962) who examined and reported the major biochemical characteristics of H.aphrophilus isolates. These studies have agreed on the major biochemical characteristics of this organism, and H.aphrophilus is described as catalase and indole negative, and positive for nitrate reduction. The organism ferments a number of carbohydrates (glucose, sucrose, maltose, lactose), but failed to ferment mannitol, salicin, xylose, arabinose, cellobiose, rhamnose, sorbitol, and melezitose. Variable results were obtained with other carbohydrates (mannose, raffinose trehalose, glycerol (Liljemark *et al.*, 1984; Tempro and Slots, 1986).

Ultra structure studies using electron microscopy techniques revealed that H.aphrophilus possessed a structure similar to other Gram-negative organisms (Holt *et al.*, 1980).

1.3.2 A.actinomycetemcomitans (Historical review)

A.actinomycetemcomitans was first described in 1912 by Klinger who isolated the organism from a case of cervicofacial actinomycosis. After this initial isolation, A.actinomycetemcomitans was recovered frequently from patients with infections due to Actinomyces species. Some studies indicated that some of the isolated organisms were similar in their microbiological characteristics to A.actinomycetemcomitans (Kaplan *et al.*, 1989). Although A.actinomycetemcomitans was frequently isolated from patients with actinomycosis its potential to initiate and cause an infection was not widely accepted (Kaplan *et al.*, 1989). In 1951 Holm suggested that A.actinomycetemcomitans alone might cause disease in human as he frequently isolated the organism from actinomycosis lesions after the elimination of Actinomyces israelii by penicillin (Holm, 1951). Subsequent studies labelled A.actinomycetemcomitans as a non pathogenic constituent of the oral microflora which rarely caused medical infections (Kaplan *et al.*, 1989). In 1962 King and Tatum (1962) reported 32 cases of human infections due to A.actinomycetemcomitans and

provided evidence for the ability of A.actinomycetemcomitans to cause disease.

Morphological and cultural characteristics of A.actinomycetemcomitans

The morphological and cultural characteristics of A.actinomycetemcomitans have been examined by a number of workers. The colonial morphology of the organism when grown on solid media is usually described as small (1mm in diameter after 2-3 days growth), circular, strongly adherent to the medium on primary isolation with a star-shaped appearance (Slots, 1982; Zambon, 1985). However, not all A.actinomycetemcomitans isolates possess this colonial morphology and smoother, non-adherent and non-star-shaped variants have been described (Rosan *et al.*, 1988). Furthermore, after primary isolation and a number of subcultures in the laboratory, the star-shaped characteristic can be lost (Rosan *et al.*, 1988). The star-shaped colonies (rough type) of A.actinomycetemcomitans grow in broth and produce granules which adhere to the bottom and to the sides of the tube, leaving clear broth on top while the smooth type colonies produce a uniform turbidity (Zambon, 1985).

A.actinomycetemcomitans is described as a non motile Gram-negative, coccobacillus that after several subcultures shows a tendency to become more rod-shaped (Zambon, 1985).

Holt, Tanner and Socransky (1980) examined the ultrastructure of A.actinomycetemcomitans by transmission electron microscopy. This revealed that the organism was similar morphologically to those of other Gram-negative bacteria, with several strains possessing some adherent, extracellular polymeric material. Numerous structures morphologically identical to lipopolysaccharide vesicles originated from and were continuous with the surface of the outer membrane and were also present in the external environment (Holt, Tanner and Socransky, 1980; Lai, Listgarten and Hammond, 1981). Scanning electron microscopy revealed that the organism had surface projections and an amorphous surface material which connected and covered adjacent cells. This material was thought to be a carbohydrate microcapsule (Zambon, Slots and Genco, 1983). Scannapieco, Kornman and Coykendall (1983), reported the presence of extracellular appendages resembling fimbriae on A.actinomycetemcomitans isolates. The freshly isolated strains of

A.actinomycetemcomitans were found to possess fimbriae as revealed by using electron microscopy examination. The presence of fimbriae correlated with colonial morphology as the star-shaped colonies were associated with fimbriae while non star colonies were shown to be devoid of fimbriae (Rosan *et al.*, 1988). Further it has been showed in the same study that isolates that possess fimbriae adhered more to hydroxyapatite and saliva-coated hydroxyapatite than their non-fimbriated variants (Rosan *et al.*, 1988).

Metabolic activity of A.actinomycetemcomitans

A.actinomycetemcomitans grew poorly on blood agar aerobically and required CO_2 for growth if the medium did not contain blood (King and Tatum 1962). Neither V nor X factor was required for growth and the optimum temperature was 37 °C. A number of media have been developed for the selective recovery of A.actinomycete-comitans from subgingival plaque samples. For example Slots, Reynolds and Genco (1980) developed a medium containing Trypticase soy agar supplemented with heat inactivated serum and bacitracin, which supported the growth of more colonies of A.actinomycetemcomitans than other standard media for example enriched brain heart

infusion blood agar. This selective medium also suppressed the growth of other oral microorganisms by 3 to 6 log counts and A.actinomycetemcomitans colonies could be distinguished from the other bacterial species that proliferated (Slots, Reynolds and Genco, 1980). Mandell and Sockransky (1981) developed a selective medium that contained malachite-green which inhibited the growth of A.actinomycetemcomitans strains even at low concentration (Slots, 1982A). At present, the most commonly used selective medium for the selective recovery of this organism was developed by Slots (1982), and contains Trypticase-Soy-Agar supplemented with heat inactivated horse serum, bacitracin and vancomycin (TSBV). The TSBV medium suppresses the growth of many oral bacteria and A.actinomycetemcomitans can be distinguished from the few species that grow on this medium for example Neisseria species. A.actinomycetemcomitans can be differentiated from the closely related H.aphrophilus and other capnophilic bacteria that may grow on the same medium such as Capnocytophaga species by distinct colonial morphology and positive catalase reaction (Slots, 1982).

Biochemical characteristics of A.actinomycetemcomitans

The biochemical characteristics of A.actinomycetemcomitans have been investigated in a number of studies, and it is usually described as non-haemolytic, indole and urease negative. All strains reduce nitrate and ferment glucose; variable results are recorded for maltose, galactose, mannose, dextran, starch, xylose, mannitol and no reactivity noted for lactose, trehalose, sucrose, glycerol or raffinose. Catalase production was reported positive for all strains by most workers (Tanner *et al.*, 1982; Miyasaki, Wilson and Genco, 1986) (King and Tatum, 1962; Slots, 1982A). Some of the reported positive results in one study have been reported as variable in another study (Slots, 1982 A), the author related this differences between studies to the differences in composition of the basal medium utilized and methods employed, he also indicated that strains of closely related bacteria such as H.aphrophilus may have been mistaken for A.actinomycetemcomitans (Slots, 1982 A).

1.3.3 Capnocytophaga species (Historical review)

Capnocytophaga species was the name proposed for a group of Gram-negative non-flagellated, fusiform

bacteria, that possess distinct cellular and morphological features. They are able to glide over solid surfaces, and require carbon dioxide for growth. Since they were not readily equitable with members of any recognized genus, the name Capnocytophaga was proposed for the first time in 1979 by Leadbetter, Holt and Socransky. Further information about this group of bacteria was presented in further papers (Leadbetter, Holt and Socransky, 1979; Holt, Leadbetter and Socransky, 1979; Socransky *et al.*, 1979). Although Capnocytophaga species have been mentioned in a number of studies, reports that addressed the biochemical characteristics are rare since the distinct colonial and morphological characteristics are used for the isolation of this bacteria, for example Papapaou *et al.*, (1993) isolated Capnocytophaga species from the subgingival plaque samples of a randomly selected subjects based on its cultural characteristics and Gram stain reaction only and no biochemical characterization was performed except for motility.

The morphological and cultural characteristics of Capnocytophaga species

Capnocytophaga species comprise a group of three different species namely C.ochracea, C.sputigena and C.gingivalis. These strains were separated originally by morphological and physiological features (Leadbetter, Holt and Socransky, 1979). Colonies of all isolates were described as flat and thin with an even edge that spread with time. Three different types of colonies were recognized when the organism was grown on blood agar, normally grey or white colonies, pink colonies and yellow colonies. Some isolates adhered to the agar surface and were difficult to remove. However, several factors influenced these characteristics, notably the composition of the medium. All members of this group possess the colonial characteristics similar to those recognized for gliding bacteria which include genera such as Myxococcus and Flexibacter (Leadbetter, Holt and Socransky, 1979). Cells of Capnocytophaga species were described as Gram-negative, fusiform, long or short rods, the size ranged from about 4.8-5.8 by 0.42-0.6μm for large cells and about 2.4-4.2 by 0.38-0.5μm for small cells though in addition to the differences in size among different isolates identical

isolates varied in size when grown on different complex media (Holt, Leadbetter and Socransky, 1979). Scanning electron microscopy of the leading edge of the spreading colony on the agar plates revealed a large mass of cells at the periphery of the expanding colonies which were devoid of flagellae, fimbriae or pili (Newman *et al.*, 1979). However, contrary to the previous report, Scannapieco, Kornman and Coykendall (1983) reported that one Capnocytophaga strain possessed fimbriae-like appendages when examined within a group of dental plaque bacteria by agar filtration and negative stain electron microscopy.

Metabolic and growth requirement of Capnocytophaga species

Capnocytophaga species grow well anaerobically (Hydrogen and Nitrogen when present did not affect the growth though 100% of either inhibited the growth of Capnocytophaga species) and in air supplemented with 5-10 % CO_2 at 37°C. However, no detectable growth was evident in air or on MacConkey agar (Leadbetter, Holt and Socransky, 1979). No growth was observed at 25°C or at 45°C, but visible proliferation was noted at 30°C and at 35°C (Socransky *et al.*, 1979). A medium has been

developed for the selective recovery of Capnocytophaga species from plaque and was formulated based on susceptibility studies to several antibiotics. This medium, designated TBBP contained Trypticase Soy Agar, sheep blood, bacitracin and polymyxin B and was reported by (Mashimo *et al.*, 1983). The medium supported the growth of 34 Capnocytophaga stock cultures very well and suppressed the growth of a number of test stock cultures of common oral Gram-positive and Gram-negative bacteria, the medium was shown to have high recovery efficiency for Capnocytophaga species from deep subgingival plaque samples from subjects with severe periodontitis and insulin-dependent diabetes mellitus, than non selective medium such as Trypticase Soy agar supplemented with sheep blood.

Biochemical characteristics of Capnocytophaga species

The major biochemical characteristics of Capnocytophaga species are presented and discussed in detail (Socransky *et al.*, 1979). Other studies in which the biochemical characteristics were addressed include a description of C.sputigena strain isolated from a patient with haematological malignancy. This strain was

described as indole, urease, catalase and oxidase negative. The strain reduced nitrate and gelatine hydrolysis was also positive. The strain fermented a number of carbohydrates (glucose, maltose, sucrose) but did not ferment (lactose, galactose, raffinose) (Gomez-Garces *et al.*, 1994). The biochemical characteristics described for the C.sputigena strain more or less similar to those described by Socransky *et al.*, (1979). All strains of Capnocytophaga species were inhibited by several bacterial inhibitors like basic fuchsin (0.004%), crystal violet (0.0004%), sodium azide (0.05%), sodium chloride (4.0%), sodium fluoride (0.05%), 10% bile and malachite green (0.00002%) (Socransky *et al.*, 1979). The three species of Capnocytophaga were distinguishable on the basis of certain biochemical characteristics, such as the fermentation of additional carbohydrates, hydrolysis of polymers and reduction of nitrite, though this can prove very difficult since the results of Socransky *et al.*, (1979) indicate great similarities between the species with regard to a number of biochemical characteristics. There is also the variability of the results obtained with different species and within the single species.

1.3.4 Comparison between A.actinomycetemcomitans and H.aphrophilus

A.actinomycetemcomitans and H.aphrophilus are Gram-negative capnophilic organisms and biochemical characterization indicates that they are very similar, since they share a number of cultural and biochemical characteristics. However, recent studies of the genetic relation ships of these organisms using multilocus enzyme electrophoresis analysis, has shown no significant overall genetic similarity between the two species (Caugant, Selander and Olsen, 1990). Also Brondz and Olsen (1993) by means of multivariate chemosystematic analysis methods showed that A.actinomycetemcomitans was a non-homogeneous group and could be divided into two groups; both groups were distinct from H.aphrophilus. Only a few biochemical reactions that can be used to differentiate between the two organisms (Slots, 1982A). Biochemical characteristics that have commonly been used to separate and distinguish between the two organisms include the catalase reaction, fermentation of lactose, starch, sucrose and trehalose and the resistance to sodium fluoride (Slots, 1982 A).

1.4 Sensitivity to antibacterial agents

The testing of suspected periodontopathogenic organisms for their susceptibility to antibiotics is important since it has been shown that the use of antimicrobials in the treatment of periodontitis can result in clinical improvement. This may possibly occur through inhibition of the suspected aetiologic agents in each disease such as A.actinomycetemcomitans in the case of localized juvenile periodontitis (Slots and Rosling, 1983; Christersson and Zambon, 1993). In the case of juvenile periodontitis tetracyclines have been widely and successfully used for treatment (Smith *et al.*, 1994). Furthermore, Pavicic *et al.*, (1994) reported that combined mechanical debridement together with antibiotics (metronidazole and amoxicillin) was successful in eliminating A.actinomycetemcomitans and this was accompanied by an improvement of the periodontal conditions of the patients.

A number of studies have been performed to investigate the sensitivity of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species to antibiotics in vitro (Baker *et al.*, 1985). The sensitivity of A.actinomycetemcomitans to antibiotics is the most frequently studied, since it has been most clearly

implicated as an aetiologic agent in some forms of periodontal disease especially localized juvenile periodontitis (Kaplan *et al.*, 1989). A.actinomyc-etemcomitans has been shown generally sensitive to chloramphenicol, doxycycline, minocycline, tetracycline, gentamicin and ciprofloxacin (Slots, 1982A; Baker *et al.*, 1985; Eng *et al.*, 1986). The most tested antibiotic is tetracycline and it has been shown to be a useful adjunct to conventional treatment of periodontal disease (Seymour and Heasman, 1995). A.actinomycetemcomitans is generally resistant to vancomycin, spiramycin, and bacitracin, while variably resistant to clindamycin, erythromycin, kanamycin, streptomycin and penicillin.

Capnocytophaga species has been reported as sensitive to penicillin G, erythromycin, cefoxitin, clindamycin and chloramphenicol (Baker *et al.*, 1985; Holt and Kinder, 1989). Capnocytophaga species have been shown to be resistant to colistin, kanamycin, nalidixic acid, gentamicin and tobramycin and variably resistant to cephalothin, cefazolin, vancomycin and oxacillin.

The sensitivity of H.aphrophilus to a number of antibiotics was examined by Baker *et al.*, (1985); strains were generally sensitive to tetracycline,

erythromycin and penicillin, while resistant to spiramycin and tyrothricin.

Thus in previous studies the 3 organisms under study were shown to be sensitive to the tetracycline the most commonly used antibiotic in the treatment of chronic periodontitis.

1.5 Prevalence of Haemophilus aphrophilus in the oral flora

Haemophilus aphrophilus is considered to be part of the normal oral flora and is present in dental plaque (Albritton, 1982). Haemophilus species and other facultative Gram-negative rods have not received adequate attention in most of the early studies on the oral microflora and were considered to account for a negligible part of the oral bacterial flora (Kilian and Schiott 1975). Sims (1970) reported that Haemophilus species can be isolated in large numbers from saliva and oral mucous membranes, especially when semi-selective media were used for isolation. However, Sims (1970) did not mention H.aphrophilus even though it was part of the control group organisms (used for comparisons). Kilian, Heine-Jensen and Bülow (1972), studying nasopharyngeal material from hospitalized

children for Haemophilus using a selective medium, found 137 strains of Haemophilus, but H.aphrophilus was not mentioned. In 1972 Kraut *et al.*, (1972) reported the isolation of H.aphrophilus from gingival scrapings and interdental material in 35.5% of 45 subjects tested using a selective medium contained Tryptic Soy agar, hemin, bacitracin (TSAH). Furthermore, they concluded that H.aphrophilus was part of the oral microflora and that in the case of H.aphrophilus infections the mouth is probably the source of the organism. However, it should be mentioned that the dental oral health of the subjects used in this study was not determined. H.aphrophilus was also isolated from 5 out of 14 tonsils (Bieger, Brewer and Washington, 1978), and in 1976 Kilian, Prachyabrued and Theilade reported the isolation of H.aphrophilus from the smooth surface of teeth. Brown (1976) studied the incidence of Haemophilus species by analysing about 4247 respiratory samples and found that H.aphrophilus was present in 1.5% of the samples. Liljemark *et al.*, (1984) studied the distribution of Haemophilus species in supragingival and subgingival plaque samples collected from healthy maxillary first molars of a group of young adults. They found H.aphrophilus in 7 subgingival sites

versus 4 of the supragingival plaque sites out of the 14 sites tested.

1.6 Infections due to H.aphrophilus

H.aphrophilus was first isolated from a case of endocarditis in 1940 (Khairat, 1940). Since then, several reports have described the isolation of H.aphrophilus from different infections. H.aphrophilus has been cultured from brain abscesses, meningitis, sinusitis, pneumonia, empyema, otitis media, wound and post operative infections, arthritis and osteomyelitis. H.aphrophilus have been isolated also from endocarditis, paronychia, osteomyelitis of the jaw, appendicitis ((King and Tatum, 1962; Sutter and Finegold, 1970; Bieger, Brewer and Washington, 1978; Ellner *et al.*, 1979). H.aphrophilus may be isolated from the urine and vagina from normal humans as other Haemophilus organisms (Kilian and Biberstein, 1984). However, the two most common infections associated with H.aphrophilus are endocarditis and brain abscess. Cases of other clinical infections due to H.aphrophilus have been reported, including sinusitis (Page and King, 1966). Soft tissue or wound infections are particularly notable in that they are not commonly reported with

other Haemophilus species (Page and King, 1966; White *et al.*, 1981).

Kilian and Schiott (1975) reported the isolation of H.aphrophilus from dental plaque, whereas Kraut *et al.*, (1972) isolated H.aphrophilus from samples of gingival scrapings of 16 out of 45 sampled healthy adults. The dental health of all subjects was unknown. They concluded that the mouth is the likely source in infections due to H.aphrophilus. Alberton (1982) presumed that the oral flora was the source of H.aphrophilus in both endocarditis and brain abscess infections.

In spite of the fact that H.aphrophilus possess the potential to cause infection it is evident from the reviewed literature, that little is known about the pathogenicity mechanisms or virulence factors involved. Furthermore, at present it is generally accepted that although H.aphrophilus is a part of the oral microflora its causal role in periodontitis is unknown but believed to be low (Tempro and Slots, 1986).

1.7 Prevalence of A.actinomycetemcomitans in the oral flora

A.actinomycetemcomitans has been isolated frequently from the oral cavity. It was considered to be a non-pathogenic constituent of the oral microflora which could rarely cause medical infections (Zambon, 1985). In the later years A.actinomycetemcomitans have been isolated from dental plaque samples (Kilian and Schiott, 1975). Since these early reports there have been a number of studies indicating that the prevalence of A.actinomycetemcomitans from different parts of the oral flora of healthy volunteers and patients with different forms of periodontal disease varies considerably. In 1980 Slots, Reynolds and Genco, reported the isolation of this organism from subgingival plaque, tongue, cheek mucosa and saliva with the highest numbers present in the subgingival area. The authors concluded that dental plaque and the periodontal pocket area are the main oral sites for A.actinomycetemcomitans. Asikainen, Alaluusua and Saxen (1991) reported the isolation of A.actinomycetemcomitans from stimulated saliva, tongue and subgingival sites in healthy and diseased subjects. Papapanou *et al.*, (1993) reported the isolation of

A.actinomycetemcomitans from 25% of subgingival plaque samples of 171 randomly selected subjects.

1.8 Systemic infections due to A.actinomycetemcomitans

A.actinomycetemcomitans has been associated with several human infections. These include abscesses of the hand; face; brain; chest wall; submandibular; mandibular; mediastinal; pulmonary and thyroid gland. It has also been associated with dentoalveolar abscesses and cervicofacial actinomycosis. The majority of extra-oral infections involving A.actinomycetemcomitans are related to the heart, with more than 50 reported cases of infective endocarditis in the literature (Page and King, 1966; Zambon, 1985; Kaplan *et al.*, 1989). Most of these infections occur in subjects with history of rheumatic heart disease or prosthetic valve replacement (Zambon, 1985). Fifteen additional cases such as empyema, cervical adenitis and septic embolus, together with those previously reported cases are reviewed by Kaplan *et al.*, (1989).

1.9 Oral infections related to A.actinomycetemcomitans

A.actinomycetemcomitans strains have been isolated and identified in plaque samples from supragingival and

subgingival sites from individuals with healthy gums or with different forms of periodontitis (Kilian and Schiott, 1975; Newman and Socransky, 1977; Tanner *et al.*, 1979; Slots, Reynolds and Genco, 1980; Mandell and Socransky, 1981). A.actinomycetemcomitans has been implicated in the aetiology of localized juvenile periodontitis in a number of studies (Slots *et al.*, 1982; Christersson and Zambon, 1993). The association of A.actinomycetemcomitans with other forms of periodontal disease has been shown also. For example it has been shown that A.actinomycetemcomitans may be important in severe adult periodontitis and refractory periodontitis (Zambon, 1985; Rodenburg *et al.*, 1990). The possible transmission of A.actinomycetemcomitans in the case of adult periodontitis has also been shown (Petit *et al.*, 1993). However, a more detailed review the evidence for the involvement of A.actinomycetemcomitans in periodontitis is presented in Section 1.13.

1.10 Prevalence of Capnocytophaga species in the oral flora

Simpson, Leadbetter and Holt (1975) reported the isolation of anaerobic gliding bacteria from human

dental plaque. Two other groups of workers (Savitt *et al.*, 1975; Newman and Socransky, 1977) reported isolation of the same organism from plaque associated with periodontal diseases. A few years later reports were published giving detailed descriptions of the morphology, biochemistry and taxonomy of these organisms which were named Capnocytophaga species (Leadbetter, Holt and Socransky, 1979; Holt, leadbetter and Socransky, 1979; Socransky *et al.*, 1979; Williams and Hammond, 1979). It has been shown that the dental plaque is its primary ecological niche (Mashimo *et al.*, 1983; Slots and Genco, 1984) and Capnocytophaga species have been isolated from 32% of the subgingival plaque of a randomly selected subjects though it was not identified to the species level (Papapanou *et al.*, 1993).

1.11 Infections due to Capnocytophaga species

Forlenza, Newman and Blachman (1979) have reported that Capnocytophaga species can cause systemic disease in compromised patients. They reported that six patients with granulocytopenia and malignancy, 5 juveniles with leukaemia and 1 adult with carcinomas had sepsis, at the time of sepsis and when the organism isolated from

blood no mature granulocytes were detected. Four episodes of sepsis were caused by C.sputigena and 2 episodes with C.ochraceus. malignancies. Furthermore, all patients had; fever, mucosal ulceration, poor oral hygiene and most of them had gingival bleeding which may have served as an entry route of the bacteria to the blood stream. Infections have been reported in seven patients with leukaemia and one patient with an adenocarcinoma (Forlenza *et al.*, 1980; Gilligan, McCarthy and Bissett, 1981; Warwood, 1981). In addition there is a single report of a patient with Hodgkin's disease who developed Capnocytophaga sepsis (Appelbaum, Ballard and Eyster, 1979). The probable site of entry in all these cases was almost certainly the oral cavity as all these patients demonstrated signs of periodontal disease. More recently a case of bacteremia due to C.sputigena in a patient with a haematological malignancy has been reported (Gomez-Garces *et al.*, 1994).

1.12 Oral infections related to Capnocytophaga species

In the nineteen seventies Capnocytophaga species were implicated in destructive forms of human periodontal disease (Newman *et al.*, 1976; Newman and Socransky,

1977). Socransky *et al.*, (1979) reported the presence of each of the three Capnocytophaga species in healthy gingival sulci as well as periodontal pockets from different forms of periodontal disease. However, no correlation was made between specific forms of periodontal disease and particular species. Capnocytophaga species have been implicated in localized juvenile periodontitis (Newman *et al.*, 1976; Slots, 1976), and in periodontitis associated with insulin-dependent diabetes mellitus (Mashimo *et al.*, 1983). Although Capnocytophaga species have been linked in the aetiology of periodontal disease in some early studies (Mashimo *et al.*, 1983A), currently the role of these oral organisms in periodontal disease is believed to be doubtful, in spite of the fact that strains of Capnocytophaga species have been shown to produce potential virulence factors for example an IgA1 protease, In a comprehensive review of the microbial aetiological agents of destructive periodontal disease Haffajee and Socransky (1994), did not list Capnocytophaga species amongst the suspected periodontopathogens.

1.13 Periodontopathogens

The search for the aetiological agents of periodontal disease is long standing but the detection of an aetiological agent of destructive periodontal disease is complex and hampered by technical difficulties (reviewed by Socransky *et al.*, 1987; Socransky and Haffajee, 1990). Based on the evidence obtained using the criteria described in Section 1.2.4, several periodontopathogens have been implicated in some forms of periodontal disease. However, it is likely that in due course some of the species currently being investigated will be removed from the list and that others will be added as further research work is published (Socransky and Haffajee, 1992).

The general approach used by investigators to define a suspected periodontopathogen, includes the demonstration of an association between disease activity and the potential pathogen usually by pilot clinical microbiological culture studies, which if positive are verified in larger study groups by the use of selective media. In the past 8-10 years the use of molecular techniques to relate potential pathogens to periodontitis has been used commonly (Haffajee and Socransky, 1994). Based on these types of studies a

range of bacterial species has been implicated in adult forms of the disease, and others have been implicated in the juvenile form of disease. All these groups of organisms and the evidence supporting their suspected role in periodontal disease were reviewed by Haffajee and Socransky (1994).

Currently there are more data to support pathogenic roles for A.actinomycetemcomitans and P.gingivalis than for other suspected periodontopathogens. A.actinomycetemcomitans was first recognized as a possible periodontopathogen by its increased frequency of detection and higher numbers in lesions of localized juvenile periodontitis compared with other periodontal, gingivitis conditions and health (Chung *et al.*, 1989; Slots, Reynolds and Genco, 1980; Christersson and Zambon, 1993; Barr-Agholme *et al.*, 1992). Another criterion for the selection of a periodontopathogen is fulfilled by A.actinomycetemcomitans since elevated serum and local antibody levels have been detected in patients with localized juvenile periodontitis (Ebersole, Taubman and Smith, 1985; Ebersole *et al.*, 1991; Taubman *et al.*, 1992). The elimination or the substantial reduction in the numbers of A.actinomycetemcomitans by local and antimicrobial

therapy in patients with localized juvenile periodontitis has been linked with clinical success (Slots and Rosling, 1983; van Winkelhoff, Tijhof and de Graaff, 1992). A.actinomycetemcomitans induced disease in gnotobiotic rats as the injection of A.actinomycetemcomitans subcutaneously caused abscess at the injection site and the abscess ulcerated two days after injection (Chen *et al.*, 1991). Furthermore, A.actinomycetemcomitans strains have been reported to produce a range of virulence factors which include leukotoxin, collagenase, endotoxin, epitheliotoxin, fibroblast inhibitory factor and bone resorption inducing factor (Socransky and Haffajee, 1990; Socransky and Haffajee, 1992). These factors are listed in Table 1.2 and reviewed later in this chapter A.actinomycetemcomitans has also been implicated in adult forms of periodontitis although its role is less clear (Haffajee and Socransky, 1994).

The evidence for a role for P.gingivalis in periodontitis consists of the detection of the organism in the lesions of adult periodontitis compared to its rare intraoral presence in health (Moore *et al.*, 1991; Papapanou *et al.*, 1993). Also when P.gingivalis is eliminated by therapy clinical improvement occurs

(McNabb, Mombelli and Lang, 1992). An elevated level of serum and local antibodies in disease indicates its ability to induce host responses (Choi *et al.*, 1990) and P.gingivalis can produce a number of potent virulence factors which include collagenase, trypsin-like protease activity, fibrinolysin, other proteases, phospholipase A, phosphatases, endotoxin, H_2S, ammonia, fatty acids and factors which adversely affect polymorphonuclear leukocytes (reviewed by Haffajee and Socransky, 1994). In experimental mixed infections in monkeys and dogs P.gingivalis has been shown to play an important role (Socransky and Haffajee, 1990; Ebersole and Kornman 1991).

In recent years and with the advances in molecular biology techniques, workers have been employing new technology to search for aetiological agents in periodontal disease. In the past most of the studies that investigated the bacterial composition of subgingival dental plaque samples used cultural techniques which are laborious, time-consuming and may be limited in their outcome, and subject to variability (Haffajee and Socransky, 1994). Recently workers have been using poly- or mono-clonal antibodies or specific DNA probes to detect specific organisms in dental

plaque. These techniques reduce the time required for sample analysis and therefore more samples can be processed in a shorter period of time, though they cannot be used to isolate new species and accurate quantification is not yet achievable (Haffajee and Socransky, 1994).

1.14 Virulence factors and pathogenicity

The search for aetiological agents in periodontitis as indicated above is complex and dependent on a number of host and microbial factors that require to occur and interact at the same time. It is now recognized that a pathogen may colonize a site but no disease progression occurs (Socransky and Haffajee, 1992; Socransky and Haffajee, 1993) due perhaps to the absence of or defects in virulence factors or the presence of powerful host defence factors. The possible host parasite interactions that may occur are many and complex, for example a bacterial species may be present in a site that enhances infection or inhibits the pathogen's activity. The pathogen must be able to multiply and reach numbers sufficient to initiate or cause infection. The expression of virulence factors produced by a pathogen could be altered by the

environment and it is now clear that not all clonal types of a pathogenic species are equally virulent for a number of medically important infectious agents (Socransky and Haffajee, 1993).

The whole process of colonization prior to tissue destruction and disease depends on several factors including interactions between bacteria and the host and between the pathogen and other microorganisms present in the site. A range of factors are produced by periodontopathogens including some which aid colonization, others which assist in multiplication and growth. In addition there are factors that overcome the host defences and others that cause tissue destruction (Socransky and Haffajee, 1991). These are collectively known as virulence factors, which may be defined as the set of unique properties which allow the organism to colonize and defend itself but may subsequently cause damage to host tissues (Socransky and Haffajee, 1991). There are a number of virulence factors produced by periodontopathogenic organisms. Table 1.2 contains a list of a number of virulence factors produced by A.actinomycetemcomitans, an organism examined in this study. However other periodontopathogens such as P.gingivalis were shown to produce an array of potent

factors. These factors include some that assist in the colonization of a site since it is generally accepted that the ability of a periodontopathogen to adhere to the host surfaces is an important step for colonization prior to subsequent tissue damage (Slots and Genco, 1984).

Adherence

Clearly the ability of a periodontopathogen to adhere to a host surface is an essential step for colonization and subsequent tissue damage (disease) (Gibbons and van Houte, 1975; Slots and Genco, 1984). P.gingivalis has been shown to adhere to oral tissues, as it has been showed that P.gingivalis was able to synthesise surface haemagglutinins which function as adhesins by which this organism attaches to host tissue or by using other mechanisms like the coaggregation with other oral bacteria (for example F.nucleatum) (Kolenbrander and Andersen, 1989; Progulske-Fox *et al.*, 1993), and also to invade oral epithelial tissue culture cell lines (KB) in vitro (Sandros, Papapanou and Dahlen, 1993).

Table 1.2 Virulence factors produced by A.actinomycetemcomitans

Virulence factor	Reference
Leukotoxin	Taichman *et al.*, (1991)
Fibroblast inhibitory factor	Helgeland and Nordby (1993)
Bone resorbing factor	Meghji *et al.*, (1994)
endotoxin	Kily and Holt (1980)
epitheliotoxin	Birkedal-Hansen *et al.*, (1982)
invades epithelial cells in vitro	Meyer, Sreenivasan and Five-Taylor (1991)
Collagenase	Rosanies *et al.*, (1983)
Fc-binding components	Tolo and Helgeland (1991)
alkaline and acid phosphatase	Slots (1981)

Interactions with PMNLs

The interactions between human PMNLs and periodontopathogens are probably very important in the pathogenesis of periodontal disease since it have been shown that the neutrophils are the predominant leukocytes in the oral cavity and provide a first line of defence against attack by periodontal organisms (Van Dyke and Vaikuntam, 1994). When PMNLs and periodontopathic bacteria interact a number of outcomes are possible; the PMNLs can interact directly and kill the pathogen by phagocytosis or by releasing their lysosomal enzymes into the pockets (Van Dyke and Vaikuntam, 1994). In contrast certain pathogens can protect themselves by either producing toxic substances that damage the neutrophils or protect themselves by means of various surface components (for example a capsule) which can protect the organism against phagocytosis. Examples of these two mechanisms are the capsule produced by P.gingivalis (Okuda and Takazoe, 1988), and the leukotoxin produced by certain A.actinomycetemcomitans strains (Taichman *et al.*, 1991). Furthermore, it has been shown that bacteria have adopted different mechanisms of interfering with phagocytosis and the killing mechanisms of human PMNLs

(Yoneda, Maeda and Aono, 1990). For example lipopolysaccharide from P.gingivalis has been shown to inhibit PMNL chemotaxis in vitro thus avoiding the phagocytic activity of PMNLs (Seow *et al.*, 1992). In 1992 Gillespie *et al.*, (1992) showed that certain strains of the oral organism Campylobacter rectus were able to produce an extracellular material that killed HL60 cells and human PMNLs as measured by the trypan blue exclusion assays. It was shown also that this toxic material was a protein with a molecular weight of 104 kilo-daltons as determined by SDS-PAGE. However, cell suspension of the bacterium were not toxic. This finding is significant for the involvement of C.rectus in periodontal disease since it is the second organism that produces a toxin which kills human PMNLs.

IgA-protease production by periodontopathogens

Another virulence factor that has been shown to be produced by certain periodontopathogens is IgA1 protease, which is capable of cleaving human IgA (subclass IgA1). This immunoglobulin is recognized as an important line of defence against invading organisms (Kilian, 1981), and a range of bacteria have been shown

to possess such activity. These include bacteria that colonize mucosal surfaces such as Neisseria meningitidis, Neisseria gonorrhoeae, and other bacteria important in dental plaque formation such as Streptoccus sanguis. Other pathogenic bacteria such as H.influenzae and S.pneumoniae also possess such activity. An example of an oral organism is Capnocytophaga species which has been shown to cleave human IgA1 in vitro as well as in vivo (Frandsen *et al.*, 1995).

Fibroblast inhibition factors

A few putative periodontopathogens produce fibroblast inhibiting factor(s) that interferes with the proliferation of cultured human fibroblast cell lines. For example the lipopolysaccharide prepared from P.gingivalis can inhibit the proliferation of cultured human gingival fibroblasts (Layman and Diedrich, 1986; Larjava *et al.*, 1987). In addition Scragg, Cannon and Williams (1994) have shown that cultured fibroblast cell lines (Vero cells and human gingival fibroblasts) were affected by different concentrations of short chain fatty acids produced by anaerobic periodontal bacteria (for example P.gingivalis), as have sonic

extracts prepared from certain spirochetes such as Treponema denticola. A dose-dependent inhibition of both human and murine fibroblasts has been demonstrated with these extracts and the inhibitory factor was heat labile and not endotoxin (Boehringer, Taichman and Shenker, 1984). A number of periodontopathogens have been shown to produce enzymes that are capable of destroying host connective tissues an example of these enzymes is collagenase which can destroy collagen which is the principle component in human gingiva and its most important structural component (Page, 1972). Porphyromonas gingivalis (P.gingivalis) is collagenase positive and this property has been proposed as a virulence factor (Slots, 1981; Grenier and Mayrand, 1987; Robertson *et al.*, 1982; Toda *et al.*, 1984). Also Birkedal-Hansen *et al.*, (1988) have reported that strains of P.gingivalis have collagenolytic activity capable of degrading collagen types I, II and III. Smally, Birss and Shuttleworth (1988) reported the purification of a trypsin-like enzyme from cell and cell-free culture supernatants from P.gingivalis strain W50. This purified enzyme, together with extracellular membrane vesicles, degraded collagen substrate and human plasma fibronectin. Their results

have indicated that the release of proteolytic enzymes by P.gingivalis, either soluble or associated with extracellular membrane structures, may be important in the pathogenesis of periodontal disease. Smalley, Shuttleworth and Birss (1989) also reported that the extracellular vesicle fraction of B.gingivalis strain W50 and the avirulent variant W50/BE1 have collagenolytic activity. However, the rate of collagen depolymerization was greater with the extracellular vesicle fraction of W50 compared to that of the variant strain W50/BE1 (90% and 5% substrate degradation respectively) over a 6 hour incubation period.

In the above section the role of bacteria in the aetiology of periodontal disease has been reviewed briefly together with a summary of the known virulence factors produced by a number of suspected periodontopathogens. In the following sections the main virulence factors produced by the three organisms under study will be presented and discussed.

1.14.1 Bacterial adhesion

The mechanisms of bacterial adhesion have been studied extensively. The attachment of a bacterium to one of

the available host or microbial surfaces in the mouth is an important step for colonization and survival in the periodontium. Host surfaces include the tooth or host-derived substances binding to the tooth, the sulcular epithelium, while attachment may also occur to other adherent bacteria (Socransky and Haffajee, 1991). Adherence occurs when specific receptors are present on the host cells or saliva coated tooth surface to which specific molecules known as adhesins present on the bacterial surface can bind or attach. A number of receptors on several host surfaces have been identified. Clark *et al.*, (1986) showed that only strains of Actinomyces that possessed Type 1 fimbriae adhered to tooth surfaces and that fimbriae constituted the principle adhesin involved in this interaction. The Actinomyces strains possess different antigenically distinct fimbriae (Type 1 and Type 2 fimbriae). The type 2 fimbriae have been shown to be involved in the mediation of lactose sensitive coaggregation. Many human oral isolates possess similar lectin activity and this has been associated with the presence of fimbriae that are genetically related (Clark *et al.*, 1986). Winkler *et al.*, (1988) showed that strains from periodontopathogenic bacteria such as P.gingivalis and

A.actinomycetemcomitans adhered more strongly to the basement membrane of a connective tissue in vitro, than other Gram-positive oral microorganisms. Mangan *et al.*, (1989) showed that Fusobacterium nucleatum possesses lectin-like adherence factors and that Fusobacterium nucleatum adhered to human PMNLs. The adherence was inhibited by N-acetyl-D-galactosamine or lactose. Adhesins identified on the surface of subgingival bacteria include fimbriae (Clark *et al.*, 1986; Isogai *et al.*, 1988) and cell associated proteins (Mangan *et al.*, 1989). Coaggregation is another mechanism of attachment, in which some species unable to bind directly to a surface are able to attach to another bacterial species that is already attached to the host. Coaggregation between species has been shown to be a specific process both in vitro and in vivo and has been reported by (Kolenbrander, Andersen and Moore, 1989); Kaufman and Dirienzo, 1989).

Adherence of A.actinomycetemcomitans

Slots and Genco (1984) discussed in detail how putative periodontopathogenic bacteria interact with and colonize the host tissue and then destroy them, despite apparently effective host defences.

A.actinomycetemcomitans was included in this study. A.actinomycetemcomitans can colonize oral mucosal surfaces, supragingival plaque and periodontal pockets (Genco, Zambon and Christersson, 1986; Zambon, 1985). Gibbons and Etherden (1983) have reported the ability of some A.actinomycetemcomitans strains to adhere to saliva-treated hydroxyapatite as have Kagermeiler and London (1985). In an electron microscopic study of the interbacterial matrix of subgingival plaque samples from patients with periodontal disease, Scannapie, Korman and Coykendal (1983) showed that all plaques contained varying amounts of extracellular appendages, morphologically identical to bacterial fimbriae, these plaque samples contained a group of bacteria and A.actinomycetemcomitans was among the group. They concluded that bacterial extracellular appendages resembling fimbriae are common components of bacteria freshly isolated from plaque samples and that they may play a significant role in adhesive interactions within plaque studied. Freshly isolated strains of A.actinomycetemcomitans possess fimbriae and adhere to hydroxyapatite, while non fimbriated variants of the same strains attach less well Rosan *et al.*, (1988). Other workers have also reported that

A.actinomycetemcomitans can adhere to buccal epithelial cells (Sweet, MacFarlane and Samaranayake 1988; Taher and MacFarlane, 1991). Okuda *et al.*, (1991) reported that lipopolysaccharide from A.actinomycetemcomitans adhered to both saliva-coated and serum-coated hydroxyapatite beads and agglutinated human erythrocytes. Recently Meyer and Fives-Taylor (1993) reported that extracellular microvesicles together with a highly proteinaceous material associated with a leukotoxic strain of A.actinomycetemcomitans enhanced the adherence of a weakly adherent strain. The authors hypothesised that both extracellular vesicles and an extracellular amorphous material play an important role in the bacterial adhesion to epithelial cells. In addition the surface material is also important in the complex interactions that occur between Gram-positive and Gram-negative oral species during the formation of dental plaque (Meyer and Fives-Taylor, 1993). Mintz and Fives-Taylor (1994) reported that A.actinomyceytemcomitans has been shown to adhere to a cultured human oral epithelial cell line (KB cells), as detected by an enzyme-linked immunosorbent assay and (^{3}H)thymidine labelled bacteria. Adhesion was shown to be time and bacterial-concentration dependent and inhibited by

saliva or by the pre-treatment of A.actinomycetemcomitans with a protease (Mintz and Fives-Taylor, 1994). It has also been reported that A.actinomycetemcomitans can invade gingival tissue (Christersson *et al.*, 1987 A; Christersson *et al.*, 1987; Zambon, 1985). This finding was further confirmed by Meyer, Sreenivasan and Fives-Taylor (1991) who showed that A.actinomycetemcomitans could invade human oral epithelial cell lines (KB cell line) as determined by the recovery of viable organisms from gentamicin treated cultured KB cells and by electronic and light microscopy examination. They also showed a correlation between the invasiveness of A.actinomycetemcomitans and its colonial morphology, as smooth variants were shown to invade more proficiently than rough variants (Meyer, Sreenivasan and Fives-Taylor, 1991).

Adhesion of H.aphrophilus

There are very few studies that have investigated the adhesion of H.aphrophilus strains to host tissues. In a study reported by Sweet, MacFarlane and Samaranayake (1988) a single strain of H.aphrophilus was reported as adhering to human buccal epithelial cells.

Adhesion of Capnocytophaga species

The characteristics which aid Capnocytophaga species to colonize the oral cavity and survive in the periodontal pocket were reviewed in detail by Slots and Genco (1984). C.sputigena strains possess surface components which consists of thick amorphous material, long fibrils and vesicles on the external surface of the outer membrane. This surface material appears to make this organism more adherent than other Capnocytophaga species as they possess less extracellular material (Slots and Genco, 1984). In an electron microscopic examination of the interbacterial matrix of subgingival plaque samples from periodontal disease patients, Capnocytophaga species have been shown to possess extracellular appendages resembling fimbriae and it was concluded by the authors that these components may play a role in the adhesive interactions within plaque (Scannapie, Korman and Coykendal, 1983). Sweet, MacFarlane and Samaranayake (1988) showed that certain Capnocytophaga species were able to adhere to human buccal epithelial cells though in lower numbers than other species present in the periodontium such as P.gingivalis or Veillonella freshly isolated strains.

1.14.2 Interactions with human polymorphonuclear leukocytes (PMNLs)

Human PMNLs represent an early line of defence against microbial attack, and individuals with defective PMNLs are predisposed to acute infections. PMNLs are recognized as important in the protection of the periodontium and the interactions between periodontopathogens and PMNLs are an important factor in periodontitis. In this section both the basic principles of phagocytosis and the leukotoxin produced by A.actinomycetemcomitans will be discussed separately.

Phagocytosis

Phagocytosis is the name given to the process by which a specialist group of white blood cells capture and digest particulate matter, bacteria and viruses. These cells are of myeloid origin and are produced initially from haemopoietic stem cells in the bone marrow. They are of two types, granulocytes and monocytes (macrophages). The granulocytes are classified as neutrophils (these constitute more than 90% of the total leukocytes), eosinophils or basophils. The mononuclear phagocytes occur as nearly spherical

monocytes in the blood and as irregularly shaped, non-circulating macrophages in organs such as the liver and spleen. The neutrophils and monocytes play an important role in removing foreign matter which may gain access to the body. The macrophages play an important role in filtering the blood as it passes through the organs in which they are present and this is a major line of defence against invading bacteria (Roitt, 1994). The bactericidal activity of the phagocytic cells depends on two different mechanisms, degranulation and the generation of highly toxic radicals. The steps of phagocytosis are illustrated in Figure 1.1. Phagocytosis consists of an attachment and ingestion phase, the ingestion of particles (including bacteria) is dependent on surface properties of the particle and the presence on the surface of the specific serum ligands, antibody and complement. These then will interact with receptors on the phagocyte surface and mediate ingestion (Horwitz, 1982). After particles are ingested, they become enveloped within the white cell membrane and an intracellular vesicle called a phagosome is formed. Lysosomes present in white blood cell then migrate to the phagosomes and fuse with it to form a phagolysosome. The lysosome

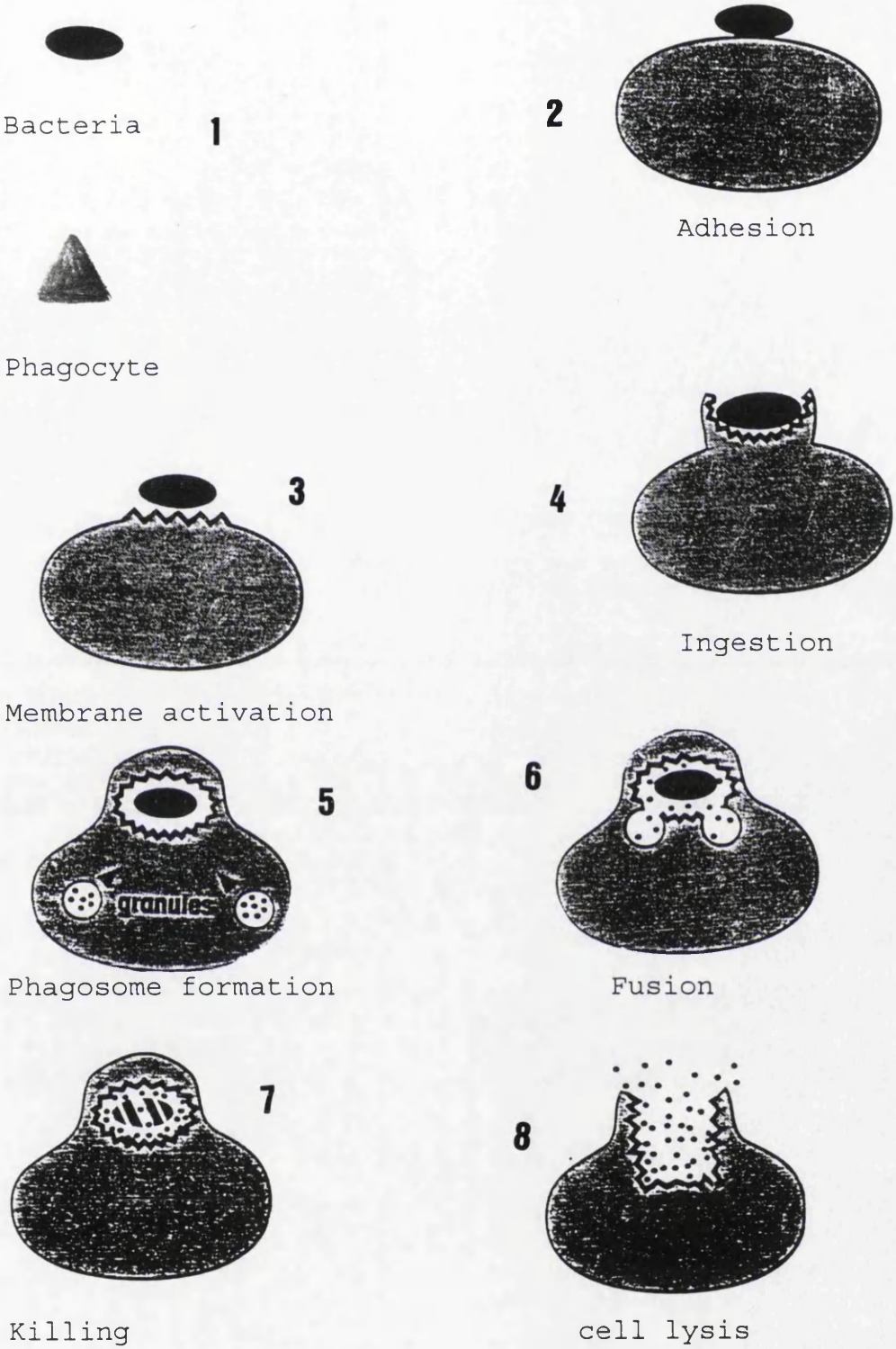


Figure 1.1 Diagrammatic illustration of the phagocytosis steps

contents (cytoplasmic granules which include enzymes and other substances capable of killing bacteria are released into the phagolysosome (degranulation). Highly reactive oxygen radicals namely superoxide anion, singlet oxygen, hydrogen peroxide and hydroxyl radicals are produced. The latter process is termed the respiratory burst (Babior, 1978). The killing of bacteria subsequently occurs. The production of these compounds is associated with the production of light and was first reported by Allen, Stjernholm and Steele (1972). The amount of light produced is very small and difficult to measure but can be enhanced and thus measured by the use of luminol (5-amino-2,3-dihydro1,4 phthalazinedione), (Allen and Loose 1976). Allen and Loose (1976) demonstrated that chemiluminescence was produced following the phagocytosis of bacteria. Other studies using luminol-enhanced chemiluminescence have directly correlated the bactericidal ability of neutrophils with chemiluminescence production (Easmon *et al.*, 1980; Welch, 1980; Seymour, Whyte and Powell, 1985).

Periodontal disease is an inflammatory disease in which human PMNLs play an important role, as they accumulate in the affected sites (Attström 1971;

Schroeder, 1973). Interactions of bacteria with human PMNLs at the disease site will activate the latter and result in the formation of highly toxic oxygen products within lysozomes which are powerful bactericidal agents. However, if the factors responsible for intracellular killing are released extracellularly damage to the host tissue may result, and the role of PMNLs in periodontal disease has therefore, been considered to be both protective and destructive (Miller, Lamster and Chasens, 1984). Robinson *et al.*, (1984) examined the chemiluminescence response of normal human PMNLs to a number of different pathogenic microorganisms and they reported that each microorganism caused a reproducible response pattern. The factors influencing the normal chemiluminescence response to these microorganisms were also studied and defined. Passo *et al.*, (1982) examined a number of Gram-negative bacteria isolated from plaque samples including Capnocytophaga species and found that only Fusobacterium nucleatum initiated a chemiluminescence response in the absence of serum. Holm and Kalfas (1993) examined a group of A.actinomycetemcomitans and H.aphrophilus strains for their ability to produce a chemiluminescence response

in human PMNLs and activate the oxygen dependent bactericidal mechanism in these cells. They reported that there were strain-dependent differences among A.actinomycetemcomitans in their ability to induce the oxygen-dependent bactericidal mechanisms of human PMNLs whereas the chemiluminescence response patterns of H.aphrophilus strains were all the same. They further reported that several environmental factors such as salivary compounds and activated complement affected the interactions between these two organisms and human PMNLs (Holm and Kalfas, 1993)

Leukotoxin

PMNLs are present in periodontal sites and they represent a very important defence against periodontitis. The ability of a periodontopathogen to interfere with or inhibit the bactericidal activity of PMNLs will subsequently enhance the virulence of the microorganism (Van Dyke, Levine and Genco, 1982). Baehni *et al.*, (1979) reported that a strain of A.actinomycetemcomitans isolated from a patient with juvenile periodontitis (strain Y4) produced a leukotoxin capable of killing human PMNLs and

monocytes. Subsequently these workers examined several strains of A.actinomycetemcomitans for their ability to produce leukotoxin (Baehni *et al.*, 1981). The study included both Type strains and fresh oral isolates, and demonstrated that 7 out of 10 fresh isolates, in addition to 3 Type strains (ATCC 29522, ATCC 29524 and Y4), were able to produce a leukotoxin capable of killing human PMNLs. They also reported that the addition of autologous human serum, either fresh or heat-inactivated, enhanced the killing by the leukotoxic stains. However, it had no effects on the non-leukotoxic A.actinomycetemcomitans strains (Baehni *et al.*, 1981). It has also been shown that the addition of serum from juvenile periodontitis patients to the assay system inhibited leukotoxicity. Similarly, rabbit antiserum against PMNL-membrane binding components of strain Y4 neutralized leukotoxicity, while antisera raised against a non-leukotoxic strain (ATCC 29523) failed to do so (Baehni *et al.*, 1981). Serum from normal individuals or those with other types of periodontal disease enhanced leukotoxicity in assays while sera from individuals with juvenile periodontitis inhibited leukotoxicity (Tsai *et al.*, 1981; McArthur *et al.*, 1981). However,

Zambon *et al.*, (1983) reported that human serum derived from healthy individuals did not enhance leukotoxicity against the tissue culture cell line HL60 in contrast to the findings for human PMNLs. Several other studies have shown that A.actinomycetemcomitans can produce a leukotoxin (Taichman, Dean and Sanderson, 1980; Taichman and Wilton, 1981; Tsai *et al.*, 1979; Zambon *et al.*, 1983). The latter examined the susceptibility of the promyelocytic HL60 cell line to leukotoxin from A.actinomycetemcomitans. They reported that HL60 cells were susceptible to lysis by A.actinomycetemcomitans leukotoxin and that the HL60 cells were slightly more sensitive to the toxin than were human PMNLs. Leukotoxic activity against HL60 cells was shown to be dose-and temperature-dependent, reaching a maximum after 45 minutes at 37°C. Another study using PMNLs as targets has indicated that the maximum was reached after 60 minutes exposure to A.actinomycetemcomitans sonicates (Tsai *et al.*, 1979) whereas a longer exposure (120 minutes) was needed when monocytes were the targets (Taichman, Dean and Sanderson, 1980).

The prevalence of leukotoxic A.actinomycetemcomitans was shown to be higher in periodontal disease patients especially in juvenile periodontitis patients and, therefore, Zambon *et al.*, (1983) correlated the leukotoxin production with virulence. A few H.aphrophilus strains were tested in this study but proved to be together with non-leukotoxic A.actinomycetemcomitans strains, unable to damage either HL60 or human PMNL target cells. Tsai *et al.*, (1981) and Taichman and Wilton (1981) have reported that the majority of juvenile periodontitis patients have a high serum titre of anti-leukotoxin antibodies produced against A.actinomycetemcomitans. Tsai *et al.*, (1982) also reported that younger patients (less than 12 years of age) were more frequently infected with leukotoxic A.actinomycetemcomitans than were older patients.

Tsai *et al.*, (1984) reported that A.actinomycetemcomitans leukotoxin had a molecular weight of 115,000 daltons as determined by the SDS-PAGE and was susceptible to heat, since incubation of leukotoxin at 56°C for 30 minutes caused more than 90% inactivation and heating for 30 minutes at 70°C or 100°C resulted in

complete inactivation. They also showed that leukotoxin was trypsin-sensitive. Purified leukotoxin killed human but not rat or guinea pig PMNLs and it was inactive against human erythrocytes. Leukotoxic activity was inhibited by pooled serum from juvenile periodontitis patients (Tsai *et al.*, 1984).

Thus several studies have indicated that leukotoxin from A.actinomycetemcomitans was capable of killing human PMNLs, monocytes and certain human myelomonocytic leukaemia cell lines (HL60 and U937). PMNLs and monocytes from the great apes and old world monkeys are also susceptible to leukotoxin produced by A.actinomycetemcomitans (Baehni *et al.*, 1981; Taichman *et al.*, 1984). Epithelial and endothelial cells, fibroblasts, erythrocytes and platelets proved to be non-susceptible (Taichman, Dean and Sanderson, 1980; Taichman and Wilton, 1981). Rabie, Lally and Shenker (1988) reported that A.actinomycetemcomitans leukotoxin was able to exert a non-lethal effect on human lymphocytes, which could compromise the ability of the host defences to cope with infection in the gingival crevice. In addition it is known that A.actinomycetemcomitans leukotoxin kills mature human T and B lymphocyte cell lines (Simpson, Berthold, and

Taichman 1988), and Taichman *et al.*, (1991) have reported that leukotoxin purified from A.actinomycetemcomitans destroyed a subpopulation of human lymphocytes. The authors indicated that Rabie, Lally and Shenker (1988) were unable to detect lethal activity by the A.actinomycetemcomitans leukotoxin against human lymphocytes may be related to the fact that the killing of lymphocytes does not involve decimation of the entire population as it appears that only a subpopulation is susceptible.

The A.actinomycetemcomitans leukotoxin gene has been cloned in E.coli and it has been shown that A.actinomycetemcomitans leukotoxin is similar to E.coli α-haemolysin and to Pasteurella haemolytica leukotoxin (Lally *et al.*, 1989). Furthermore, when the complete nucleotide sequence of the A.actinomycetemcomitans leukotoxin gene was determined it was found to be closer to the α-haemolysin of E.coli than to P.haemolytica leukotoxin (Kraig, Lally and Kolodrubetz, 1990). This group of cytotoxins is referred to as the RTX group (Repeats in Toxin) as they are characterized by a series of glycine-rich repeat units at the C-terminal end of each protein, in

addition to other features (Coote, 1992). However, they differ in their target specificity and the A.actinomycetemcomitans leukotoxin tends to remain cell-associated and is not secreted into culture supernatant as has been reported for other RTX toxins (Coote, 1992). However, it can be released into the culture supernatant by polymyxin B treatment (Tsai *et al.*, 1984; Ohta *et al.*, 1993). Most of the leukotoxic activity of A.actinomycetemcomitans has been detected on the cell surface when it is cultured under controlled conditions in a chemostat (Ohta *et al.*, 1993), and Berthold *et al.*, (1992) reported that it was localized either in the cell envelope or in membranous vesicles on the outer surface of the bacterial cell by an experiments using monospecific poly-clonal antibody against A.actinomycetemcomitans leukotoxin combined with electron immunocytochemical techniques. Ohta *et al.*, (1991) reported that the leukotoxin could be extracted from whole cells by nuclease digestion. Furthermore, Ohta *et al.*, (1993) reported that the association of A.actinomycetemcomitans leukotoxin with the cell surface is mediated by nucleic acids and that the extracellular secretion of

leukotoxin occurs in growing cultures with an increased ionic strength of buffers or media.

Mode of action of leukotoxin

Four genes are required for the synthesis, activation and secretion of the RTX toxins, and they are designated C, A, B and D in the order of the genetic organization. In the case of A.actinomycetemcomitans leukotoxin, these are lktC, lktA, lktB and lktD (Coote 1992). The A gene is the structural toxin gene, the C gene is involved in activation, and the B and D genes are required for secretion (Coote, 1992). Calcium has been shown to have an important role in the activity of RTX toxins. Lally *et al.*, (1991) showed that A.actinomycetemcomitans leukotoxin binds Ca^{2+} in a dose-dependent pattern. The leukotoxin of A.actinomycetemcomitans binds to cell surface receptors and injures the plasma membrane, although certain mono-and di-saccharides with mannose and galactose configuration provided some protection to target cells from toxins (Simpson, Berthold and Taichman, 1988). They further reported that cellular resistance to the toxin in non-susceptible target

cells may be due to a lack of or decrease in the number of leukotoxin receptors or alternatively due to impairment of cytopathic events that occur after the initial binding between toxin and receptor. Iwase *et al.*, (1990) studied different aspects of the mechanisms of action of A.actinomycetemcomitans leukotoxin and reported that it altered the permeability of HL60 target cells leading to colloid osmotic lysis. They further suggested that the toxin acts as a membranolytic agent producing pores in the target cells which could be protected by certain saccharides. In a similar study Taichman *et al.*, (1991) reported that the leukotoxin isolated from A.actinomycetemcomitans rapidly binds to susceptible target cells and alters membrane permeability. Resistant target cells either fail to bind to the toxin or resist permeability after binding. Similar events have been shown to occur in the lysis of mammalian erythrocytes and leukocytes by other RTX toxins derived from Gram-negative bacteria (eg Pasteurella haemolytica leukotoxin).

It has been shown that substantial differences in the level of leukotoxin expression among A.actinomycetemc-

omitans strains exist, though all strains carry the complete toxin gene operon (Poulsen *et al.*, 1994).

It is evident from the reviewed literature that the ability of certain A.actinomycetemcomitans strains to produce leukotoxin and suppress lymphocyte function may well contribute to the pathogenesis of juvenile periodontitis and possibly other forms of periodontitis. However, the production of leukotoxin is not the only factor required for pathogenesis, since leukotoxic and non-leukotoxic A.actinomycetemcomitans strains were isolated from patients with juvenile periodontal disease (Slots *et al.*, 1982).

1.14.3 Chemotaxis inhibition by periodontopathic bacteria

As mentioned in the Section (1.13.2), human PMNLs play a major role in defending the gingival site against potential pathogenic bacteria. The ability of any putative periodontopathogen to interfere with the bactericidal function of PMNLs will contribute to the pathogenicity of that species. Several bacterial species produce substances that are toxic for neutrophils (ie leukotoxin; leucocidin) (Baehni *et al.*, 1979; Scharmann, Jacob and Portendorfen, 1976),

and can inhibit specific neutrophil functions such as chemotaxis and phagocytosis (Church and Nye, 1979; Shurin *et al.*, 1979; Ingham *et al.*, 1977).

Chemotaxis is the movement of PMNLs towards the site of infection in response to specific chemical agents. Van Dyke *et al.*, (1982) examined the ability of sonic extracts of A.actinomycetemcomitans to inhibit peripheral blood neutrophil chemotaxis, and reported that it specifically inhibited chemotaxis. The authors concluded that such an ability to inhibit neutrophil function may be an important determinant in virulence. Cutler *et al.*, (1991) in a study of PMNL functions in adult insulin-dependent diabetic patients with severe recurrent periodontitis, reported that the chemotactic ability of PMNLs was depressed and that the subgingival microflora had significant numbers of several periodontopathic bacteria including A.actinomycetemcomitans. However, Kinane *et al.*, (1989) reported that PMNLs isolated from patients with juvenile periodontitis and rapidly progressive periodontitis did not show any defective chemotaxis and further concluded that the variation which exists in the literature with respect to PMNL locomotion in periodontal disease was due to differences in the

methods used for analysis. A more surprising finding has been reported by Holm, Kalfas and Holm (1989) in which they showed that certain strains of A.actinomycetemcomitans were able to evoke the chemotaxis of PMNL in vivo. The authors related this contradictory result to the methodology employed such as the source and pre-treatment of PMNL, and additional biological factors in the in vitro assay. In another study A.actinomycetemcomitans was shown to inhibit the chemotaxis of neutrophil in vitro and thus may contribute to the pathogenesis of localized juvenile periodontitis (Ashkenazi, White and Dennison, 1992). In addition Ochiai *et al.*, (1989) have reported that sonic extracts from periodontopathic bacteria, including strains of A.actinomycetemcomitans, have an immunosuppressive effect when tested in vivo with an experimental mouse model. They related this immunosuppressive activity to a heat-labile protein in the extract. Further, they concluded that this immunosuppressive activity produced by A.actinomycetemcomitans could enhance survival not only of the initial periodontopathogen but also other opportunistic organisms present in the gingival crevice.

There is little information regarding the ability of H.aphrophilus to interfere with PMNLs, though in a study reported by Holm, Kalfas and Holm (1989) it has been shown that certain strains of H.aphrophilus evoked the chemotaxis of PMNL in vivo. Lindhe and Socransky (1979) showed that Capnocytophaga species are less chemotactic for human PMNL than some other oral bacteria both in vitro and in vivo. Shurin *et al.*, (1979) showed that peripheral PMNL from patients with Capnocytophaga intra orally exhibit distinctive abnormalities in morphology and locomotion, and the removal of the organism produced an improvement with a restoration of normal function. Finally sonic extracts of Capnocytophaga species have produced factors that specifically inhibited peripheral blood neutrophil chemotaxis, (Van Dyke *et al.*, 1982).

The interactions between A.actinomycetemcomitans and human PMNLs has been studied more extensively in contrast with both H.aphrophilus and Capnocytophaga species and there is not much information regarding the latter two organisms.

1.14.4 Immunoglobulin A protease(IgA protease)

Immunoglobulin A (IgA) is found in human serum in concentrations that range from 1.4 to 4 mg /ml. There are two subclasses of IgA (IgA1 and IgA2) of which IgA1 constitutes 80-90% of the total. IgA is the predominant immunoglobulin class in secretions and it is present in saliva, tears, nasal fluids, sweat, colostrum and secretions of the lung, genitourinary and gastrointestinal tracts (Roitt, 1994). Serum IgA occurs mostly in the form of monomers, composed of two heavy and two light chains with molecular weights of about 53,000 and 22,500 respectively. A minor portion of the IgA in the sera of normal adults is found in a polymeric form, in which monomers are connected by disulphide bonds and linked to an additional polypeptide called a J chain (Koshland, 1975). In external secretions most of the IgA occurs in a polymeric form; dimeric IgA predominates, but milk and saliva also contain tetramers (Zikan *et al.*, 1972; Halpern and Koshland, 1973). IgA in external secretions is usually present as a dimer linked to secretory component (secretory IgA). The function of this secretory component may be to protect IgA molecules from proteases (Roitt, 1994).

IgA is considered to be an important defence against invading microorganisms (Kilian, 1981), and acts by neutralizing toxins, enzymes and viruses and by inhibiting the adherence of bacteria to mucosal surfaces, hence preventing them from attaching and possible entering host tissues (McNabb and Tomasi 1981; Tomasi, 1984).

Plaut, Wistar and Capra (1974) suggested that IgA proteases may be an important factor in the pathogenesis of mucosal infections. Certain bacteria which colonize mucosal surfaces such as Neisseria meningitidis, Nesseria gonorrhoeae, and other bacteria important in dental plaque formation such as Streptococcus sanguis, produce an extracellular enzyme which cleaves human serum IgA1 (Mulks and Plaut, 1978; Plaut, Wistar and Capra, 1974; Nyvad and Kilian, 1990). Furthermore, it has been suggested that this enzymatic activity may be involved in the pathogenesis of infections with Neisseria gonorrhoeae and Neisseria meningitidis since it was not produced by non-pathogenic strains. Haemophilus influenzae and Streptococcus pneumoniae, both cause bacterial meningitis, and produce an IgA protease which specifically cleaves human IgA1 to yield intact Fab

and Fc fragments (Kilian, Mestecky and Schrohenloher, 1979; Male, 1979). Several periodontopathogens are also capable of producing an IgA protease which cleaved IgA1 in the hinge region to produce intact Fab and Fc fragments (Kilian, 1981). These included Capnocytophaga and Bacteroides strains. Furthermore, Kilian suggested that the presence of such organisms can cause damage to the immune defence mechanisms which will result in the penetration and spread of several toxic products released by subgingival microflora. As discussed earlier, of the organisms under study, only Capnocytophaga species have been shown to cleave human IgA1 both in vitro and in vivo (Frandsen *et al.*, 1995). There is little information in the literature regarding the ability of the two other species (A.actinomycetemcomitans and H.aphrophilus) to produce IgA1 protease.

1.14.5 Fibroblast inhibition

Periodontal diseases in general, and juvenile periodontitis in particular, are inflammatory diseases which lead to the destruction of the tooth supporting tissues (Manson and Lehner, 1974; Saxen, 1980). The effects of dental plaque or dental plaque extracts on

mammalian cells have been studied in vitro. For example Smalley and Birss (1986) showed that P.gingivalis strain W50 possess a surface membrane vesicles when examined by electron microscopy, and these vesicles were cytotoxic to human gingival fibroblasts, the authors concluded that such activity may contribute to the pathogenicity of P.gingivalis. Gingival fibroblasts maintain connective tissues in the periodontium, and may be affected positively or negatively by interacting both with inflammatory cells and their products and also directly with bacterial products (Larjava *et al.*, 1987).

The inhibition of fibroblast proliferation by A.actinomycetemcomitans, Capnocytophaga and H.aphrophilus

The ability of A.actinomycetemcomitans to inhibit the proliferation of fibroblasts has been examined in a number of studies. Shenker, Kushner and Tsai (1982) examined sonic extracts of A.actinomycetemcomitans strain Y4 for their ability to interfere with the proliferation of human and murine fibroblast cell lines. The extracts inhibited the proliferation of both cell lines, and it was suggested that this

activity of A.actinomycetemcomitans may be an important pathogenicity factor of the organism. Clearly inhibition of fibroblast proliferation could extend and depress healing and contribute to the loss of supportive collagen fibres associated with certain forms of periodontal disease, especially juvenile periodontitis. The ability of capsular material and lipopolysaccharides isolated from A.actinomycetemcomitans strains to inhibit the proliferation of human gingival fibroblasts was examined by Kamin *et al.*, (1986). They reported that capsular material inhibited the proliferation of human gingival fibroblasts by inhibiting the synthesis of DNA and collagen. They also reported that lipopolysaccharide from the same bacterium caused only modest inhibition of DNA synthesis and it had no effect on collagen synthesis. Furthermore, Kamin *et al.*, (1986) reported that this inhibitory activity was not accompanied by cell death as there was no lactate dehydrogenase release by fibroblasts. Lipopolysaccharides from A.actinomycetemcomitans has been examined for its ability to interfere with cultured fibroblasts and compared with lipopolysaccharides prepared from several other bacterial species including Salmonella enteritidis and

Bacteroides (Porphyromonas) gingivalis, (Bartold and Millar, 1988). The lipopolysaccharide preparations inhibited the proliferation of human gingival fibroblasts, with the lipopolysaccharide from A.actinomycetemcomitans proving more potent than others. Based on these results Bartold and Miller (1988) concluded that the potential virulence of a periodontopathogen may vary as a result of the variation in the potency of lipopolysaccharides preparation and because of the ability of lipopolysaccharides.

The ability of A.actinomycetemcomitans to interfere with fibroblasts was further tested by Stevens and Hammond (1988), who examined the cytotoxicity of sonic extracts from A.actinomycetemcomitans and other oral bacteria for human gingival fibroblasts. They used three different criteria for the assessment of cytotoxicity including direct counts, inhibition of DNA synthesis and microscopical examination for morphological effects. A.actinomycetemcomitans and F.nucleatum were shown to be the most potent of all tested bacteria by all three criteria. Helegeland and Nordby (1993) reported the isolation of a toxin from the culture medium of A.actinomycetemcomitans and

showed that the toxin inhibited the proliferation of human gingival fibroblasts.

A few studies have investigated the ability of Capnocytophaga species to inhibit the proliferation of fibroblasts and a fibroblast inhibitory factor has been reported (Sasaki, 1979; Stevens and Hammond, 1982). In the study reported by Stevens and Hammond (1988) sonic extracts from C.sputigena inhibited the proliferation of human gingival fibroblasts, although the inhibitory activity of C.sputigena was less than that expressed by A.actinomycetemcomitans.

The ability of H.aphrophilus strains to inhibit the proliferation of fibroblasts, has not previously been investigated.

It is evident from the reviewed literature that fibroblasts play a fundamental role in maintaining the health of the periodontal tissues. The ability of periodontopathogenic organisms to interfere with this function could indeed contribute to periodontitis with A.actinomycetemcomitans being most active followed by Capnocytophaga species.

1.14.6 Collagenolytic activity of A.actinomycetemcomitans H.aphrophilus and Capnocytophaga species

As indicated in previous sections a number of periodontopathogens have been shown to produce collagenase that will cause damage to connective tissue by disrupting collagen. Localized juvenile periodontitis is characterised by extensive loss of collagen from the gingival connective tissue, a feature which distinguishes this form of periodontal disease from other types (Zambon, 1985). Several studies have demonstrated that the level of degradation of extracellular connective tissue which occurs in localized juvenile periodontitis is greater than that which occurs in adult periodontitis (Gillet and Johnson, 1982; Liljenberg and Lindhe, 1980). Certain strains of A.actinomycetemcomitans have been shown to produce collagenase (Roertson *et al.*, 1982; Rozanis and Slots, 1982; Rozanis *et al.*, 1983), and this activity combined with collagenase released from neutrophils lysed by leukotoxin may be involved in tissue destruction (Zambon, 1985). Furthermore, certain A.actinomycetemcomitans strains produce a fibroblast inhibitory factor (Shenker, Kushner and

Tsai, 1982; Steven and Hammond, 1982) which results in a loss in collagen production. Thus in combination with production of collagenase these two factors can destroy subgingival connective tissue and affect the production of new collagen (Stevens and Hammond, 1982).

There is little information available regarding collagenase production by H.aphrophilus and Capnocytophaga species.

1.14.7 Miscellaneous virulence factors produced by A.actinomycetemcomitans strains and or Capnocytophaga species

This section will include a brief presentation of various additional factors produced by the group of organisms under study, since they may contribute to the pathogenesis of periodontitis.

Birkedal-Hansen *et al.*, (1982) reported that A.actinomycetemcomitans strains produce an inhibitory factor against epithelial cells (epitheliotoxin), and Kamen, (1983) described a heat-labile sonic extract prepared from a non-leukotoxic A.actinomycetemcomitans isolate that inhibited the proliferation of cultured keratinocytes in a dose-dependent pattern as measured by direct observation of cultures (cell counts) and by incorporation of ^{3}H-thymidine. If these effects occurred in vivo such depression of epithelial cell proliferation could produce a profound effect on the integrity and healing of periodontal tissues. Capnocytophaga species has been shown to produce a similar factor (Kamen, 1982). Taichman *et al.*, (1984 A) tested soluble extracts of A.actinomycetemcomitans and Capnocytophaga for their ability to alter the proliferation of cultured endothelial cells. They

reported that all A.actinomycetemcomitans strains tested produced a dose- dependent and heat-labile factor that inhibited DNA and RNA synthesis in human and bovine endothelial cells. This inhibitory factor was not leukotoxin since it was produced by both leukotoxic and non-leukotoxic strains. They also reported that sera from patients with juvenile periodontal disease neutralized this inhibitory factor. They concluded that the alteration of the endothelial cells which include a decreased endothelial cells proliferation as evident by the presence of a fewer labelled nuclei compared to control cells and also there was no increase in total cell numbers though cell viability was unaltered, may be of pathologic significance to the development of periodontal disease (Taichman *et al.*, 1984A). It is interesting to note that Capnocytophaga sputigena was reported as stimulating the proliferation of endothelial cells (Taichman *et al.*, 1984A).

Both A.actinomycetemcomitans and Capnocytophaga species were among a group of Gram-negative bacteria isolated from periodontally diseased sites that produced B-cell activating factor which activates the B-lymphocytes to produce a poly clonal antibody and

the release of osteolytic factors. These responses would lead to increased inflammation in the disease site, activation of bone resorption and loss of periodontal support for the teeth (Bick *et al.*, 1981). A.actinomycetemcomitans has been shown to possess lipopolysaccharide and capsular material that induce a bone resorption in vitro (Kiley and Holt, 1980; Nishihara *et al.*, 1989). Wilson, Kamin and Harvey, (1985) also reported that purified capsular material isolated from A.actinomycetemcomitans was a potent bone resorption mediator. Ishihara *et al.*, (1991) reported that lipopolysaccharide isolated from A.actinomycetemcomitans strain Y4 induced bone resorption in a BALB/c mouse calvarial system, and they further added that this activity may be mediated by interleukin-1 and prostaglandin. Meghji *et al.*, (1994) reported that a saline extracted surface associated material from A.actinomycetemcomitans induced a bone resorbing activity. Lipopolysaccharide of Capnocytophaga species has also been shown to induce bone resorption activity (Sasaki, 1979; Stevens *et al.*, 1980).

Tolo and Helgeland, (1991) reported that A.actinomycetemcomitans (strain ATCC 33384) can

produce and release components capable of binding to the Fc part of the immunoglobulin G (IgG), and that the activity was present in whole bacteria, capsular material and medium from broth cultures. The authors concluded that the Fc-binding components may contribute to the pathogenesis of periodontitis, since they interfere with the phagocytic activity of the granulocytes and with complement. Furthermore, it has been shown that A.actinomycetemcomitans produces potent endotoxin which augments the survival of the organism which kills macrophages (Kily and Holt, 1980). It has also been shown that these bacteria can produce factors that interfere with the function of human blood lymphocytes since it was shown that the function of both B and T-lymphocytes were impaired by soluble extracts of this organism and immune suppression was observed in vitro, by means of soluble heat-labile factor (Shenker, McArture and Tsai, 1982; Shenker, Tsai and Taichman, 1982). A.actinomycetemcomitans strains were shown to produce alkaline and acid phosphatase, which can induce bone resorption and thus cause damage to the periodontal tissue (Slots, 1981). A.actinomycetemcomitans strains have been shown to produce a catalase capable of

degrading hydrogen peroxide from the host immune cells and therefore, provides protection against oxidative bacterial killing (Slots, 1982A).

Capnocytophaga species were shown to possess a high aminopeptidase activity as tested by API ZYM AP system (Nakamura and Slots, 1982). This activity may contribute to collagen breakdown following initial splitting of collagen by host or bacterial collagenase, and it may also be important for the survival of Capnocytophaga species in dental plaque as the ability of this bacterium to degrade numerous peptides may provide essential energy and cellular constituents for continued growth. Such activity may play a role in the pathogenesis of periodontitis (Nakamura and Slots, 1982).

In the above Sections a number of factors that may contribute to the pathogenic potential of the group of bacteria under study, were presented. Although both A.actinomycetemcomitans and Capnocytophaga species have been shown to possess a number of possible virulence factors, A.actinomycetemcomitans has been studied more extensively than the other two organisms. There is substantial evidence to support a role for A.actinomycetemcomitans in periodontal disease

especially the localized form of juvenile periodontitis. However, the evidence supporting the role of Capnocytophaga species in periodontitis is far from complete and it is clear that more studies are needed. In contrast there is very little information about the possible virulence of H.aphrophilus, in periodontal disease, even although it has been implicated in other systemic infections (for example endocarditis and brain abscess).

1.15 Aims of this study

As outlined in the review of literature the search for aetiologic agents of periodontal disease is complex and far from certain. A number of suspected periodontal pathogens have been implicated in the aetiology of different forms of periodontal disease based on defined criteria which include association, elimination, induction of immune response, causing disease in experimental animals and producing virulence factors. Based on evidence obtained by using the described criteria, A.actinomycetmcomitans has been strongly associated with the juvenile form of periodontal disease. Although other Gram-negative capnophilic organisms like Capnocytophaga species were implicated in the aetiology of periodontal disease in some early studies, the evidence supporting this is far from complete and additional studies are needed. The possible role of H.aphrophilus, another Gram-negative capnophilic rod which is frequently isolated from the dental plaque is not clear since only a few studies have addressed this question. Therefore, this study was undertaken to examine a number of Type and wild oral isolates of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species for their

possible role in periodontitis. The virulence factors that were studied consisted of leukotoxin production, fibroblast inhibitory activity and the production of IgA-protease. The ability of the test bacteria to cause haemagglutination of human erythrocytes was also examined since colonization and adherence to oral surfaces is probably an important step for colonization and survival in the subgingival area. The ability of the test bacteria to trigger a chemiluminescence response from human PMNLs was also tested.

Chapter 2

Material and Methods

2.1 Isolation of Haemophilus aphrophilus, Actinobacillus actinomycetemcomitans and Capnocytophaga species from subgingival plaque samples

Subgingival plaque samples were collected from groups of patients with different forms of periodontal disease. These included a group of a 23 patients diagnosed with chronic periodontitis and a group of 14 patients with pockets which persisted following hygiene phase therapy. However, the clinical diagnosis of the latter group was unknown. Nine patients with juvenile and rapidly progressive periodontitis and a group of 3 patients with unknown clinical diagnosis completed a the total of 49 patients used for this study. Subgingival plaque was removed from multiple sites and placed in 1 ml of Anaerobic Blood Broth (ABB), or Fastidious Anaerobic Broth (FAB), and transferred to the laboratory immediately after collection. Samples were dispersed by vortex mixing for 60 seconds, then serially diluted in FAB, and finally 50µl of each dilution were inoculated onto the following media: Colombia blood agar plates (CBA), Tryptic Soy Serum Bacitracin Vancomycin agar (TSBV), a selective medium for A.actinomycetemcomitans (Slots, 1982) and Tryptic

Soy Serum Bacitracin Vancomycin Fluoride agar (TSBVF), the selective medium for H.aphrophilus, (Tempro and Slots, 1986). Inoculation was performed using a spiral plater (Don Whitley, Shipley, England)(Figure 2.1). Neat and 10^{-1} dilutions of plaque were inoculated onto TSBV and TSBVF plates and 10^{-2} and 10^{-3} dilutions were inoculated onto CBA plates. All plates were incubated at 37°C in 5% CO_2 in air, for 3 days. Counts were made by using the grid supplied by the manufacturer (Don Whitley, Shipley, England) which is divided into sections of different sizes in which known volumes of the sample were spread. The number of colony forming units/ml was then calculated. After incubation, plates were processed as follows: CBA plates were used for the isolation of Capnocytophaga species. All suspected Capnocytophaga colonies (ie large, flat, spreading, grey, yellow or pink) were transferred to fresh CBA for purity and further identification. TSBV and TSBVF plates were used as semi-selective media for the isolation of A.actinomycetemcomitans and H.aphrophilus respectively. However, colonies of both species on both media were very similar and therefore, suspected colonies (ie those which were small, convex, entire, cream in colour with irregular edges, and sometimes

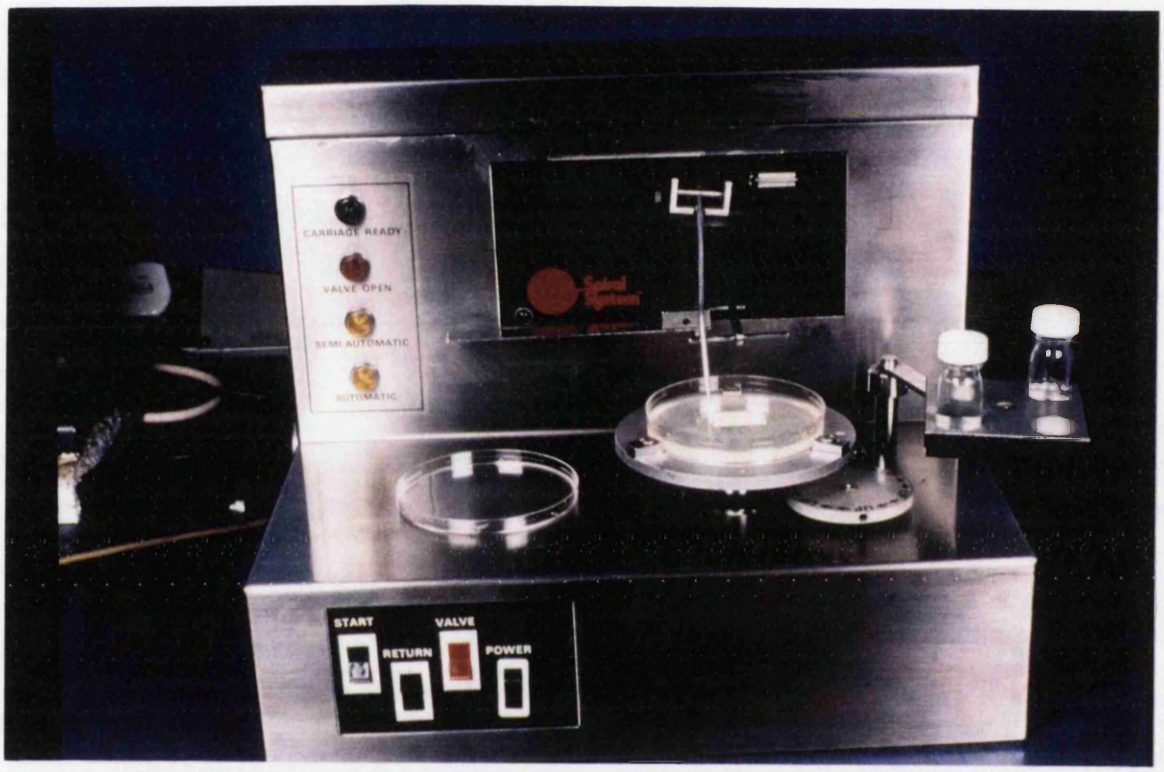


Figure 2.1 Spiral plater used for inoculating agar plates

with star-shaped inclusion) were transferred to fresh CBA plates for purity before identification.

2.2 Identification of isolated organisms

2.2.1 Identification of A.actinomycetemcomitans and H.aphrophilus

After incubation (3days), plates were checked for purity and all plates that contained a pure growth were used for identification. From pure cultures smears were prepared and stained by Gram's stain. Cultures that consisted of Gram-negative short rods or coccobacilli were tested for catalase production. The catalase test was carried out by emulsifying a few colonies of test bacteria grown on CBA for 18-24 hours in coloured catalase reagent (Bio Mérieux, Marcy-l'Etoile, France) on a clean glass slide with a platinum loop. Positive strains broke down hydrogen peroxide and bubbles of oxygen were released within a few seconds. All A.actinomycetemcomitans strains are known to be catalase positive, while all H.aphrophilus strains are catalase negative. This test was, therefore, one of the tests used to differentiate between these two closely related organisms. However, some exceptions were reported in the literature and

they will be discussed in the discussion section of this study. Cultures were then retained and identified biochemically by using the API 20A system.

API 20 A system

The API 20A system (Bio Mérieux, Marcy-l'Etoile, France) comprises a strip of 20 different biochemical tests, that differentiate between microorganisms by their abilities to utilize certain carbohydrates and produce certain enzymes (Figure 2.2). API 20 A identification strips were inoculated as recommended by the manufacturer. Briefly, the test organism was suspended in sterile broth (API Lab, France), to give an initial turbidity of McFarland opacity scale 3. All microtubes were then filled carefully with the suspension from a Pasteur pipette and the strips incubated for 24 to 48 hours at 37°C in 5% CO_2 in air. The results were then read and identification made after comparing the biochemical profile of the test strain to those of well characterized reference strains. These included H.aphrophilus NCTC 5886, A.actinomycetemcomitans NCTC 9710 and A.actinomycetemcomitans Y4.

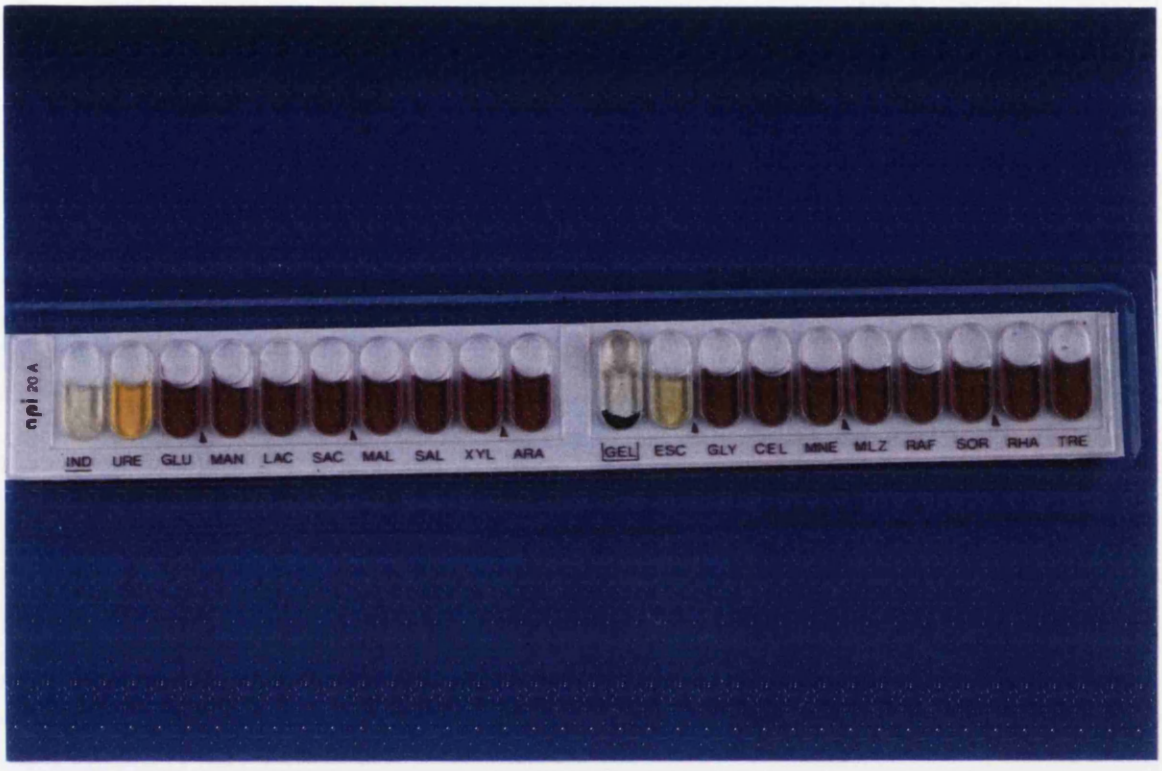


Figure 2.2 API 20A strip used for the biochemical identification of both A.actinomycetemcomitans and H.aphrophilus strains (uninoculated strip).

2.2.2 Identification of Capnocytophaga species

After incubation, plates were checked for purity and used for identification. The morphological characteristics of Capnocytophaga species are distinct and make the preliminary identification possible. Smears were prepared from pure growth and stained by Gram's stain. All Gram-negative long fusiform rods were retained and identified as Capnocytophaga species. A biochemical characterization was attempted using the Minitek Anaerobe Identification System (Becton Dickinson, Cowley, England). The identification to a species level was not possible using the Minitek system, as it was not possible to reproduce the results with all isolates, with the exception of the C.ochracea strains which can be identified using the Minitek system. Therefore, it was decided not use the system and alternatively all colonies with the morphological characteristics outlined in section 2.1 were identified as Capnocytophaga and the term Capnocytophaga species was used in this thesis.

2.3 Storage of microorganisms

After isolation and identification, bacterial isolates were stored on Protect beads (Lab M, Topley House, Bury, England). Bacterial suspensions were prepared by harvesting the growth on CBA plates with a sterile cotton wool swab and suspending the organisms in a vial of Protect beads in a cryopreservative fluid suspension. The vials were mixed by inverting them several times as recommended by the supplier and excess liquid was removed with a sterile Pasteur pipette. Each isolate was given a number and storage vials were numbered accordingly. They were stored in triplicate at -70°C in a freezer.

2.4 Retrieval of bacteria

Whenever a fresh culture was required, the relevant vial was removed from the freezer, one or two beads removed aseptically and inoculated onto fresh CBA plates by rolling the beads gently over the plate surface. The plates were incubated at 37 °C in 5% CO_2 in air for 24 to 48 hours. The identity of each organism was confirmed by morphological and biochemical examination and subcultured onto fresh CBA plates before use.

2.5 Sensitivity to antibacterial agents (antibiotics)

Isolates of H.aphrophilus, A.actinomycetemcomitans and Capnocytophaga species were tested for their sensitivity to selected antibiotics by Stokes method (Stokes and Waterman, 1972). Microorganisms were grown on CBA plates for 48 hours at 37°C in 5% CO_2 in air. A loopful of each organism was spread across the middle of a CBA plate as a broad band (Figure 2.3). Staphylococcus aureus (Oxford reference strain NCTC 6571) was used as a control (Figure 2.3). A suspension of S.aureus was prepared in peptone water (Oxoid Unipath Ltd, Basingstoke, Hampshire, England)by suspending five colonies of a 24 hour CBA culture in 3 ml peptone water then vortex mixing for 10 seconds. A sterile cotton swab was used to inoculate the control suspension on either side of the test inoculum without touching it (Figure 2.3). Antibiotic discs (Table 2.1) (Mast Lab, Merseyside, England) were placed, using a sterile needle, on the plates on the space between the reference strain and test bacteria. All plates were incubated at 37°C in 5% CO_2 in air for 48 hours. After incubation, results were recorded either as (i)sensitive, where the zone of inhibition was equal

to, greater or not more than 3 mm smaller than the control zone, (ii) moderately sensitive where the zone of inhibition was more than 3mm but smaller than the control by more than 3mm or (iii) resistant if the zone of inhibition was less than 3mm. Zones of inhibition were measured from the edge of the disc to the edge of the zone. Tests were performed in duplicate on a single occasion.

Table 2.1 LIST OF ANTIBIOTICS USED

ANTIBIOTIC	CONCENTRATION	ABBREVIATION
TETRACYCLINE	10μg	T
ERYTHROMYCIN	10μg	E
PENICILLIN	1UNIT	PG
AMPICILLIN	10μg	AP

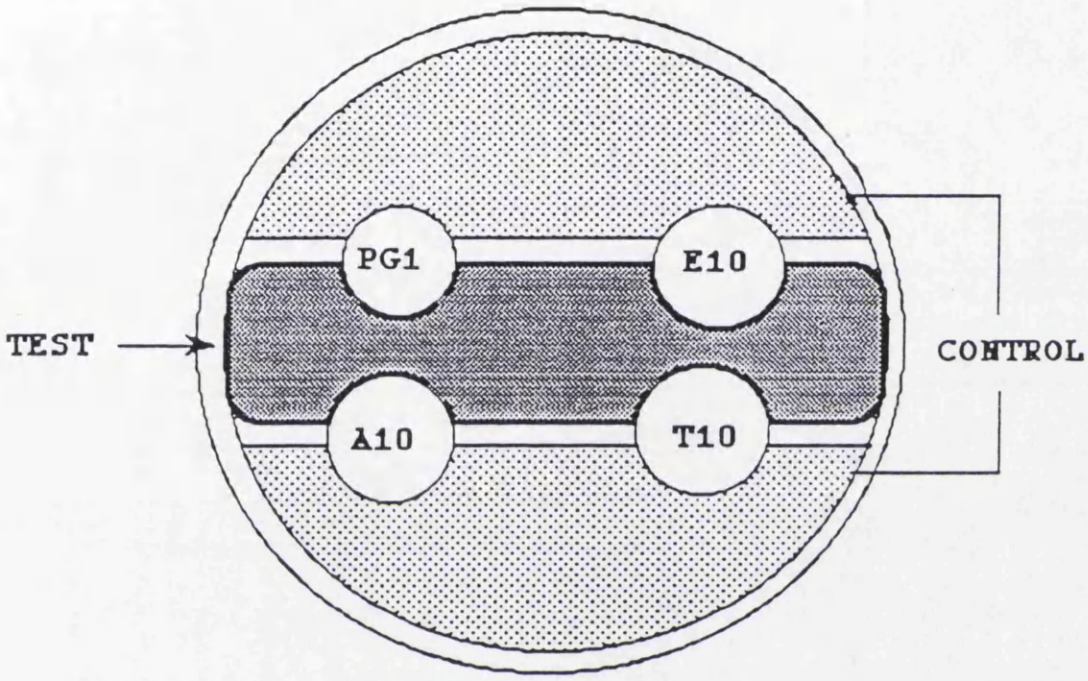


Figure 2.3 Diagrammatic illustration of an antimicrobial sensitivity plate using Stokes method

2.6 Haemagglutination of human group A and group O erythrocytes by capnophilic bacteria

2.6.1 Bacteria employed

The microorganisms used in this study included both National Type Cultures and fresh isolates from subgingival plaque samples taken from patients with periodontal disease who attended Glasgow Dental Hospital and School for treatment (Tables 3.1, 3.2, 3.3). Stock cultures at -70^{o}C were revived by transfer to CBA and incubated for 24 hours, at 37^{o}C in 5% CO_2 in air, followed by subculture onto fresh CBA and incubation for a further 18-24 hours.

E.coli (NCTC 10418) was used as a positive control in the assay and was grown on CBA for 24 hours at 37^{o}C under aerobic conditions. Bacterial suspensions were prepared according to a method described by Majeed and Macrae (1994). Briefly, the growth from a CBA plate was harvested on a sterile cotton wool swab and suspended in 0.6 ml of PBS pH7.2 and vortexed for 10 seconds.

Viable counts of these suspensions were determined using a spiral plater as described in Section 2.1. The bacterial concentration of suspensions prepared in this way was determined in preliminary experiments by viable culture technique, and regularly a figure of about

$5x10^{10}$ organisms/ml was recorded. Subsequently viable counts were performed and suspensions with lower counts were not considered for the experiments.

2.6.2 Erythrocyte preparation

Human group A and group O erythrocytes were supplied by the West of Scotland Blood Transfusion Service in Alsever solution (sodium chloride 0.42%, trisodium citrate (dihydrate) 0.8%, and glucose 2.05%). For use in the assays, erythrocytes were diluted 1:10 in PBS pH 7.2 and washed 3 times by centrifugation at 500 g for 10 minutes. The red cells were resuspended in PBS pH 7.2 to a final concentration of 1% (v/v) before use in the assay.

2.6.3 Haemagglutination assay

The haemagglutination assay was performed as described by Parry and Porter (1978) with slight modification. One hundred microlitres of each bacterial suspension were added to the wells of the first row of a 96-well microtitre plate (Nunc, Intermed, Denmark). Doubling dilutions of bacteria in PBS were made by transferring 50 µl of each suspension from the first row to 50 µl of PBS previously added to the wells in the second row;

after mixing, 50 µl were transferred and added to wells in a third row. The previous step was repeated until all rows were completed, giving a dilution range from neat to 1 : 128. Group A or group O red blood cells (50µl) were added to all wells. The plate was shaken gently to mix the contents, and then incubated statically at room temperature for 2 hours. The test results were compared with the positive and negative controls and the highest dilution giving complete haemagglutination was recorded as the titre. A negative control consisting of 50µl PBS + 50µl of either group A or group O erythrocytes was included in every plate. The positive control consisted of E.coli NCTC 10418.

Three different carbohydrates were tested for their ability to inhibit the haemagglutination of human red blood cells by the tested microorganisms. Stock solutions (10% w/v) of fucose, galactose and mannose all supplied by (Sigma Chemical Company, Fancy Road, Poole, Dorset, England) were prepared in sterile distilled water and stored at $4^{o}C$ until needed. Working solutions of 1% of the carbohydrates in PBS were prepared immediately prior to the experiments. The effect of these sugars was tested by including the sugar solution in the assay mixture as a diluent

instead of PBS as described above. Once again Escherichia coli NCTC 10418 strain was included in the assay as a positive control, together with a PBS negative control.

2.7 Detection of leukotoxic activity in strains of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species

2.7.1 Bacterial strains and growth conditions

Test strains of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species are listed in Tables 3.1, 3.2, 3.3, and include Type strains together with fresh isolates from subgingival plaque samples taken from patients with periodontal disease who attended Glasgow Dental Hospital and School for treatment. Stock cultures were revived from Protect beads stored at -70^{o}C by inoculation onto CBA for 24-48 hours at 37^{o}C in 5% CO_2 in air. The growth from two blood agar plates was removed with sterile cotton wool swabs and transferred to 20 ml of tryptic soy broth(Gibco BRL Life Technologies Ltd, Paisley, Scotland). Cultures were incubated for 18 hours at 37^{o}C in 5% CO_2 in air, then the cells harvested by centrifugation at 1500g for 10 minutes at room

temperature (MSE centrifuge, Crawley, Sussex, England). The cells were washed twice in 10ml ice cold PBS pH 7.2 and resuspended in PBS to give a concentration of about $2x10^9$ organisms/ml. The concentration of bacterial cells in the suspensions was determined by viable counts (see Section 2.1) in pilot experiments performed to standardize the assay conditions. The number of viable bacteria present in all the suspensions tested was routinely performed and if values were found subsequently to be less than $2x10^9$ organism/ml, the experimental results obtained were excluded. Each cell suspension was then divided into 1.5ml aliquots; one labelled as whole cells and used in the trypan blue exclusion assay and the other disrupted with an ultrasonic probe (Heat System Inc, New York, USA) (Figure 2.4), at 20% power output for 2 minutes on ice. Particulate material was pelleted by centrifugation in Eppendorf tubes in a microcentrifuge (MSE Microcentaur, Crawley, Sussex, England) at 8000 g for 5 minutes. The supernatant was transferred to a clean Eppendorf tube and stored on ice for about 30-45 minutes prior to use in a chemiluminescence assay (Section 2.7.6).

Figure 2.4 Heat systems ultrasonic probe

2.7.2 Isolation of human polymorphonuclear leukocytes (PMNLS)

Human peripheral blood PMNLS were isolated from heparinized venous blood from 3 normal adult donors, by means of density gradient centrifugation using Histopaque (Sigma Diagnostics, Fancy Road, Poole, Dorset, England). Equal parts (3ml each) of Histopaque solutions 1119 and 1077 were layered on top of each other and an equal volume of whole blood (6ml) was layered on top of this gradient. All tubes were centrifuged at 700 g for 30 minutes at room temperature. Granulocytes formed at the lower opaque layer on top of the sedimented erythrocytes (Figure 2.5). Material on top of the granulocyte layer was removed carefully and the granulocytes then aspirated with a pasteur pipette to avoid disturbing the sedimented erythrocytes. After resuspension in PBS and washing twice by centrifugation at 300 g for 10 minutes at room temperature, the cells were finally resuspended in 0.5 ml PBS. The number of cells was counted as follows in a haemocytometer chamber: the glass chamber (The Improved Neubauer) was cleaned and a clean cover slip pressed down over the grooves (counting area). Pelleted cells were suspended in 100-200µl volume, mixed well and then, 20µl-

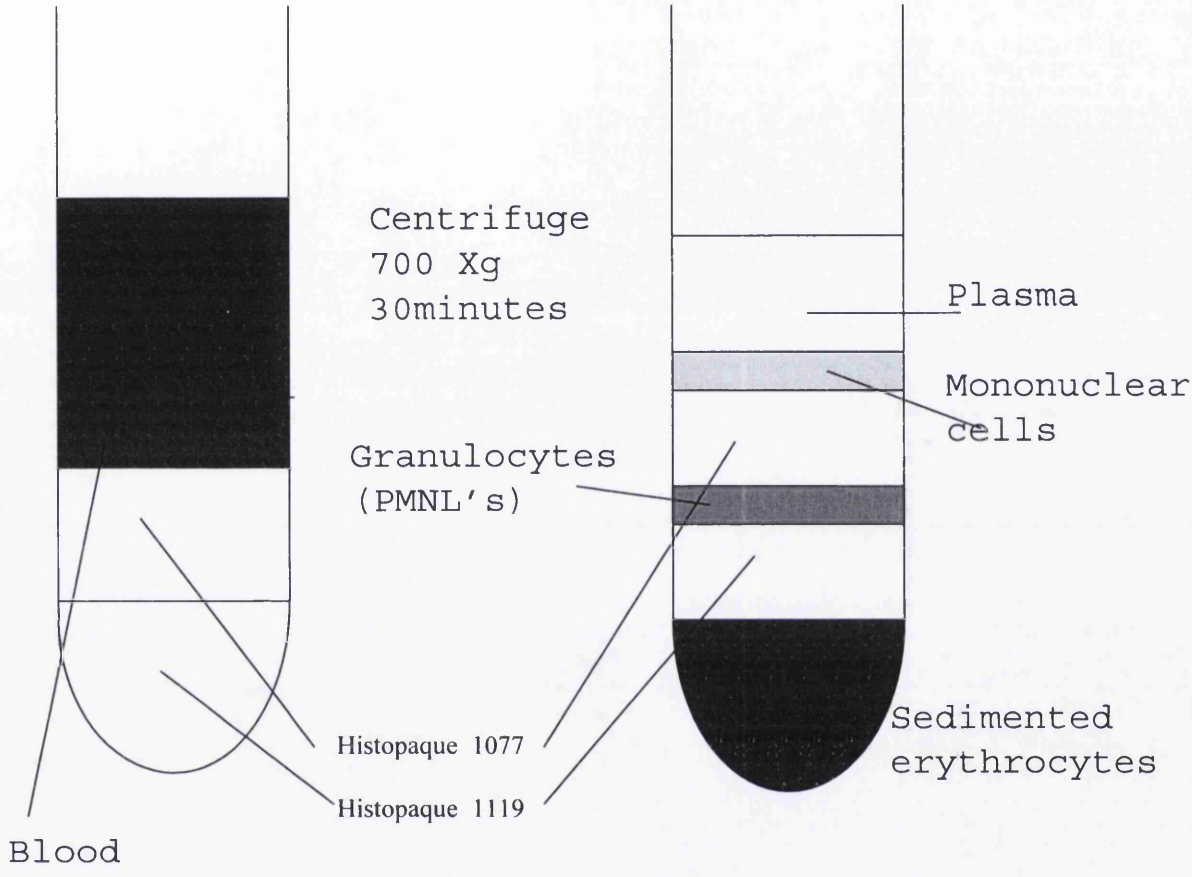


Figure 2.5 Diagramatic illustration of the separation of human PMNLs by using Histopaque gradient

50µl of cells were transferred to the haemocytometer chamber. The haemocytometer was placed on the stage of a microscope (Olympus Optical Co, Japan) and examined at 400 times magnification. The haemocytometer consists of grid with a side of 1.0mm divided into 400 squares of $0.0025mm^2$ each. The number of cells lying within a $1mm^2$ area (large square bounded by three parallel lines) was counted. The number of cells per ml of neat sample was calculated by using the following formula:

Cells/ml= Number of cells counted × 10^4.

Some samples were very concentrated and, therefore, they were diluted with PBS before counting and thus, the formula used was:

Cells/ml= Number of cells counted × Dilution factor ×10^4

Finally the cells were diluted to $2x10^5$ -$2x10^6$ cells/ml.

2.7.3 Preparation of HL60 cells

The HL60 cells are human myeloid cells that were established by Collins, Gallo, and Gallagher (1977) and derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukaemia. HL60 cells express surface markers and components which are associated with

mature granulocytes (Gallagher et al., 1979). The HL60 cells used in this study were obtained from the European Collection of Animal Cell Cultures (ECACC) held at the PHLS Centre for Applied Microbiology and Research, Porton, Salisbury, Wilts, England. The cells were received as a suspension in RPMI 1640 medium supplemented with 10% foetal calf serum. Cells were handled as recommended by the supplier. Briefly, cells were checked microscopically and then incubated overnight at $37^{O}C$ in 5% CO_2 in air (CO_2 incubator). After incubation, cells were passaged aseptically in a tissue culture cabinet(M.D.H Ltd, Andover, Hants, England) by decanting cells into a sterile plastic universal container. Cells were pelleted by centrifugation at 70 g (MSE Centaur 2 Centrifuge, Crawley, Sussex, England), then suspended in 1-2ml of freshly prepared RPMI 1640 growth medium supplemented with 10% foetal calf serum (Gibco Life Technologies Ltd, Paisley, Scotland). Cells were counted using a haemocytometer as described in Section 2.7.2 and suspended in freshly prepared RPMI 1640 medium at $1\text{-}3x10^{5}$ cells/ml. Cells were passaged every 4-5 days. Alternatively and when the cells were not required they were passaged weekly by decanting most of the medium

aseptically, leaving the cells suspended in 5-10 ml which were then diluted with fresh growth medium and reincubated.

For leucotoxicity assays, HL60 cells were harvested after 4-5 days growth by centrifugation (70 g, for 6 minutes), then washed twice in PBS. Finally, cells were resuspended in 0.5 ml PBS, counted in a haemocytometer as described in Section 2.7.2, and diluted to $5x10^6$ to $1x10^7$ /ml. The viability of cells was determined before they were used in the assays by the exclusion of trypan blue. This was performed by diluting the cells (1:1) with 0.4% trypan blue. Dead cells were stained with the trypan blue dye while live cells excluded the dye. Both viable and dead cells were easily distinguishable when examined by light microscopy (Olympus Optical Company, Japan) by using the X40 objective. One hundred cells were counted as described in Section 2.7.5, in duplicate and the percentage viability was calculated using the following formula:

$$\% \text{ Viability} = \frac{\text{Total cells counted - Dead cells}}{\text{Total cells counted}} \times 100$$

Cell suspension with viability greater than 80% were used.

2.7.4 Trypan blue exclusion assay

Trypan blue exclusion was used to detect the leukotoxic activity of A.actinomycetemcomitans strains by Brogan et al., (1994). Zambon et al., (1983) used the promyelocytic HL60 cell line as targets for the A.actinomycetemcomitans leukotoxin. In this study the trypan blue exclusion assay was used for the detection of the leukotoxic activity of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species, and HL60 cells were used as targets. HL60 cells were prepared for the assay as described in Section 2.7.3. Bacterial strains were prepared as described in Section 2.7.1. Two hundred microlitres of each test strain (whole cells) were pipetted into a sterile plastic bijou bottle, to which 200 µl of HL60 cells were added. The ratio of bacterial cells to target cells was upto 400 bacterial cells/target cell. The contents of each bottle were mixed, giving a total volume of 400 µl. A negative control(200µl of HL60 cells + 200 µl PBS) and a positive control (200 µl HL60 cells + 200 µl of 0.1% Triton X100) were included in every assay (see Table 2.2). The bottles were incubated in a shaking water bath at 37^{O}C (Heto Birköd, Denmark) for one hour, after which they were placed on ice for 5 minutes, before counting.

2.7.5 Counting of HL60 cells

After storage for 5 minutes, 100 µl of each mixture were removed and added to an Eppendorf tube containing 100 µl of 0.4% (w/v) trypan blue which was prepared in PBS. After hand mixing several times, 50µl from each tube was placed on a clean glass microscope slide and a cover slip applied. Counts were made by light microscopy at a magnification of 400x (Olympus Optical Company, Japan). The microscope stage was moved with a staggered motion, to avoid counting the same cells more than once. A minimum of 100 cells were counted. Cells were counted only if they appeared morphologically typical of healthy HL60 control cells. Each strain was tested in duplicate and on two separate occasions. The final results were expressed as the average percentage death of HL60 cells, which was calculated using the following formula:

$$\%\text{HL60 death} = \frac{\text{Total dead cells - Negative control}}{\text{Total cells counted}} \times 100$$

2.7.6 Chemiluminescence assay

A luminol-dependent chemiluminescence assay described for the detection of leukotoxin produced by Pasteurella haemolytica (Chang et al., 1986), was used to detect the leukotoxic activity of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains, with a slight modification.

In this assay the leukotoxic activity was measured as the percentage reduction of chemiluminescence emission

by phorbol myristate acetate (PMA)-stimulated human PMNLs. It was necessary to perform a series of preliminary investigations in order to optimize the experimental conditions. The assay finally employed was as follows. The reaction mixtures had a final volume of 1.0ml and the constituents of the test, PMA control and blank tubes are shown in Table 2.3. Luminol (5-amino-2,3dihydro-1,4-phthlazindione) (BDH Laboratory Supplies, Poole, England) was prepared by dissolving 1.77mg in 1ml dimethyl sulphoxide (DMSO) (BDH Laboratories Supplies, Poole, England), to give a concentration of 10^{-2} M. This stock solution was further diluted to give a working concentration of 10^{-4} M. Phorbol 12-myristate-13-acetate (PMA) (Sigma Chemical Company, Fancy Road, Poole, Dorset,

England) was prepared by dissolving 2mg PMA in 1 ml of DMSO. This stock solution was diluted further to a working strength solution by adding 50µl to 10ml of PBS. Leukocytes were used at concentrations sufficient to produce high measurable chemiluminescence after stimulation, and this was normally between $2x10^5$ to $2x10^6$ cells/ml. Leukocytes at the appropriate concentrations were mixed with PBS, luminol and test microorganisms (at about $2x10^9$ organism/ml) in cuvettes. All cuvettes were incubated at 37^oC for a period of 40 minutes, after which the PMA stimulus was added to all cuvettes, except the blanks. The responses were recorded before and after the addition of PMA in an LKB Wallac Luminometer connected to an IBM PC computer. The assay results were printed out in a graphical and tabulated form, and chemiluminescence emission was measured in millivolts (mv). Each sample was measured in duplicate and on two separate occasions.

2.8 Phagocytosis of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains by human PMNLs

2.8.1 Bacterial strains and growth conditions

Leukotoxic and non-leukotoxic Type and freshly isolated strains of A.actinomyctemcomitans, H.aphrophilus and Capnocytophaga species were selected for use in this part of the study (Table 2.4). Bacterial suspensions were prepared as described in Section 2.7.1, and final suspensions were diluted to an absorbance of 1.0 at 550nm.

Table 2.2 Trypan blue assay-tube contents

	TEST	NEGATIVE CONTROL	POSITIVE CONTROL
HL60(5x 10^6 - 1 x 10^7 /ml)	200µl	200µl	200µl
Organisms(2x10^9 - 2x10^{10} /ml)	200µl	0.00µl	0.00µl
Triton X100 (0.1%)	0.00µl	0.00µl	200µl
PBS	0.00µl	200µl	0.00µl

Table 2.3 Chemiluminescence assay-tube contents

	BLANK	PMA CONTROL	TEST
Luminol (10^{-4} M in DMSO)	200µl	200µl	200µl
PMA (10mg /ml)	0.00µl	200µl	200µl
PMNLs (2x10^5- 2x 10^6 /ml)	100µl	100µl	100µl
Organisms(2x10^7 o- 2x10^9 /ml)	0.00µl	0.00µl	100µl
PBS	700µl	500µl	400µl

2.8.2 Sera for opsonization

Blood was collected from 2 healthy donors, allowed to clot at room temperature for 90 minutes and then centrifuged at 500 g for 10 minutes. The serum was removed carefully and added in 1ml amounts to sterile plastic bijou bottles, after which part of the serum was heat inactivated for 30 minutes at 56^{O}C using a heating module (Reacti-Therm heating module, Pierce and Warriner UK Limited, Chester), and other parts remained unheated. Tubes were labelled then stored at -20^{O}C until required.

Table 2.4 List of H.aphrophilus, A.actinomycetemcomitans and Capnocytophaga strains used for the phagocytosis experiments

STRAIN	SOURCE	STRAIN	SOURCE
Ha 40	GDH	Aa 115	GDH
Ha 44	GDH	Aa 120	GDH
Ha 46	GDH	Aa E24	GDH
Ha 79	GDH	Aa E79	ATCC29523
Ha 80	GDH	Aa E80	ATCC29524
Ha 82	GDH	Aa E81	Y 4
Ha 83	GDH	Aa E83	NCTC9710
Ha 97	GDH	Aa E88	JP2
Ha 104	GDH	Cap 13	GDH
Ha 5886	NCTC	Cap 52	GDH
Aa 100	GDH	Cap 90	NCTC11153

2.8.3 Opsonization of bacterial suspensions

In order to opsonize the bacteria, 0.9ml of the cell suspension was mixed with 0.1ml of human serum. Tubes were incubated at 37°C for 30 minutes in an incubator, placed on ice and then immediately used in experiments. To examine whether the opsonization was essential in the phagocytosis process, both opsonized and unopsonized bacterial suspensions were tested at the same time.

2.8.4 Phagocytosis assay

The constituent parts of the phagocytosis assay are listed in Table 2.5. Two hundred microlitres of opsonized or non-opsonized bacteria and 200µl of human polymorphonuclear leukocytes (PMNLs) at $1x10^6$ cells/ml were mixed together in polystyrene cuvettes (Clinicon). An equal volume of luminol(200µl)(10^{-4} M in DMSO) was then added and the reaction mixtures were made up to 1000µl by adding appropriate volumes of PBS. After 40 minutes aerobic incubation at 37°C, 200µl PMA (10mg /ml) was added.

The cuvettes, were placed in a LKB Wallac Luminometer, at 37°C, and the initial chemiluminescence was measured. Almost immediately PMA was added to all tubes except for the blanks to measure the residual activity of the PMNLs. The responses were recorded in an LKB Wallac Luminometer connected to an IBM PC computer. Results were printed out in a graphical and tabulated form, chemiluminescence was measured in millivolts (mv), and each sample was measured in duplicate and on two separate occasions.

Table 2.5 Tube contents (phagocytosis study)

	BLANK	**PMA CONTROL**	**TEST**
LUMINOL 10^{-4}M	200µl	200µl	200µl
PMA 10 mg/ ML	0.00µl	200µl	200µl*
Human PMNLs $1x10^{6}$	200µl	200µl	200µl
Opsonized or unopsonized OD 1.0	0.00µl	0.00µl	200µl
PBS	600µl	400µl	200µl

* PMA was added after the initial incubation period as described in the text.

2.9 The cytotoxicity of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains for a human skin fibroblast cell line(IBR3)

2.9.1 Human skin fibroblast cell line-IBR3

Cells of the IBR3 human skin fibroblast cell line were obtained from the European Collection of Animal Cell Cultures (ECACC). Cells were derived originally from a skin biopsy from a normal adult male. Cells were received as a monolayer in a tissue culture flask with Eagle's Minimum Essential Medium with Earl's balanced salts (EMEM-EBSS). Cells were handled as recommended by the supplier. Briefly, the flask was checked under an inverted microscope upon arrival and incubated overnight at 37^{O}C in 5% CO_2 in air (CO_2 Incubator, Leec Ltd, Nottingham, England). After incubation, the medium was decanted, and cells were trypsinized by adding 0.25% trypsin in EDTA (Gibco Life Technologies Ltd, Paisley, Scotland). Trypsin was added in volumes sufficient to wet the monolayer(1.5-2.0ml), and the flask was incubated at 37^{O}C in 5% CO_2 in air, for 5-15 minutes. When the cells became detached they were dispersed by pipetting and placed in a centrifuge tube containing about 1ml of foetal calf serum. The tubes

were centrifuged at 90 g for 8 minutes (MSE Minor S Centrifuge, Crawley, Sussex, England), the supernatant discarded and the pelleted cells resuspended in fresh tissue culture medium (EMEM-EBSS)(Gibco Life Technologies Ltd, Paisley, Scotland). The medium was supplemented with 1% penicillin/ streptomycin mixture and 1% non-essential amino acids (Gibco Life Technologies Ltd, Paisley, Scotland). Cells were counted using a haemocytometer as described in Section 2.7.2, and seeded at about 1.5x 10^5 cells/ml. Flasks were incubated at 37^{o}C in 5% CO_2 in air (CO_2 incubator), and were initially passaged every 3-4 days. However, when the cells were not required they were passaged weekly. Stock cultures were prepared after the second passage of cells, and stored at -80^{o}C in a freezing medium containing the cryoprotective agent dimethyl sulphoxide (90% growth medium + 10% dimethyl sulphoxide).

For the cytotoxicity experiments, cells were used between passages 6 and 18. The growth medium was changed every second day, and flasks were checked for contamination and deterioration on an almost daily basis. Single cell suspensions of trypsinized

fibroblasts were seeded into wells of 96 well flat bottomed microtitre plates (Nunc, Intermed, Denmark) at a concentration of 5-6 $x10^3$ cells/100µl.

2.9.2 Bacterial strains and growth conditions

The microorganisms used in this part of the study are listed in Tables 3.1, 3.2 and 3.3. Stock cultures were revived from Protect beads stored at -70°C by inoculation onto CBA plates. They were subcultured onto fresh CBA and incubated at 37°C, in 5% CO_2 in air for two days before use in assays.

2.9.3 Preparation of whole bacterial cells

In preliminary experiments the growth on blood agar plates was harvested on sterile cotton wool swabs and transferred into 20 ml of tryptic soy broth (Gibco Life Technologies Ltd, Paisley, Scotland). Following incubation for 18 hours at 37°C in 5% CO_2 in air, the cells were sedimented by centrifugation (1500 g for 10 minutes) then resuspended and washed twice with PBS. Six ml of PBS were added to the pellet to produce the final bacterial cell suspension. Viable counts were

determined by serially diluting the bacterial suspensions, and plating out with a spiral plater (Figure 2.1). Plates were incubated at $37^{O}C$ in 5% CO_2 in air for 3 days (CO_2 incubator). Determination of viable counts was undertaken as described in Section 2.1. The number of viable bacteria present in all the suspensions was routinely performed and if the cell numbers were found to be less than $4x10^9$ to $1x10^{10}$ cells/ml, the experimental results obtained were excluded.

2.9.4 Preparation of cell free extracts from bacteria

Bacteria were cultured on CBA, as described in Section 2.9.2, and harvested from two CBA plates with sterile cotton wool swabs into 2ml of sterile distilled water, followed by vortex mixing for 20 seconds. Ballotini beads, size 12 (Jencons Scientific Ltd, Leighton Buzzard, England) (0.3 g) were placed in glass containers and the bacterial suspension added. The containers were fitted in a Mickle High Speed Vibratory Tissue Disintegrator (The Mickle Laboratory Engineering Co Ltd, Surrey, England) (Figure 2.6) for 1 hour, after which the containers were centrifuged at 1500 g for 10 minutes to separate beads and debris. Cleared

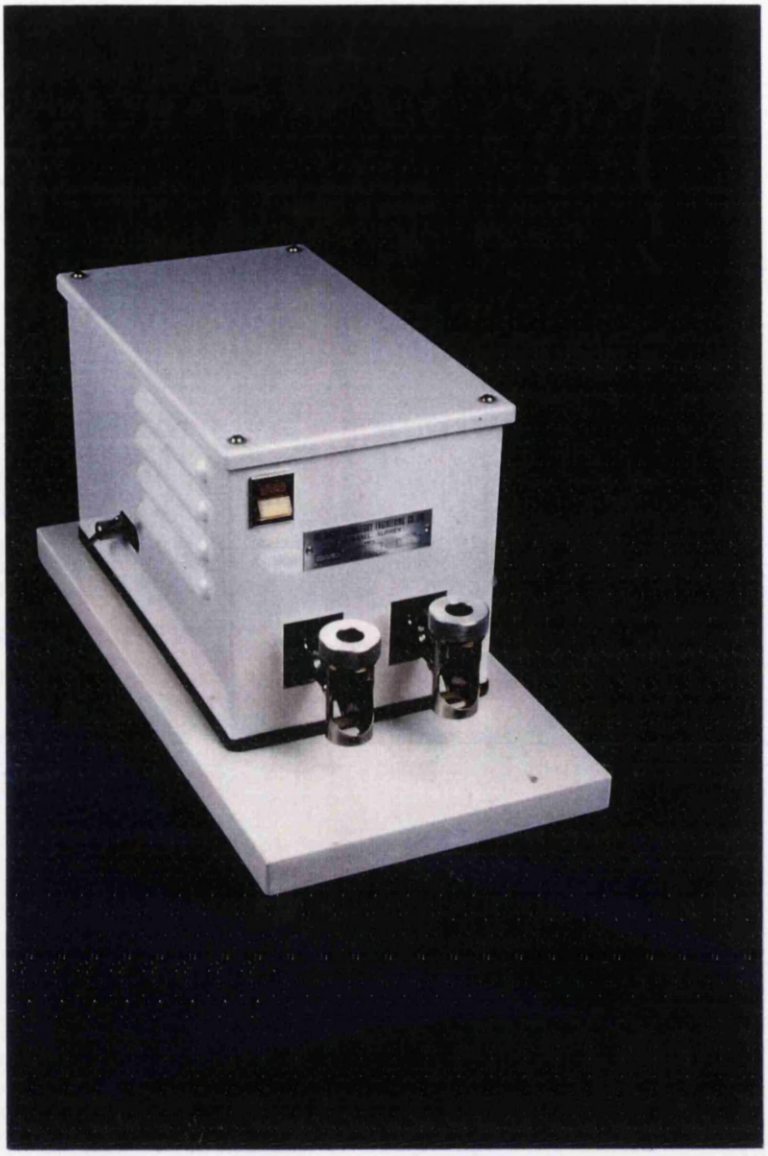


Figure 2.6 The Mickle high speed vibratory tissue disintegrator

supernatants were filtered through Minisart filters of 0.2μm pore diameter (Minisart NML, Sartorius, Sartorius AG, Gottingen, Germany). The filtrates were designated cell-free extracts and were used in the cytotoxicity assays.

2.9.5 The Bioassay

Mosmann (1983) described a rapid colourimetric assay for mammalian cell survival and proliferation, using a tetrazolium salt. The basis of the same method was used to test the cytotoxicity of A.actinomycetemcomitans, H.aphrophilus and Capnoctytophaga species on a cultured human skin fibroblast cell line (IBR3). The experimental work for this section of the thesis was carried out in two parts. The first part was carried out with whole viable bacterial cells, and was basically a pilot study. The second part contained the main experimental study and was performed using bacterial cell-free extracts. Plates for the assay were prepared as described in Section 2.9.1. The plates were incubated at $37^{O}C$ in 5% CO_2 in air(CO_2 incubator) for two days and the medium changed daily. After 48 hours, three different volumes of whole bacterial cells (50, 100,150μl) were added in

triplicate and then all volumes were made up to 200µl by adding appropriate volumes of growth medium. Plates were then incubated for 24 hours at $37^{O}C$ in 5% CO_2 in air. After incubation the contents of the plate were decanted aseptically and 100µl of fresh growth medium were added to all wells, followed by 10µl of freshly prepared 3-[4,5-methylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Company, Fancy Road, Dorset, England). MTT was prepared by dissolving powder at a concentration of 5mg/ml in PBS and then filtering to remove all undissolved materials.

The plates were incubated for a further 4 hours, after which their contents were decanted and 100µl of dimethyl sulphoxide (DMSO) were added to all wells to solubilize any formazan crystals that may have formed. The plates were mixed by gentle agitation on an orbital shaker for 5-10 minutes and plates were read as described at the end of this section. During the course of these experiments using whole bacterial cells, organisms were observed microscopically to adhere to the cultured fibroblasts.

In the second part of these experiments, cell-free extracts were used instead of whole bacterial cells.

The same procedures were followed as with the whole cells except that a washing step using PBS (pH 7.2) was introduced after the fibroblasts had been incubated with the bacterial extracts for 24 hours. This was performed by decanting the plate's contents then washing by adding 100µl of PBS to all wells and decanting the contents of the plates. This step was repeated 3 times. The cytotoxicity or interference with fibroblast proliferation was expressed as percentage reduction in the optical density obtained with control wells containing cultured fibroblasts only. The optical density of the plates was measured at 570 nm by using a Dynatech MR5000. The percentage reduction in the metabolic activity of fibroblasts was calculated by using the following formula:

$$\%\text{inhibition} = 100 - \frac{\text{Optical density of test}}{\text{Optical density of control}} \times 100$$

2.10 Human Immunoglobulin A (IgA) protease production by A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species.

2.10.1 Bacterial strains and culture conditions

The microorganisms tested are listed in Tables 3.1, 3.2, 3.3. They were revived as described in Section 2.4, then cultured on CBA plates for 3 days at 37 °C in 5% CO_2 in air. H.influenzae (NCTC 12194) was included in these experiments as a positive control since it is known to produce a protease that specifically cleaves human IgA1 to Fab and Fc fragments (Kilian et al., 1980). H.influenzae (NCTC 12194) was revived from a lyophilized culture by hydration with FAB, then inoculation onto chocolate agar plates. The plates were incubated at 37°C in 5% CO_2 in air (CO_2 incubator) for 48 hours, then subcultured onto fresh media and prepared for storage as described in Section 2.3. When required H.influenzae was revived as described in Section 2.4, except that chocolate agar plates were used in place of CBA plates. H.influenzae cultures were discarded after they had been subcultured on more than 3-4 occasions.

2.10.2 Detection of cleavage of human Immunoglobulin A (IgA)

The screening of bacterial IgA protease activity was performed according to methods described by Kilian (1981) and Cole and Hale (1991) with slight modifications. Briefly, human myeloma serum was obtained from Dr I. Franklyn of the Glasgow Royal Infirmary and used as the substrate in IgA protease assays for screening A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga. However, commercial human secretory IgA (SIgA)(Cappel Organon Teknika Corporation, 100 AKZO Avenue, Durham, NC 27704) was also used as the substrate when Capnocytophaga isolates were tested. Human myeloma serum was diluted 1:300 in PBS (pH 7.2) and then two loopfuls of each test organism grown on either CBA or chocolate agar (H.influenzae) were emulsified in 70µl of diluted serum in an Eppendorf tube. All assay tubes were incubated at 37^{o}C for 48 hours, after which bacteria were sedimented by centrifugation. The cleared supernatants were separated and stored at -20^{o}C, until further analysis. Cleavage products of IgA and SIgA by bacterial proteases were detected by using sodium dodecyl

sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting techniques.

2.10.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), was performed according to Laemmli (1970). Slab gels were prepared by mixing 12.5 ml of 30% acrylamide/0.8% bis-acrylamide(w/v), 7.5ml of (1.5 M) Tris buffer (pH 8.8) and 10 ml of distilled water. Dissolved oxygen was removed under vacuum, after which 0.3ml of 10% SDS(w/v), 15 µl of N,N,N',N'-tetramethyl-ethylenediamine (TEMED), and 150µl of 10% (w/v) ammonium persulphate in distilled water(freshly prepared) were added to initiate polymerization. The mixtures were poured as 1.5 mm x 12.5 cm x 14.5 cm slab gels. A layer of 1.5ml isopropanol was pipetted onto the gel and removed after polymerization. A 5% polyacrylamide stacking gel was prepared by mixing 1.5 ml of 30% acrylamide/0.8% bis-acrylamide(w/v), 2.5 ml of stacking gel buffer (0.5 M) Tris pH 6.8, and 6.0 ml of distilled water. Dissolved oxygen was removed under vacuum, after which 100µl of 10% SDS, 10 µl of TEMED, and 50µl of 10% ammonium persulphate were added. The

mixtures were layered onto the 12.5% gels and the sample comb was inserted. After polymerization, the comb was removed carefully. Samples were prepared as follows. Cleared supernatants prepared as described in Section 2.10.2, were diluted (1:1) with sample buffer containing 0.125 M Tris buffer (pH 6.8), 2% SDS (w/v), 5% 2 mercaptoethanol(v/v), 10% glycerol (v/v), and 0.05% bromophenol blue (w/v), and heated at 100^{O}C for 4 minutes. Molecular weight markers (BDH Laboratory Supplies, Poole, England) which included cytochrome C (12300 daltons), myoglobin (17200 daltons), carbonic anhydrase (30000 daltons), ovalbumin (42700 daltons), albumin (66200 daltons) and ovotransferrin (78000 daltons), were dissolved in the sample buffer and included as an track in all experiments. Each sample(40-50 µl) was loaded into the appropriate well, and the gel was placed in a vertical electrophoresis tank (Hoefer Scientific Instruments, San Francisco, California, USA). Both lower and upper tanks were filled with tank buffer (25 mM Tris (Sigma Chemical Co), 192 mM glycine and 0.1% SDS w/v). Electrophoresis was carried out at 30 milliampere (mA) per gel at 10^{O}C, and took about 4-4.5 hours to complete.

Completed gels were then removed carefully and stained by soaking overnight in a staining solution composed of 0.1% Coomassie brilliant blue R250(BDH Laboratory Supplies, Poole, England) in 50% methanol (v/v) (Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, England) and 10% acetic acid (Prolabo, Rue Pélée, Paris, France) in water. After staining, gels were rinsed with water and soaked in destaining solution composed of 50% methanol and 10% acetic acid in water. Gels were then dried or stored in sealed bags until photographed.

2.10.4 Immunoblotting

Polyacrylamide gels were run as described previously (Section 2.10.3), then removed and soaked in distilled water for a few minutes. Filter paper (Whatman 3MM) and a sheet of nitrocellulose membrane were cut to the size of the gel (12.5 cm x 14.5 cm). Six sheets of filter paper and one sheet of nitrocellulose membrane were used per gel. The nitrocellulose membrane was soaked in water for a few minutes and the absorbent sheets were wetted by soaking in blotting buffer composed of Tris 48 mM, glycine 39 mM, SDS 0.037% (v/v), and 20% methanol in distilled water. Three sheets of absorbent

paper soaked in transfer buffer were placed on the bottom plate of the blotting apparatus (anode) (LKB Produkter AB, Bromma, Sweden) followed by one nitrocellulose membrane (Whatman Ltd,) wetted in distilled water. The polyacrylamide gel, slightly wetted in water, was placed carefully on top of the nitrocellulose membrane and the remaining 3 absorbent sheets were placed on top of the polyacrylamide gel. Air bubbles were removed from the assembled sandwich by rolling a pipette over the sandwich. Excess buffer and water on the plate surrounding the sandwich were removed, and the upper electrode (the cathode) was placed carefully on top of the stack. The electrodes were connected and transfer undertaken at 0.8 mA per cm^2 (140 mA per gel), which took between 45-60 minutes to complete. After transfer the nitrocellulose membrane was removed and processed as described below.

2.10.5 Staining of blots

Blots on the nitrocellulose membrane were removed after transfer was completed and stained for several minutes with 0.5% (w/v) Ponceau S stain (Hopkin and Williams Ltd, Chadwell Heath, Essex, England) in 1% acetic acid to reveal molecular weight markers. The blots were

washed with distilled water and blocked for one hour using a blocking buffer composed of phosphate buffered saline (PBS), 0.15% Tween 20 (T20), and 10% dried milk. After blocking, blots were incubated for one hour in blotting buffer (PBS, 0.15% T20, 10% dried milk) containing primary antibody diluted 1:200 (goat anti human SIgA, α chain specific) (Cappel Laboratories). After incubation, blots were washed three times for 10-15 minutes with PBS containing 0.15% T20 and 1% dried milk. After the final wash, blots were incubated in blotting buffer containing the secondary antibody diluted 1:2000, which was a peroxidase-conjugated rabbit anti goat immunoglobulin (Dako, DK-2600 Glostrup, Denmark) for one hour at room temperature. After incubation with the secondary antibody, blots were washed three times for 10-15 minutes with PBS containing 0.15% T20 and 1% dried milk. Finally, blots were developed using a fresh solution of 98 ml PBS containing 30 mg diaminobenzidine (Sigma Chemical Company, Fancy Road, Poole, Dorset, England), 2ml of 1% aqueous solution of cobalt chloride and 100 µl of hydrogen peroxide (Sigma Chemical Company), for several minutes, until the bands became clear. Blots were then

washed with distilled water and stored in the dark until photographed.

Chapter 3

Results

3.1 Isolation of H.aphrophilus, A.actinomycetemcomitans, and Capnocytophaga species from subgingival plaque samples

A total of 170 subgingival plaque samples from 49 patients with different forms of periodontal disease were received and analysed as described in section 2.1. H.aphrophilus was isolated from 59 (35%) of the 170 test sites. A.actinomycetemcomitans was isolated from only 11 samples (6%). Of the 170 sites tested, 90 sites (53%) were positive for Capnocytophaga species. All isolated organisms were stored as described in section 2.3. A selection of these clinical isolates together with isolates from the Glasgow Dental Hospital (GDH) collections and Type strains were used for the different experiments described in the following sections. Tables 3.1, 3.2 and 3.3 contain a list of all isolates used.

Table 3.1 List of Actinobacillus actinomycetemcomitans strains used in this study

STRAIN Number	SOURCE	STRAIN Number	SOURCE
Aa 33	GDH	Aa E 35	GDH
Aa 34	GDH	Aa E 54	GDH
Aa 94	GDH	Aa E 56	GDH
Aa 96	GDH	Aa E 57	GDH
Aa 100	GDH	Aa E74	GDH
Aa 112	GDH	Aa E75	GDH
Aa 113	GDH	Aa E76	GDH
Aa 115	GDH	Aa E79	ATCC29523
Aa 120	GDH	Aa E80	ATCC29524
Aa E1	GDH	Aa E81	Y 4
Aa E8	GDH	Aa E83	NCTC9710
Aa E24	GDH	Aa E88	JP2
Aa E33	GDH		

Table 3.2 List of H.aphrophilus strains used in this study

STRAIN NUMBER	SOURCE	STRAIN NUMBER	SOURCE
Ha 40	GDH	Ha 76	GDH
Ha 41	GDH	Ha 77	GDH
Ha 42	GDH	Ha 78	GDH
Ha 43	GDH	Ha 79	GDH
Ha 44	GDH	Ha 80	GDH
Ha 46	GDH	Ha 82	GDH
Ha 58	GDH	Ha 83	GDH
Ha 59	GDH	Ha 86	GDH
Ha 60	GDH	Ha 92	GDH
Ha 61	GDH	Ha 97	GDH
Ha 62	GDH	Ha 99	GDH
Ha 63	GDH	Ha 104	GDH
Ha 67	GDH	Ha 109	GDH
Ha 68	GDH	Ha 110	GDH
Ha 75	GDH	Ha 5886	NCTC

Table 3.3 List of Capnocytophaga species used in this study

STRAIN NO	SOURCE	STRAIN NO	SOURCE
Cap 52	GDH	Cap 174	DH
Cap 53	GDH	Cap 176	DH
Cap 72	GDH	Cap 179	DH
Cap 81	GDH	Cap 183	DH
Cap 84	GDH	Cap 90	CTC11153
Cap 170	GDH		

3.2 Biochemical characteristics of the freshly isolated strains of H.aphrophilus and A.actinomycetemcomitans

3.2.1 Biochemical characteristics of the freshly isolated A.actinomycetemcomitans strains

The biochemical characteristics were obtained using the API 20 A System, which provided a simple, and reproducible way of identification.

All A.actinomycetemcomitans strains fermented glucose, maltose and mannose. Variable results were obtained with mannitol and xylose. No strains of A.actinomycetemcomitans fermented lactose, saccharose, salicin, arabinose, glycerol, cellobiose, melezitose, raffinose, sorbitol, rhamnose or trehalose. A.actinomycetemcomitans strains did not produce indole or urease, and were unable to hydrolyse gelatine and aesculin. Catalase was produced by all strains. A summary of the results obtained with a representative number of fresh isolates of A.actinomycetemcomitans strains is presented in table 3.4. Type strains of A.actinomycetemcomitans yielded very similar results compared to the fresh isolates in all biochemical tests. A typical API 20A profiles obtained with

A.actinomycetemcomitans type strains are summarised in Table 3.4A.

3.2.2 Biochemical characteristics of H.aphrophilus strains

The biochemical characteristics were obtained using the API 20 A System which provided a simple and reproducible way of identification. All H.aphrophilus strains fermented glucose, lactose, saccharose and maltose. A number of H.aphrophilus strains fermented mannitol, salicin, xylose, arabinose, glycerol, cellobiose, rhamnose, sorbitol, and melezitose. None of the strains produced urease, formed indole, or hydrolysed gelatine or aesculin. Of 24 strains, 21 (87%) fermented mannose, 20 (83%) fermented raffinose and 19 (79%) fermented trehalose. All strains were catalase negative.

The biochemical characteristics of H.aphrophilus strains are listed in Table 3.5.

Table 3.4 Biochemical characteristics of A.actinomycetemcomitans

Test	Reaction	Result	% positive
Indole	Indole formation	Negative	0%
Urea	Urease production	Negative	0%
Glucose	Fermentation	Positive	100%
Mannitol	Fermentation	Variable	20%
Lactose	Fermentation	Negative	0%
Saccharose	Fermentation	Negative	0%
Maltose	Fermentation	Positive	100%
Salicin	Fermentation	Negative	0%
Xylose	Fermentation	Variable	80%
Arabinose	Fermentation	Negative	0%
Gelatine	Hydrolysis	Negative	0%
Aesculin	Hydrolysis	Negative	0%
Glycerol	Fermentation	Negative	0%
Cellobiose	Fermentation	Negative	0%
Mannose	Fermentation	Positive	100%
Melezitose	Fermentation	Negative	0%
Raffinose	Fermentation	Negative	0%
Sorbitol	Fermentation	Negative	0%
Rhamnose	Fermentation	Negative	0%
Trehalose	Fermentation	Negative	0%
Catalase	Catalase production	Positive	100%

Table 3.4 A Biochemical characteristics of A.actinomycetemcomitans Type strains

Test	Reaction	Result	% positive
Indole	Indole formation	Negative	0%
Urea	Urease production	Negative	0%
Glucose	Fermentation	Positive	100%
Mannitol	Fermentation	Variable	60%
Lactose	Fermentation	Negative	0%
Saccharose	Fermentation	Negative	0%
Maltose	Fermentation	Positive	100%
Salicin	Fermentation	Negative	0%
Xylose	Fermentation	Variable	60%
Arabinose	Fermentation	Negative	0%
Gelatine	Hydrolysis	Negative	0%
Aesculin	Hydrolysis	Negative	0%
Glycerol	Fermentation	Negative	0%
Cellobiose	Fermentation	Negative	0%
Mannose	Fermentation	Positive	100%
Melezitose	Fermentation	Negative	0%
Raffinose	Fermentation	Negative	0%
Sorbitol	Fermentation	Negative	0%
Rhamnose	Fermentation	Variable	60%
Trehalose	Fermentation	Negative	0%
Catalase	Catalase production	Positive	100%

Table 3.5 Biochemical characteristics of H.aphrophilus

Test	Reaction/enzyme	result	% positive
Indole	Indole formation	Negative	0%
Urea	Production of urease	Negative	0 %
Glucose	Fermentation	Positive	100 %
Mannitol	Fermentation	Negative	0 %
Lactose	Fermentation	Positive	100 %
Saccharose	Fermentation	Positive	100 %
Maltose	Fermentation	Positive	100 %
Salicin	Fermentation	Negative	0 %
Xylose	Fermentation	Negative	0 %
Arabinose	Fermentation	Negative	0 %
Gelatine	Hydrolysis	Negative	0 %
Esculin	Hydrolysis	Negative	0 %
Glycerol	Fermentation	Negative	0 %
Cellobiose	Fermentation	Negative	0 %
Mannose	Fermentation	Variable	87 %
Melezitose	Fermentation	Negative	0 %
Raffinose	Fermentation	Variable	83 %
Sorbitol	Fermentation	Negative	0 %
Rhamnose	Fermentation	Negative	0 %
Trehalose	Fermentation	Variable	79 %
Catalase	Catalase production	Negative	0 %

3.3 Sensitivity to antibacterial agents

All A.actinomycetemcomitans strains were sensitive to tetracycline, 92% were sensitive to ampicillin (8% being moderately sensitive) 12% of strains were sensitive to penicillin, (8% moderately sensitive) and 80% were sensitive to erythromycin (20% being moderately sensitive) (Table 3.6).

All H.aphrophilus strains tested were sensitive to tetracycline and ampicillin, 87% were fully sensitive to penicillin (with 13% being moderately sensitive) while 73% were fully sensitive to erythromycin (27% moderately sensitive) (Table 3.7).

All Capnocytophaga species were sensitive to tetracycline, ampicillin and erythromycin; 50% were sensitive to penicillin and 50% were moderately sensitive to penicillin (Table 3.8).

Table 3.6 Antimicrobial sensitivity results for 25 strains of

A.actinomycetemcomitans

Antibiotic*	% sensitive strains	% moderately sensitive strains	% resistant strains
Tetracycline	100 %	0 %	0 %
Ampicillin	92 %	8 %	0 %
Penicillin	12 %	8 %	80 %
Erythromycin	80 %	20 %	0 %

Table 3.7 Antimicrobial sensitivity results for 30 strains of H.aphrophilus

Antibiotic*	% sensitive strains	% moderately sensitive strains	% resistant strains
Tetracycline	100 %	0 %	0 %
Ampicillin	100 %	0 %	0 %
Penicillin	87 %	13 %	0 %
Erythromycin	73 %	27 %	0%

* Supplied by Mast Laboratory, Merseyside, England.

Table 3.8 Antimicrobial sensitivity results for 10 strains of

Capnocytophaga species

Antibiotic*	**% sensitive strains**	**% moderately sensitive strains**	**% resistant strains**
Tetracycline	100 %	0 %	0 %
Ampicillin	100 %	0 %	0 %
Penicillin	50 %	50 %	0 %
Erythromycin	100 %	0 %	0 %

* Supplied by Mast Laboratory, Merseyside, England

3.4 Haemagglutination activity of H.aphrophilus, A.actinomycetemcomitans and Capnocytophaga species

3.4.1 Haemagglutination by A.actinomycetemcomitans

Of the 25 A.actinomycetemcomitans strains tested, 18 (72%) showed positive haemagglutination. Most of the strains tested gave low titres. However, about 30% of the tested strains had a titre of 8 (Table 3.9).

3.4.2 Haemagglutination by H.aphrophilus

Of the 30 H.aphrophilus strains tested for their ability to agglutinate group A and group O human erythrocytes, 28 (93%) showed positive haemagglutination. However, the titres (calculated as the reciprocal of the highest dilution giving positive haemagglutination) were low (Table 3.10). There was no difference in the pattern of haemagglutination for type A and type O red cells. All strains were tested on at least 4 occasions and even though some titres varied in different assays (for example a certain strain may give a titre of 2 in one assay and a titre of 4 in a second assay), the results obtained were consistent in terms of positives and negatives. The majority of tested

strains (16 strains) gave a titre of 4, 10 strains gave a titre of 2, while, only 2 strains gave a titre of 8. The positive control yielded a consistent titre of 64.

3.4.3 Haemagglutination by Capnocytophaga species

Ten strains of Capnocytophaga species were tested for their ability to haemagglutinate group A and group O human erythrocytes. Of the 10 strains tested, 3 strains had a titre of 64 with both blood groups, 2 strains had a titre of 32, 1 strain of 16 and 4 strains of 8. Generally the titres obtained with most of the Capnocytophaga strains were higher than those obtained with other bacteria (H.aphrophilus and A.actinomycetemcomitans) included in this study. The results obtained with Capnocytophaga strains are listed in Table 3.11.

Table 3.9 Haemagglutination activity of 25 strains of A.actinomycetemcomitans with human groupA and groupO cells

STRAIN	Haemagglutination titre*		STRAIN	Haemagglutination titre*	
	Group A	Group O		Group A	Group O
Aa 33	8	8	Aa E35	4	4
Aa 34	2	2	Aa E54	4	4
Aa 94	8	8	Aa E56	8	8
Aa 96	4	4	Aa E57	4	4
Aa 100	8	8	Aa E74	2	2
Aa 112	8	8	Aa E75	0	0
Aa 113	8	8	Aa E76	0	0
Aa 115	8	8	Aa E79	0	0
Aa 120	8	8	Aa E80	0	0
Aa E1	4	4	Aa E81	0	0
Aa E8	4	4	Aa E83	0	0
Aa E24	0	0	Aa E88	0	0
Aa E33	8	8	Negative control	0	0
E.coli	64	64			

* Titre was calculated as the reciprocal of the highest dilution that gave positive haemagglutination.

Table 3.10 Haemagglutination activity of 30 strains of H.aphrophilus with human groupA and O cells

STRAIN	Haemagglutination titre*		STRAIN	Haemagglutination titre*	
	Group A	Group O		GroupA	Group 0
Ha 40	4	4	Ha 75	4	4
Ha 41	4	4	Ha 76	2	2
Ha 42	4	4	Ha 77	2	2
Ha 43	4	4	Ha 78	2	2
Ha 44	4	4	Ha 79	2	2
Ha 46	4	4	Ha 80	2	2
Ha 58	4	4	Ha 82	4	4
Ha 59	4	4	Ha 83	4	4
Ha 60	4	4	Ha 86	4	4
Ha 61	2	2	Ha 92	0	0
Ha 62	2	2	Ha 97	4	4
Ha 63	0	0	Ha 99	4	4
Ha 67	8	8	Ha 104	4	4
Ha 68	8	8	Ha 109	2	2
Ha 5886	2	2	Ha 110	2	2
E.coli	64	64	Negative control	0	0

* Titre was calculated as the reciprocal of the highest dilution that gave positive haemagglutination.

Table 3.11 Haemagglutination activity of 10 strains of Capnocytophaga species with human group A and group O cells

STRAIN	Haemagglutination titre*		STRAIN	Haemagglutination titre*	
	Group A	Group O		Group A	Group O
Cap 52	32	32	Cap 174	8	8
Cap 53	64	64	Cap 176	8	8
Cap 72	64	64	Cap 179	8	8
Cap 81	16	16	Cap 183	64	64
Cap 170	8	8	Cap 90	32	32
E.coli	64	64	Negative control	0	0

* The titre was calculated as the reciprocal of the highest dilution that gave positive haemagglutination

3.4.4 Haemagglutination inhibition of Capnocytophaga species

The ability of carbohydrates to inhibit haemagglutination was tested with some haemagglutination-positive strains from each group of bacteria. A number of H.aphrophilus and A.actinomycete-mcomitans strains were tested in preliminary experiments and because of the low titres of haemagglutination obtained with these strains the results of these experiments were not conclusive. Therefore, the experiment was restricted to the use of mannose only and it was tested with the 3 strongly positive Capnocytophaga strains only. Of the 3 strains tested, all were mannose-sensitive as compared with E.coli positive control, since the titres of all 3 strains were reduced from 64 to 4 (Table 3.12).

Table 3.12 Inhibition of haemagglutination activity of three strains of Capnocytophaga by mannose

STRAIN	Haemagglutination titre*		Haemagglutination inhibition by mannose (titre)	
	GroupA	Group O	Group A	Group O
Cap 53	64	64	4	4
Cap 72	64	64	4	4
Cap 183	64	64	4	4
E.coli	64	2	64	2
Negative control	0	0	0	0

* The titre was calculated as the reciprocal of the highest dilution that gave positive haemagglutination.

3.5 Leukotoxic activity in H.aphrophilus, A.actinomycetetcomitans and Capnocytophaga species as measured by the Trypan blue exclusion and chemiluminescence assays

3.5.1 Leukotoxic activity of A.actinomycetemcomitans as measured by trypan blue exclusion assay

The results showing the leukotoxic activity of 25 strains of A.actinomycetemcomitans are presented in Table 3.13. Five strains (20%) were leukotoxic to HL60 cells and these included 2 (10%) out of 20 fresh oral A.actinomycetemcomitans isolates (strains E24 and 34) and 3 Type strains, (strains Y4, ATCC 29524 and JP2). Cell killing was in the range of 37% to 64%, with the Type strain JP2 being the most toxic (64% killing) and strain 34 the least toxic strain (37% killing) (Table 3.13).

Table 3.13 Leukotoxicity of A.actinomycetemcomitans strains as measured by trypan blue exclusion assay with HL60 cells

Strain	Number of HL 60 cells		
	Dead (± SEM)	Live (± SEM)	% Death
Aa 33	20.25 (1.90)	79.75(1.90)	0.00
Aa 94	26.25 (3.14)	73.75 (3.14)	6.25
Aa 96	38.50 (2.76)	61.50 (2.76)	18.50
Aa 100	35.00 (5.18)	65.00 (5.18)	15.00
Aa 112	44.00 (4.89)	56.00 (4.89)	24.00
Aa 113	45.50 (4.55)	54.50 (4.55)	25.50
Aa 115	28.00 (6.27)	72.00 (6.27)	8.00
Aa 120	36.50 (6.04)	63.50 (6.04)	16.50
Aa E1	36.00 (2.16)	64.00 (2.16)	16.00
Aa E8	34.75 (4.65)	65.25(4.65)	14.75
Aa E33	28.75 (4.30)	71.25 (4.30)	8.75
Aa E35	36.50 (6.22)	63.50 (6.22)	16.50

Table 3.13 continued

Strain	Number of HL60 cells		
	Dead (± SEM)	Live (± SEM)	%Death
Aa E54	33.25 (3.10)	66.75 (3.10)	13.25
Aa E56	33.25 (3.69)	66.75 (3.69)	13.25
Aa E57	38.00 (5.65)	62.00 (5.65)	18.00
Aa E74	37.75 (8.58)	62.25 (8.58)	17.75
Aa E75	37.50 (7.31)	62.50 (7.31)	17.50
Aa E76	33.50 (4.55)	66.50 (4.55)	13.50
Aa E79	26.75 (0.98)	73.25 (0.98)	6.75
Aa E83	41.25 (7.96)	58.75 (7.96)	21.25
Aa E80	74.00(2.00)	26.00(2.00)	54.00
Aa E81	70.00(3.60)	30.00(3.60)	50.00
Aa E88	84.00(9.34)	16.00(9.34)	64.00
Aa E24	81.00(3.85)	19.00(3.85)	61.00
Aa 34	57.00(6.72)	43.00(6.72)	37.00
PBS control	20.00(2.47)	80.00(2.47)	20.00

3.5.2 Leukotoxic activity of H.aphrophilus as measured by the trypan blue exclusion assay

The leukotoxic activity of 30 H.aphrophilus strains for HL60 cells was measured by using the trypan blue exclusion assay. Results obtained are shown in Table 3.14. None of the H.aphrophilus strains tested were leukotoxic to HL60 cells based on the criteria used in this study (ie death of 30% or more of the target cells), even at the highest bacteria/target cell ratio employed (400 bacteria/HL60 cell). The range of percentage death of target cells ranged from 0% to 18%, and results obtained with these experiments were consistent and reproducible. All tested strains were examined on two separate occasions and in duplicate. It is also important to indicate that a random selection of strains from the 3 test groups of bacteria (H.aphrophilus, A.actinomycetemcomitans and Capnocytophaga) were examined together in a single experiment by using the same population of target cell.

Table 3.14 Leukotoxicity of H.aphrophilus strains as measured by the trypan blue exclusion assay with HL60 cells

Strain	Number of HL60 cells		
	Dead (± SEM)	Live(± SEM)	% Death
Ha 40	29.25(5.21)	70.75 (5.21)	9.25
Ha 41	31.25 (0.70)	69.00 (0.70)	11.25
Ha 42	30.50 (5.28)	69.50 (5.28)	10.50
Ha 43	27.00 (2.94)	73.00 (2.94)	7.00
Ha 44	32.75 (3.50)	76.25 (3.50)	12.75
Ha 46	29.25 (7.26)	70.75 (7.26)	9.25
Ha 58	28.50 (3.78)	71.50 (3.78)	8.50
Ha 59	29.50 (5.50)	70.50 (5.50)	9.50
Ha 60	19.00 (1.56)	81.00 (1.56)	0.00
Ha 61	16.00 (2.98)	84.00 (2.98)	0.00
Ha 62	19.25 (4.75)	80.75 (4.75)	0.00
Ha 63	22.25 (3.81)	77.75 (3.81)	2.25
Ha 67	29.25 (5.21)	70.75 (5.21)	9.25
Ha 68	35.00 (2.40)	65.00 (2.40)	15.00
Ha 75	32.75 (4.14)	67.25 (4.14)	12.75
PBS control	20.00(2.47)	80.00(2.47)	20.00

Table 3.14 Continued

Strain	Number of HL 60 cells		
	Dead(± SEM)	Live (± SEM)	% Death
Ha 76	35.50 (2.42)	64.50 (2.42)	15.50
Ha 77	36.75 (5.93)	63.25 (5.93)	16.75
Ha 78	31.25 (4.43)	68.75 (4.43)	11.25
Ha 79	21.25 (2.42)	78.75 (2.42)	1.25
Ha 80	19.00 (1.56)	81.00 (1.56)	0.00
Ha 82	32.75 (3.17)	67.25 (3.17)	12.75
Ha 83	27.50 (2.42)	72.50 (2.42)	7.50
Ha 86	35.50 (8.43)	64.50 (8.43)	15.50
Ha 92	34.50 (5.00)	65.50 (5.00)	14.50
Ha 97	38.0 (2.00)	62.00 (2.00)	18.00
Ha 99	22.00 (2.53)	78.00 (2.53)	2.00
Ha 104	28.25 (6.93)	71.75 (6.93)	8.25
Ha 109	29.25 (3.41)	70.75 (3.41)	9.25
Ha 110	33.50 (6.11)	66.50 (6.11)	13.50
Ha 5886	19.75 (5.38)	80.25 (5.38)	0.00
PBS control	20.00(2.47)	80.00(2.47)	20.00

3.5.3 Leukotoxic activity of Capnocytophaga species as measured by the trypan blue exclusion assay

None of the Capnocytophaga species were leukotoxic to HL60 cells (Table 3.15).

The results outlined in this section have indicated substantial differences between these group of organisms with respect to their leukotoxicity. A.actinomycetemcomitans were the most toxic and Capnocytophaga strains the least leukotoxic.

Table 3.15 Leukotoxicity of Capnocytophaga species as measured by the trypan blue exclusion assay with HL60 cells

Strain	Number of HL 60 cells		
	Dead (± SEM)	Live (± SEM)	% Death
Cap 15	28.50 (3.34)	71.50 (3.34)	8.50
Cap 30	17.75 (2.20)	82.25 (2.20)	0.00
Cap 45	17.00 (2.74)	83.00 (2.74)	0.00
Cap 52	23.50 (2.84)	76.50 (2.84)	3.50
Cap 53	19.50 (2.60)	80.50 (2.60)	0.00
Cap 65	21.00 (1.56)	79.00 (1.56)	1.00
Cap 81	29.75(3.35)	70.25 (3.35)	9.00
Cap 90	19.50 (1.45)	80.50 (1.45)	0.00
PBS control	20.00(2.47)	80..00(2.47)	20.00

3.5.4 Leukotoxic activity of A.actinomycetemcomitans as measured by the inhibition of chemiluminescence produced by human PMNLs

Of the 25 A.actinomycetemcomitans strains tested, 5 were leukotoxic to human PMNLs as indicated by their ability to inhibit the chemiluminescence produced by PMA- stimulated human PMNLs (Figure 3.1; Table 3.16). These strains were A.actinomycetemcomitans E80, E81, E88, E24 and 34. It is clear from figures 3.1 and 3.2 that the A.actinomycetemcomitans E88 strain caused the greatest inhibition of luminol-dependent chemiluminescence compared with the positive control (from 350mv to 15mv) while A.actinomycetemcomitans strain 34 reduced the chemiluminescence response from 350 to 105 mv. The results obtained with this assay were concordant with the trypan blue exclusion assay, since the same 5 leukotoxic strains were found to be leukotoxic. The reduction in chemiluminescence ranged from 65% (strain 34) to over 95% (A.actinomycetemcomitans E88). Figure 3.2 shows the results obtained with the positive control (PMA-stimulated human PMNLs) and the negative control (human PMNLs without PMA). Representative non-leukotoxic

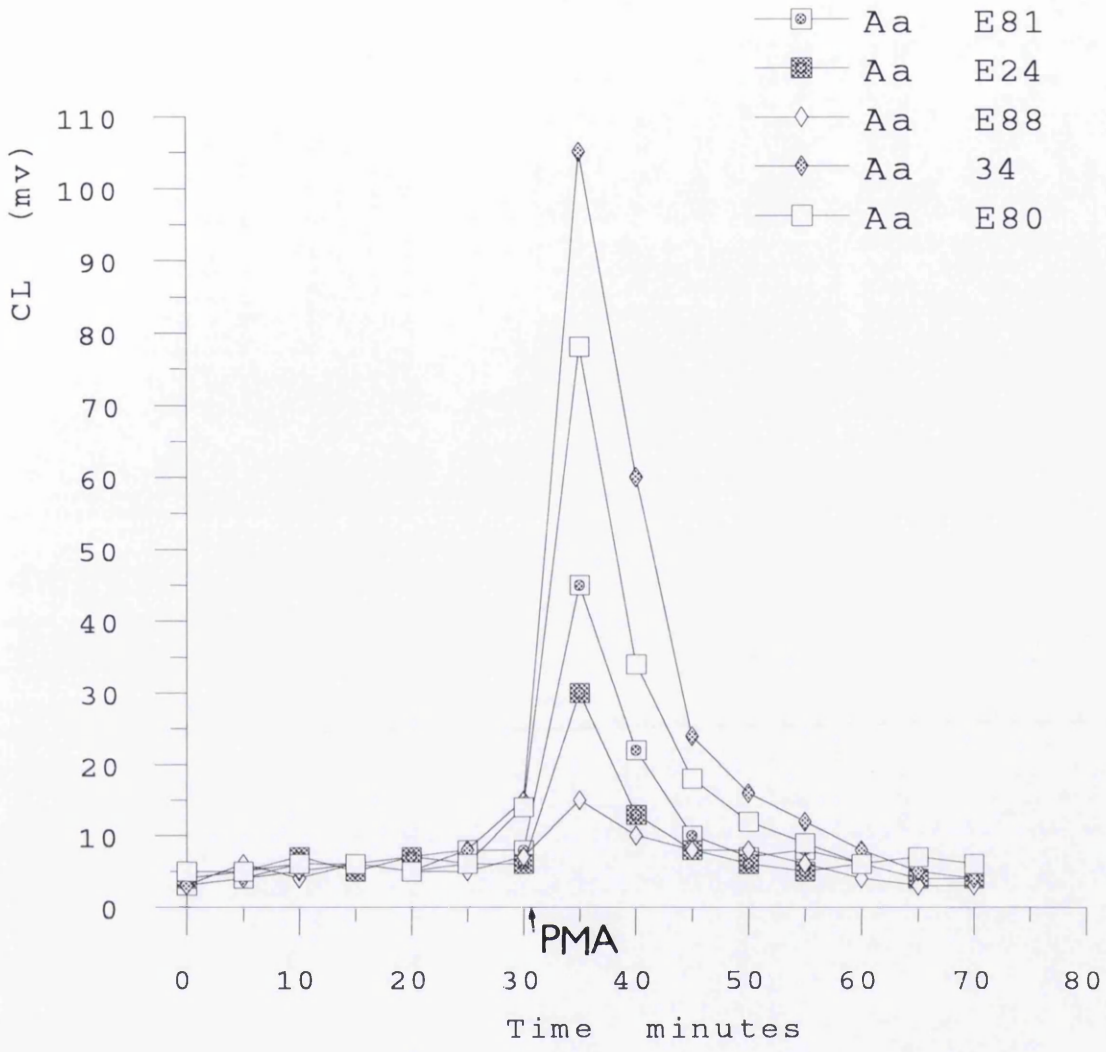


Figure 3.1 Detection of leukotoxic activity among strains of Aa as measured by the inhibition of luminol-dependent chemiluminescence. The five leukotoxic strains are shown with strain JP2 being the most toxic and strain 34 the least toxic.

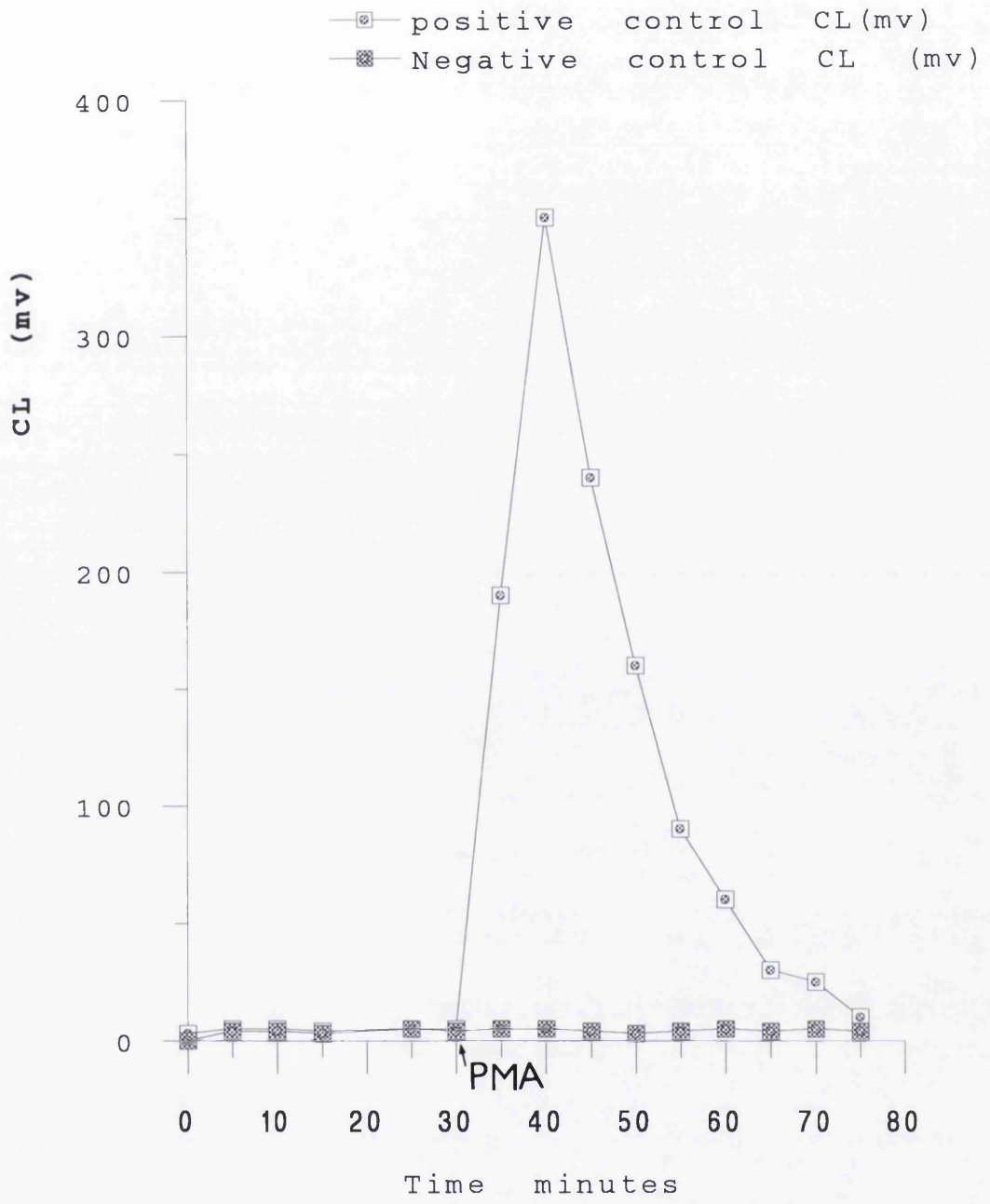


Figure 3.2 A positive and negative control for the luminol-dependent chemiluminescence inhibition assay used for the detection of leukotoxic activity among Aa, Ha and Capnocytophaga strains.

A.actinomycetemcomitans strains as measured by chemiluminescence are shown in Figure 3.3.

3.5.5 Leukotoxic activity of H.aphrophilus as measured by the inhibition of chemiluminescence produced by human PMNLs

None of the tested H.aphrophilus strains were leukotoxic as indicated by their failure to inhibit the level of chemiluminescence produced by PMA-stimulated control PMNLs. Representative non-leukotoxic H.aphrophilus strains are shown in Figure 3.4, and it is clear that no strains inhibited the chemiluminescence response of the positive control. It is also clear from Figure 3.4 that certain non-leukotoxic H.aphrophilus strains caused an increase in the level of chemiluminescence response produced by the positive control. A similar observation was recorded with the non-leukotoxic A.actinomycetemcomitans strains and may represent a response to lipopolysaccharide.

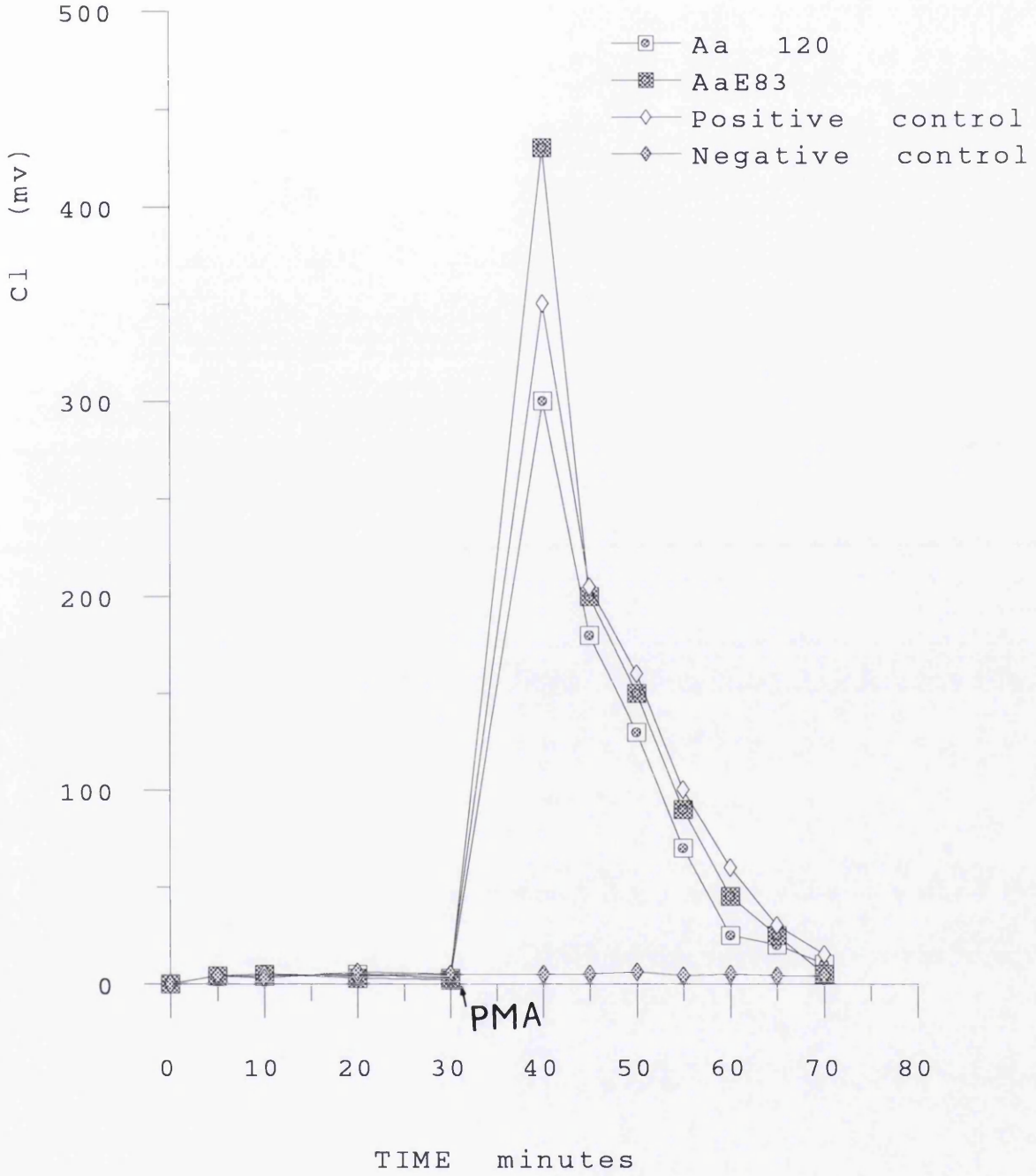


Figure 3.3 Detection of leukotoxic activity among strains of Aa as measured by the inhibition of luminol-dependent chemiluminescence. Two representative non-leukotoxic strains (E83 and 120) are shown.

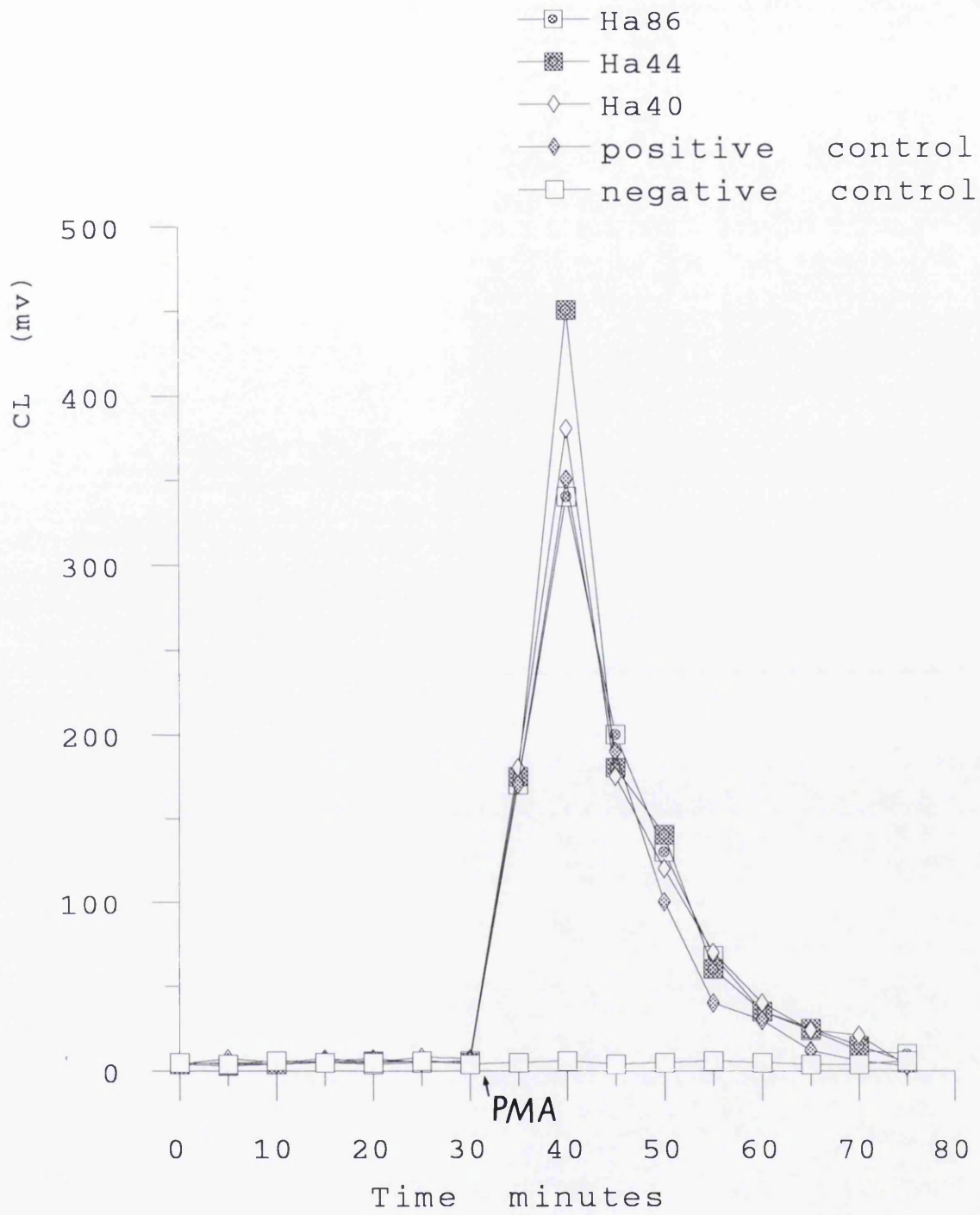


Figure 3.4 Detection of leukotoxic activity among strains of Ha as measured by the inhibition of luminol-dependent chemiluminescence. Three representative non-leukotoxic strains (GDH 40, 44,86) are shown.

Table 3.16 Leukotoxic activity of toxic A.actinomycetemcomitans strains as measured by the reduction in chemiluminescence (CL) of human PMNLs

Strain	Chemiluminescence (mv)	% Reduction in Chemiluminescence (mv)
Aa E 80	78	74.00
Aa E88	15	95.00
Aa 34	105	65.00
Aa E81	45	85.00
Aa E24	30	90.00

3.5.6 Leukotoxic activity of Capnocytophaga species as measured by the inhibition of chemiluminescence produced by human PMNLs

None of Capnocytophaga species tested with the chemiluminescence assay were leukotoxic. Representative non-leukotoxic Capnocytophaga strains are shown in Figure 3.5.

3.6 Results of the phagocytosis of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species by human PMNLs using the luminol-dependent chemiluminescence assay

3.6.1 Phagocytosis of A.actinomycetemcomitans strains

Two patterns of chemiluminescence responses were observed when opsonized A.actinomycetemcomitans strains were incubated with human PMNLs. The first pattern was produced by the majority of strains of A.actinomycetemcomitans, that is an immediate, exponential, fast, well-defined chemiluminescence peak and was exhibited by eight of the nine strains (90%) (A.actinomycetemcomitans strain E24, E79, E80, E81, E83, 100, 115 and 120). A representative strain

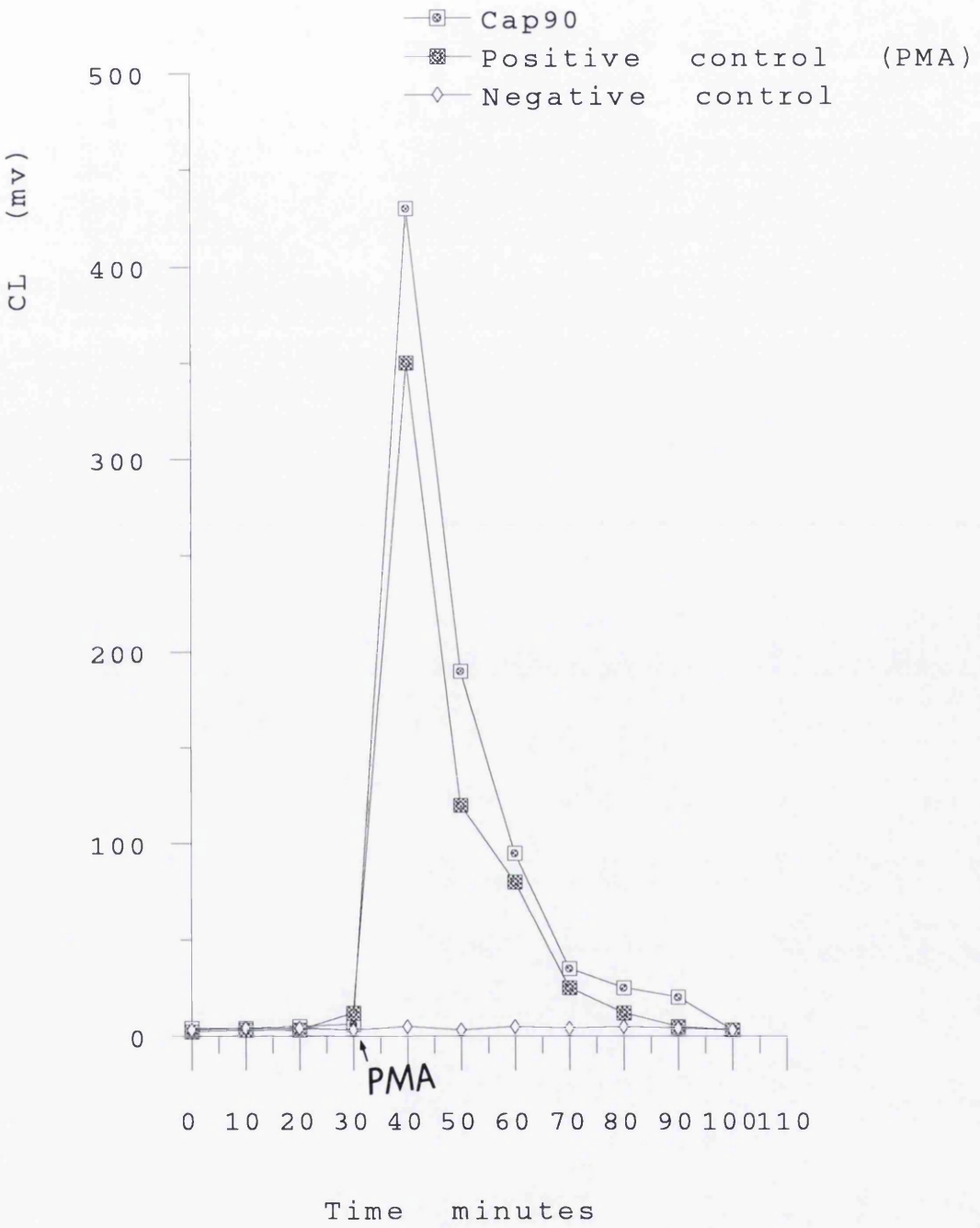


Figure 3.5 Detection of leukotoxic activity among strains of Capnocytophaga species by the inhibition of luminol-dependent chemiluminescence. Representative non-leukotoxic strain (90).

producing this pattern is shown in Figure 3.6 and chemiluminescence values obtained with all tested strains are listed in Table 3.17. Unopsonized cells of the same strains did not stimulate human PMNLs and no chemiluminescence response was recorded. The second pattern of chemiluminescence response was observed with only one strain (E88) and it was recorded when an opsonized A.actinomycetemcomitans strain E88 was incubated with human PMNLs under the same conditions. This strain produced a relatively fast and small chemiluminescence peak, but no further response was recorded when PMA was added. This indicated that the PMNLs were not functioning properly, perhaps due to leukotoxic activity (Figure 3.7). However, other leukotoxic strains were different, since the chemiluminescence response produced by these strains (E24, E81, E80) indicated that they were phagocytosed by human PMNLs. The reason for this difference is not clear but it could be due to differences in the surface components expressed by these different strains. All non-leukotoxic A.actinomycetemcomitans strains were able to trigger a chemiluminescence response, though it was strain-dependent, since certain strains produced higher chemiluminescence responses than others. For

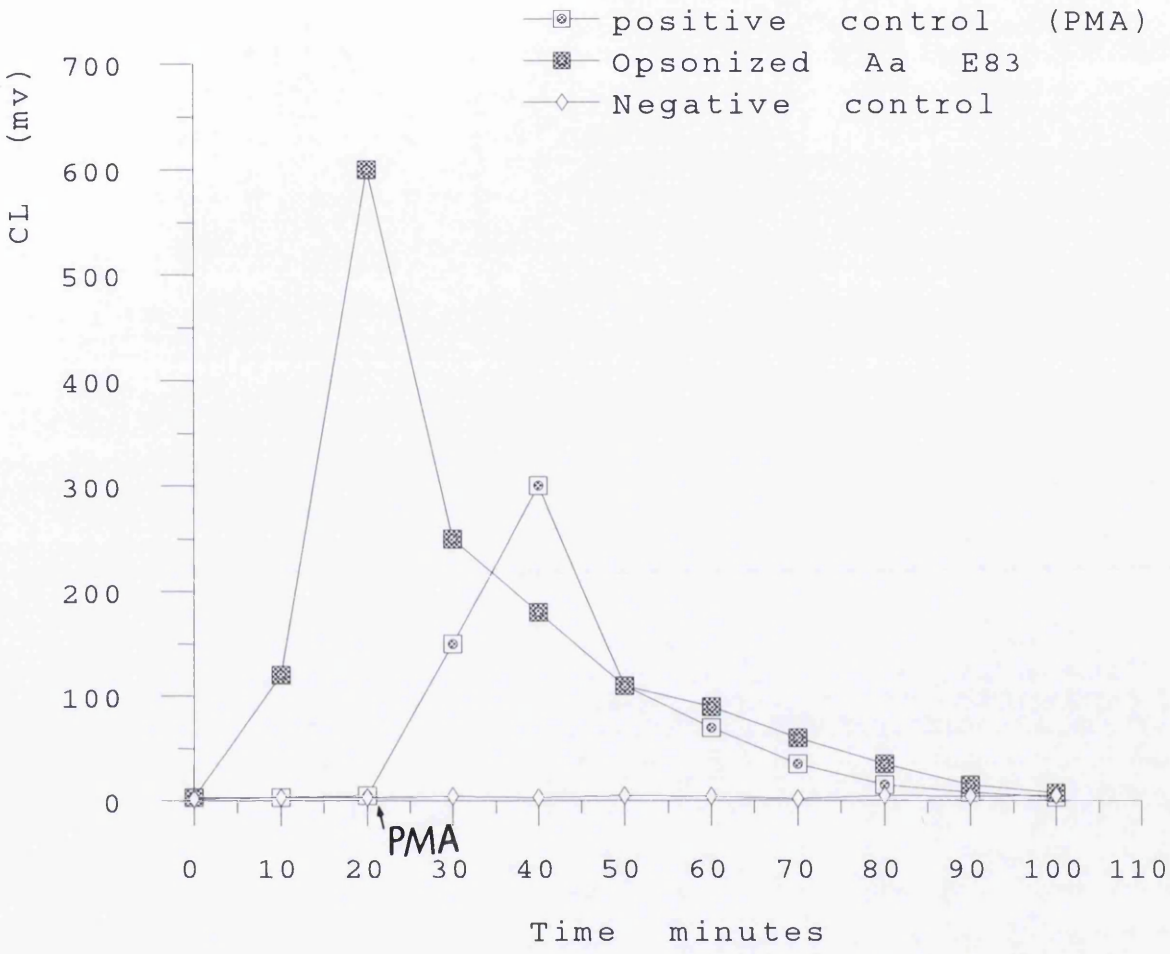


Figure 3.6 Representative opsonized Aa strain (E83) used for the phagocytosis experiments.

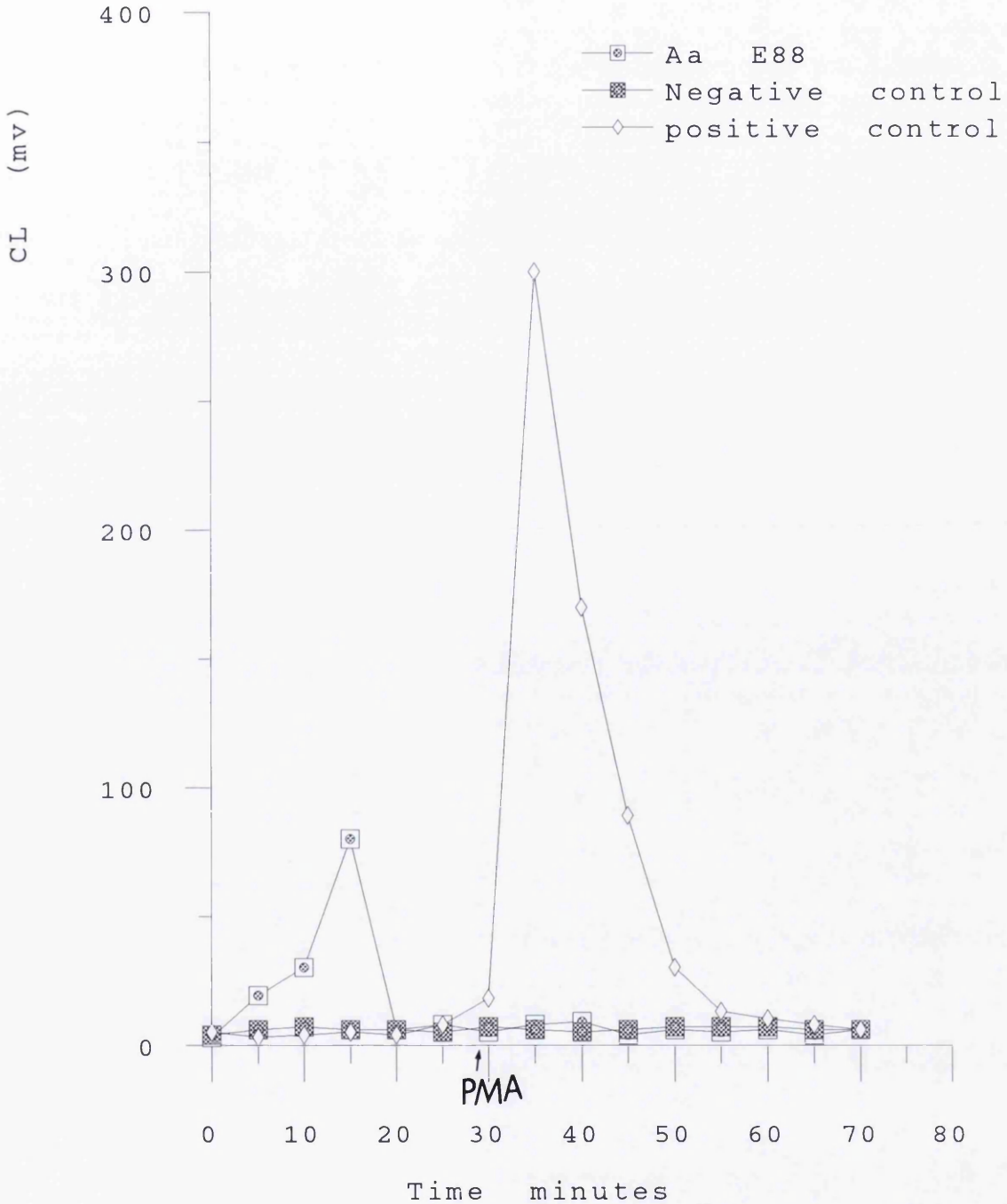


Figure 3.7 The chemiluminescence response triggered by the opsonized Aa leukotoxic JP2 strain used for the phagocytosis experiments.

example, the A.actinomycetemcomitans strain E83 triggered a chemiluminescence response about 3 times greater than that produced by A.actinomycetemcomitans strain 100. Unopsonized cells of the same strains failed to initiate similar responses which indicates the opsonic activity of serum.

3.6.2 Phagocytosis of H.aphrophilus

The incubation of ten strains of H.aphrophilus with human PMNLs resulted, after serum opsonization, in an immediate exponential chemiluminescence response, indicating phagocytic activity of human PMNLs (Figure 3.8). These strains included H.aphrophilus 40, 44, 46, 79, 80, 82, 83, 97,104 and 5886 (NCTC strain), chemiluminescence values obtained with all tested strains are listed in Table 3.17. Unopsonized cells of the same strains failed to initiate similar responses when incubated under the same conditions. However, prolonged incubation times with unopsonized cells resulted in very broad and substantially smaller chemiluminescence peaks (Figure 3.9).

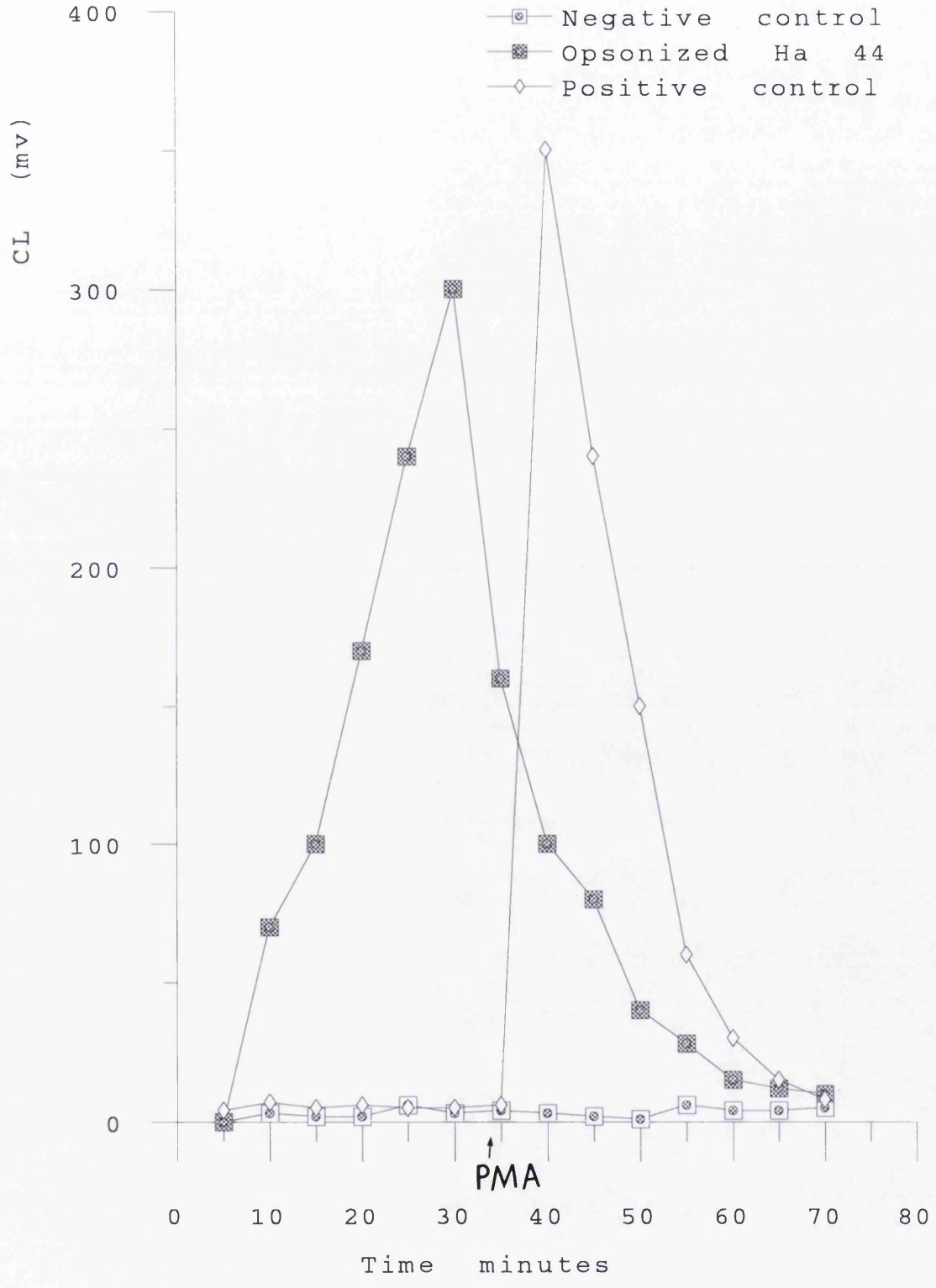


Figure 3.8 Representative opsonized Ha strain (44) used for the phagocytosis experiments

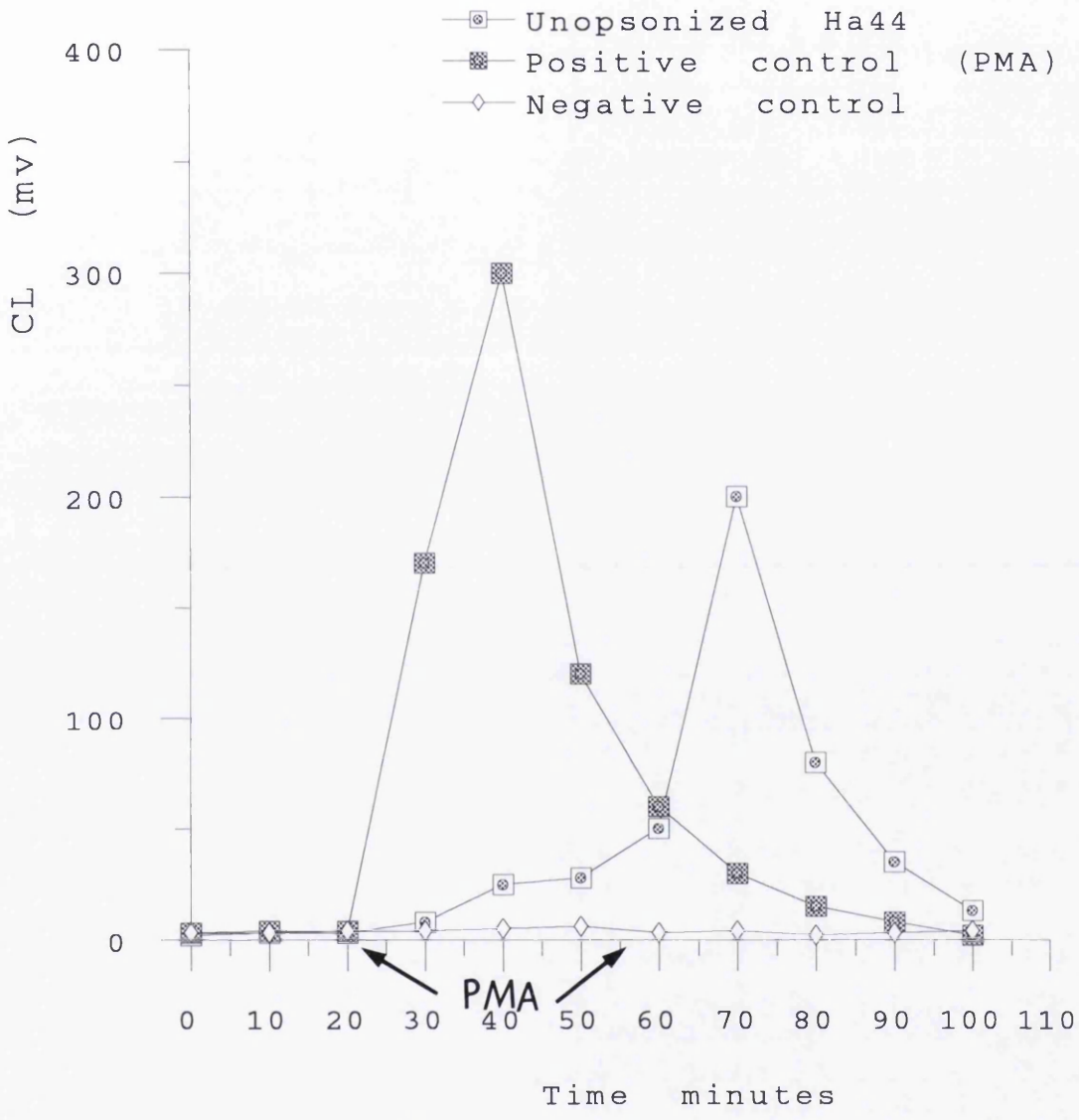
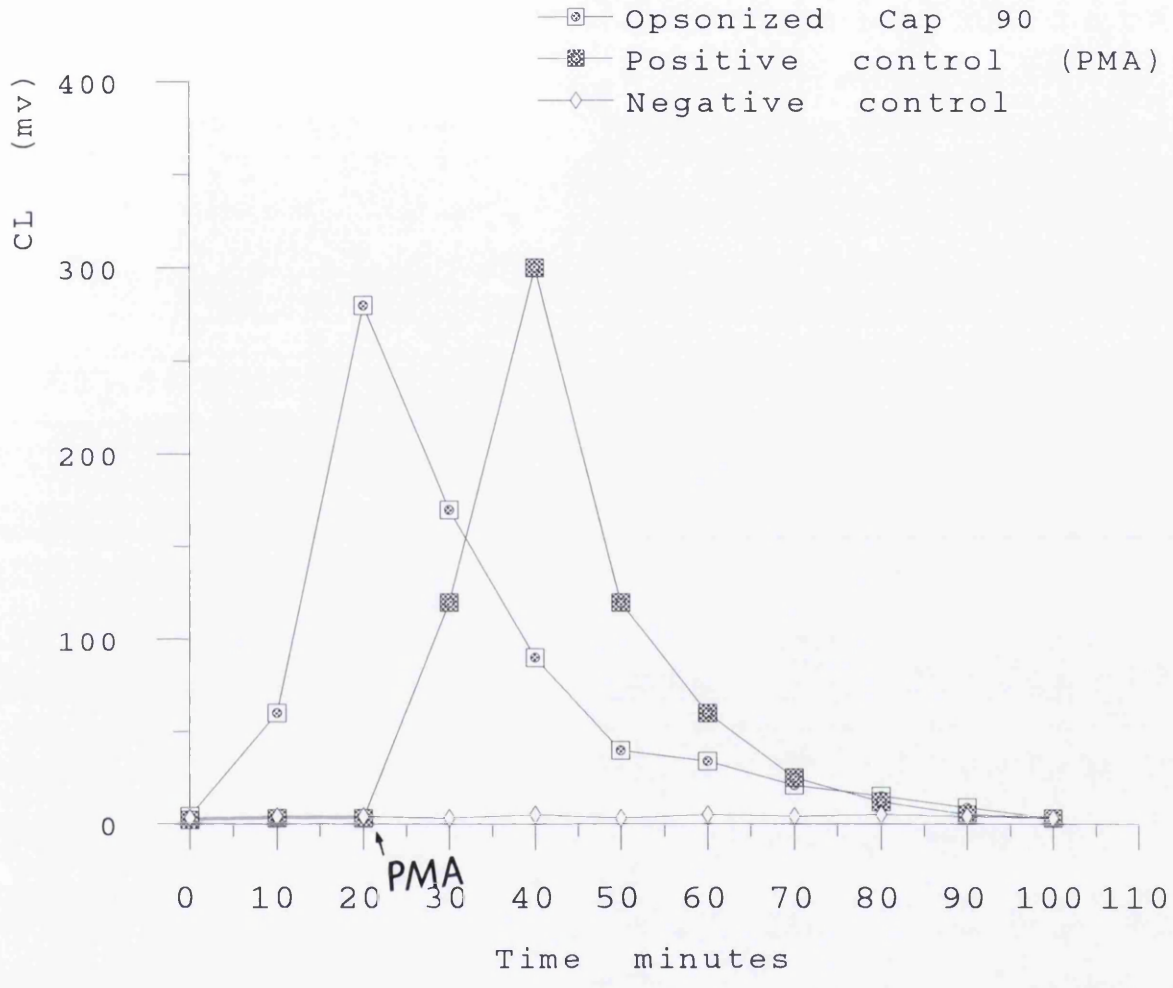


Figure 3.9 Representative unopsonized Ha strain (44) used for the phagocytosis experiments.

3.6.3 Phagocytosis of Capnocytophaga species

Incubation of opsonized Capnocytophaga species with human PMNLs resulted in fast, exponential chemiluminescence responses similar to those observed with H.aphrophilus strains (Figure 3.10). These strains included Capnocytophaga strain 13, 52 and 90 (NCTC strain). These results indicate the ability of Capnocytophaga species to activate the respiratory burst by human PMNLs, list of chemiluminescence values recorded for Capnocytophaga strains are shown in Table 3.17.



Figur 3.10 Representative opsonized Capnocytophaga strain (90) used for the phagocytosis experiments

Table 3.17 Chemiluminescence values recorded with strains of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species

Strain	Chemiluminescence		Strain	Chemiluminescence	
	opsonized	unopsonized		opsonized	unopsonized
Ha 40	280mv*	0.00	Aa 115	375mv	0.00
Ha 44	3oomv	0.00	Aa 120	260mv	0.00
Ha 46	3600mv	0.00	Aa E24	518mv	0.00
Ha 79	230mv	0.00	Aa E79	440mv	0.00
Ha 80	290mv	0.00	Aa E80	280mv	0.00
Ha 82	320mv	0.00	Aa E81	175mv	0.00
Ha 83	320mv	0.00	Aa E83	600mv	0.00
Ha 97	380mv	0.00	Aa E88	65 mv	0.00
Ha 104	400mv	0.00	Cap 13	420mv	0.00
Ha5886	290mv	0.00	Cap 52	350mv	0.00
Aa 100	120mv	0.00	Cap 90	295mv	0.00

* millivolts

3.7 The cytotoxicity of H.aphrophilus, A.actinomycetemcomitans and Capnocytophaga species for cultured human skin fibroblasts

3.7.1 The cytotoxicity of H.aphrophilus, A.actinomycetemcomitans and Capnocytophaga species for cultured human skin fibroblasts (Whole live cells)

In the first part of the cytotoxicity experiments, the ability of whole cells of some H.aphrophilus, A.actinomycetemcomitans and Capnocytophaga strains to interfere with the growth of the cultured human skin fibroblast cell line IBR3 was examined. Results obtained from these preliminary experiments indicated that some strains from all species were able to interfere with and reduce the metabolic activity of cultured fibroblasts. This reduction occurred in a dose-dependent manner. However, a major technical problem arose especially in wells with higher ratios of fibroblast growth medium to bacteria (50µl bacterial cells/150µl growth medium). The problem involved the formation of a dark coloured precipitate, which interfered with the final optical density measurements. The results of a representative A.actinomycetemcomitans strain illustrating this problem are shown in Figure

3.11. A number of experiments were performed to resolve the problem. These included dilution of the medium with PBS, medium modification (ie removal of serum) and medium replacement with RPMI 1640 medium. The results of these preliminary experiments indicated that the precipitation occurred as a result of an interaction between bacterial cell components and the fibroblast growth medium. An attempt to use cell-free bacterial extracts was therefore made. Cell-free extracts were prepared either by sonication or disintegration of bacteria for 20 minutes. The results of these experiments indicated the suitability of the preparation obtained by disintegration, though some viable cells were detected by viable counts. The time of disintegration was increased to 1 hour and the system was kept cool for the period of 1 hour by placing an ice box on top of the disintegrator. The resultant cell-free extracts were tested in an additional group of experiments. Satisfactory cytotoxicity results were achieved and, therefore, cell-free extracts were used in the second part of the study.

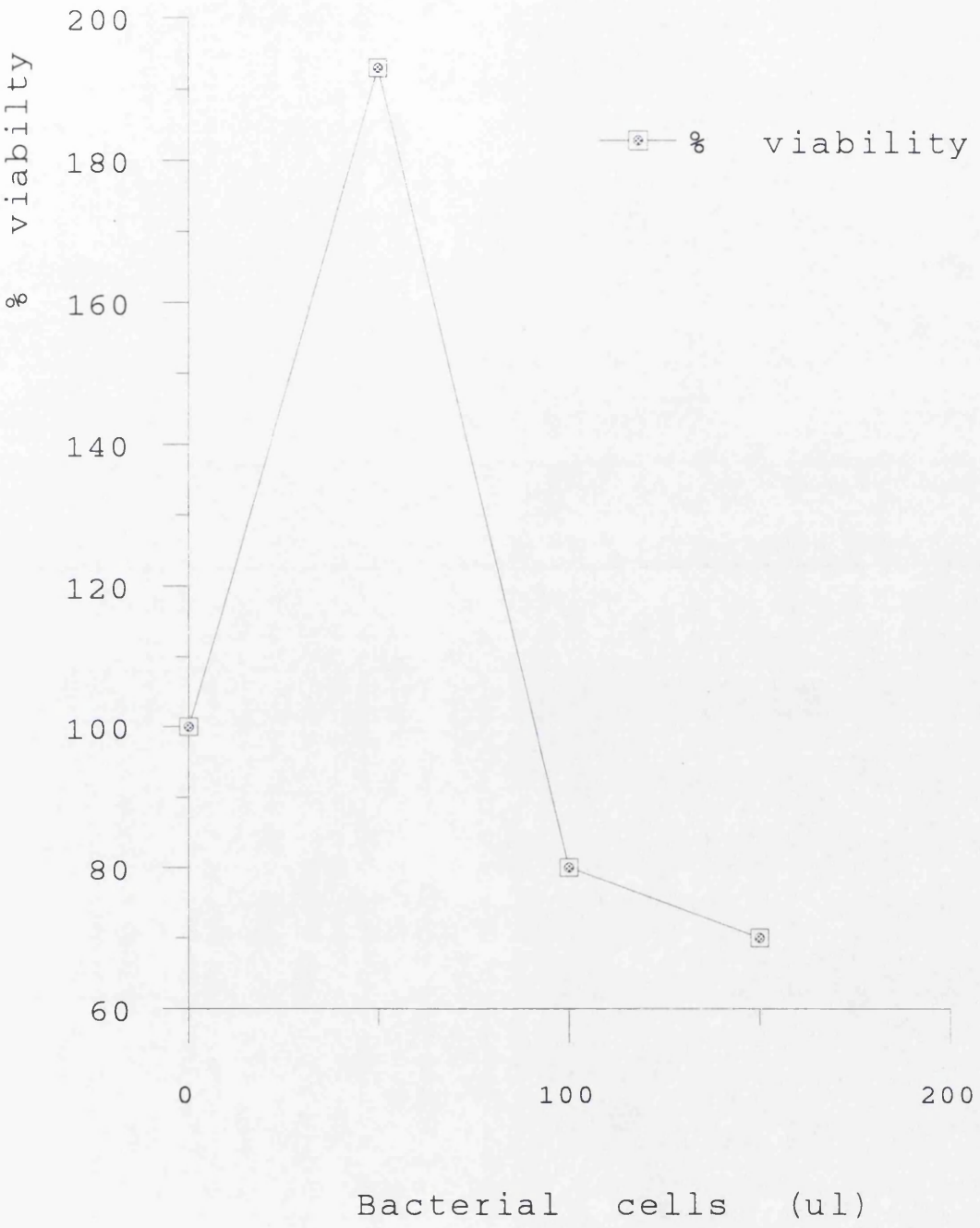


Figure 3.11 representative Aa strain (115) whole cells when used for the cytotoxicity experiments. To illustrate the problem of using whole bacterial cells.

3.7.2 The cytotoxicity of A.actinmycetemcomitans cell-free extracts for cultured human skin fibroblasts

Twenty five A.actinomycetemcomitans strains were tested for their ability to depress the growth of human skin fibroblasts. Of those tested, 24/25 were cytotoxic and reduced the optical density of cultured fibroblasts by more than 50% (Table 3.18). The optical density reduction by these cytotoxic strains ranged from 64% to 83%, which suggests different degrees of cytotoxic activity among A.actinomycetemcomitans strains. The group of A.actinomycetemcomitans strains tested in the present study included a number of leukotoxic and non-leukotoxic strains. There was no correlation between the leukotoxicity and fibroblast inhibition, as certain non-leukotoxic strains were more cytotoxic to the cultured fibroblast cell line. The non-cytotoxic strain was A.actinomycetemcomitans E79 (Table 3.18). Furthermore, before the termination of the cytotoxicity cultures the plates were examined microscopically for any morphological changes. It was observed that most cytotoxic strains caused some morphological changes, which included gross changes in the appearance of cells and a clear reduction of cell density (Figure 3.12).

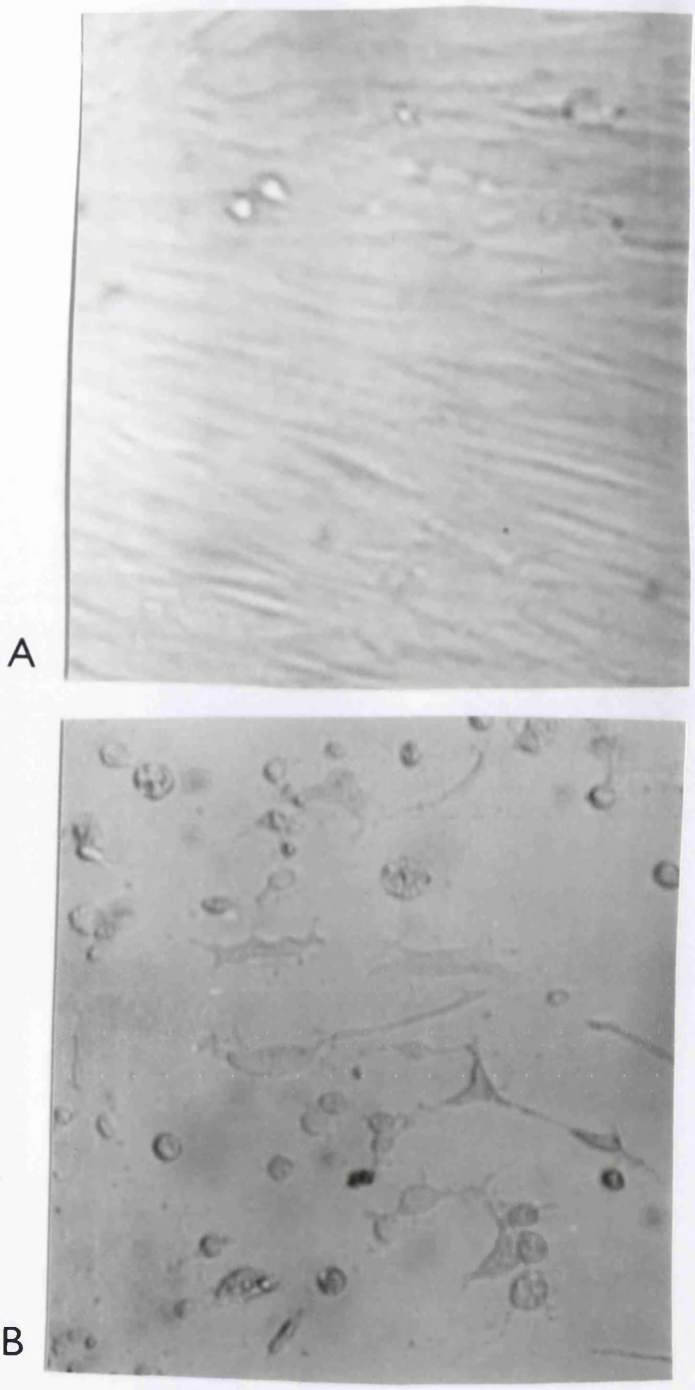


Figure 3.12 (A and B) Morphological changes in cultured human skin fibroblasts caused by a strain of A.actinomycetemcomitans (115). Photograph A shows cultured fibroblasts not exposed to bacteria (control culture). Photograph B shows cultured fibroblasts exposed to a strain of A.actinomycetemcomitans (115)

Table 3.18 List of all A.actinomycetemcomitans strains used for the cytotoxicity assay and their effects on the fibroblast cell line

Strain	OD 570nm (± SEM)	% Reduction*	Strain	OD570 nm (± SEM)	% Reduction*
Aa 33	0.166(0.0235)	76%	Aa E35	0.123(0.0030)	69%
Aa34	0.331(0.0794)	66%	Aa E54	0.128(0.0014)	68%
Aa 94	0.185(0.0016)	79%	Aa E56	0.189(0.0230)	75%
Aa 96	0.191(0.0059)	77%	Aa E57	0.175(0.0104)	76%
Aa 100	0.183(0.0057)	79%	Aa E74	0.211(0.0225)	71%
Aa 112	0.166(0.0076)	78%	Aa E75	0.196(0.0312)	77%
Aa 113	0.159(0.0125)	64%	Aa E76	0.180(0.047)	70%
Aa 115	0.156(0.0047)	83%	Aa E79	0.523(0.0140)	46%
Aa 120	0.324(0.0223)	70%	Aa E80	0.230(0.0606)	63%
Aa E1	0.230(0.0399)	77%	Aa E81	0.256(0.0013)	67%
Aa E8	0.143(0.0104)	81%	Aa E83	0.211(0.0272)	71%
Aa E24	0.151(0.0087)	80%	Aa E88	0.177(0.0138)	75%
Aa E33	0.178(0.0180)	81%			

* Reduction in optical density proportionate to degree of cytotoxicity

3.7.3 The cytotoxicity of H.aphrophilus cell free extracts for cultured human skin fibroblasts

Of the 30 H.aphrophilus strains tested for their ability to depress the growth of a human skin fibroblast cell line IBR3, 26 (87%) were recorded as cytotoxic since they reduced the optical density of cultured fibroblasts by more than 50% (Table 3.19). The remaining four strains (13%) reduced the optical density of cultured fibroblasts by less than 50% and were, therefore, considered non-cytotoxic. These strains were H.aphrophilus strains 58, 59, 5886 (NCTC strain) and 42.

3.7.4 The cytotoxicity of Capnocytophaga cell free extracts for cultured human skin fibroblasts

Of the 9 Capnocytophaga strains, 5 (55%) were cytotoxic to cultured human skin fibroblasts (strain 52,53,72,183 and 90) (Table 3.20). Capnocytophaga strains 170, 174, 176 and 179 were non-cytotoxic. Morphological changes were evident though to a lesser extent than those caused by A.actinomycetemcomitans cell-free extracts.

Table 3.19 List of H.aphrophilus strains used for the cytotoxicity assay and their effects on the fibroblast cell line

Strain	OD 570 nm (± SEM)	% Reduction*	Strain	OD 570nm (± SEM)	%Reduction*
Ha 40	0.194(0.0406)	75%	Ha 76	0.145(0.0075)	76%
Ha 41	0.130(0.0050)	84%	Ha 77	0.146(0.015)	73%
Ha 42	0.211(0.0063)	47%	Ha 78	0.120(0.0015)	83%
Ha 43	0.134(0.0064)	66%	Ha 79	0.238(0.0199)	71%
Ha 44	0.140(0.0150)	88%	Ha 80	0.203(0.0332)	73%
Ha 46	0.120(0.0015)	85%	Ha 82	0.164(0.0052)	78%
Ha 58	0.527(0.043)	46%	Ha 83	0.132(0.0124)	85%
Ha 59	0.329(0.035)	45%	Ha 86	0.195(0.0023)	67%
Ha 60	0.149(0.0126)	79%	Ha 92	0.139(0.0018)	66%
Ha 61	0.182(0.047)	71%	Ha 97	0.157(0.0024)	61%
Ha 62	0.303(0.074)	55%	Ha 99	0.109(0.0043)	87%
Ha 63	0.290(0.068)	58%	Ha 104	0.203(0.011)	79%
Ha 67	0.178(0.0172)	73%	Ha 109	0.112(0.0025)	86%
Ha 68	0.155(0.0115)	76%	Ha 110	0.135(0.0115)	85%
Ha 75	0.175(0.0054)	70%	Ha 5886	0.525(0.030)	24%

* Reduction in optical density proportionate to degree of cytotoxicity

Table 3.20 List of all Capnocytophaga strains used for the cytotoxicity study and their effects on the fibroblast cell line

Strain	OD570 nm (± SEM)	% reduction*	Strain	OD 570 nm (± SEM)	% reduction*
Cap 52	0.201(0.0238)	61%	Cap 176	0.129(0.0156)	13%
Cap53	0.126(0.0009)	68%	Cap 179	0.375(0.0190)	8.5%
Cap 72	0.202(0.0169)	55%	Cap 183	0.262(0.045)	57%
Cap 170	0.323(0.0120)	22%	Cap 90	0.197(0.0039)	65%
Cap 174	0.299(0.0192)	33%			

* Reduction in optical density proportionate to degree of cytotoxicity

3.7.5 Adherence of certain A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains to cultured human skin fibroblast cell line

In the first part of the cytotoxicity study and during the course of the preliminary experiments with the whole live cells, it was observed that certain A.actinomycetemcomitans and H.aphrophilus strains adhered to the surface of the cultured fibroblast monolayer. Figure 3.13 shows the results obtained with a representative positive and negative strains. The adherent cells formed clumps on the top of the monolayer. An attempt to remove the attached cells from the monolayers by washing with PBS did not remove the attached cells. This further confirms this observation, since the non-adherent cells were removed very easily from the plates even without washing, simply by inverting the plates. Although it was not possible to quantify the number of attached bacteria, it was possible to distinguish between adherent and non-adherent strains visually when examined microscopically, as the non-adherent cells formed a homogenous layer on top of the monolayer, while the adherent cells formed clumps. Results obtained with representative strains of all 3 species under study are

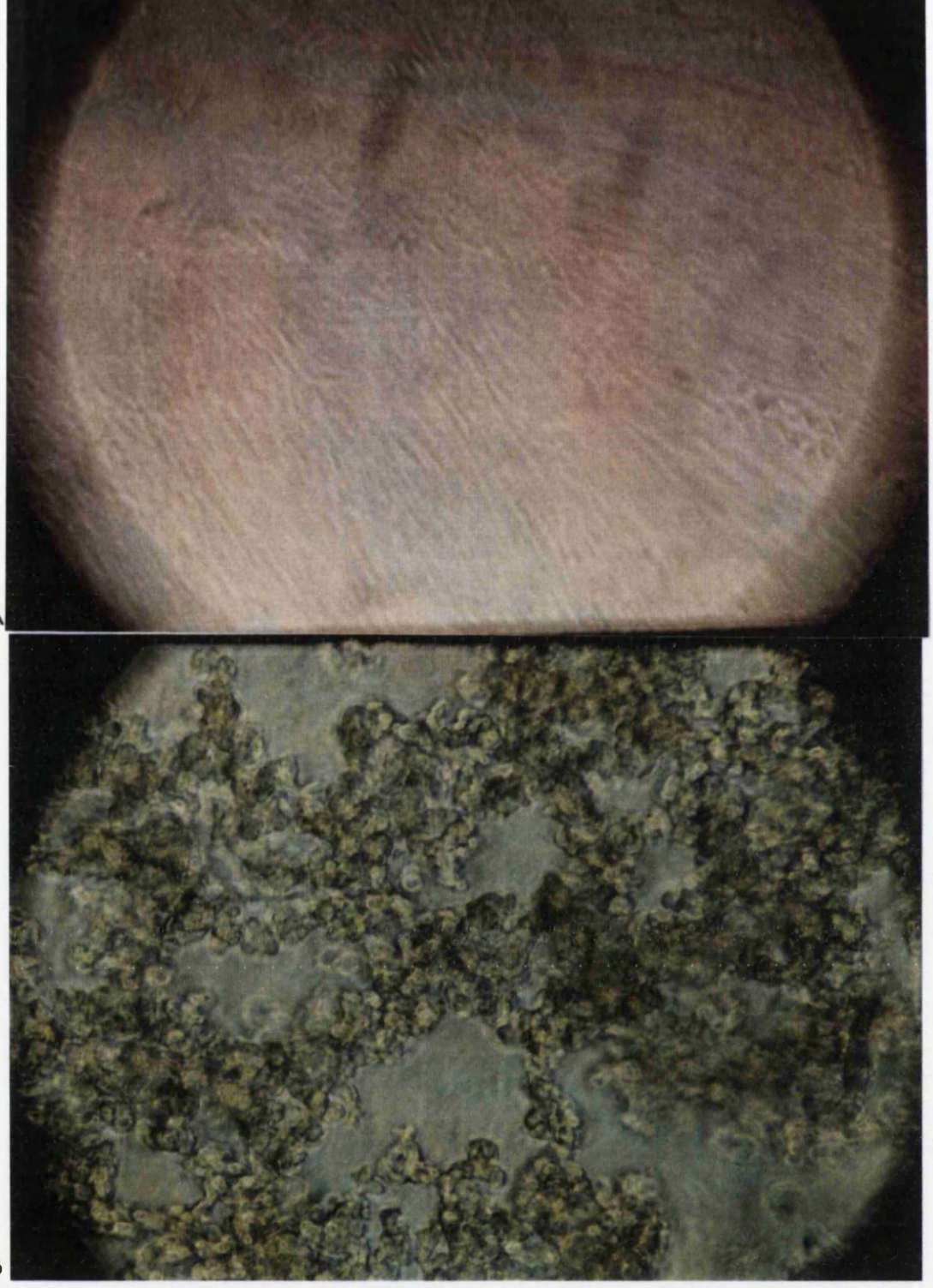


Figure 3.13 (A and B): A representative strain (115) of A.actinomycetemcomitans adhering to cultured human skin fibroblasts. Photograph A shows a negative control culture. Photograph B A.actinomycetemcomitans adhering to cultured fibroblasts.

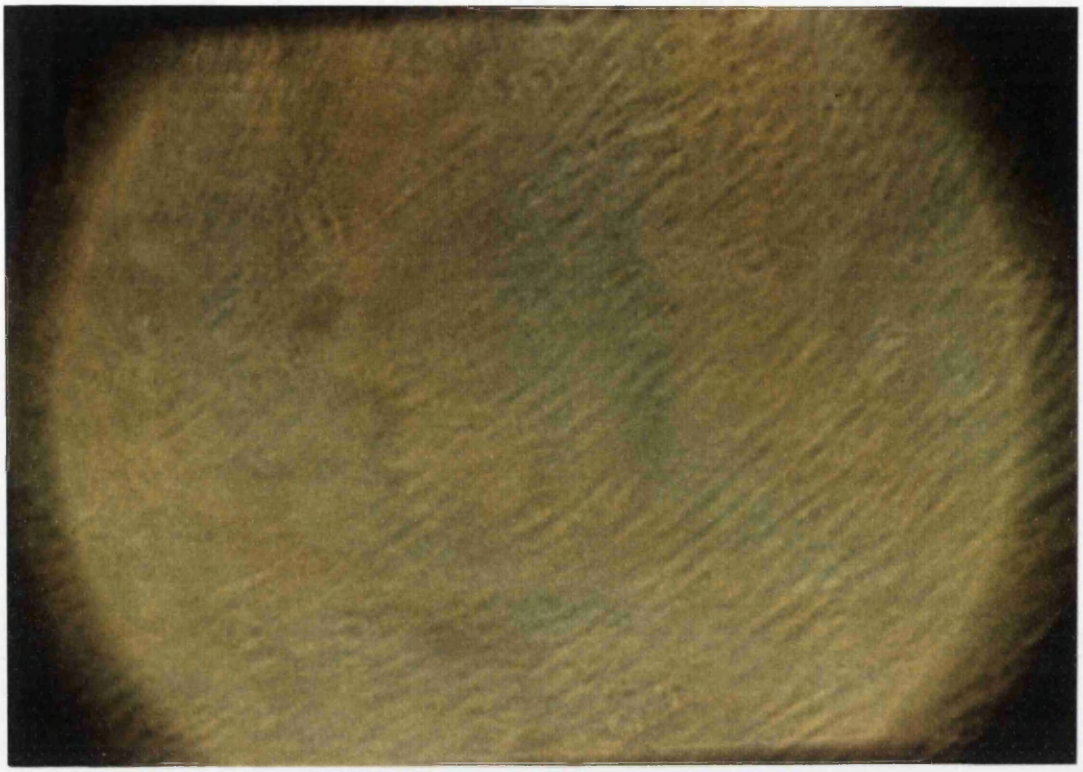


Figure 3.13 A : A representative non-adherent A.actinomycetemcomitans strain (E88) to cultured human skin fibroblast

presented in Table 3.21. There was a correlation with the colonial morphology of the strain, since the cells of the rough type colonial morphology adhered more than those with smooth colonies. The ability of strains to adhere was variable, as certain strains adhered almost immediately or after several hours, while others did not adhere even after 24 hours.

3.21 A comparison of the colonial morphology and adherence of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains to cultured human skin fibroblasts

Strain	colonial type	Adherence
Ha 42	Smooth	-
Ha 43	Smooth	-
Ha 44	Smooth	+
Ha 82	Rough	++
Ha 83	Smooth	+
Ha 67	Rough	++
Ha 68	Rough	++
Aa E35	Rough	+++
Aa E54	Rough	+++
Aa E76	Smooth	-
Aa E88	Smooth	-
Aa 112	Rough	+++
Aa 113	Rough	+++
Aa 115	Rough	+++
Cap 81	Smooth	-
Cap 90	Smooth	-

* +++ = adherence occurred almost immediately; ++ adherence occurred after 4 hours; + = weak adherence occurred within 24 hours; - = no adherence even after 24 hours

3.8 Human Immunoglobulin A protease production by A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species

3.8.1 Cleavage of human myeloma IgA by A.actinomycetemcomitans strains

Twenty five strains of A.actinomycetemcomitans were examined for their ability to cleave human IgA myeloma serum. SDS-PAGE and immunoblotting analysis revealed that none of the strains tested cleaved IgA even after 48 hours of incubation (Figure 3.14)

3.8.2 Cleavage of human myeloma IgA by H.aphrophilus strains

SDS-PAGE and immunoblotting analysis of IgA myeloma serum incubated with 30 H.aphrophilus strains revealed that none of the strains tested cleaved IgA even after 48 hours incubation (Figures 3.15)

3.8.3 Cleavage of human myeloma IgA by Capnocytophaga species.

The incubation of all 10 Capnocytophaga strains with human IgA myeloma serum under the same experimental conditions resulted in the cleavage of IgA as revealed

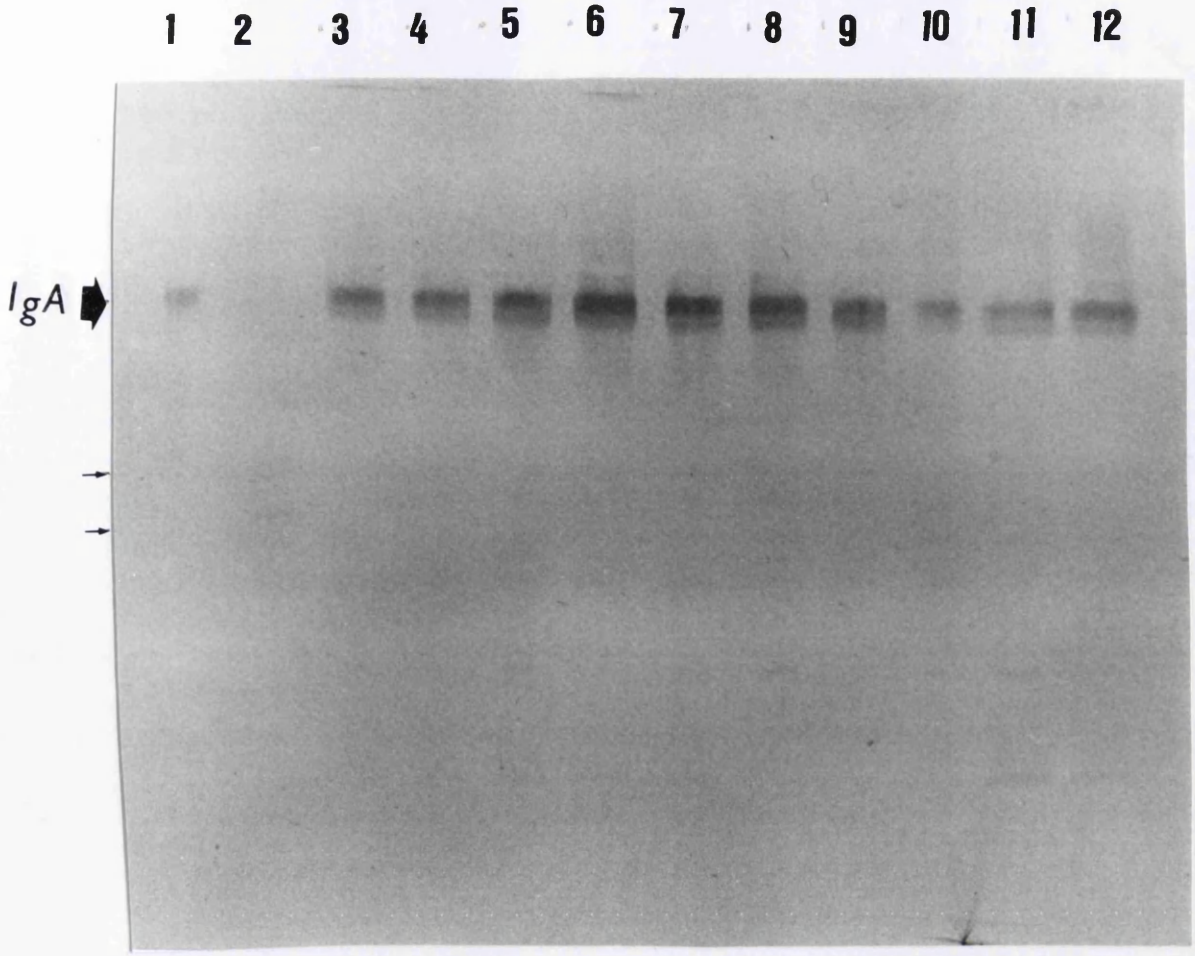


Figure 3.14 Western blot of human myeloma IgA before and after incubation with strains of A.actinomycetemcomitans. Lane 1 IgA incubated in PBS. Lane 2 IgA after incubation with the positive control H.influenzae IgA band was cleaved to two smaller fragments (arrows). Lanes 3-12 IgA after incubation with strains of A.actinomycetemcomitans, no cleavage as the IgA band (arrow) still present.

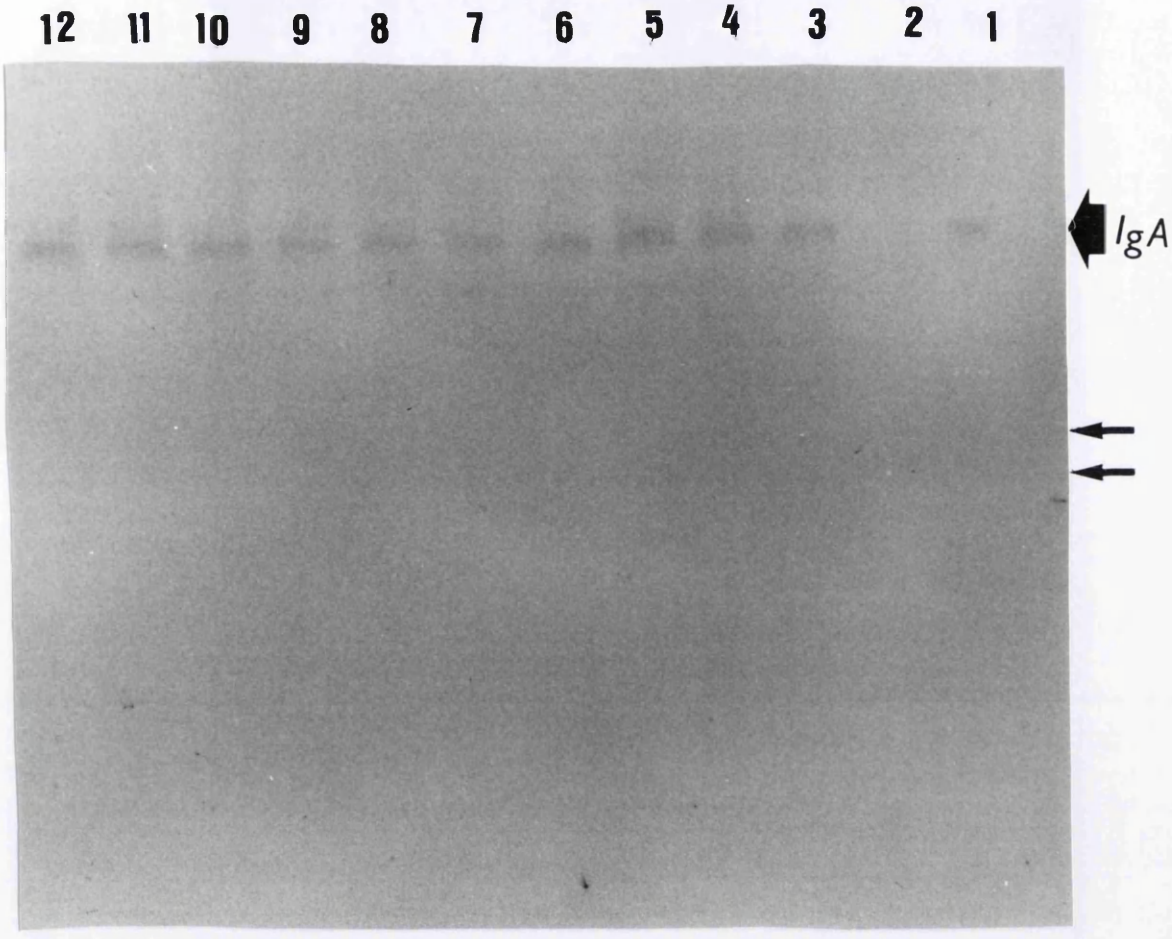


Figure 3.15 Western blot of human myeloma IgA before and after incubation with strains of H.aphrophilus. Lane 1 IgA incubated in PBS. Lane 2 IgA after incubation with the positive control H.influenzae IgA band was cleaved to two smaller fragments (arrows). Lanes 3-12 IgA after incubation with strains of H.aphrophilus, no cleavage occured as the IgA band (arrow) still present.

by immunoblotting (Figure 3.16). Capnocytophaga strains cleaved IgA to yield 2 smaller fragments, similar to the pattern obtained with H.influenzae (NCTC 12194), the positive control (Figure 3.17). However, in some experiments there was non-specific reactivity between the commercial antisera used for blotting and some bacterial proteins, as evident by the presence of unknown bands in some blots. This reactivity was stronger with the Capnocytophaga strains than with the H.influenzae positive control. Furthermore, H.influenzae cleaved a commercially purified human secretory IgA (SIgA) in a similar pattern. All Capnocytophaga strains cleaved human SIgA in the same pattern (Figure 3.17). Furthermore, the incubation of certain Capnocytophaga strains with SIgA, resulted in the cleavage of the secretory component. However, whether this is due to the action of IgA protease or another proteolytic enzyme is not clear (Figure3.18).

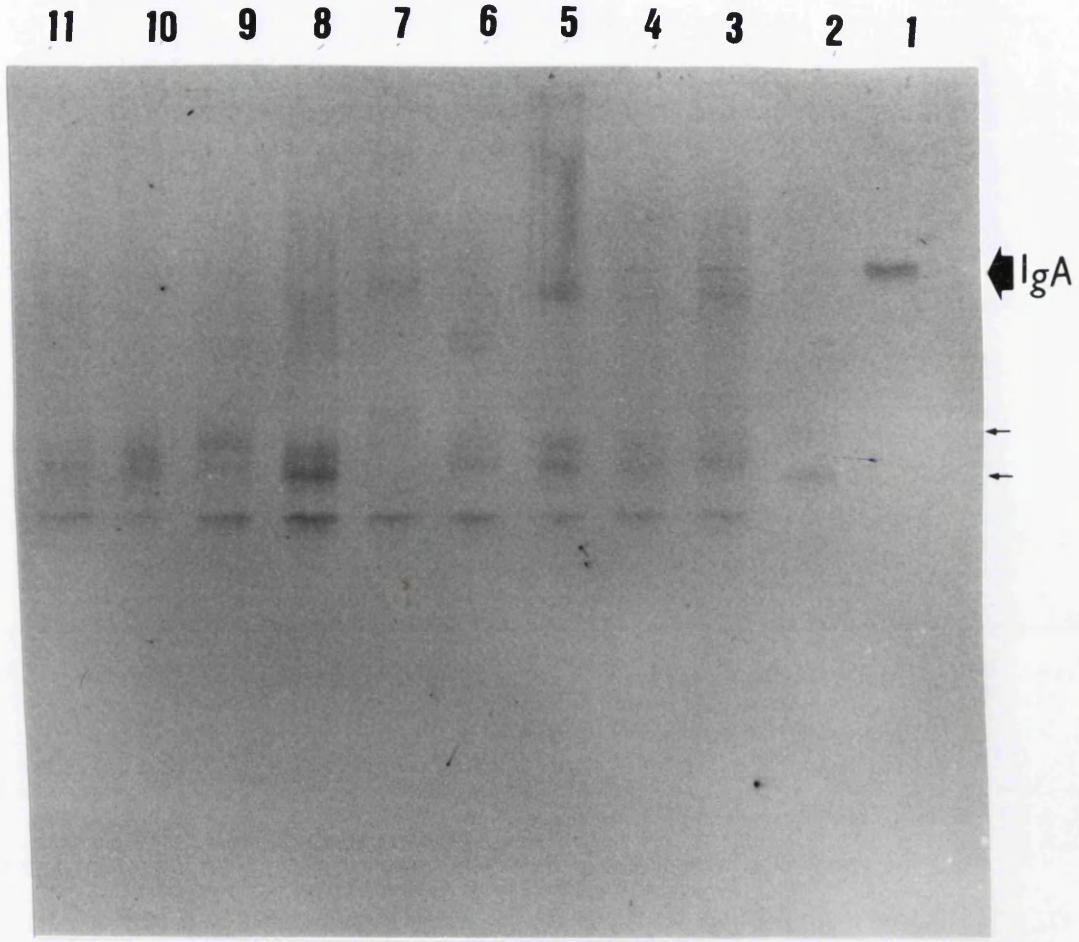


Figure 3.16 Western blot of human myeloma IgA before and after incubation with Capnocytophaga strains. Lane 1 IgA incubated in PBS. Lane 2 IgA after incubation with H.influenzae (positive control). Lanes 3-11 IgA after incubation with Capnocytophaga strains, all strains cleaved IgA as the major IgA band (arrow) was cleaved to two smaller fragments.

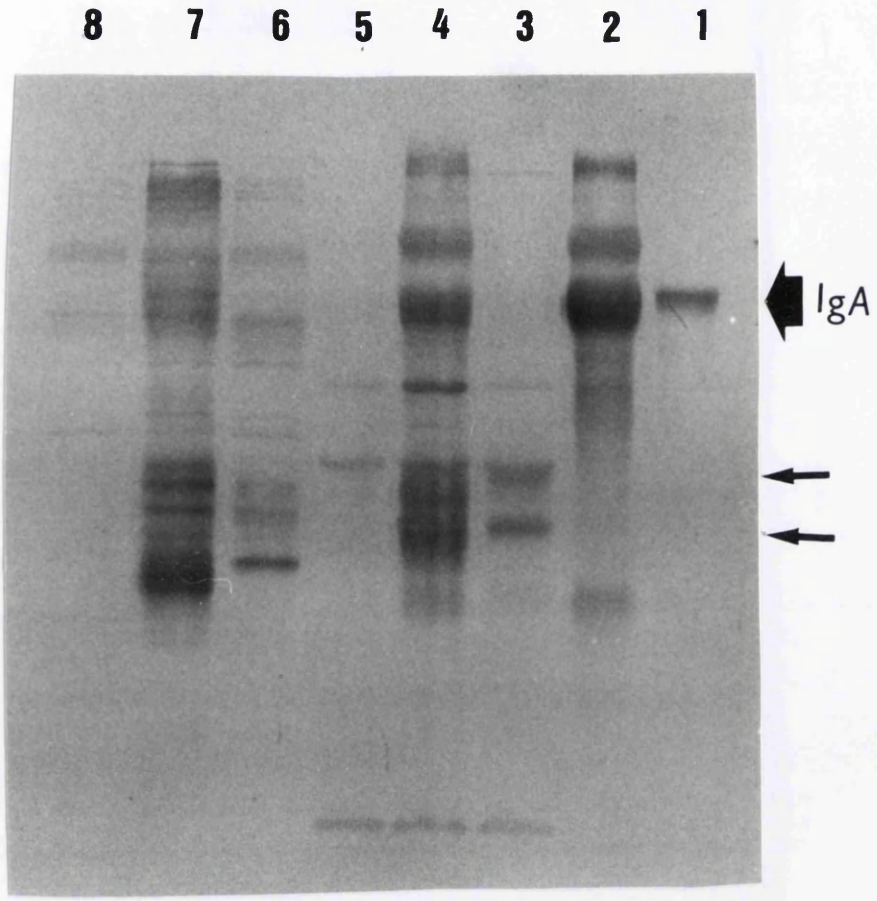


Figure 3.17 Western blot of human myeloma IgA and human SIgA before and after incubation with a strain of H.influenzae (positive control) and Capnocytophaga strain (52). Lane 1 IgA in PBS. Lane 2 human SIgA in PBS. Lane 3 IgA cleaved by H.influenzae, IgA band removed (arrows).Lane 4 SIgA incubated with H.influenzae. Lane 5 H.influenzae incubated in PBS. Lane 6 IgA after incubation with Capnocytophaga strain 52 (IgA band removed). Lane 7 SIgA after incubation with Capnocytophaga strain. Lane 8 Capnocytophaga incubated in PBS.

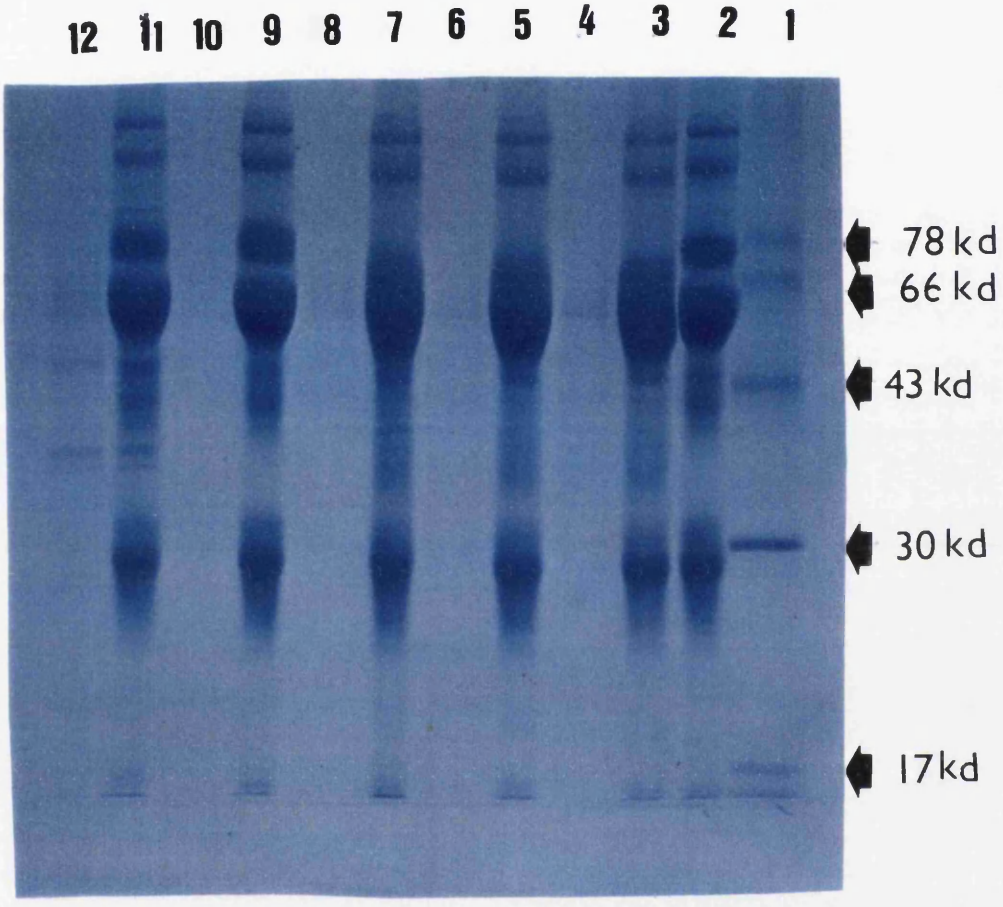


Figure 3.18 SDS-PAGE of human S-IgA before and after incubation with strains of Capnocytophaga species. Lane 1 molecular weight markers. Lane 2 S-IgA in PBS. Lane 3 SIgA after incubation with Capnocytophaga strain 52. Lane 4 strain 52 in PBS. Lane 5 strain SIgA after incubation with Capnocytophaga strain 53. Lane 6 strain 53 in PBS. Lane 7 SIgA after incubation with Capnocytophaga strain 54. Lane 8 strain 54 in PBS. Lane 9 SIgA after incubation with H.aphrophilus strain 44. Lane 10 strain 44 in PBS. Lane 11 SIgA after incubation with H.aphropilus strain 40. Lane 12 strain 40 in PBS. Secretory component (SC) arrows, is cleaved by all 3 Capnocytophaga strains

3.9 Summary of virulence factors produced by A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species

Twenty five A.actinomycetemcomitans, 30 H.aphrophilus and Capnocytophaga species were examined in the current study for their ability to produce a number of virulence factors. These included the ability of the strains to haemagglutinate human erythrocytes, possession of leukotoxic activity, the ability to resist phagocytosis, the ability to cleave human IgA by means of IgA-protease and finally the ability to exert cytotoxic effects on cultured human skin fibroblasts.

3.9.1 A.actinomycetemcomitans

The results obtained in the current study showed that 68% of tested strains possessed a haemagglutinating factor since they were able to haemagglutinate both human group A and group O erythrocytes. Five (20%) of tested strains killed both HL60 cells and human PMNLs as measured by the trypan blue and the inhibition of luminol-dependent chemiluminescence assays. All tested strains but one triggered a chemiluminescence response, these strains including both leukotoxic and non-leukotoxic strains. The single A.actinomycetemcomitans

strain that failed to elicit a chemiluminescent response and which would therefore escape phagocytosis was the highly toxic JP2 strain. All tested strains but one inhibited the proliferation of cultured human skin fibroblasts, though none of the tested strains produced IgA protease. A summary of all results obtained with the tested strains is shown in Table 3.22.

3.9.2 Capnocytophaga species

All tested Capnocytophaga strains were able to haemagglutinate human erythrocytes and to cleave human myeloma IgA. Three strains were tested for their susceptibility to phagocytosis by human PMNLs, and all 3 strains triggered a chemiluminescence response indicative of phagocytic activity. Fifty five percent of tested Capnocytophaga strains were able to interfere with the proliferation of cultured human skin fibroblasts. None of the tested Capnocytophaga strains possessed leukotoxic activity. A summary of all results obtained with Capnocytophaga species are shown in Table 3.23.

3.9.3 H.aphrophilus

The majority of H.aphrophilus strains agglutinated human erythrocytes. None of the tested strains possessed leukotoxic activity as measured by both assays employed in the current study, though all tested strains triggered a chemiluminescence response and appeared sensitive to phagocytosis. The majority of strains exerted cytotoxic effects against cultured human skin fibroblasts. None of the tested strains produced IgA-protease. A summary of all results obtained with H.aphrophilus strains is shown in Table 3.24.

It is clear from the summarised results that A.actinomycetemcomitans is capable of producing a range of virulence factors that may contribute to the pathogenesis of periodontitis. The results obtained in the present investigation suggest that A.actinomycetem-comitans possesses the highest potential of the three bacterial species studied to cause infection. Capnocytophaga species also produced an array of potent virulence factors that may damage the host tissues (for example the IgA-protease). The results obtained with H.aphrophilus strains indicate that the potential of this bacterium to cause damage to the host is less than

for A.actinomycetemcomitans or Capnocytophaga species. However, the majority of tested H.aphrophilus strains were able to produce a fibroblast inhibitory factor and possessed haemagglutination activity, in keeping with their ability to cause infection under certain circumstances. Additional studies are needed to define the true role of these capnophilic organisms in the aetiology of periodontitis.

Table 3.22 Summary of the virulence factors produced by A.actinomycetemcomitans strains tested in the current study

Strain	Haemagglutination	Leukotoxicity	Phagocytosis	Cytotoxicity	IgA-protease
Aa 33	+	-	ND	+	-
Aa 34	+	+	ND	+	-
Aa 94	+	-	ND	+	-
Aa 96	+	-	ND	+	-
Aa 100	+	-	+	+	-
Aa 112	+	-	ND	+	-
Aa 113	+	-	ND	+	-
Aa 115	+	-	+	+	-
Aa 120	+	-	+	+	-
Aa E1	+	-	ND	+	-
Aa E8	+	-	ND	+	-
Aa E24	-	+	+	+	-
Aa E33	+	-	ND	+	-

ND not determined

Table3.22 Continued

Strain	Haemagglutination	Leukotoxicity	Phagocytosis	Cytotoxicity	IgA-protease
Aa E35	+	-	ND	+	-
Aa E54	+	-	ND	+	-
Aa E56	+	-	ND	+	-
Aa E57	+	-	ND	+	-
Aa E74	+	-	ND	+	-
Aa E75	-	-	ND	+	-
Aa E76	-	-	ND	+	-
Aa E79	-	-	+	-	-
Aa E80	-	+	+	+	-
Aa E81	-	+	+	+	-
Aa E83	-	-	+	+	-
Aa E88	-	+	-	+	-

ND not determined

Table 3.23 Summary of virulence factors produced by Capnocytophaga species tested in the current study

Strain	Haemagglutination	Leukotoxicity	Phagocytosis	Cytotoxicity	IgA-protease
Cap 52	+	-	+	+	+
Cap 53	+	-	ND	+	+
Cap72	+	ND	ND	+	+
Cap 81	+	-	ND	ND	+
Cap 170	+	ND	ND	-	+
Cap 174	+	ND	ND	-	+
Cap 176	+	ND	ND	-	+
Cap 179	+	ND	ND	-	+
Cap183	+	ND	ND	+	+
Cap 90	+	-	+	+	+
Cap 15	ND	-	+	ND	ND
Cap 30	ND	-	ND	ND	ND
Cap 45	ND	-	ND	ND	ND
Cap 65	ND	-	ND	ND	ND

ND not determined

Table 3.24 Summary of the virulence factors produced by *H.aphrophilus* strains tested in the current study

Strains	Haemagglutination	Leukotoxicity	Phagocytosis	cytotoxicity	IgA-protease
Ha 40	+	-	+	+	-
Ha 41	+	-	ND	+	-
Ha 42	+	-	ND	+	-
Ha 43	+	-	ND	+	-
Ha 44	+	-	+	+	-
Ha 46	+	-	+	+	-
Ha 58	+	-	ND	-	-
Ha 59	+	-	ND	-	-
Ha 60	+	-	ND	+	-
Ha 61	+	-	ND	+	-
Ha 62	+	-	ND	+	-
Ha 63	-	-	ND	+	-
Ha 67	+	-	ND	+	-
Ha 68	+	-	ND	+	-

ND not determined

Table 3.24 continued

Strain	Haemagglutination	Leukotoxicity	Phagocytosis	Cytotoxicity	IgA-protease
Ha 75	+	-	ND	+	-
Ha 76	+	-	ND	+	-
Ha 77	+	-	ND	+	-
Ha 78	+	-	ND	+	-
Ha 79	+	-	+	+	-
Ha 80	+	-	+	+	-
Ha 82	+	-	+	+	-
Ha 83	+	-	+	+	-
Ha 86	+	-	ND	+	-
Ha 92	-	-	ND	+	-
Ha 97	+	-	+	+	-
Ha 99	+	-	ND	+	-
Ha 104	+	-	+	+	-
Ha 109	+	-	ND	+	-
Ha 110	+	-	ND	+	-
Ha5886	+	-	+	-	-

ND not determined

Chapter 4

Discussion

4.1 The isolation of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species from subgingival plaque samples

4.1.1 The isolation of A.actinomycetemcomitans

The fresh A.actinomycetemcomitans strains used in this study were either freshly isolated from subgingival plaque samples from patients with different forms of periodontal disease or were previously isolated strains stored at -70^{O}C at Glasgow Dental Hospital and School. A.actinomycetemcomitans was isolated from only 11 (6%) of 170 sites from 49 subjects with different forms of periodontal disease and even though, the initial diagnosis was available and of the 49 subjects, 23 were diagnosed initially with chronic periodontitis, 9 patients with rapidly progressive periodontitis, 14 patients having persisting pockets following a hygiene phase therapy and their clinical diagnosis was unknown and 3 patients with unknown clinical diagnosis, the complete clinical diagnoses of the subjects investigated in the present study were not completely known. The prevalence of A.actinomycetemcomitans in these patients was lower than expected and substantially lower than what has been reported

previously in the literature (Slots et al., 1982; Zambon, 1985). Slots et al., (1982) reported that A.actinomycetemcomitans was isolated from 26 of 27 patients with juvenile periodontitis compared to only 24 of 142 healthy individuals. Petit et al., (1993) reported the isolation of A.actinomycetemcomitans from 16 subjects with adult periodontitis patients out of 13 families participated in a study regarding the transmission of A.actinomycetemcomitans in families with adult periodontitis, though 5 subjects were children and the total number of subjects consisted of 13 couples and 26 children. The reason for this lower prevalence of A.actinomycetemcomitans among the subjects used in the present study was not clear. One explanation is the frequent isolation of Streptococcus species from the subgingival plaque samples used in the present study, since it was observed that in all samples where A.actinomycetemcomitans was isolated it was isolated in almost pure culture. It has been shown that certain Streptococcus species can suppress the growth of A.actinomycetemcomitans (Slots, 1982; Hillman and Socransky, 1989; Socransky and Haffajee, 1992). Streptococcus species were present in virtually all

other samples, suggesting that bacterial antagonism against A.actinomycetemcomitans may have occurred.

The selective medium TSBV was employed for the recovery of A.actinomycetemcomitans from the subgingival plaque samples. This medium has been used by a number of workers for the isolation of A.actinomycetemcomitans (Taher, 1990; Asikainen, Alaluusua and Saxen, 1991; Papapanou et al., 1993), since it has been shown that the TSBV medium resolved a number of problems encountered when other media, for example Chocolate agar medium or Malachite green medium, were used for the isolation of this organism. Chocolate agar medium is not sufficiently selective and supports the growth of a number of organisms like Haemophilus species, Streptococcus species and Neisseria species, which hamper the isolation of A.actinomycetemcomitans. Malachite green inhibits the growth of A.actinomycetemcomitans strains even at low concentrations (Slots, 1982). An additional advantage of the TSBV medium is that it contains no haem and, therefore, the catalase test, which is an important test used to distinguish A.actinomycetemcomitans from the closely related H.aphrophilus, can be performed directly on colonies on the plate. Taher (1990) also

found that the TSBV medium was substantially better than the malachite green medium for the isolation of A.actinomycetemcomitans from subgingival plaque samples. Although the use of TSBV medium reduced the number of contaminating bacteria present in the samples, in the present study it still supported the growth of Streptococcus species, in spite of the presence of vancomycin. This was contrary to a report by Slots (1982).

4.1.2 The identification of A.actinomycetemcomitans

In the present study A.actinomycetemcomitans isolates were identified on the basis of their colonial morphology, catalase production and biochemical characteristics.

Colonial morphology of A.actinomycetemcomitans

The colonial morphology of A.actinomycetemcomitans is an important feature used for the isolation of this organism from primary cultures. The colonial morphology of A.actinomycetemcomitans on TSBV medium in primary cultures is distinct. The colonies are adherent to the agar surface and are star-shaped. In a number of reported studies, this was the only colonial type

recognised as A.actinomycetemcomitans (Slots, 1982; Kaplan et al., 1989) though, in the present study and in previously conducted studies in this laboratory (Taher, 1990) both star-shaped and non star-shaped colonies were produced by A.actinomycetemcomitans on primary isolation. Furthermore, Taher (1990) showed that more A.actinomycetemcomitans isolates produced non star-shaped colonies than those produced the star-shaped colonies. It was also observed in this study that organisms other than A.actinomycetemcomitans and H.aphrophilus were capable of producing a star-shaped colony including some Gram-positive cocci. Therefore, the selection of only star-shaped colonies may result in under reporting of samples which are positive for A.actinomycetemcomitans. Representative star-shaped colonies of A.actinomycetemcomitans, H.aphrophilus and one unknown Gram-positive organism are shown in Figure 4.1. The star-shaped colonies usually lost this characteristic after subculturing an observation already reported in the literature (Slots, 1982).

Catalase test

Catalase production is an important characteristic in identifying A.actinomycetemcomitans isolates on primary

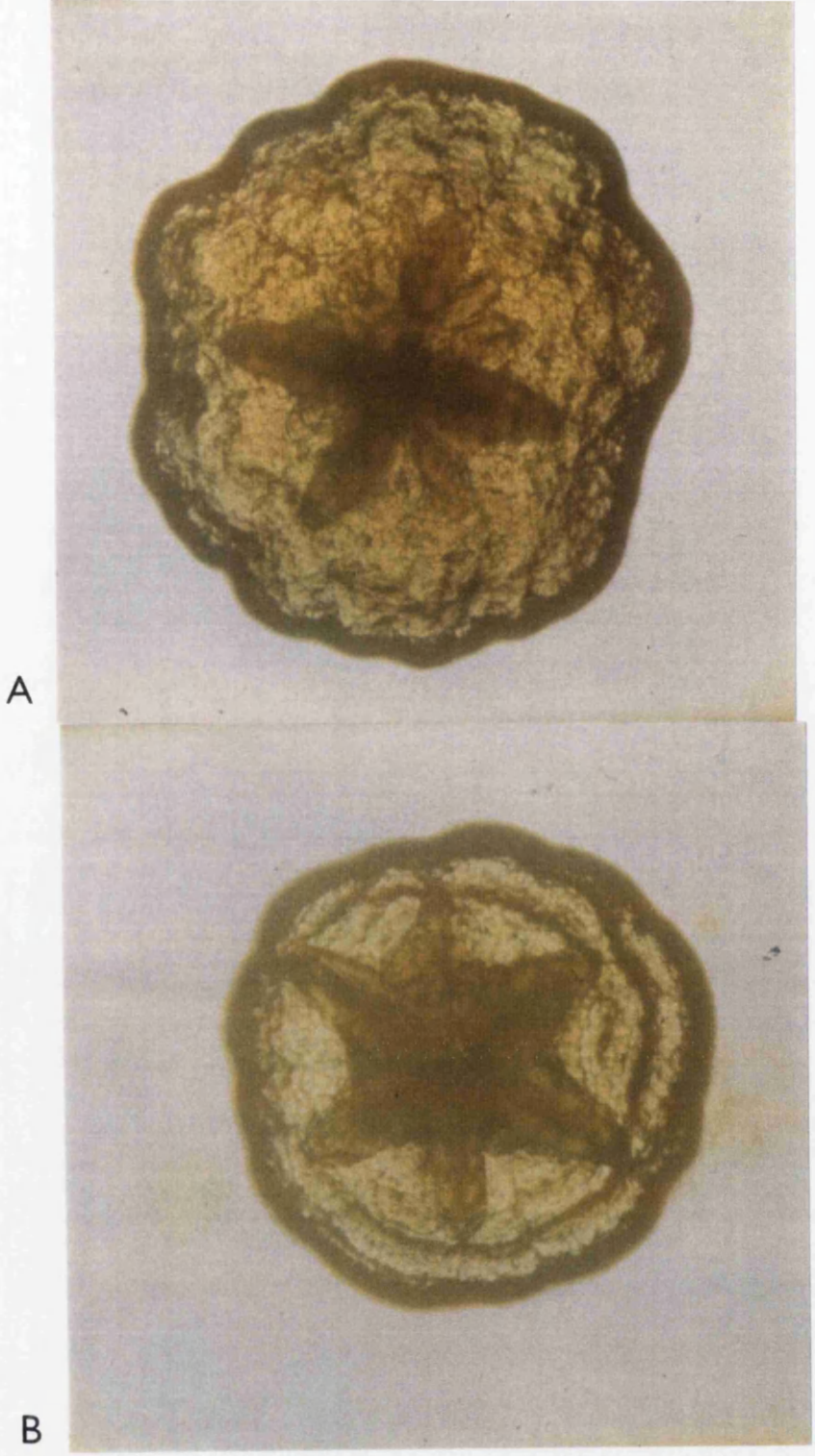


Figure 4.1 (A and B) A representative A.actinomycetemcomitans star-shaped colony (photopraph a) and representative H.aphrophilus star-shaped colony (photograph b).

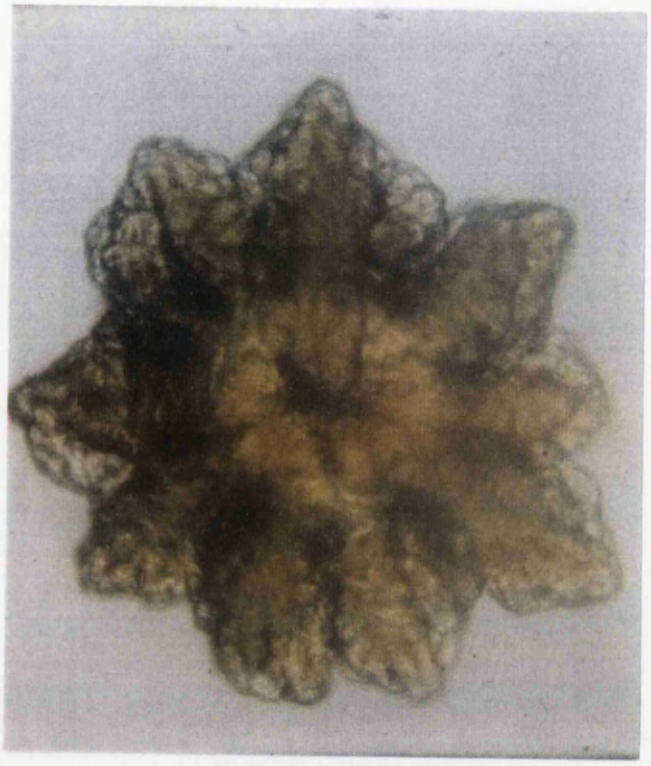


Figure 4.1 (C) A representative star-shaped colony of an identified Gram-positive coccus

cultures. The test is used to distinguish between the very similar colonies produced by A.actinomycetemcomitans and H.aphrophilus. It is generally accepted that A.actinomycetemcomitans strains are catalase producers. (Zambon, 1985; Kaplan, 1989; Papapanou et al., 1993). This was in agreement with the results obtained in the present study, as all A.actinomycetemcomitans strains isolated were catalase positive. However, it is important to mention that catalase negative A.actinomycetemcomitans strains have been isolated infrequently from subgingival plaque samples (Tanner et al., 1982; Miyasaki, Wilson and Genco, 1986) . Baehni et al., (1979) reported that the A.actinomycetemcomitans strain Y4 was catalase negative which is very surprising as other studies, including the present study, found the strain Y4 to be a catalase producer. Tanner et al., (1982) reported that 3 of 15 A.actinomycetemcomitans strains were catalase negative, though no information was given about the test and how it was performed. Miyasaki, Wilson and Genco (1986) also reported the variability of A.actinomycetemcomitans strains with respect to catalase activity. If the catalase production is not a consistent feature of A.actinomycetemcomitans, then a large number of the

reported A.actinomycetemcomitans prevalence studies are questionable in their findings, since most workers have used catalase production as a key test for identification of the organism. Indeed, some studies have used the colonial morphology and catalase production only for the identification of A.actinomycetemcomitans strains isolated from subgingival plaque samples (Papapanou et al., 1993). Biochemical tests are essential for the formal identification of these bacteria.

Biochemical characteristics of A.actinomycetemcomitans

In the present study the commercial system API 20A was used for the biochemical characterization of the isolated A.actinomycetemcomitans strains. The system contains 20 different biochemical tests that can be inoculated with a standard suspension of the organism. The system is a very convenient and rapid way of testing large numbers of strains. The biochemical characteristics of A.actinomycetemcomitans are listed in Table 3.4. Results obtained with the system were in agreement with some of the reported studies. Tanner et al., (1982) reported similar results for carbohydrate fermentation, though they reported negative catalase

results for some strains. The results obtained in the present study were in agreement with those reported by King and Tatum (1962) and Kaplan et al., (1989).

It is clear from the ongoing discussion that there are some differences in certain biochemical characteristics of A.actinomycetemcomitans. These differences are more evident with the reported variable tests rather than the confirmed positive or negative tests. This may be related to differences in the experimental methods, the type and source of the basic chemicals and media used for these tests. The API 20A system resolved a number of these problems and it provided a very reliable, rapid way of identifying this organism.

4.1.3 The sensitivity of A.actinomycetemcomitans strains to antibiotics

The susceptibility of A.actinomycetemcomitans strains to four antibiotics was tested in vitro. The results obtained in the present study showed that all tested strains were sensitive to tetracycline, the majority of the strains were sensitive to ampicillin and erythromycin but a large number of strains were resistant to penicillin. The results of the present study are in agreement with those reported by Slots et

al., (1980) for tetracycline sensitivity, as they showed that all tested strains (59) were susceptible to tetracycline at small concentrations (2µg/ml). However, there were differences with regard to other antibiotics. For example, in the present study 92% of strains were sensitive to ampicillin while in a study by Slots et al., (1980) 83% of the strains were sensitive to ampicillin but at a much higher concentration (about 3 times the concentration used in the present study). Similarly, in the current study 80% of the strains were highly susceptible to erythromycin while Slots et al., (1980) reported that only 53% of the strains were sensitive to the same antibiotic at a comparable concentration. However, the results of the present study were substantially different from those of Slots et al ., (1980) with regard to penicillin sensitivity. Thus the results of this study showed that only 12% of the test strains were sensitive

to 1 unit (0.6 µg/ml) of penicillin whereas Slots et al., (1980) found that about 54% of strains were sensitive to penicillin at 4µg/ml. The differences in the susceptibility is probably due to the difference in the concentration of antibiotic used, although differences in the assay methods employed and the

number of strains tested in each of the studies may also be involved. Kaplan et al., (1989) in a review of the literature reported that A.actinomycetemcomitans strains were highly susceptible to tetracycline, which is in agreement with the results of the present study. However, they reported that about 50-80% of 145 A.actinomycetemcomitans strains in the reviewed studies were susceptible to ampicillin and about 50-80% of 100 A.actinomycetemcomitans tested strains were susceptible to penicillin. In the present study 92% and 12% of the 25 A.actinomycetemcomitans strains tested were sensitive to ampicillin and penicillin respectively. Furthermore, Kaplan et al., (1989) reported that less than 30% of A.actinomycetemcomitans strains were sensitive to erythromycin a finding which was not supported by the results of the present study, in which about 80% of the tested strains were sensitive. The reasons for these differences were not clear, though they may be related to methodology, concentrations of antibiotic, or natural changes in the antibiotic susceptibility of this organism as a result of the clinical use of antibacterial agents.

4.1.4 The isolation of H.aphrophilus from subgingival plaque samples

The H.aphrophilus strains used in this study were isolated from subgingival plaque samples taken from patients with different forms of periodontal disease. A total of 170 sites from 49 subjects were sampled and processed; 59 (35%) proved to be positive for H.aphrophilus, most of the sites positive for this bacterium showed heavy growth.

There is very little information about the prevalence of H.aphrophilus strains in periodontal disease or about their clinical significance. Only a few studies have addressed the role of H.aphrophilus and this was merely for reasons of comparison, since it is very closely related to A.actinomycetemcomitans. In the present study, H.aphrophilus was initially isolated by using the selective medium TSBVF (Tempro and Slots, 1986). A.actinomycetemcomitans was reported as sensitive to sodium fluoride and inhibited on TSBVF medium (Tempro and Slots, 1986). This medium is very similar to the TSBV medium, but in addition contains sodium fluoride. In the present study the TSBVF medium was used initially together with the TSBV medium, to selectively isolate H.aphrophilus and

A.actinomycetemcomitans respectively. However, it was observed during the early stages of the study that there was not much difference between the organisms cultured on both media, contrary to what Tempro and Slots (1986) had reported. For this reason it was decided that only the TSBV medium would be used, since it supported the growth of both organisms equally well. Tempro and Slots (1986) reported the isolation of H.aphrophilus from 52% of subgingival plaque samples collected from 11 out of a group of 14 subjects. Its contribution to the total subgingival microflora at diseased sites was lower than at healthy sites and the authors concluded that H.aphrophilus plays no role in advanced periodontal disease (Tempro and Slots, 1986). In the present study no attempt was made to calculate the proportion of H.aphrophilus in the total microflora of the subgingival plaque samples, though the overall prevalence of H.aphrophilus in the subgingival sites 35% was slightly lower than the 52% reported by Tempro and Slots (1986). Liljemark et al., (1984) in a study to demonstrate the distribution of oral Haemophilus species in dental plaque reported the isolation of H.aphrophilus from 7 (50%) of the subgingival sites

sampled among 14 subjects and from 4 (29%) supragingival sites from the same 14 subjects.

4.1.5 Identification of H.aphrophilus

H.aphrophilus was isolated and identified on the basis of colonial morphology, negative catalase reaction and biochemical characterization.

The colonial morphology of H.aphrophilus is very similar to the colonial morphology of A.actinomycetemcomitans on primary culture. Cultures were examined for both star-shaped and non star-shaped colonies which were purified by culture; after a few subcultures all colonies lost the star-shaped characteristic. Tempro and Slots (1986) in their characterization of H.aphrophilus oral isolates did not mention the ability of H.aphrophilus colonies to form the star-shape on primary culture. Their description of the smooth type colonies, however, is in agreement with the appearance of these colonies in the current study.

The catalase test was used to differentiate between the colonies of both H.aphrophilus and A.actinomycetemcomitans. In the present study all H.aphrophilus isolates were catalase negative, in agreement with all reported studies in the literature (Tempro and Slots, 1986).

Biochemical characterization of H.aphrophilus strains

In the present study the identification of H.aphrophilus was performed by using the API 20A system. The identification of H.aphrophilus was based on the fermentation of glucose, lactose, saccharose and maltose together with a negative catalase test. The results obtained with the API 20A system for H.aphrophilus oral isolates were in agreement with all reported studies (King and Tatum, 1962; Liljemark et al., 1984; Tempro and Slots, 1986).

4.1.6 The sensitivity of H.aphrophilus to antibiotics in vitro

In the present study, 4 antibiotics were tested against H.aphrophilus strains isolated from subgingival plaque samples. The results showed that the H.aphrophilus strains were susceptible to all the antibiotics tested. Though, larger number of strains were sensitive to tetracycline and ampicillin and the remaining strains were moderately sensitive. Results obtained in the present study were in agreement with the findings of Baker et al., (1985). This group (Baker et al., 1985) studied 17 antibiotics, three of which (tetracycline,

erythromycin and penicillin) were used in the current study. H.aphrophilus strains were found to be highly sensitive to tetracycline and erythromycin, though a higher concentration of penicillin was required according to Baker et al., (1985). Similar results were obtained in the current study.

4.1.7 The isolation of Capnocytophaga species from subgingival plaque samples

The identification of Capnocytophaga species was based on colonial morphology and Gram stain reaction only. Colonies of Capnocytophaga species on CBA plates were distinct and easily distinguishable from other colonies on the plate. Biochemical characterization was attempted by using the Minitek Anaerobe Identification System but it was unsuccessful with poor reproducibility. The identification of the Capnocytophaga isolates to the species level was not, therefore, possible by using the Minitek system. The isolation of Capnocytophaga species was based on the distinct colonial and morphological characterization. Similar identification procedures were used by Papapanou et al., (1993) as these authors used only

cultural and morphological characteristics and a motility test for the isolation of Capnocytophaga species. It has to be mentioned though that the Minitek system can be used for the identification of certain Capnocytophaga species (for example C.ochracea) though not other Capnocytophaga species. In the current study Capnocytophaga species were isolated from a large proportion (53%) of the 170 subgingival plaque samples. Some early reports in the literature suggested that Capnocytophaga species might be important in the aetiology of periodontal disease (Newman et al., 1976; Slots and Genco, 1984) but the evidence supporting this claim is contradictory and not conclusive. Furthermore, in a recent review of the currently suspected periodontal pathogens (Socransky and Haffajee, 1994) Capnocytophaga species were not included. In fact it has been shown that some Capnocytophaga species, for example C.ochracea, may be beneficial to the host in some cases (Socransky and Haffajee, 1992). Papapanou et al., (1993) reported that Capnocytophaga species were isolated from subgingival plaque samples of 32% of 171 randomly selected individuals, though there was no correlation between the isolation of Capnocytophaga species and the periodontal conditions of the subjects.

In the present study the prevalence of Capnocytophaga species was slightly higher than that reported by Papapanou et al., (1993). Mashimo et al., (1983) also reported the isolation of Capnocytophaga species from 15 subgingival plaque samples from 5 patients with advanced periodontitis, a higher prevalence than that obtained in the present study. However, Mashimo et al., (1983) used a selective medium for the recovery of Capnocytophaga species as opposed to the non-selective medium (CBA) used in the current study. This may account for the differences observed between the two studies. A number of studies have reported the isolation of Capnocytophaga species from subgingival plaque samples taken from patients with different periodontal conditions and from healthy individuals. For example, Moore et al., (1983) reported the isolation of Capnocytophaga species from subgingival plaque samples from patients with moderately sever chronic periodontitis. However, they were considered unimportant as direct causative agents since they were isolated in equal or greater numbers from healthy individuals (Moore et al, 1983).

4.1.8 The sensitivity of Capnocytophaga species to antibiotics

The Capnocytophaga species isolated in the present study were tested for their susceptibility to four antibiotics. The results showed that all tested Capnocytophaga strains (34) were highly susceptible to tetracycline, erythromycin and ampicillin. Susceptibility to penicillin varied among strains, as some strains were sensitive and others were only moderately sensitive. Mashimo et al., (1983) reported that Capnocytophaga strains were highly sensitive to tetracycline, ampicillin, erythromycin and penicillin, in agreement with the results obtained in the current study. Baker et al., (1985) also tested a number of Capnocytophaga strains for their susceptibility to a range of antibiotics. They demonstrated the sensitivity of this organism to tetracycline, penicillin and erythromycin, though a higher concentrations of tetracycline relative to the other two antibiotics was required, since Baker et al., (1985) reported that 25μM of tetracycline were required to inhibit 90% of the tested strains, compared to only 1.6μM/ml and 3.2μM/ml for penicillin and erythromycin respectively. This result is in agreement with that obtained in the

present study. Recently Garces et al., (1994) reported the isolation of a C.sputigena strain which was resistant to a number of β-lactam antibiotics, including ampicillin. The authors concluded that sensitivity testing should be performed on isolates from all patients with infections caused by Capnocytophaga species or any other capnophilic organisms present in their oral microflora.

4.1.9 Conclusions

H.aphrophilus was isolated from 35% of 170 subgingival plaque samples from 49 subjects with different periodontal conditions.

A.actinomycetemcomitans was isolated from only 6% of 170 subgingival plaque samples by using the selective medium TSBV.

Capnocytophaga species were isolated from the larger proportion (53%) of 170 subgingival plaque samples.

All three organisms, which form the subject of this thesis, were sensitive to tetracycline, erythromycin and ampicillin, though the susceptibility to penicillin was variable.

4.2 Haemagglutination of human erythrocytes of blood groups A and O by A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains

The adherence of pathogenic bacteria to host surfaces is an essential step for colonization, invasion and subsequent production of disease. In the present study the ability of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains to adhere to oral surfaces was addressed by testing the ability of these groups of organisms to haemagglutinate human group A and group O erythrocytes. Others have already shown that the ability of microorganisms to haemagglutinate erythrocytes from different sources can be used as a model to study the interaction between bacterial cells and other cell types, for example epithelial cells (Duguid, Clegg and Wilson, 1979). The results obtained in the current study for the A.actinomycetemcomitans strains showed that 18 (72%) of the 25 tested strains were able to haemagglutinate both group A and O human erythrocytes. About 30% of the positive strains had a titre of 8, while the remaining 42% of positive strains had a titre of 2 or 4. Haemagglutination titres of 2 or 4 are extremely low and some would suggest that they be viewed as negative. However, in other reported studies

the ability of certain organisms to haemagglutinate erythrocytes has been measured visually without calculating titres (Majeed and Macrae, 1994). There are a number of studies in the literature which indicate the ability of A.actinomycetemcomitans to adhere to host tissues. Rosan et al., (1988) illustrated the ability of A.actinomycetemcomitans to adhere to hydroxyapatite by using fimbriated and non-fimbriated variants. Taher and MacFarlane (1991) reported the ability of A.actinomycetemcomitans strains to adhere to human buccal epithelial cells in vitro. Mintz and Fives-Taylor (1994) also reported the ability of A.actinomycetemcomitans to adhere to the cultured human oral cell line KB as measured by an enzyme linked immunosorbent assay or (H^3)thymidine labelled adhesion assay. Okuda and Kato (1987) demonstrated the ability of certain lipopolysaccharides prepared from a number of subgingival plaque bacteria, including A.actinomycetemcomitans to haemagglutinate human and animal erythrocytes. They reported that lipopolysaccharide from the strain Y4 agglutinated human erythrocytes of groups A and O (Okuda and Kato, 1987). Several mechanisms have been suggested for adherence of A.actinomycetemcomitans to host tissues.

For example, fimbriae are believed to play a role in some cases (Rosan et al., 1988), but extracellular microvesicles derived from some strains of A.actinomycetemcomitans have also been shown to function in adhesion (Meyer and Fives-Taylor, 1993). The results of the present study regarding the ability of A.actinomycetemcomitans to haemagglutinate human erythrocytes also indicate ability of some strains to bind to host tissue.

Of the 30 H.aphrophilus strains tested in the present study, 28 (93%) were recorded as haemagglutination positive though only 7% had a titre of greater than 4, and overall the haemagglutination activity was lower than for A.actinomycetemcomitans. There are few studies in which the question of H.aphrophilus adherence has been addressed. Sweet, MacFarlane and Samaranayake (1988) examined a number of oral bacteria for their ability to adhere to human buccal epithelial cells in vitro, and H.aphrophilus was among the test group. They reported that H.aphrophilus adhered to buccal epithelial cells, though not strongly. Furthermore, it was shown that freshly isolated strains of A.actinomycetemcomitans were more hydrophobic than the freshly isolated strains of H.aphrophilus (Holm and

Kalfas (1991) and a correlation between hydrophobicity and the ability of oral bacteria to adhere is well recognised (Taher, 1990). The haemagglutination results for H.aphrophilus may, therefore, reflect the ability to adhere.

Ten Capnocytophaga species were tested for their ability to haemagglutinate human erythrocytes. All tested strains were able to haemagglutinate both group A and group O human erythrocytes, producing very high titres, as 50% of the strains had a titre of 32 or more, while the remaining 50% had a titre of 8 or 16. These results may reflect the ability of this organism to adhere and colonize the host tissues, since it has been shown that certain Capnocytophaga strains were able to adhere to human buccal epithelial cells in vitro (Sweet, MacFarlane and Samaranayake, 1988). There are no similar studies regarding the ability of Capnocytophaga species to haemagglutinate human erythrocytes, though the lipopolysaccharide isolated from C.gingivalis strain ATCC 33624 has been shown to haemagglutinate human erythrocytes. No haemagglutination was observed when the lipopolysaccharide from C.ochracea strain ATCC 33596 was tested.

The results obtained in the present study regarding the ability of these groups of organisms to haemagglutinate human erythrocytes were variable, with Capnocytophaga species being the strongest and H.aphrophilus the weakest.

Various sugars can inhibit the attachment of certain oral bacteria to surfaces. For example binding of E.corrodens to human buccal epithelial cells is inhibited by sugars containing D-galactose and N-acetyl-D galactoseamine. Mannose inhibited the binding of E.corrodens only moderately (Yamazaki, Ebisu and Okada, 1981), which suggests that carbohydrate receptors on either the bacterial surface or the target cells to which they adhere are important determinants of adherence. Haemagglutination inhibition by certain sugars was attempted in this study, though the results obtained with both A.actinomycetemcomitans and H.aphrophilus were not conclusive, perhaps because of the lower titres in the absence of sugars. However, not all such interactions involve carbohydrate receptors. For example the attachment of the oral pathogen B.gingivalis to erythrocytes was resistant to sugars (Okuda et al., 1986). The authors related this result to the fact that the haemagglutinin of B.gingivalis

could bind specifically to proteinaceous material associated with amino acid residues, and similar ability may also exist among strains of the A.actinomycetyemcomitans and H.aphrophilus.

The ability of mannose to inhibit the haemagglutination of three Capnocytophaga strains with very strong haemagglutination activity was tested in the present study. The results indicated that all 3 strains were sensitive to mannose suggesting that the bacterial lectin-like surface components are important determinants of adherence and the attachment of Capnocytophaga species to erythrocytes.

4.2.1 Conclusions

Of the 25 A.actinomycetemcomitans strains tested 18 (72%) were able to haemagglutinate human erythrocytes of groups A and O.

Of the 30 H.aphrophilus strains tested 28 (93%) were shown to possess haemagglutination activity against human erythrocytes.

All tested Capnocytophaga strains were shown to possess strong haemagglutinating activity against human erythrocytes.

The haemagglutination activity of 3 Capnocytophaga strains was sensitive to mannose.

4.3 Leukotoxic activity in A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains as measured by the trypan blue exclusion and chemiluminescence assays

4.3.1 Experimental methods

A number of methods have been used for the detection of leukotoxic activity in A.actinomycetemcomitans. These include the inhibition of ^{51}Cr release, lactate dehydrogenase release, trypan blue exclusion and less frequently used electron microscopic examination methods (Baehni et al., 1981; Rabie, Lally and Shenker, 1988; Tsai et al., 1984). Other techniques have been employed for the detection of related toxins (eg Pasteurella haemolytica leukotoxin), including the reduction of the tetrazolium dye MTT (Vega et al., 1987) and a luminol-dependent chemiluminescence inhibition assay (Chang and Renshaw, 1986; Chang et al., 1986).

In this study two different assay systems, the trypan blue exclusion with HL60 cells and luminol-dependent inhibition with human PMNLs assays were used to detect

leukotoxic activity among strains of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species.

4.3.2 Criteria used to determined the leukocytotoxicity by both assay systems

Trypan blue exclusion assay

In the trypan blue assay, dead cells are stained with trypan blue while live cells exclude the dye. The percentage of dead target cells was calculated by directly counting dead and live cells by light microscopy. There are only a few studies where the trypan blue exclusion was used as an indicator of leukotoxicity to HL60 cells and human PMNLs and no criteria have been agreed by which a strain can be defined as leukotoxic. Although a number of investigators have used trypan blue to measure the leukotoxicity of A.acinomycetemcomitans, they have not defined clearly the cut-off point employed. For example Ohta et al., reported a value of 30% or more, while Tsai et al., (1979) reported a value of < 95% for human PMNLs though the cut-off point was not indicated in both studies. Furthermore some of these workers did not

even show any data related to the trypan exclusion assay (Baehni et al., 1981). The percentage of dead cells reported for human PMNLs are different: Ohta et al., (1987) reported a value of 30% or more, while Tsai et al., (1979) reported a value of more than 95% when human PMNLs were exposed to A.actinomycetemcomitans strain Y4 and more than 90% when human monocytes were the targets. Taichman and Wilton (1981) examined the ability of sonic extracts prepared from A.actinomycetemcomitans strain Y4 to destroy human PMNLs by leukotoxin and showed that the strain Y4 was leukotoxic to human PMNLs (56% kill) as measured by the trypan blue exclusion assay. Zambon et al., (1983) reported a value of more than 60% death for HL60 cells when they were exposed to the leukotoxic strain Y4, compared to 15% for HL60 cells unexposed to leukotoxin. Rabie, Lally and Shenker (1988) reported a percentage death of 80-85% of the total target cells exposed to leukotoxin, using human monocytes as targets.

In this study any strain which killed 30% or more of the target cells after subtracting the negative control value was considered leukotoxic. Although other workers have reported higher values, they failed to mention the threshold value for leukotoxicity, and it was

considered reasonable in this study to accept a kill of 30% as the lower limit of leukotoxicity. This same figure has been reported by other workers (Ohta et al., 1987), although, it was obtained with a single A.actinomycetemcomitans leukotoxic strain, and it was not considered as the cut-off point for deciding whether a strain is leukotoxic or non-leukotoxic.

Luminol-dependent chemiluminescence inhibition assay

Chemiluminescence has not been employed previously to distinguish between leukotoxic and non-leukotoxic strains of A.actinomycetemcomitans and no figures are available to indicate the lower limit of significant activity. In the present study the cut-off-point was set at about 50% reduction or inhibition of the chemiluminescence peak produced by PMA-stimulated human PMNLs (control cells) at $37^{O}C$ for 40 minutes. The 50% reduction was chosen to allow for the variability in PMNL chemiluminescence responses that may occur, since it was observed that PMNLs responses vary from one individual to another and also from the same individual at different times (Stevens, Winston and Van Dyke, 1978). Such variability was also observed during the

course of these experiments and by other workers (Dr. M.Saddati, personal communication).

4.3.3 Leukotoxic activity of A.actinomycetemcomitans Type strains

In the present study a total of 5 A.actinomycetemcomitans type strains were tested for their leukotoxic activity by using the trypan blue exclusion assay. Based on the criteria used in this thesis, three strains [A.actinomycetemcomitans Y4 (E81), JP2 (E88) and ATCC 29524 (E80)] were leukotoxic. Strain JP2 was the most toxic of all strains, killing more than 63% of HL60 cells, while the two other type strains were non-toxic [ATCC 29523 (E79) and NCTC 9710 (E83)]. The leukotoxicity of these type strains have been studied by a number of workers and even though some of these strains were investigated more frequently than others, in several studies similar results have been reported (Table4.1). The leukotoxic type strains in this study have been shown to be leukotoxic in other studies (Baehni et al., 1981; Simpson, Berthold and Taichman, 1988; Iwase et al., 1990). However, Tsai et al., (1978) reported that A.actinomycetemcomitans strain Y4 was not

cytotoxic to human PMNLs, but in a later report (Baehni et al., 1979) by the same group strain Y4 was recorded as leukotoxic to human PMNLs. These workers related the discrepancy to the growth medium and conditions under which the non-cytotoxic strain was grown (Baehni et al., 1979). The significance of this finding is not clear, since virtually all other studies using the same strain (Y4) have indicated that it is leukotoxic to both commonly used targets (human PMNLs and HL60 cells) under a number of different experimental conditions (Baehni et al., 1981; Zambon et al., 1983; Taichman et al., 1991)(Table 4.1).

There are several studies where the leukotoxicity of these type strains were reported and in spite of the fact that the experimental conditions employed in the present study were different from those reported in other studies, (for example the growth medium and conditions employed, together with the criteria by which a leukotoxic strain is defined), the results obtained were similar. However, the reported values for certain strains varied from one study to another as shown in Table 4.1, for example the value reported for the strain Y4 was about 96% in a study reported by Baehni et al., (1979), while Taichman and Wilton (1981)

Table 4.1 Leukotoxic activity of A.actinomycetemcomitans strains

Study reference	No of strains		No of positive		%death Try. blue		Concentration used	Target cells	Culture medium*
	Type	Fresh	Type	Fresh	Type	Fresh			
Tsai et al.,(1978)	Y4		0		ND		25-100	PMNL's	TSA
Baehni et al., (1979)	Y4		1		85-96%		50-200	PMNL's	TGB
Tsai et al., (1979)	Y4		1		>95%		0.01-1.0 mg/ml	PMNL's	TGB
Taichman et al., (1980)	Y4, ATCC 29522,23,24		3		ND		10-50 µg/ml	Monocytes	TGB
Baehni et al., (1981)	Y4,ATCC 29522,23,24	10	3	7	ND		5-200 or 0.1-0.8mg/ml	PMNL's	TGB
McArthur et al., (1981)	Y4		1		ND		1mg/ml	PMNL's	TGB

Table 4.1 continued

Study reference	No of strains		No of positive		%dead tryp an blue		Concentrat ion used	Target cells	Culture medium*
	Type	Fresh	Type	Fresh	Type	Fresh			
Zambon et al., (1983)	Y4, ATCC 29522, 23,24, Aa 67	100	3	49*	60%(Y4) (HL60)		OD_{540} 0.3-1.0	HL60 cells and PMNL	TGB
Tsai et al., (1984)	Y4, JP2, 627, 511, 652		5		ND		0.1-10µg/ml	PMNL	TGB
Ohta et al., (1987)	ATCC 29522, 23, 24	32	2	8	30%		1.7-3.6 mg/ml	PMNL's	TGB
Lally et al., (1989)	JP2		1		ND				PYG
Chung et al., (1989)	ATCC 29523,Y4, SUNYaB67	46	3	10	ND		50 bacte. cells/PMNL	PMNL's	BHIB
Iwase et al., (1990)	JP2		1		ND		0.6-10ng toxin	HL60 cells	TGB

Table 4.1 continued

Study reference	No of strains Type	Fresh	No of Positive Type	Fresh	%dead cells Trypan blue Type	Fresh	Conc. used	Target cells	Culture medium*
Iwase et al., (1990)	JP2		1		ND		0.6-10ng toxin	HL60 cells	TGB
Taher (1990	Y4, NCTC 9709, 9710, 10980, 109, 81, 10979	12	0	1		33%	0-200	HL60 and PMNL's	TGB
Taichman et al., (1991)	Y4		1		ND		5-20ng toxin	HL60 cells, PMNL, U937	TGP
Brogan et al., (1994)	17 strains		2		LD_{50} 2.5-50x10^{6**}		0-250/ml	HL60 cells	PYG
Present study	Y4, ATCC 29523, 24, NCTC 9710, JP2	20	3	2	37-63%		400/ml	HL60	TSB

* See abbreviations table

reported a value of 56% though the target cells in both studies was human PMNLs. In spite of the different conditions used in both studies as in the first study whole cells were used while in the second sonic cell extract was used. Ohta et al., (1987) reported a value of 30% kill. Furthermore, Zambon et al., (1983) reported a value of only 60% though the target cells were HL60 which were shown to be slightly more sensitive than PMNLs. The reasons for this variability are not clear, but perhaps it is not surprising due to the lack of defined criteria for leukotoxicity and consistency in assay methodology in different studies. The latter may be especially important since it has been shown that the sensitivities of a number of commonly used assays for the detection of leukotoxicity differ in their sensitivity (Chang and Renshaw, 1986).

There is not much information in the literature concerning the trypan blue exclusion assay which was used in the present study, especially when HL60 cells are employed as the target cells. However, a few studies have reported the use of trypan blue exclusion as an indicator of leukotoxicity (Table 4.1). Zambon et al., (1983) reported that the trypan blue staining of

HL60 cells exposed to A.actinomycetemcomitans strain Y4, revealed that more than 60% of HL60 cells were killed compared to 15% for the unexposed HL60 cells. In Zambon et al., (1983) study A.actinomycetemcomitans strain Y4 was the only type strain stained with trypan blue, though 3 other type strains were examined in the study. The authors did not refer to or discuss the suitability of using trypan blue exclusion with HL60 cells for the detection of leukotoxic activity of A.actinomycetemcomitans, considering the variability between the reported values for the same strain Y4 in different studies. The results obtained in the present study were comparable with those reported by Zambon et al., (1983), since 50% killing of HL60 was the value obtained for the same Y4 strain.

More recently Brogan et al., (1994) examined a number of A.actinomycetemcomitans strains for their leukotoxic activity against the HL60 cell line and the trypan blue exclusion was used as an indicator for leukotoxic activity. A number of type strains were included and some of these strains were also tested in the current study (JP2, Y4, ATCC 29523, ATCC 29524 and NTCC 9710). The leukotoxic activity was measured by calculating the LD_{50} which was defined as the number of bacteria

required to kill 50% of $2x10^5$ HL60 cells in 1 hour at $37^{O}C$. The results reported indicated that strain JP2 was the most toxic strain. The remaining strains were less toxic, as even the commonly reported leukotoxic Y4 strain expressed 20-fold lower toxicity to HL60 cells while strain ATCC 29524 expressed about 10-fold lower toxicity than the JP2 strain. This result compares to the results obtained in the present study in spite of the differences in the experimental conditions employed. However, the more surprising result reported by Brogan et al., (1994) was that of the strain NCTC 9710 which was shown to be leukotoxic to HL60 cells and also with higher potency than the known leukotoxic strain Y4. The results obtained in the present study indicated that it is a non-leukotoxic strain as measured by the trypan blue exclusion assay.

The reason for this variability is not clear, though it may be the criteria used to identify leukotoxicity.

The two type strains used in the present study and found to be non-leukotoxic were strain ATCC 29523 and NCTC 9710. There was some variability with respect to the leukotoxicity of these type strains since most of the reported results of the ATCC 29523 strain have indicated variability of the leukotoxicity of this

strain. Thus, it was originally reported as leukotoxic to human PMNLs (Baehni et al., 1979; Spitznagel, Kraig and Kolodrubetz, 1991) though negative results against both human PMNLs and HL60 cells have also been reported (Zambon et al., 1983). More recently Ohta et al., (1993) have shown that the strain ATCC 29523 was leukotoxic when it was grown in a chemostat culture. More surprising was the finding that the reported non-leukotoxic strain ATCC 33384, the equivalent of NCTC 9710, was also found to be leukotoxic (Ohta et al., 1993). Recently, in a study reported by Haubek et al., (1995) it has been shown that a different epidemiological situation may exist between Europe and USA regarding the virulence of A.actinomycetemcomitans isolates. Particularly virulent clones, with high level production of leukotoxin, may be present in the USA but not in Northern Europe. This was previously reported by Brogan et al., (1994) as they examined 97 A.actinomycetemcomitans strains originating from both Europe and USA and found that only two strains expressed high leukotoxic activity, both of which originated from the USA.

It is clear from the discussion above that there is some variation in the reported leukotoxicity values for

A.actinomycetemcomitans type strains. This may result from the different criteria used to identify leukotoxicity when the trypan blue exclusion was used, since it varied from 30% (Ohta et al., (1987) to 96% (Baehni et al., 1979), and it may be also due to the different detection systems and their sensitivities. It may also be important to mention that most of the reported studies have examined only a few strains, and sometimes only one strain was used. In spite of the reported differences between different studies, the results obtained in the present study with A.actinomycetemcomitans type strains were comparable to those reported for the similar type strains in several other studies. Unless a standardized method taking into consideration all aspects of the assay, is established it will be difficult to compare results from different studies.

4.3.4 Leukotoxic activity of freshly isolated oral A.actinomycetemcomitans strains

Of the 20 fresh A.actinomycetemcomitans isolates, only two (10%) were leukotoxic to HL60 cells as measured by the trypan blue exclusion assay: strains E24 and 34 produced values of 61% and 37% respectively. There are

a number of studies where the leukotoxicity of fresh A.actinomycetemcomitans isolates has been measured. However, only a few of them used the trypan blue exclusion assay: Taher, for example, (1990) examined 12 fresh A.actinomycetemcomitans isolates and showed that only one strain was leukotoxic, and killed about 33% of the target cells.

The leukotoxic activity among the fresh isolates tested in this study was not previously determined. However, similar studies using A.actinomycetemcomitans fresh isolates were performed by a number of researchers.

The prevalence of leukotoxic and non leukotoxic A.actinomycetemcomitans fresh isolates was examined by Zambon et al., (1983). They reported that 6% of 13 fresh A.actinomycetemcomitans isolates from 11 healthy individuals, were toxic to PMNLs, as were 43% of 14 strains isolated from 13 patients with adult periodontitis, 75% of 4 strains isolated from 4 patients with juvenile diabetic periodontitis, 66% of 3 strains isolated from 2 patients with generalized juvenile periodontitis and 55% of 66 isolates from 25 patients with localized juvenile periodontitis. However, data obtained with these leukotoxic fresh oral isolates were not shown as the authors indicated what

percentage of tested strains were leukotoxic (Zambon et al., 1983). Furthermore, they have shown that a single individual harboured both leukotoxic and non-leukotoxic A.actinomycetemcomitans strains. These findings of Zambon et al., (1983) clearly indicate that leukotoxic and non-leukotoxic strains were found within each subject group, though leukotoxic strains were found in significantly higher proportions in diseased subjects, especially among juvenile periodontitis patients. Baehni et al., (1981) examined 10 fresh A.actinomycetemcomitans isolates for leukotoxic activity and they reported that the majority (70%) of tested strains were leukotoxic to human PMNLs. However, the trypan blue exclusion test was not used as an indicator for leukotoxicity (Table 4.1). Ohta et al., (1987) reported that 8 (25%) of 32 freshly isolated A.actinomycetemcomitans strains from 3 patients with rapidly progressive periodontitis, gingivitis and juvenile periodontitis were leukotoxic to human PMNLs. Chung et al., (1989) reported that 10 (22%) out of 46 A.actinomycetemcomitans oral isolates were leukotoxic to human PMNLs. Therefore overall, the majority of wild strains of A.actinomycetemcomitans tested for leukotoxicity have proved to be negative.

leukotoxicity is a characteristic not shared by all A.actinomycetemcomitans strains.

Even though an objective comparison between the current study and those available in the literature is difficult, since the majority of these studies have used different detection systems (for example trypan blue was not commonly used), the prevalence of leukotoxicity among the fresh isolates used in this study was lower than those reported in the literature, since only 2 (10%) of 20 fresh isolates were found leukotoxic to HL60 cells as measured by the trypan blue exclusion assay. There are several possible reasons for this disparity. These factors will be discussed separately in the following sections.

4.3.5 Leukotoxic activity of H.aphrophilus strains

A total of 30 H.aphrophilus strains were tested for their leukotoxic activity against either HL60 cells as measured by trypan blue exclusion assay or against human PMNLs as measured by the luminol-dependent chemiluminescence inhibition assay. None of the strains tested were leukotoxic based on the criteria employed in this study. The percentage death of the target cells as measured by the trypan blue exclusion assay ranged

from (0% to 18%) though none was considered leukotoxic, since the values fell below the 30% cut-off point chosen for this study. There are only a few studies where H.aphrophilus leukotoxic activity has been reported and even then only a small number of strains were included for the purpose of comparison with A.actinomycetemcomitans. Zambon et al., (1983) included one strain (H.aphrophilus ATCC 5908) and it was found to be non-leukotoxic to HL60 cells as measured by the lactate dehydrogenase release assay. To further confirm the point about the difficulty in deciding on the criteria used to distinguish between leukotoxic and non-leukotoxic strains in the absence of a well standardized one, Zambon et al., (1983) considered the 3.3% and 3.7% lactate dehydrogenase release by a H.aphrophilus strain from HL60 cells and human PMNLs respectively as non-significant. On the other hand Chung et al., (1989) when testing a group of A.actinomycetemcomitans strains, considered a 2.5% release of lactate dehydrogenase was sufficiently high to designate a strain as leukotoxic. Baehni et al., (1981) tested 7 H.aphrophilus plaque isolates for their leukotoxic activity and reported that none of the tested strains were leukotoxic as measured by the

lactate dehydrogenase assay at a ratio of 100 bacteria/ PMNL. Sonic extracts of 3 H.aphrophilus strains caused the release of 3% of lactate dehydrogenase by human PMNLs when used at a concentration of 0.4mg protein/10^7 PMNLs. Tsai et al., (1984) tested one H.aphrophilus strain for its leukotoxic activity as measured by a ^{51}Cr release assay and reported that the tested strain was non-leukotoxic. Ohta et al., (1987) tested two H.aphrophilus type strains (H.aphrophilus NCTC 5906 and NCTC 5908) for their leukotoxic activity and reported that both strains were non-leukotoxic.

In this study 30 H.aphrophilus strains were tested for their leukotoxic activity as measured by two different assays. In the present study the results obtained from both assay methods were concordant and indicated that all tested strains were non-leukotoxic. This was the first time that a large number of H.aphrophilus strains were actually tested for their leukotoxic activity, as most if not all other studies included a few strains of H.aphrophilus for comparison reasons only.

4.3.6 Leukotoxic activity of Capnocytophaga species

Capnocytophaga species have been implicated in some forms of periodontal disease in a number of studies and

it has been shown that this organism is capable of producing a number of virulence factors (for example IgA1 protease) (Slots and Genco, 1984). Although leukotoxin is not believed to be produced by Capnocytophaga species, few studies have actually been reported. One study examined one C.gingivalis strain for leukotoxic activity and was shown to be non-leukotoxic as determined by observing morphological changes on human PMNLs (Ohta et al., 1987). In the present study, 8 Capnocytophaga strains were examined for their leukotoxic activity by two different techniques but none proved to be leukotoxic. Since these strains were not identified to species level it is still possible that a particular species possesses leukotoxic activity, but it was not present in the 8 strains that we examined.

Luminol-dependent chemiluminescence inhibition assay

The luminol-dependent chemiluminescence inhibition assay has not been used previously for the detection of the leukotoxic activity of A.actinomycetemcomitans strains and in order to assess the suitability of the assay a number of preliminary experiments were performed by using the leukotoxic E88 strain together

with some representative non-leukotoxic strains of A.actinomycetemcomitans and H.aphrophilus.

The incubation time was investigated and the results obtained from these experiments indicated that maximum inhibition of chemiluminescence was achieved after about 30 minutes at 37^{o}C with no substantial changes for upto 45 minutes. Cells were, therefore, incubated for 40 minutes. The stimulation of human PMNLs by using opsonized zymosan and PMA, and the possibility of using HL60 cells as targets in this assay, were also examined in these preliminary experiments since these cells express surface markers and components which are associated with mature granulocytes (Gallagher et al., 1979). The results obtained from these experiments indicated that opsonized zymosan was not an effective stimulus for human PMNLs, as it produced a broad and not very well defined chemiluminescence peak, over a longer period of time. By contrast the peak produced by the use of PMA was well defined and appeared quickly. There was no chemiluminescence produced when HL60 cells were used, even when the number of HL60 cells was increased substantially. However, the results of these experiments were not conclusive as the experiment was performed only once and the stage of growth at which

the HL60 cells were used was uncertain. This may be an important factor in determining whether the HL60 cells produce a chemiluminescent response or not. It would be very interesting to see if HL60 cells can produce chemiluminescence when exposed to appropriate stimulation, as this would enhance the assay substantially, because of the lengthy procedures needed for the isolation of human PMNLs and the need for fresh PMNLs.

Other important parameters that may affect the leukotoxicity assay outcome were tested in these preliminary experiments. These were the dose-dependency and the effect of heat on the leukotoxic activity of A.actinomycetemcomitans strains, since a number of studies have shown that the activity of A.actinomycetemcomitans leukotoxin was dose-dependent and inactivated by heat (Zambon et al., 1983; Ohta et al., 1987; Simpson et al., 1988). For this purpose the highly toxic A.actinomycetemcomitans JP2 strain and representative non-leukotoxic A.actinomycetemcomitans and H.aphrophilus strains were used. The results obtained from these experiments demonstrated that the leukotoxicity of the JP2 strain was dose-dependent, since an increase in the number of bacteria added was

accompanied by an increase in the inhibition of the chemiluminescence responses produced by the PMA-stimulated human PMNLs. The heated fractions of (JP2) strain for $90^{o}C$ for 20 minutes had no effect on chemiluminescence and similar results were obtained with suspensions of non-leukotoxic strains of A.actinomycetemcomitans and H.aphrophilus, treated in the same way as the JP2 strain.

Chemiluminescence has been used to investigate the functional activity of human PMNLs by a number of workers (Robinson et al., 1984). The function of PMNLs in periodontal disease patients was also assessed by chemiluminescence (Gomez et al., 1994), though chemiluminescence has not been used previously for detection of the leukotoxic activity of periodontopathic organisms like A.actinomycetemcomitans However, a chemiluminescence assay was developed and used for the detection of the Pasteurella haemolytica leukotoxin (Chang et al., 1986; Chang and Renshaw, 1986). The assay was also used to investigate different factors affecting the leukotoxic activity of P.haemolytica. The direct comparison of the chemiluminescence assay with other commonly used assays for the detection of P.haemolytica leukotoxin revealed

that the chemiluminescence assay was the most sensitive. It could detect leukotoxic activity at much higher dilutions than other assays. For example it was not possible to detect leukotoxic activity with the trypan blue exclusion assay at a dilution of 1:64, while it was still possible to detect (21% inhibition) leukotoxicity at a dilution of 1:16384 when the chemiluminescence assay was used (Chang and Renshaw, 1986). The sensitivity of the assay may relate to the fact that it measures the metabolic activity of PMNL rather than cell death.

For both assay systems employed in the current study there are advantages and disadvantages. The trypan blue exclusion assay has been used frequently to examine the viability of cells. It is technically easy and does not require the use of any expensive equipment or reagents. Further, the use of HL60 cells as targets in the assay may be advantageous since HL60 cells are probably more sensitive to the A.actinomycetemcomitans leukotoxin than are human PMNLs (Zambon et al., 1983). The maintenance and preparation of HL60 cells in the laboratory is also simpler and more convenient than the isolation and preparation of human PMNLs. In relation to disadvantages the visual counting of dead cells

makes it laborious to perform if a large number of samples is to be counted. Variability may occur if counting is performed by different individuals. In contrast the luminol-dependent chemiluminescence inhibition assay was shown to be very sensitive. The elimination of visual counting makes it more objective, it is rapid to perform as multiple samples can be tested simultaneously and a number of factors can be examined in a single experiment. It is highly reproducible, but it requires the use of relatively expensive equipment (Luminometer). Another major difference between the two assays is the need for freshly isolated human PMNLs with the latter assay, as it can be used only as a measurement of the functional activation of phagocytic cells (PMNL). Unfortunately HL60 cells can not be used in this assay. The fact that the results obtained by using both assays for all tested organisms were concordant, indicates the suitability of chemiluminescence for the detection of leukotoxic activity of oral organisms.

4.3.7 Factors affecting leukotoxicity

It is evident from the ongoing discussion that there was some variability regarding the prevalence of

leukotoxic A.actinomycetemcomitans strains. Several factors are thought to affect the leukotoxic activity of A.actinomycetemcomitans, a number of which will be presented and discussed in the following sections.

Growth medium and cultural conditions

A number of workers have correlated the growth medium and cultural conditions for A.actinomycetemcomitans to be important factors in demonstrating toxicity. Most workers have employed thioglycollate broth as the medium of choice with anaerobic culture conditions (Baehni et al., 1979; Baehni et al., 1981; Zambon et al., 1983; Iwase et al., 1990; Taichman et al., 1991) (Table 4.1). These studies reported positive leukotoxic activity for the majority of the tested strains though only small number of strains were tested and mostly type strains in some of these studies. The strains that have been studied most frequently are Y4 and JP2. Other media used by researchers to grow A.actinomycetemcomitans for the detection of leukotoxic activity have included PYG medium, TSA medium and BHIB medium; the leukotoxic activity of all leukotoxic strains was detected equally well in spite of using different

growth media (Lally et al., 1991; Brogan et al., 1994; Chung et al., 1989).

In this study, for the detection of leukotoxic activity of A.actinomycetemcomitans strains by both assays, bacteria were initially grown on CBA plates for 48 hours and then in TSB for 18 hours at $37^{O}C$ in 5% CO_2 in air. As indicated in the results only two fresh isolates were leukotoxic as measured by both assays and the prevalence of leukotoxicity among these strains was lower than that reported by other workers (Baehni et al., 1981; Zambon et al., 1983). It seems unlikely that growth medium and assay conditions were the reason for the low prevalence of leukotoxicity in the fresh isolates since these conditions were suitable for the detection of leukotoxic activity in known leukotoxic type strains.

Thioglycollate broth (TGB) was not used in the present study for a number of reasons. The medium was not readily available and TSB combined with CO_2 culture gave satisfactory results in preliminary experiments and successfully identified known toxic and non-toxic strains. Furthermore, in a previous study from this laboratory the TGB medium was used for growing A.actinomycetemcomitans for the detection of leukotoxic

activity, and in spite of the fact that some of the tested strains had been shown previously as leukotoxic they were found to be non-leukotoxic after growth in this medium (Taher, 1990). Furthermore, other researchers have used media other than the thyioglycollate broth and have been able to demonstrate leukotoxicity of A.actinomycetemcomitans strains (Lally et al., 1991; Brogan et al., 1994). Therefore, the importance of growth medium and culture conditions in the demonstration of leukotoxin is probably low.

Toxin concentration (dose-dependency)

For the detection of leukotoxic activity among A.actinomycetemcomitans strains, whole live cells, (bacteria/target cell), soluble sonic extracts (mg protein/ml) and purified toxin mg/dry weight) have been used. Leukotoxic activity of A.actinomycetemcomitans strains has been shown to be dose-dependent in a number of studies. However, experiments have been designed specifically to compare the suitability of using whole cells or soluble sonic extracts in the determination of leukotoxic activity of this organism. Tsai et al., (1979) showed that sonic extracts prepared from strain Y4 (concentration of 10^9 cells/ml) were toxic to both

human PMNLs and monocytes in a dose-dependent pattern, since the release of lactate dehydrogenase which was used as an indicator of leukotoxicity, increased directly (0-60%) with the increase in the Y4 sonic extract concentration from 0-1.5mg [dry weight]/ml after 1 hour incubation at $37^{o}C$. Similarly the incubation of sonic extract of Y4 with human PMNLs or monocytes for 1 hour at $37^{o}C$, resulted in killing of more than 95% of human PMNLs and more than 90% of human monocytes, as measured by trypan blue exclusion. Baehni et al., (1981) examined both fresh clinical isolates and type strains of A.actinomycetemcomitans for their leukotoxic activity and reported that both sonic extracts and whole cells prepared from leukotoxic strains of A.actinomycetemcomitans caused dose-dependent cytotoxicity to human PMNLs as measured by the lactate dehydrogenase assay. Taichman and Wilton (1981) also reported that a sonic extract prepared from the leukotoxic Y4 strain killed target cells in a dose-dependent pattern since an increased concentration of sonic extract (0.001-1.0μg/ml) resulted in increased killing of human PMNLs as measured by the trypan blue exclusion assay. Several reports confirmed the finding that the leukotoxicity of A.actinomycetemcomitans was a

dose-dependent (Zambon et al., 1983; Iwase et al., 1990).

In the current study a ratio of 400 bacterial cells to one HL60 cell was used. This ratio was higher than that used by some other workers, for example Baehni et al., (1981) who used a maximum ratio of 200 bacteria/PMNL. Tsai and Taichman (1986) used a concentration of 5-100 bacteria/PMNL, while Chung et al., (1989) used a dose of 50 bacteria/PMNL. The higher ratio was chosen since the leukotoxicity of H.aphrophilus strains and the fresh oral A.actinomycetemcomitans strains examined in this study was not known and a high bacterial cell concentration would ensure the detection of leukotoxic activity in any that possessed only low or moderate activity. In preliminary experiments the highly toxic A.actinomycetemcomitans JP2 strain was examined at different concentrations of whole cells, and the number of HL60 cells killed increased with higher concentrations of A.actinomycetemcomitans JP2 cells.

Although a high bacteria/target cell ratio was used, only 2 out of 20 fresh A.actinomycetemcomitans strains were leukotoxic as measured by both assays employed in this study. It, therefore, seems unlikely that this low prevalence of leukotoxic activity among fresh

A.actinomycetemcomitans isolates was due to the dose of toxin available.

From the discussion above it was clear that whole cells, sonicates or pure toxin can be used for the detection of leukotoxicity and that the leukotoxic activity is dose-dependent.

Exposure time

The incubation (exposure) time required to achieve maximum killing of the target cells (human PMNLs or HL60 cells) in the leukotoxic activity assays was shown to be of great importance. A number of studies have demonstrated that the leukotoxic activity of A.actinomycetemcomitans is time-dependent. Tsai et al., (1979) reported that the leukotoxicity of strain Y4 sonic extract was time-dependent, since the percentage of lactate dehydrogenase released from human PMNLs exposed to one dose (1.0mg/ml) of Y4 leukotoxin increased when the time was increased (0-60 minutes). This finding was also confirmed by Taichman, Dean and Sanderson (1980), who showed that the activity of A.actinomycetemcomitans leukotoxin against human monocytes was time dependent. Taichman, Dean and Sanderson (1980) reported that the maximum lactate

dehydrogenase release was reached after 120 minutes. Zambon et al., (1983) showed that exposure of whole A.actinomycetemcomitans strain Y4 to HL60 cells or human PMNLs resulted in maximum killing after 45 minutes at 37^{o}C as measured by the lactate dehydrogenase release. Chung et al., (1989) reported that the leukotoxic activity of A.actinomycetemcomitans reference strains was time-dependent and they used a 60 minute incubation time in their experiments. The exposure time used in the present study was 1 hour when the trypan blue exclusion assay was employed, as a number of published studies using HL60 cells and human PMNLs as targets have reported maximum killing within 45-60 minutes (Chung et al., 1989; Zambon et al., 1983). However, other incubation times have been used. It should be mentioned that prolonged preparation times for the target cells, extended incubation times or delays in cell counting after the assay is complete can cause damage to the target cells, as observed during the course of current experiments.

Temperature

The temperature of incubation in the leukotoxic activity detection assays is a critical factor. It is known that the leukotoxic activity of A.actinomycetemcomitans strains is temperature dependent (Tsai et al., 1979; Zambon et al., 1983), most indicating that 37^{O}C is the temperature most suitable for the detection of leukotoxicity. To determine the temperature of incubation for this study preliminary experiments were performed using the leukotoxic strain A.actinomycetemcomitans JP2 and the non-leukotoxic strain 100. The results showed that there was no detectable leukotoxic activity with the highly toxic JP2 strain when tested at 4^{O}C (ice cold cells) and minimal activity was detected at 22^{O}C (room temperature). All experiments were therefore, performed at 37^{O}C. Similar observations were reported in the literature, as Tsai et al., (1979) reported that there was no detectable leukotoxic activity at 4^{O}C, but at 37^{O}C the leukotoxic activity was detectable. Furthermore, Zambon et al., (1983) showed that the temperature was an influencing factor in the detection of leukotoxic activity in A.actinomycetemcomitans.

Target cells (target cell specificity)

Another important factor in the assay is the target cell employed. A number of studies have been conducted to determine the target cell specificity of A.actinomycetemcomitans leukotoxin. The results of these studies indicated that human skin fibroblasts, erythrocytes, platelets, and a number of non-human cells were non-susceptible to leukotoxin, while human monocytes and PMNLs were shown to be sensitive (Tsai et al., 1979; Tsai et al., 1984; Taichman et al., 1984). Additional studies revealed that PMNLs and monocytes from monkeys were susceptible to A.actinomycetemcomitans leukotoxin (Taichman et al., 1984). Taichman et al., (1987) showed that PMNL from humans, the great apes, and most Old World monkeys were susceptible to A.actinomycetemcomitans leukotoxin. The most commonly used target cells for the detection of A.actinomycetemcomitans leukotoxin have been human PMNLs and the leukaemic cell line HL60 (Zambon et al., 1983; Simpson, Berthold and Taichman, 1988; Iwase et al., 1990; Taichman et al., 1991; Brogan et al., 1994). The target cells employed in this study for the trypan blue exclusion assay were the leukaemic cell line HL60,

for the following reasons. HL60 cells have been previously used by a number of workers (Zambon et al., 1983; Simpson, Berthold and Taichman, 1988). Furthermore, the susceptibility of the HL60 cell line to A.actinomycetemcomitans leukotoxin and their availability provided a very convenient alternative to human PMNLs. Human PMNLs were used as targets when the luminol-dependent chemiluminescence inhibition assay was used since HL60 cells were not suitable for this assay. Simpson, Berthold and Taichman, (1988) studied the susceptibility of several cell lines to leukotoxin purified from A.actinomycetemcomitans strain JP2 and reported that a number of human tumour cell lines were susceptible to leukotoxin. These included the leukaemic cell lines HL60, U937 and KG-1, which can be used as models to study the mechanism of action of A.actinomycetemcomitans leukotoxin.

Other factors that may affect leukotoxicity

Some workers have reported that the age of the donor patient is an important factor in the leukotoxicity of A.actinomycetemcomitans isolates. Tsai and Taichman (1986) reported that 100% of the A.actinomycetemcomitans strains isolated from young

patients (6-12 years of age) were leukotoxic, but only 23% of strains isolated from a group of older patients (13-25 years of age) were leukotoxic. However, the significance of this finding is doubtful, since a number of other studies have indicated that leukotoxic strains of A.actinomycetemcomitans can be isolated from older patients (Chung et al., 1989).

The serotype of a particular A.actinomycetemcomitans strain or even the geographical location of the patients from which the strains were isolated were thought to be important factors in the determination of leukotoxicity of A.actinomycetemcomitans. Some studies have associated the A.actinomycetemcomitans serotype b with localized juvenile periodontitis in North American patients and hence with leukotoxicity, as the isolation of leukotoxic strains in these group is more common (Slots et al., 1982; Zambon et al., 1983; Zambon, 1985). However, Chung et al., (1989) reported that there was no correlation between serotype b and localized juvenile periodontitis nor with leukotoxicity in a group of Korean patients.

4.3.8 Conclusions

In summary, in the present study, three capnophilic Gram-negative microorganisms were tested for their leukotoxicity by using two assay systems. Results obtained indicated substantial differences in the leukotoxicity of all three organisms.

Only 2 of the 20 freshly isolated A.actinomycetemcomitans strains and 3 of the 5 type strains were leukotoxic to both HL60 cells and human PMNLs as measured by the trypan blue exclusion assay and the luminol-dependent chemiluminescence inhibition assay respectively.

The prevalence of leukotoxic strains among the fresh isolates of A.actinomycetemcomitans tested in this study was lower than that reported in the literature for fresh A.actinomycetemcomitans isolates. However, the results obtained with type strains were in agreement with those reported.

Dose of toxin, temperature of incubation and exposure time were the most important factors influencing the outcome of the detection of leukotoxic activity in A.actinomycetemcomitans.

This was the first time the leukotoxic activity of A.actinomycetemcomitans was detected by using the luminol-dependent chemiluminescence inhibition assay.

Leukotoxin production was not detected in any of 30 strains of H.aphrophilus. However, certain strains of H.aphrophilus killed small numbers of HL60 cells as measured by the trypan blue assay but they were considered as non leukotoxic based on the criteria used in the present study.

None of the Capnocytophaga strains was leukotoxic as measured by both methods.

The comparison of results obtained in different studies proved to be very difficult due to the large number of variables. A standard method, taking into consideration all these variables, is essential to allow comparison of results of different studies and secondly to be certain of the results obtained.

4.4 Phagocytosis of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species by human PMNLs in vitro

In the present study the ability of human PMNLs to phagocytose selected strains of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga was tested by using

a luminol-dependent chemiluminescence assay. (Allen, Stjernholm and Steele, 1972). The initial light emission by the phagocytic cells is very small to measure, requiring a large number of cells and a very sensitive light detector. Luminol has been used to enhance the light emission, thus improving the sensitivity of the assay (Allen and Loose, 1976; Stevens, Winston and Van Dyke, 1978). Furthermore, it has been shown also that the chemiluminescence response of normal PMNLs is positively correlated to the phagocytic ability of the PMNLs (Grebner et al., 1977; Stevens, Winston and Van Dyke, 1978; Easmon et al., 1980). The results obtained in the present study showed that two chemiluminescence response patterns were produced by human PMNLs. The first pattern was initiated by all opsonized H.aphrophilus strains, 9 of the 10 opsonized A.actinomycetemcomitans strains and all opsonized Capnocytophaga strains. In this pattern, a rapid exponential chemiluminescence response was observed over a short period of time, indicating the ability of these strains to trigger a chemiluminescence response when incubated with human PMNLs. This suggests that human PMNLs can phagocytose these strains under the experimental conditions employed in this study,

though this was not confirmed by any other test. However, a positive correlation between chemiluminescence response and the bactericidal activity of human PMNLs against A.actinomycetemcomitans has been shown by Sjöström et al., (1992), while Holm and Kalfas (1993) and Holm, Kalfas and Holm (1993) also reported that the chemiluminescence triggered by H.aphrophilus strains positively correlated with the degree of killing by human PMNLs. Unopsonized cells of the same strains failed to trigger similar responses, indicating the importance of serum opsonic activity. A similar finding was reported by Sjöström et al., (1992) as they showed that the serum from both rapidly progressive periodontitis patients and normal individuals enhanced the chemiluminescence response and killing by human PMNLs against A.actinomycetemcomitans. Furthermore, it has been shown by Sjöström et al., (1992) that complement was an effective opsonin and that the removal of complement resulted in substantially reduced chemiluminescence with A.actinomycetemcomitans, although only one strain (A.actinomycetemcomitans strain Y4) was studied. This finding was also supported by results obtained from preliminary experiments performed in the present study.

In these experiments the serum used for opsonization was heated at 56°C for 30 minutes to inactivate complement, resulting in a minimal chemiluminescence response. Holm and Kalfas (1993) also reported that the highest degree of killing and chemiluminescence response by H.aphrophilus strains were observed in the presence of active serum, and that both parameters were reduced when heated serum was present. The opsonic activity of human serum IgG from patients with localized juvenile periodontitis against A.actinomycet-emcomitans has also been reported (Baker and Wilson, 1989).

The second pattern was obtained with one leukotoxic A.actinomycetemcomitans strain (JP2). In this pattern, a very rapid chemiluminescence peak response and decay were observed. The peak was substantially smaller than those observed in the first pattern and no residual activity of human PMNLs was observed after the addition of PMA to the reaction mixture. This may indicate that the leukotoxin destroyed the human PMNLs and prevented the phagocytosis process, though the viability of the human PMNLs was not formally checked. The addition of PMA to all other test mixtures resulted in a second chemiluminescence peak, indicative of residual PMNL

activity. In the present study, both leukotoxic and non-leukotoxic A.actinomycetemcomitans strains were tested. Among the leukotoxic strains tested only the JP2 strain appeared to damage the PMNLs in this system. This finding was contrary to that reported by Clark, Leidal and Taichman (1986) as they showed that A.actinomycetemcomitans strain JP2 leukotoxin was inactivated by exposure to myeloperoxidase, H_2O_2 and a halide, the main bactericidal system generated by human PMNLs during stimulation (respiratory burst). The reason for the discrepancy is not clear though, it may be related to the fact that in the current study whole bacterial cells were used, while Clark, Leidal and Taichman (1986) used pure toxin. Miyasaki et al., (1986) showed that human PMNLs were capable of killing both leukotoxic and non-leukotoxic A.actinomycetemcomitans strains but in Miyasaki et al., (1986) study a very small bacteria:PMNL ratio was used (10 bacteria:1PMNL), though when an increased ratio to a one commonly used for the detection of leukotoxic activity (100 bacteria/PMNL), an increase in trypan blue uptake was noted by the authors, indicative of toxicity. Kalmar, Arnold and Van Dyke, (1987) also showed that A.actinomycetemcomitans strain Y4 was

phagocytosed and killed by human PMNLs in the presence of serum, prior to the killing of PMNLs by leukotoxin, though in the absence of serum the PMNLs were killed by leukotoxin. Sjöström et al., (1992) reported that the viability of human PMNLs was not affected by exposure to the nominally leukotoxic A.actinomycetemcomitans Y4 strain during the course of their experiments. Holm, Kalfas and Holm (1993) also reported that leukotoxin production by A.actinomycetemcomitans strains was not a major factor influencing the susceptibility of these bacteria to the phagocytic killing of human PMNLs since both leukotoxic and non-leukotoxic strains were killed. The results obtained in the current study with regard to the leukotoxic A.actinomycetemcomitans strain Y4 were in agreement with those reported by Miyasaki et al., (1986) and Sjöström et al., (1992). The chemiluminescence response produced by A.actinomycetem-comitans strains used in the current study was strain-dependent, as certain strains produced very large chemiluminescence peaks (for example, Strain (E83) and others produced smaller chemiluminescence peaks (for example, Strain 100). Similar observations have been reported for other species of bacteria, for example different responses were observed for three different

Klebsiella pneumoniae strains, the authors related this to differences in membrane or cell wall characteristics (Robinson et al., 1984).

Holm and Kalfas (1993) reported the ability of certain A.actinomycetemcomitans and H.aphrophilus strains to induce a chemiluminescence response from human PMNLs in the presence of active serum and saliva. They showed that while all H.aphrophilus strains produced a similar chemiluminescence response pattern, A.actinomycetemcomitans strains produced strain-dependent chemiluminescence patterns. This was in agreement with the results obtained in the current study. Holm, Kalfas and Holm (1993) in a separate study tested the susceptibility of the same A.actinomycetemcomitans and H.aphrophilus strains to phagocytic killing by human PMNLs and reported that certain A.actinomycetemcomitans strains were resistant to phagocytic killing in spite of their ability to trigger a chemiluminescence response in the earlier study. In addition, A.actinomycetemcomitans strains that triggered a strong chemiluminescent response (for example A.actinomycetemcomitans strain ATCC 29523) showed a degree of killing that was comparable to strains which failed to induce a chemiluminescence response (for example strain ATCC

29522). The reverse was observed with H.aphrophilus strains as there was a positive correlation between chemiluminescence production and the degree of killing by human PMNLs (Holm and Kalfas, 1993). Holm and Kalfas (1993) concluded that while H.aphrophilus strains are susceptible to phagocytic killing by oxygen-dependent mechanisms, A.actinomycete-mcomitans strains are killed mainly by non-oxygen-dependent mechanisms. The conclusion of Holm and Kalfas (1993) with regard to the susceptibility of A.actinomycetemcomitans strains to killing by human PMNLs is different from the findings reported by Miyasaki et al., (1986), who showed that A.actinomycetemcomitans strains were susceptible to both oxygen and non-oxygen-dependent killing mechanisms by human PMNLs. Holm, Kalfas and Holm (1993) related the discrepancy to differences in the experimental methodology employed in the two studies. A.actinomycetemcomitans strains were shown to be different with regard to their susceptibility to oxidative killing by the different oxidants produced by the xanthine-xanthine oxidase system, hydrogen peroxide proving the most bactericidal against A.actinomycetemcomitans (Dongari and Miyasaki, 1991). H.aphrophilus was also examined and shown to be in-

sensitive to the oxidant products of the xanthine-xanthine oxidase system (Dongari and Miyasaki, 1991). Although direct phagocytic killing of H.aphrophilus strains was not measured, the results obtained in the present study regarding the phagocytosis of H.aphrophilus strains were in agreement with a number of the reported studies (Holm and Kalfas, 1993; Holm, Kalfas and Holm, 1993).

Robinson et al., (1984) examined the luminol-dependent chemiluminescent response of human PMNLs to a number of pathogenic microorganisms and reported that each test organism induced a reproducible chemiluminescence peak and pattern. Differences in chemiluminescence response between strains of the same organism were related to the different cell wall and membrane structures that may exist between different strains (Robinson et al., 1984). However, Robinson et al., (1984) did not correlate the chemiluminescence response to the phagocytic and bactericidal activity of human PMNLs. Their findings are similar to those obtained in the present study with respect to the ability of microorganisms to induce a luminol-dependent chemiluminescence response from human PMNLs and to the strain-dependent differences. Robinson et al., (1984)

also showed that zymosan was a good activator of PMNLs as it produced a well defined peak in a short period of time. Results obtained from preliminary experiments performed in the present study did not support this finding, since the use of zymosan to stimulate human PMNLs resulted in a very broad small peak over a long period of time (90 minutes). This was also observed in other studies, though opsonized zymosan was used to stimulate some non human PMNLs (Dr. M. Saddati, personal communication). The reasons for the discrepancies observed are not clear, though they may relate to the zymosan preparation method, PMNLs employed or the assay conditions used.

All Capnocytophaga species tested in the current study produced the first described pattern of chemiluminescence response. This suggests that human PMNLs can phagocytose this organism and also show the need for serum opsonization. One previous study has examined the ability of Capnocytophaga species to trigger a chemiluminescence response in human PMNLs (Passo, Syed and Silva, 1982). Passo, Syed and Silva (1982) examined a group of Gram-negative microaerophilic or anaerobic bacteria including Capnocytophaga species, isolated from dental plaque, for their ability

to produce chemiluminescence from human PMNLs. The addition of serum enhanced the chemiluminescence response to all tested organisms, including Capnocytophaga species.

4.4.1 Conclusions

All tested H.aphrophilus strains triggered a chemiluminescence response by human PMNLs in vitro. Opsonization was shown to be essential for the interactions between H.aphrophilus strains and human PMNLs in vitro.

A.actinomycetemcomitans triggered chemiluminescence in a strain-dependent manner.

Leukotoxin activity was not a major factor in the phaogocytosis of A.actinomycetemcomitans strains, since both leukotoxic and non-leukotoxic strains triggered a chemiluminescent response from human PMNLs.

Opsonization of A.actinomycetemcomitans strains was shown to enhance substantially the chemiluminescence response by human PMNLs.

All Capnocytophaga strains triggered a chemiluminescence response from human PMNLs. This response was enhanced by opsonization of Capnocytophaga strains.

4.5 The cytotoxicity of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga species for cultured human skin fibroblasts

4.5.1 Experimental methods

The cytotoxicity of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains for cultured human skin fibroblasts was tested by using a colorimetric MTT (Tetrazolium salt) cleavage assay. The assay, which was based on the cleavage of MTT by the mitochondrial enzyme succinyl-dehydrogenase to form coloured formazan, was first developed by Mosmann (1983). The assay had not been used previously for testing the cytotoxicity of any of these groups of organisms for cultured fibroblasts. However, it had been used in a number of other cytotoxicity studies, for example those of Visconti et al., (1991) who used the assay for testing the cytotoxic effects of Fusarium mycotoxins on cultured human cell lines (K-56 and MIN-GL1). Smith et al., (1992) also used the assay for testing the cytotoxicity of fungal spores on cultured human embryonic diploid fibroblast lung cells. Scragg, Cannon and Williams (1994) used the reduction of the tetrazolium salt MTT assay to assess the cytotoxic

effects of a number of short-chain fatty acids produced by some periodontal pathogens such as P.gingivalis on two cultured fibroblast cell lines (Vero cells and human gingival fibroblasts). They showed that Vero cells were more sensitive than human gingival fibroblasts to both morphological and metabolic changes.

A number of preliminary experiments were performed in the current study to determine the final experimental conditions and to examine the suitability of this assay. In the early experiments, whole live bacterial cells were used and the results suggested that A.actinomycetemcomitans strains were cytotoxic for cultured fibroblasts. However, it became clear that it was not possible to use whole, live bacterial cells since they caused a very dark coloured precipitate which interfered with the final absorbance readings. This made the interpretation of the results difficult. The results of an additional group of preliminary experiments performed to resolve this problem indicated that the precipitate was the result of an interaction between bacterial components and the fibroblast growth medium. However, it is not clear whether the interfering factor(s) present in only whole viable

cells or also in dead intact cells. The use of cell-free preparations was, therefore, attempted. A group of preliminary experiments were performed with sonicated cell extracts. In these experiments bacterial cells were sonicated by using the Heat systems probe at 20% power output for 20 minutes on ice. The results of these experiments were not conclusive because there were still some live cells in the preparations, as evident from viable counts, and this resulted in the same problem of precipitation. An attempt to use a cell free extract was then made by disintegrating the bacterial cells for 20 minutes, which proved to be no more successful. The time of disintegration was then increased to 1 hour. The preparation obtained from this procedure resulted in a cell-free extract that contained no live cells and produced satisfactory results when used for the cytotoxicity experiments. It should be mentioned that the use of whole viable cells of H.aphrophilus and Capnocytophaga strains caused the same problem.

The bio-assay employed in the present study represented a rapid, reproducible, straightforward assay for evaluating the cytotoxicity of these groups of organisms. The use of the multiwell scanning

spectrophotometer (ELISA reader) provided the means by which a large number of samples could be read accurately at the same time.

4.5.2 The criteria used to assess the cytotoxicity of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains for cultured human skin fibroblasts

The bio-assay employed in the present study was based on the ability of metabolically active cultured fibroblasts, but not dead fibroblasts, to cleave the MTT. Control fibroblast cultures, unexposed to any test organisms, were included in all test plates and their optical density measurement was considered as 100%. Since there was no precedent for the use of this assay with these bacterial species, the cut-off point was taken to be a 50% reduction of the optical density obtained with control cultures. It is not clear, though, whether the reduction of 50% in the optical density of control cultures means that 50% of cultured cells were inhibited by test organism or just an overall reduction of the metabolic activity of the whole cultured cells. In a study reported by Smith et al., (1992) using the same methodology to measure the cytotoxicity of fungal spores, the cut off point used

was 10%. The assay requires about 5 days to complete, during which time a number of steps are performed including media changes, washing the cells and adding toxins. These treatments of the cultured cells over 5 days may affect their activity, thus considering a cut-off point of 10% reduction as an indicator of toxicity appears to be very low. Furthermore, Smith et al., (1992) expressed the cytotoxicity as mortality which is not an accurate expression since no direct cell counts were performed and the viability of the cells was not assessed. Scragg, Cannon and Williams (1994) reported different values for different acids. This was in a range of 60%-90% though it is not clear whether this was a cut-off point or reductions in the metabolic activity obtained. In the current study if the 50% cut-off point used is reduced by only 5% to 45% then all A.actinomycetemcomitans strains will become positive, while only one H.aphrophilus strain will remain negative though the results obtained with Capnocytophaga strains will not change.

4.5.3 The cytotoxicity of A.actinomycetemcomitans to cultured human skin fibroblasts

The cytotoxic activity was recorded for almost all of the 25 A.actinomycetemcomitans strains tested (96%). This was probably the largest number of strains tested for their ability to interfere with cultured human fibroblasts in a single study. The positive strains reduced the optical density of the control cultures in a range from 63% to 83%, indicating possible differences in potency among the cytototoxic strains. Similar findings were reported by Shenker, Kushner and Tsai (1982) as they reported the ability of soluble sonic extracts of A.actinomycetemcomitans to inhibit the proliferation of cultured murine and normal human skin fibroblasts as assessed by DNA and RNA synthesis assays (incorporation of [^{3}H] thymidine and [^{3}H]uridine). They reported that the inhibitory factor was heat-labile and independent from other cytotoxic factors produced by A.actinomycetemcomitans strains, for example leukotoxin. They also reported that this inhibition was not accompanied by cell death as assessed by direct cell counts, though only four strains were tested in their study. The single non-cytotoxic strain reduced the control cultures by about

46%. The results obtained in the present study were not surprising since many studies have shown a high proportion of A.actinomycetemcomitans strains to be cytotoxic to cultured human fibroblast cell lines, in spite of the differences in experimental procedures used in different studies. Stevens and Hammond (1982) reported that two A.actinomycetemcomitans strains, one of which was leukotoxic (strain Y4) and one non-leukotoxic (strain 627) were able to inhibit the proliferation of human fibroblasts. Stevens and Hammond (1988), in a comparative study, tested the cytotoxicity of several periodontal bacteria against human gingival fibroblasts. A.actinomycetemcomitans strains were among the test organisms and the results showed that the sonic extracts of A.actinomycetemcomitans and F.nucleatum were the most toxic to cultured human gingival fibroblasts. This cytotoxicity was dose-dependent. Furthermore, Stevens and Hammond (1988), showed that sonic extracts of A.actinomycetemcomitans were able to cause morphological effects which included a reduction in cell density and alteration in cellular morphology. These latter observations were in agreement with the findings of the present study. Other bacterial components, for example lipopolysaccharides and

capsular material, were shown to be cytotoxic to cultured human fibroblasts. Kamin et al., (1986) showed that the capsular material isolated from A.actinomycetemcomitans was toxic to cultured human gingival fibroblasts. Lipopolysaccharide isolated from the same strain was also tested by Kamin et al., (1986). They reported that the lipopolysaccharide was toxic to cultured human gingival fibroblasts only at a high concentration (10-50µg/ml) compared to much lower concentrations (0.1µg/ml and above) for the capsular material. The cytotoxicity of capsular material also behaved in a dose-dependent pattern. Contrary to the previous study, Bartold and Millar (1988) reported that lipopolysaccharides from A.actinomycetemcomitans and other periodontal bacteria exerted cytotoxic effects on cultured human gingival fibroblasts and that the lipopolysaccharide from A.actinomycetemcomitans was the most toxic to cultured human gingival fibroblast at concentrations from 0.5-50µg/ml. Bartold and Millar (1988) in their discussion indicated that their finding was in agreement with most reported studies regarding the toxicity of lipopolysaccharides to human fibroblasts and failed to refer to the study reported by Kamin et al., (1986). Recently Helgeland and Nordby

(1993) reported the isolation of a toxin from the growth medium of A.actinomycetemcomitans and showed that this toxin inhibited the growth of human gingival fibroblasts, though the cells were not killed. The factor responsible for the inhibitory activity among the A.actinomycetemcomitans strains tested in the current study is not known.

4.5.4 The cytotoxicity of H.aphrophilus strains for cultured human skin fibroblasts

The ability of 30 H.aphrophilus strains to interfere with the proliferation of cultured human skin fibroblasts was tested by using the MTT bio-assay. The results showed that the majority of tested H.aphrophilus strains (87%) were cytotoxic. The positive strains reduced the optical density in a range from 55% to 87%. There is no information in the literature regarding the cytotoxicity of H.aphrophilus strains for cultured human fibroblasts, and this is the first report of cytotoxic activity by a collection of relatively large number of freshly isolated strains. Only one type strain of H.aphrophilus (NCTC 5886) was tested in this study and it was shown to be non-cytotoxic as it reduced the optical density of control

cultures by only 24% which is low compared with the majority of the fresh isolates. Whether this represents a fundamental difference between type and fresh isolates with regard to their cytotoxicity is not clear until more type strains have been tested. In spite of the frequent isolation of H.aphrophilus strains from subgingival plaque, it is generally accepted that H.aphrophilus is a part of the normal oral microflora and its role in the aetiology of periodontitis in minimal. The findings that H.aphrophilus strains exerted a cytotoxic activity against human fibroblasts is therefore, very interesting since it clearly shows that H.aphrophilus could cause damage to host tissues.

4.5.5 The cytotoxicity of Capnocytophaga species for cultured human skin fibroblasts

Nine Capnocytophaga strains were tested for their ability to alter the proliferation of cultured human skin fibroblasts. The results obtained in the present study showed that 5 out of 9 strains were cytotoxic for cultured human skin fibroblasts. The positive strains reduced the optical density of the control cultures in a range from 55% to 68%, while the negative strains reduced control cultures in a range from 8.5% to 33%. A

few previous studies have examined the cytotoxicity of this organism for cultured human fibroblasts. Stevens and Hammond (1982) showed that filter sterilized sonic extracts of certain Capnocytophaga strains inhibited the proliferation of human foreskin fibroblasts. This included all tested strains of C.sputigena, though they reported variable results regarding the cytotoxicity of C.gingivalis and C.ochracea (Stevens and Hammond, 1982). Stevens and Hammond (1988) tested a number of periodontal bacteria for their ability to interfere with cultured human gingival fibroblasts, including Capnocytophaga sputigena. They showed that C.sputigena was cytotoxic for cultured human gingival fibroblasts, though the degree of fibroblast inhibition was less than that observed with the highly toxic A.actinomycetemcomitans (Stevens and Hammond, 1988). In the current study only one strain known as C.sputigena while other strains as indicated earlier were not identified to the species level. The C.sputigena tested in the current study is cytotoxic for cultured fibroblasts and even though the experimental methods in the current study and those in the study reported by Stevens and Hammond (1988), the results agreed. The extent of cytotoxicity of

C.sputigena obtained in the present study is lower than those obtained with A.actinomycetemcomitans strains which also in agreement with Stevens and Hammond (1988).

The cytotoxicity results recorded with all tested strains from each of the test bacteria indicate that A.actinomycetemcomitans strains are the most toxic to cultured human skin fibroblast cell line used in this study followed by H.aphrophilus strains. Capnocytophaga species is the less toxic of the three.

4.5.6 Adhesion of A.actinomycetemcomitans, H.aphrophilus and Capnocytophaga strains to cultured human skin fibroblasts

During the course of the early cytotoxicity experiments using whole viable bacterial cells it was observed that certain strains of both A.actinomycetemcomitans and H.aphrophilus, but not of the Capnocytophaga species, were able to attach to the cultured human skin fibroblasts employed in the cytotoxicity study. Seven A.actinomycetemcomitans, 7 H.aphrophilus and 2 Capnocytophaga strains were tested for their ability to adhere to cultured fibroblasts. Capnocytophaga species did not adhere, while the ability of both organisms to

adhere was dependent on the colonial morphology, since the colonies that adhered to the agar surface of culture plates (rough type) attached more strongly to the cultured fibroblasts and attached cells were not removed by washing. It was also observed that certain A.actinomycetemcomitans strains (for example GDH 115) adhered almost immediately, while others adhered only after several hours. The smooth type colonies adhere weakly or not at all to both agar and fibroblasts. Although it was not possible to quantify the number of attached bacteria per fibroblast cell, it was possible to distinguish visually between adherent, weakly adherent and non-adherent cells.

The ability of certain strains of A.actinomycetemcomitans to adhere to the human oral KB cell line (epithelial cells) has been shown previously (Meyer and Fives-Taylor, 1993). These authors attributed this ability to extracellular micro-vesicles associated with the bacterial surface (Meyer and Fives-Taylor, 1993). In addition these extracellular microvesicles, together with a highly proteinaceous polymer associated with a leukotoxic test strain, enhanced the adherence of otherwise weakly adherent strains. The results

obtained in the current study may represent further evidence for the ability of A.actinomycetemcomitans to adhere to host tissues, though the mechanisms are unclear. Mintz and Fives-Taylor (1994) also reported that A.actinomycetemcomitans strains were able to adhere to a human epithelial cell line as detected by enzyme linked immunosorbent assay. The findings of the recent study by Mintz and Fives-Taylor (1994) are in agreement with the preliminary results obtained in the current study and further confirm the suitability of using cultured human cell lines for adhesion studies.

There are a few studies that have investigated the adhesion of H.aphrophilus strains to host tissues. In a study reported by Sweet, MacFarlane and Samaranayake, (1988) a single strain of H.aphrophilus was reported as adhering to human buccal epithelial cells in vitro.

4.5.7 Conclusions

Twenty four of the 25 A.actinomycetemcomitans strains tested and 26 of the 30 H.aphrophilus strains tested were cytotoxic for cultured human skin fibroblasts.

Of the 9 Capnocytophaga strains tested only 5 were cytotoxic for cultured human skin fibroblasts.

The MTT bio-assay was used for the first time to test for the cytotoxicity of these organisms and it was shown to be both rapid and reproducible.

This is the first study to determine cytotoxic activity for H.aphrophilus strains.

Adherence of A.actinomycetemcomitans and H.aphrophilus is shown to be dependent on colonial morphology. Strains with rough colonies adhered more strongly than the smooth colonies strains.

Capnocytophaga species did not adhere to cultured human skin fibroblasts.

4.6 Human IgA protease production by Capnocytophaga, A.actinomycetemcomitans and H.aphrophilus

4.6.1 Experimental methods

There are a number of established methods to demonstrate cleavage of human IgA by IgA1 protease-producing human pathogenic bacteria. These methods include SDS-PAGE, immunoelectrophoresis and immunoblotting (Kilian, 1981). In the present study the cleavage of human myeloma IgA and human secretory IgA was detected by SDS-PAGE and immunoblotting, since they

have been frequently used for the detection of cleavage products.

To determine the suitability of the methods chosen for this study, a number of preliminary experiments were performed. Both H.influenzae and selected Capnocytophaga strains were used as controls, since both organisms have been shown previously to cleave human IgA1 by producing the specific enzyme IgA (IgA1) protease (Kilian, Mestecky and Schrohenloher, 1979; Kilian, 1981). Initially the assay mixtures were incubated for 18 hours at 37^{o}C and SDS-PAGE used to detect cleavage products. The results obtained from these experiments were not conclusive, since it was extremely difficult to distinguish bacterial protein bands from IgA cleavage products. Additional experiments were performed in which the concentration of test bacteria was increased to make sure that sufficient enzyme was present. The incubation times of the assay mixtures (bacterial suspensions in diluted human myeloma serum or human SIgA) were also increased from 18 hours to 48 hours at 37^{o}C. Again SDS-PAGE was still used to detect cleavage products. Results obtained from these experiments indicated that both H.influenzae and Capnocytophaga strains cleaved human

myeloma IgA and human SIgA. However, the protein bands corresponding to IgA bands in the control lanes were still evident in the gel but with substantially reduced intensity. The difficulty of identifying cleavage products from bacterial protein bands was not resolved and thus more experiments were performed using similar incubation conditions but employing the more specific immunoblotting method for the detection of the cleavage products. Results obtained from these experiments indicated that both positive control organisms cleaved human myeloma IgA and human SIgA. However, there were extra protein bands in some blots, especially when Capnocytophaga species were used. These were probably bacterial proteins reacting non-specifically with the commercial anti-serum used for immunoblotting. This tends to be confirmed by the fact that bands with similar mobility were usually present in the identical gels when stained with Coommassie blue. Also these bands were not detected in all blots which further suggests that they are bacterial proteins and not cleavage products. Therefore, H.influenzae was used in the main experiments as the positive control. A number of changes were made to reduce the non-specific reactivity observed in some blots. The constituents of

the blocking buffer were modified by increasing the concentration of milk from 1% to 10%, and the incubation of blots was carried out at 37^{o}C instead of room temperature in some of the experiments. Dried milk was also added to the washing buffer. These measures improved the assay substantially. Immunoblotting proved to be a reproducible, relatively rapid assay since multiple samples could be examined in a single experiment.

4.6.2 Cleavage of human myeloma IgA and human SIgA by Capnocytophaga species

Ten strains of oral Capnocytophaga species were tested for their ability to produce IgA protease capable of cleaving human myeloma IgA. All tested strains cleaved human myeloma IgA as detected by SDS-PAGE and immunoblotting. Results obtained in the present study were similar to those reported in the literature. Kilian (1981) examined a number of bacterial species suspected of being involved aetiologically in human periodontal disease, including Capnocytophaga species, and reported that all tested Capnocytophaga strains were capable of producing IgA protease that specifically cleaved human IgA1 in the hinge region to

yield intact Fab and Fc fragments. Furthermore, human IgG was cleaved by all Capnocytophaga strains, but IgA2 was not cleaved (Kilian, 1981). Similar results for Capnocytophaga species were reported by Frandsen, Reinholdt and Kilian (1987), who characterized the IgA1 protease from strains of Capnocytophaga species and other potential oral pathogens (for example Bacteroides species). They reported that IgA1 protease from Capnocytophaga cleaved human IgA1 from the hinge region, leaving intact Fab and Fc fragments and they described the IgA1 protease prepared from Capnocytophaga species as a metallo-enzyme (Frandsen, Reinholdt and Kilian, 1987). Furthermore, Frandsen, Reinholdt and Kilian (1991) have shown in vivo activity of IgA1 proteases from Capnocytophaga species and other suspected oral pathogens.

During the course of the present experiments it was observed that Capnocytophaga species were able to cleave the secretory component from SIgA as evident in the SDS-PAGE gels. This degradation was complete, since there was no reactivity when samples were immunoblotted by using anti-secretory-component serum. However, whether the IgA protease itself cleaved the secretory component or whether it was cleaved by another

proteolytic enzyme is not clear. Although no specific work was performed to determine the time sequence required for the cleavage of the secretory component, it was observed in some of these experiments that cleavage occurred after 12 hours of incubation. If the cleavage was due to the action of IgA protease, then this may suggest that IgA protease cleaves the SIgA molecule in two different steps. The first would involve removal of secretory component, since the secretory component makes SIgA more resistant to cleavage by IgA proteases and other proteolytic enzymes. The second step would be cleavage of the unprotected molecule. This mechanism merits additional investigation.

4.6.3 Cleavage of human myeloma IgA by A.actinomycetemcomitans and H.aphrophilus

In the preliminary experiments discussed in the previous section, selected strains of A.actinomycetemcomitans and H.aphrophilus were tested for their ability to cleave human SIgA. Results obtained from these experiments indicated that none of the tested strains produced IgA protease capable of cleaving human SIgA and the final experiments were,

therefore, performed by using the human myeloma IgA only.

Twenty five A.actinomycetemcomitans and 30 H.aphrophilus strains were tested for their ability to cleave human myeloma IgA in vitro. None of the tested strains of either organism cleaved human myeloma IgA under the experimental conditions employed in the present study, with no evidence of cleavage products by immunoblotting. This is believed to be the first study in which H.aphrophilus strains have been tested for their ability to cleave human IgA. Kilian et al., (1983) screened a large number of microorganisms for IgA protease activity. H.aphrophilus was not included, but hundreds of different species were screened and only a few were found to possess IgA protease activity. The results obtained in the present study were not, therefore, surprising.

Microorganisms shown to produce IgA1 protease include H.influenzae, H.aegyptius, N.meningitidis, N.gonorrhoeae, S.sanguis, S.oralis and S.pneumoniae. Among the suspected periodontopathic bacteria, Capnocytophaga species and certain Porphyromonas and Prevotella (Bacteroides) species have been shown to produce IgA protease (Kilian, Mestecky and

Schrohenloher, 1979; Mulks and Plaut, 1978; Frandsen, Reinholdt and Kilian, 1987).

In the present study a number of oral microorganisms were screened for human IgA protease activity. Some of these are implicated as aetiological agents in destructive periodontal disease, namely A.actinomycetemcomitans and Capnocytophaga species. Only Capnocytophaga species were demonstrated to possess IgA protease capable of cleaving human myeloma IgA and the more resistant human SIgA.

The significance of IgA protease in virulence is not completely clear. However, there is some evidence to suggest that it has a pathogenic function, since among the hundreds of different bacterial species screened for human IgA protease activity only a few actually possess such activity and most are pathogens associated with specific infections, all these infections take place on mucosal surfaces where IgA is present and provides a means of defence for the host. The 3 leading causative agents of meningitis produce IgA protease, whereas related, non-pathogenic species do not (Senior, Loomes and Kerr, 1991). Furthermore, Frandsen, Reinholdt and Kilian (1991) investigated

whether IgA proteases from Capnocytophaga species were active in vivo by using an enzyme-linked immunosorbent assays and reported that IgA1 protease from Capnocytophaga species actually cleaved human IgA1 in vivo. Frandsen et al., (1995) examined sera by ELIZA, from a group of periodontal disease patients for the presence of antibodies against the antigenically distinct neoepitope on the IgA1 Fab fragments produced by the cleavage of IgA1 by Capnocytophaga protease. These antibodies were detected in 42 serum samples out of 92 samples analysed (Frandsen et al., 1995). It is also important to mention that an elevated level of IgA subclass IgA1 to antigens of A.actimomycetemcomitans has been demonstrated in patients with localized juvenile periodontitis. The presence of IgA1 suggests that the protective effects against A.actinomycetemcomitans could be compromised by bacterial IgA1 proteases that may be present in the same environment and which may add to the virulence of A.actinomycetemcomitans (Brown et al., 1991).

Cole and Hale (1991) also reported that H.influenzae IgA1 protease cleaved the SIgA of a chimpanzee. This finding is important since it will provide the use of

an animal model to further examine the significance of IgA1 protease production and its role in pathogenesis.

4.6.4 Conclusions

Ten Capnocytophaga species were found to possess an IgA protease capable of cleaving human myeloma IgA as detected by immunoblotting methods. The cleavage pattern was similar to that observed with H.influenzae. Five strains of Capnocytophaga species degraded the secretory component completely, as there was no reactivity detected with immunoblotting methods when probed by commercial anti secretory component serum. However, the significance of this finding is unclear and probably merits further investigation.

Twenty five A.actinomycetemcomitans strains and 30 H.aphrophilus strains were included in this study, none of which cleaved human myeloma IgA under the experimental conditions employed.

It is clear that more detailed studies are required to examine the properties of these enzymes in detail and to assess their significance in virulence. The role of IgA in immunity requires further study, since it is known in some cases that the inability to produce the

IgA protease is not accompanied by the loss of pathogenicity. Thus, certain H.influenzae serotype b strains isolated from cases of meningitis were void of IgA-protease activity (Kilian et al., 1983).

APPENDIX I
MEDIA, BUFFERS AND REAGENTS COMPOSITION

Anaerobic blood Broth (ABB)

Tryptone	10 g
Beef extract	2.0 g
Liver extract	3.0 g
Yeast extract	5.0 g
Glucose	5.0 g
Sodium chloride	5.0 g
Vitamin K	0.005 g
Haemin	0.005 g
Cysteine hydrochloride	1.0 g
Dithiothreitol	0.1 g
Sodium bicarbonate	0.9 g

Preparation

Thirty two grams of the powdered medium was dissolved in 1000 ml distilled water by heating in a Koch steamer, sterilized by autoclaving at 121^{o} C for 15 minutes and stored.

Colombia Blood Agar

Composition:

Peptone 140	13 g
Peptone 100	6 g
Yeast extract	3 g
Beef extract	3 g
Starch	1 g
Sodium chloride	5 g
Agar	13 g
Defibrinated horse blood	50 ml

Preparation

Forty four g of the powder medium was dissolved in 1000 ml of distilled water by steam heating, the medium pH was adjusted to 7.4. Dissolved medium aliquoted in 100 ml bottles and sterilized by autoclaving at 121^{O}C for 15 minutes. After sterilization the medium cooled at room temperature and then 5 ml of blood was added to each aliquot (100 ml). The medium was poured aseptically into sterile petri-dishes.

Tryptic Soy-Serum-Bacitracin-Vancomycin Agar (TSBV)

Composition:

Peptone 140	15 g
Peptone 110	5 g
Sodium chloride	5 g
Yeast extract	1 g
Horse serum	100 ml
Bacitracin	75 mg
Vancomycin	5 mg
Agar	15 g
Distilled water	1000 ml

Preparation

Fifty grams of Tryptic Soy Agar and 1 gram of yeast extract were dissolved in 1000 ml of distilled water by steam heating, the pH of the medium was adjusted to 7.4, aliquoted in 100 ml volumes and sterilized by autoclaving at 121^{O} C for 15 minutes. The medium was cooled to about 45^{O}C, after which 10 ml of serum, 1 ml of bacitracin and 1 ml of vancomycin were added aseptically and poured into sterile petri-dishes.

Tryptic Soy Serum Bacitracin Vancomycin Fluoride Agar (TSBVF)

Composition:

Peptone 140	15 g
Peptone 110	5 g
Sodium chloride	5 g
Yeast extract	1 g
Horse serum	100 ml
Bacitracin	75 mg
Vancomycin	5 mg
Sodium fluoride	50 mg
Agar	15 g
Distilled water	1000 ml

Preparation

Fifty grams of Tryptic Soy Agar and 1 gram of yeast extract were dissolved in 1000 ml of distilled water by steam heating, the pH of the medium was adjusted to 7.4, aliquoted in 100 ml volumes and sterilized by autoclaving at 121^{O} C for 15 minutes. The medium was cooled to about 45^{O}C, after which 10 ml of serum, 1.0 ml of bacitracin, 1.0 ml of vancomycin and 1.0 ml of sodium fluoride were added aseptically and medium then poured into sterile petri-dishes.

RPMI 1640 medium: Composition

Amino acids

L-Arginine	200	mg/l
L-Asparagine	50	mg/l
L-Aspartic acid	20	mg/l
L-Cystine	50	mg/l
L-Glutamic acid	20	mg/l
L-Glutamine	300	mg/l
Glycine	10	mg/l
L-Histidine	15	mg/l
L-Hydroxproline	20	mg/l
L-Isoleucine	50	mg/l
L-Luecine	50	mg/l
L-Lysine.HCl	40	mg/l
L-Methionine	15	mg/l
L-phenylalanine	15	mg/l
L-Proline	200	mg/l
L-Serine	30	mg/l
L-Threonine	20	mg/l
L-Tryptophan	5	mg/l
L-Tyrosine	20	mg/l
L-Valine	20	mg/l

Vitamins

Biotin	0.2 mg/l
D-Ca Pantothenate	0.25 mg/l
Choline Chloride	3 mg/l
Folic acid	1 mg/l
i-Inositol	35 mg/l
Nicotinamide	1 mg/l
Para-amonobenzoic acid	1 mg/l
Pyridoxine HCl	1 mg/l
Riboflavin	0.2 mg/l
Thiamine HCl	1 mg/l
Vitamin B_{12}	0.005 mg/l

Salts

$Ca\ (NO_3)_2.4H_2O$	100 mg/l
KCL	400 mg/l
$MgSO_4.7H_2O$	100 mg/l
NaCl	6000 mg/l
$NaHCO_3$	2000 mg/l
$Na_2HPO_4.7H_2O$	1512 mg/l

Other components

D-Glucose	2000 mg/l
Glutathione	1.0 mg/l
Phenol red	5.0 mg/l

RPMI 1640 medium is commercially supplied in a liquid form, and is stored at 4°C.

Phosphate Buffered Saline Dulbecco's Formula

(Modified)*

Composition

Disodium hydrogen orthophosphate	1.15 g/l
Potassium dihydrogen orthophosphate	0.2 g/l
Sodium chloride	8.0 g/l
Potassium chloride	0.2 g/l

One sachet of already made powder was dissolved in a liter of distilled water, pH was adjusted as required. The buffer was then sterilized by autoclaving and stored at room temperature.

* without magnesium and calcium

Bibliography

Ainamo, J. and Löe, H. (1966). Anatomical characteristics of gingiva. A clinical and microscopic study of the free and attached gingiva. Journal of Periodontology, **37**, 5-13

Albritton, W.L. (1982). Infections due to haemophilus species other than *H. influenzae*. Annual Reviews of Microbiology, **36**, 199-216.

Allen, R.C., Loose, L.D. (1976). Phagocytic activation of luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. Biochemical and Biophysical Research Communications, **69**, 245-252.

Allen, R.C., Stjernholm, R.L. and Steele, R.H. (1972). Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochemical and Biophysical Research Communication, **47**, 679-684.

Appelbaum, P.C., Ballard, J.C. and Eysten, M.E. (1979). Septicemia due to *capnocytophaga* (*Bacteroides ochraceus*) in Hodgkin's disease. Ann Intern Med, **90**, 716.

Ashkenazi, M., White, R.R. and Dennison, D.K. (1992). Neutrophil modulation by *Actinobacillus actinomycetemcomitans* I. Chemotaxis, surface receptor expression and F-actin polymerization. Journal of Periodontal Research, **27**, 264-273.

Asikainen, S., Alaluusua, S. and Saxen, L. (1991). Recovery of *Actinomycetemcomitans* from teeth, tongue and saliva. Journal of Periodontology, **62**, 203-206.

Atterström, R. (1971). Studies on neutrophil polymorphonulear leukocytes at the dentogingival junction in gingival health and disease. Journal of Periodontal Research, **6 (Suppl. 8)**, 7-15.

Babior, B.M. (1978). Oxygen dependent microbial killing by phagocytes. I. New England Journal of Medicine, **298**, 659-668.

Baehni, P., Tsai, C.C., McArthur, W.P., Hammond, B.F., Taichman, N.S. (1979). Interaction of inflammatory cells and oral microorganisms. VIII. Detection of leukotoxic activity of a plaque derived Gram-negative microorganism. Infection and Immunity, **24**, 233-243.

Baehni, P.C., Tsai, C.C., McArthur, W.P., Hammond, B.F., Shenker, B.J. and Taichman, N.S. (1981). Leukotoxic activity in different strains of the bacterium *Actinobacillus actinomycetemcomitans* isolated from juvenile periodontitis in man. Archives of Oral Biology, **26**, 671-676.

Baer, P.N. (1971). The case for periodontitis as a clinical entity. Journal of Periodontology, **42**, 516-520.

Baker, P. and Wilson, M.E. (1989). Opsonic IgG antibody against *Actinobacillus actinomycetemcomitans* in localized juvenile periodontitis. Oral Microbiology and Immunology, **4**, 98-105.

Baker, P.J., Evans, R.T., Slots, J. and Genco, R.J. (1985). Susceptibility of human oral anaerobic bacteria to antibiotics suitable for topical use. Journal of Clinical Periodontology, **12**, 201-208.

Barr-Agholme, M., Dahllöf, G., Linder, L. and Modéer, T. (1992). *Actinobacillus actinomycetemcomitans, Capnocytophaga and Porphyromonas gingivalis* in subgingival plaque of adolescents with Down's syndrome. Oral Microbiology and Immunology, 244-248.

Bartold, P.M. and Millar, S.J. (1988). Effect of lipopolysaccharide on proteoglycan synthesis by adult human gingival fibroblasts in vitro. Infection and Immunity, **56**, 2149-2155.

Baumgartner, J.G., Falkler, W.A. Jr. and Beckman, T. (1992). Experimentally induced infection by oral

anaerobic microorganisms in a mouse model. Oral Microbiology and Immunology, **7**, 253-256.

Behling, U.H., Sallay, C., Sanavi, F., Pham, P.H. and Noworny, A. (1981). Humoral immunity and reduced periodontal bone loss in *Eikenella corrodens*-monoassociated rats. Infection and Immunity, **33**, 801-805.

Berthold, P., Forti, D., Kieba, I.R., Rosenbloom, J., Taichman, N.S. and Lally, E.T. (1992). Electron immunocytochemical localization of *Actinobacillus actinomycetemcomitans* leukotoxin. Oral Microbiology and Immunology, **7**, 24-27.

Bick, P.H., Carpenter, A.B., Holdeman, L.V., Miller, G.A., Ranney, R.R., Palcanis, K.G. and Tew, J.G. (1981). Polyclonal B-Cell activation induced by extracts of Gram-negative bacteria isolated from Periodontally Diseased Sites. Infection and Immunity, **34**, 43-49.

Bieger, R.C., Brewer, N.S. and Washington, J.A. (1978). *Haemophilus aphrophilus* : a microbiologic and clinical review and report of 42 cases. Medicine, **57**, 345-356.

Birkedal-Hansen, H., Caufield, P.W., Wanne-Muehler, Y.M. and Pierce, R. (1982). A sensitive screening assay for epitheliotoxins produced by oral microorganisms. IADR Progress and Abstract **61**, No. 125.

Birkedal-Hansen, H., Taylor, R.E., Zambon, J.J., Barwa, P.K. and Neiders, M.E. (1988). Characterisation of collagenolytic activity from strains of *Bacteroides gingivalis*. Journal of Periodontal Research, **23**, 258-264.

Blachman, U. (1980). *Capnocytophaga* sepsis: a newly recognised clinical entity in granulocytopenic patients. Lancet **i**, 567-568.

Boehringer, H., Taichman, N.S. and Shenker, B.J. (1984). Suppression of fibroblast proliferation by oral spirochetes. Infection and Immunity, **45**, 155-159.

Brogan, J.M., Lally, E.T., Poulsen, K., Kilian, M. and Demuth, D.R. (1994). Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: Analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. Infection and Immunity, **62**, 501-508.

Brondz, I. and Olsen, I. (1993). Multivariate chemosystematics demonstrate two groups of *Actinobacillus actinomycetemcomitans* strains. Oral Microbiology and Immunology, **8**, 129-133.

Brown, T.A., Byres, L., Gardner, M. and Van Dyke, T.E. (1991). Subclass and molecular form of immunoglobin A antibodies to *Actinobacillus actinomycetemcomitans* in juvenile periodontitis. Infection and Immunity, **59**, 1126-1130.

Brown, W.J. (1976). Incidence of *Haemophilus* spp. and similar organisms in the adult respiratory tract. Abstracts of the Annual Meeting of The American Society for Microbiology, 30.

Carlton, D., Bricknell, K., Newman, M.G., Yoon, N., Woolfe, S. and Horikoshi, A. (1979). Comparison of chromatographic profiles of plaque from idiopathic juvenile periodontitis. Journal of Dental Research, **58**, 177 (abstract 334).

Caugant, D.A., Selander, R.K. and Olsen, I. (1990). Differentiation between *Actinobacillus (Haemophilus) actinomycetemcomitans*, *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* by multi-locus enzyme electrophoresis. Journal of General Microbiology, **136**, 2135-2141.

Chang, Y-F., Renshaw, H.W., Martens, R.J., Charles, W. and Livingston, jr. (1986). *Pasteurella haemolytica* leukotoxin; Chemiluminescent responses of peripheral blood leukocytes from several different mammalian species to leukotoxin- and opsonin-treated living and killed *Pasteurella haemolytica* and *Staphylococcus aureus*. American Journal of Veterinary Research, **47**, 67-74.

Chang, Y.F. and Renshaw, H.W. (1986). *Pasteurella haemolytica* leukotoxin: comparison of ^{51}chromium-

release, trypan blue dye exclusion and luminol-dependent chemiluminescence inhibition assays for sensitivity in detecting leukotoxin activity. American Journal of Veterinary Research, **47**, 134-138.

Chen,P.B., Davern, L.B., Neiders, M.E., Reynolds, H.S. and Zambon, J.J. (1991). Analysis of in vitro lymphoproliferative responses and antibody formation following subcutaneous injection of *Actinobacillus actinomycetemcomitans* and *Wolinella recta* in a murine model. Oral Microbiology and Immunology, **6**, 12-16.

Choi, J.I., Nakagawa, T., Yamada, S., Takazoe, I. and Okuda, K. (1990). Microbiological and immunological studies on recurrent periodontal disease. Journal of Clinical Periodontology, **17**, 426-434.

Christersson, L.A., Albini, B., Zambon, J.J., Wikesjo, U.M. and Genco, R.J. (1987). Tissue localization of *Actinobacillus actinomycetemcomitans* in human periodontitis. I. Light, immunofluorescence and electron microscopic studies. Journal of Periodontology, **58**, 529-539.

Christersson, L.A., Wikesjo, U.M., Albini, B., Zambon, J.J. and Genco, R.J. (1987A). Tissue localization of *Actinobacillus actinomycetemcomitans* in human periodontitis. II. Correlation between immunofluorescence and culture techniques. Journal of Periodontology, **58**, 540-545.

Christersson, L.A., Wikesjo, U.M.E., Albini, B., Zambon, J.J. and Genco, R.J. (1987) Tissue localization of *Actinobacillus actinomycetemcomitans* in human periodontitis. II. Correlation between immunofluorescence and culture techniques. Journal of Periodontology, **58**, 540-545.

Chritersson, L.A. and Zambon, J.J. (1993). Suppression of subgingival *Actinobacillus actinomycetemcomitans* in localized juvenile periodontitis by systemic tetracycline. Journal of Clinical Periodontology, 20, 395-401.

Chung, H-J., Chung, C-P., Son, S-H. and Nisengard, R.J. (1989). *Actinobacillus actinomycetemcomitans* serotypes and leukotoxicity in Korean localized juvenile periodontitis. Journal of Periodontology, **60**, 506-511.

Church, J.A. and Nye C.A. (1979). Inhibition of polymorphonuclear leukocyte chemotaxis by streptokinase-streptodornase. Annals of Allergy, **43**, 333-.

Clark, R.A., Leidal, K.G. and Taichman, N.S. (1986). Oxidative inactivation of *Actinobacillus actinomycetemcomitans* leukotoxin by the neutrophil myeloperoxidase system. Infection and Immunity, **53**, 252-256.

Clark, W.B., Wheeler, T.T., Lane, M.D., Cisar, J.O. (1986). Actinomyces adsorption mediated by type-1 fimbriae. Journal of Dental Research, **65**, 1166-1168.

Cole, M.F. and Hale, C.A. (1991). Cleavage of chimpanzee secretory immunoglobulin A by *Haemophilus influenzae* IgA1 protease. Microbial Pathogenesis, **11**, 39-46.

Collins, S.J., Gallo, R.C. and Gallagher, R.E. (1977). Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature, **270**, 347-349.

Coote, J.G. (1992). Structural and functional relationships among the RTX-toxin determinants of Gram-negative bacteria. FEMS Microbiology Reviews, **88**, 137-162.

Cutler, C.W., Eke, P., Arnold, R.R. and Van Dyke, T.E. (1991). Defective neutrophil function in an insulin-dependent diabetes mellitus patient (a case report). Journal of Periodontology, **65**, 394-401.

Dibart, S., Haffajee, A.D. and Socransky, S.S. (1993). Effect of four therapies on periodontal infections. I. Overall response. Journal of Dental Research, **72**, Abstr. No. 2049.

Dongari, A.I. and Miyasaki, K.T. (1991). Sensitivity of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* to oxidative killing. Oral Microbiology and Immunology, **6**, 363-372.

Duguid, J.P., Clegg, S. and Wilson, M.I. (1979). The fimbrial and non-fimbrial haemagglutination of *Esherichia coli*. Journal of Medical Microbiology, **12**, 213-227.

Easmon, C.S.F., Cole, P.J., Williams, A.J. and Hastings, M. (1980). The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescene. Immunology, **41**, 67-74.

Ebersole, J.L, Taubman, M.A., Smith, D.J. and Socransky, S.S. (1982). Humoral immune responses and diagnosis of human periodontal disease. Journal of Periodontal Research, **17**, 478-480.

Ebersole, J.L. and Kornman, K.S. (1991). Systemic antibody responses to oral microorganisms in the cynomolgus monkeys development of methodology and longitudinal responses during ligature-induced disease. Research Immunology, **142**, 829-839.

Ebersole, J.L., Sandoval, M.N., Steffen, M.J. and Cappelli, D. (1991) Serum antibody in *Actinobacillus actinomycetemcomitans* infected patients with periodontal disease. Infection and Immunity, **59**, 1795-1802.

Ebersole, J.L., Taubman, M.A. and Smith, D,J. (1985). Gingival crevicular fluid antibody to oral microorganisms. II. Distribution and specificity of local antibody responses. Journal of periodontal Research, **20**, 349-356.

Ebersole, J.L., Taubman, M.A., Smith, D.J., Frey, D.E., Haffajee, A.D. and Socransky, S.S. (1987). Human serum antibody responses to oral microorganisms IV. Correlation with homologous infection. Oral Microbiology and Immunology, **2**, 53-59.

Ebersole, J.L., Taubman, M.A., Smith, D.J., Genco, R.J. and Frey, D.E.(1982). Human immune responses to oral micro-organisms. 1. Association of localized

juvenile periodontitis (LJP) with serum antibody responses to *Actinobacillus actinomycetemcomitans*. Clinical and Experimental Immunology, **47**, 43-52.

Ellner, J.J., Rosenthal, M.S., Lerner, P.I. and McHenry, M.C. (1979). Infective endocarditis caused by slow-growing, fastidious Gram-negative bacteria. Medicine, **58**, 145-158.

Eng, R.H.K., Smith, S.M., Goldstei, E.J.C., Miyasaki, K.T., Quah, S-E. and Buccini, F. (1986). Failure of vancomycin prophylaxis and treatment for *Actinobacillus actinomycetemcomitans* endocarditis. Antimicrobial Agents and Chemotherapy, **29**, 699-700.

Forlenza, S.W., Newman, M.G. and Blachman, U. (1979). *Capnocytophaga* sepsis in granulocytopenic patients. Journal of Dental Research, Abstr. No. 1027, p 348.

Forlenza, S.W., Newman, M.G., Lipsey, A.I., Siegel, S.E. and Blachman, U. (1980). *Capnocytophaga* sepsis: a newly recognised clinical entity in granulocytopenic patients. Lancet **i**, 567-568.

Frandsen, E.V.G., Reinholdt, J. and Kilian, M. (1987). Enzymatic and antigenic characterization of immunoglobulin A1 proteases from *Bacteroides* and *Capnocytophaga* species. Infection and Immunity, **55**, 631-638.

Frandsen, E.V.G., Reinholdt, J. and Kilian, M. (1991). Immunoglobulin A1 (IgA1) protease from *Prevotella (Bacteroides)* and *Capnocytophaga* species in relation to periodontal diseases. Journal of Periodontal Research, **26**, 297-299.

Frandsen, E.V.G., Reinholdt, J., Kjeldsen, M. and Kilian, M. (1995). In vivo cleavage of immunoglobulin A1 by immunoglobulin A1 proteases from *Prevotella* and *Capnocytophaga species*. Oral Microbiology and Immunology, **10**, 291-296.

Gallagher, R., Collins, S., Trujillo, J., McCredie, K., Ahearn, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Ruscetti, F. and Gallo, R. (1979). Characterization of the continuous differentiating

myeloid cell line (HL60) from a patient with acute promyelocytic leukaemia. Blood, **54**, 713-733.

Genco, R.J., Zambon, J.J. and Christersson, L.A. (1986). Use and interpretation of microbiological assays in periodontal diseases. Oral Microbiology and Immunology, **1**, 73-81.

Gibbons, R.J. and Etherden, I. (1983). Comparative hydrophobicity of oral bacteria and their adherence to salivary pellicles. Infection and Immunity, **41**, 1190-1196.

Gibbons, R.J. and van Houte, J. (1975) Bacterial adherence in oral microbial ecology. Annual Reviews of Microbiology, **29**, 19-44.

Gillespie, J., De Nardin, E., Radel, S., Kuracina, J., Smutko, J. and Zambon, J.J. (1992). Production of an extracellular toxin by the oral pathogen *Campylobacter rectus*. Microbial Pathogenesis, **12**, 69-77.

Gillett, R. and Johnson, N.W. (1982). Bacterial invasion of the periodontium in a case of juvenile periodontitis. Journal of Clinical Periodontology, **9**, 93-100.

Gilligan, P.H., McCarthy, L.R. and Bissett, B.K. (1981). *Capnocytophaga ochracea* septicemia. Journal of Clinical Microbiology, **13**, 643-645.

Gomez, R.S., da Costa, J.E., Lorentz, T.M., Garrocho, A.A. and Nogueira-Machado, J.A. (1994). Chemiluminescence generation and MTT dye reduction by polymorphonuclear leukocytes from periodontal disease patients. Journal of Periodontal Research, **29**, 109-112.

Gomez-Garces, J.L., Alos, J.I., Sanchez, J. and Cogollos, R. (1994). Bacteremia by multidrug-resistant *Capnocytophaga sputigena*. Journal of Clinical Microbiology, **32**, 1067-1069.

Gorden, J.M., Walker, C.B. (1993). Current status of systemic antibiotic usage in destructive

periodontal disease. Journal of Periodontology, **64**, 760-771.

Grebner, J.V., Mills, E.L., Gray, B.H. and Quie, P.G. (1977). Comparison of phagocytic and chemiluminescence response of human polymorphonuclear neutrophils. Journal of Laboratory Clinical Medicine, **89**, 153-159.

Grenier, D. and Mayrand, D. (1987). Functional characterisation of extracellular vesicles produced by *Bacteroides gingivalis*. Infection and Immunity, **55**, 111-.

Grenier, D., Mayrand, D. and McBride, B.C. (1989). Further studies on the degradation of immunoglobulins by black-pigmented *Bacteroides*. Oral Microbiology and Immunology, **4**, 12-18.

Gunsolley, J.c., Tew, J.G., Gooss, C., Marshall, D.R., Burmeister, J.A., Schenkein, H.A. (1990). Serum antibodies to periodontal bacteria. Journal of Periodontology, **61**, 412-419.

Haffajee, A.D., Dzink, J.L. and Socransky, S.S. (1988). Effect of modified Widman flap surgery and systemic tetracycline on the subgingival microbiota of periodontal lesions. Journal of Clinical Periodontology, **15**, 255-262.

Haffajee, A.D., Socransky, S.S. and Ebersole, J.L. (1984). Clinical, microbiological and immunological features associated with the treatment of active periodontitis lesions. Journal of Clinical Periodontology, **11**, 600-618.

Haffajee, A.D., Socransky, S.S., Dzink, J.L., Taubman, M.A. and Ebersole, J.L. (1988). Clinical, microbiological and immunological features of subjects with refractory periodontal diseases. Journal of Clinical Periodontology, **15**, 390-398.

Halpern, M.S. and Koshland, M.E. (1973). The stoichiometry of J chain in human secretory IgA. Journal of Immunology, **111**, 1653-1660.

Haubek, D., Poulsen, K., Asikainen, S. and Kilian, M. (1995). Evidence for absence in Northern Europe of especially virulent clonal types of *Actinobacillus actinomycetemcomitans*. Journal of Clinical Microbiology, **33**, 395-401.

Helgeland, K. and Nordby, ø. (1993). Cell cycle-specific growth inhibitory effect on human gingival fibroblasts of a toxin isolated from the culture medium of *Actinobacillus actinomycetemcomitans*. Journal of Periodontal Research, **28**, 161-165.

Hillman, J.D. and Socransky, S.S. (1989). The theory and application of bacterial interference to oral diseases. In: Myers, H.M, ed. New Biotechnology in Oral Research. Basel: Karger. 1-17.

Holm, A. and Kalfas, S. (1991). Cell surface hydrophobicity and electrokinetic potential of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. Oral Microbiol Immunology, **6**, 236-240.

Holm, A. and Kalfas, S. (1993). Polymorphonuclear leukocyte chemiluminescence induced by *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* in serum and saliva. Scandinavian Journal of Dental Research, **101**, 350-356.

Holm, A., Kalfas, S. and Holm, S.E. (1989). In vivo chemotaxis evoked by *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. Oral Microbilogy and Immunology, **4**, 30-34.

Holm, A., Kalfas, S. and Holm, S.E. (1993). Killing of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* by human polymorphonuclear leukocytes in serum and saliva. Oral Microbiology and Immunology, **8**, 134-140.

Holm, P. (1951). Studies on the aetiology of human actinomycosis. II. Do the "other microbes" of actinomycosis possess virulence?. Acta Pathol Microbiol Scandinavian, **28**, 391-406.

Holt, S.C. and Kinder, S.A. (1989). Genus II. *Capnocytophaga* pp2050-2058. In Staley, J.T., Bryant, M.P., Pfennig, N. and Holt, J.G.(ed) Bergey's Manual of Systematic Bacteriology, **3**, Williams and Wilkins, Baltimore.

Holt, S.C., Ebersole, J., Felton, J., Brunsvold, M. and Kornman, K.S. (1988). Implantation of *Bacteroides gingivalis* in non human primates initiates progression of periodontitis. Science, **239**, 55-57.

Holt, S.C., Leadbetter, E.R. and Socransky, S.S. (1979). *Capnocytophaga*: New genus of Gram-negative gliding bacteria. II. Morphology and Ultrastructure. Archives of Microbiology, **122**, 17-27.

Holt, S.C., Tanner, A.C.R. and Socransky, S.S. (1980). Morphology and ultrastructure of oral strains of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. Infection and Immunity, **30**, 588-600.

Horwitz, M.A. (1982). Phagocytosis of Microorganisms. Reviews of Infectious Diseases, **4**, 104-123.

Iino, Y. and Hopes, R.M. (1984). The bone-resorbing activities in tissue culture of lipopolysaccharides from the bacteria *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Capnocytophaga ochracea* isolated from human mouths. Archives of Oral Biology, 29, 59-63.

Ingham, H.R., Sisson, P.R., Tharagonnet, D., Selkon, J.B. and Codd, A.P. (1977). Inhibition of phagocytosis in vitro by obligate anaerobes. Lancet **ii**, 1252.

Ishihara, Y., Nishihara, T., Maki, E., Noguchi, T. and Koga, T. (1991). Role of interleukin-1 and prostaglandin <u>in vitro</u> bone resorption induced by *Actinobacillus actinomycetemcomitans* lipopolysaccharide. Journal of Periodontal Research, **26**, 155-160.

Isogai, H., Isogai, E., Yoshimura, F., Suguki, T., Kagota, W., and Takano, K. (1988). Specific inhibition of adherence of an oral strain of

Bacteroides gingivalis to epithelial cells by monoclonal antibodies against the bacterial fimbriae. Archives of Oral Biology, **33**, 479-485.

Iwase, M., Lally, E.T., Berthold, P., Korchak, H.M. and Taichman, N.S. (1990). Effects of cations and osmotic protectants on cytolytic activity of *Actinobacillus actinomycetemcomitans* leukotoxin. Infection and Immunity, **58**, 1782-1788.

Kagermeiler, A.S. and London, J. (1985). *Actinobacillus actinomycetemcomitans* strains y4 and N27 adhere to hydroxyapatite by distinctive mechanisms. Infection and Immunity, **47**, 654-658.

Kalmar, J.R., Arnold, R.R. and van Dyke, T.E. (1987). Direct interaction of *Actinobacillus actinomycetemcomitans* with normal and defective (LJP) neutrophils. Journal of Periodontal Research, **22**, 179-181.

Kamen, P.R. (1981). The effect of bacterial sonicates on human keratinizing stratified squamous epithelium in vitro. Journal of Periodontal Research, **16**, 323-330.

Kamen, P.R. (1983). Inhibition of keratinocyte proliferation by extracts of *Actinobacillus actinomycetemcomitans*. Infection and Immunity, **42**, 1191-1194.

Kamin, S., Harvey, W., Wilson, M. and Scutt, A. (1986). Inhibition of fibroblast proliferation and collagen synthesis by capsular material from *Actinobacillus actinomycetemcomitans*. Journal of Medical Microbiology, **22**, 245-249.

Kaplan, A.H., Weber, D.J., Oddone, E.Z. and perfect, J.R. (1989). Infection due to *Actinobacillus actinomycetemcomitans*: 15 cases and review. Reviews of Infectious Diseases, **11**, 46-63.

Kaufman, J. and Di Rienzo, J.M. (1989). Isolation of a corncob (coaggregation) receptor polypeptide from *Fusobacterium nucleatum*. Infection and Immunity, **57**, 331-337.

Khairat, O. (1940). Endocarditis due to a new species of *Haemophilus*. Journal of Pathology and Bacteriology, **50**, 497-505.

Kiley, P. and Holt, S.C. (1980). Characterization of the lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Y4 and N27. Infection and Immunity, **30**, 862-873.

Kilian, M. (1981). Degradation of immunoglobulins A1, A2 and G by suspected principal periodontal pathogens. Infection and Immunity, **34**, 757-765.

Kilian, M. and Biberstein, E.L. (1984). Genus II. Haemophilus Winslow, Broadhurst, Buchnan, Krumwiede, Rogers and Smith 1917, 561 AL. In Bergey's Manual of Systematic Bacteriology, Vol, **1**, pp 558-560. Ed, Krieg, N.R and Holt, J.G. Baltimore and London: Wiliams and Wilkins.

Kilian, M. and Schitt, C.R. (1975). *Haemophili* and related bacteria in the human oral cavity. Archives of Oral Biology, **20**, 791-796.

Kilian, M., Heine-Jensen, J. and Bülow, P. (1972). *Haemophilus* in the upper respiratory tract of children: A bacteriological, serological and clinical investigation. Acta Pathologica et Microbiologica Scandinavica. [B], **80**, 571.

Kilian, M., Mestecky, J. and Schrohenloher, R.E. (1979). Pathogenic species of the genus *Haemophilus* and *Streptococcus pneumoniae* produce Immunoglobulin A1 protease. Infection and Immunity, **26**, 143-149.

Kilian, M., Mestecky, J., Kulhavy, R., Tomans, M. and Butler, W.T. (1980). IgA1 protease from *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Streptococcus sanguis*. comparative immunochemical studies. Journal of Immunology, **124**, 2596-2600.

Kilian, M., Prachyabrued, W. and Theilade, E. (1976). *Haemophili* in developing dental plaque. Scandinavian Journal of Dental Research, **84**, 16-19.

Kilian, M., Thomsen, B., Petersen, T.E. and Bleeg, H.S. (1983). Occurrence and nature of bacterial IgA proteases. Annals of New York Academy of Science, **409**, 612-624.

Kinane, D.F. and Davies, R.M. (1990). Periodontal manifestations of systemic disease. In: Oral Manifestations of Systemic Disease, pp 512-536, eds Jones, J.H and Mason, D.K., London: Bailliére-Tindall.

Kinane, D.F., Cullen, C.F., Johnston, F.A. and Evans, C.W. (1989). Neutrophil chemotactic behaviour in patients with early-onset forms of periodontitis (II). Assessment using the under agarose technique. Journal of Clinical Periodontology, **16**, 247-251.

King, E.O. and Tatum, H.W. (1962). *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. Journal of Infectious Diseases, **111**, 85-94.

Kolenbrander, P.E., Andersen, R.N. and Moore, L.V. (1989). Coaggregation of Fusobacterium nucleatum, *Selenomonas flueggei*, *Selenomonas infelix*, *Selenomonas noxia* and *Selenumonas sputigena* with strains from 11 genera of oral bacteria. Infection and Immunity, **57**, 3194-3203.

Kornman, K.S, Holt, S.C. and Robertson, P.B. (1981). The microbiology of ligature-induced periodontitis in the cynomologus monkey. Journal of Periodontal Research, **16**, 363-371.

Kornman, K.S., Siegrist, B., Soskolne, W.A. and Nuki, K. (1981). The predominant cultivable subgingival flora of beagle dogs following ligature placement and metronidazole therapy.. Journal of Periodontal Research, **16**, 251-258.

Koshland, M.E. (1975). Structure and function of j chain. Advances in Immunology, **20**, 41-69.

Kraig, E., Lally, T. and Kolodrubetz, D. (1990). Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*: homology to

the alpha-hemolysin/leukotoxin gene family. Infection and Immunity, **58**, 920-929.

Kraut, M.S., Attebery, H.R., Finegold, S.M. and Sutter, V.L. (1972). Detection of *Haemophilus aphrophilus* in the human oral flora with a selective medium. Journal of Infectious Diseases, **126**, 189-192.

Kulkarni, G.V., Lee, W.K., Aitken, S., Birek, P. and McCulloch, C.A. (1991). A randomized, placebo-controlled trial of doxycycline: effect on the microflora of recurrent periodontitis lesions in high risk patients. Journal of Periodontology, **62**, 197-202.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), **227**, 680-685.

Lai, C-H., Listgarten, M.A., Evian, C.I. and Dougherty, P. (1986). Serum IgA and IgG antibodies to *Treponema vincentii* and *Treponema denticola* in adult periodontitis, juvenile periodontitis and periodontally healthy subjects. Journal of Clinical Periodontology, **13**, 752-757.

Lai, C.H., Listgarten, M.A. and Hammond, B.F. (1981). Comparative ultrastructure of leukotoxic and non-leukotoxic strains of *Actinobacillus actinomycetem-comitans*. Journal of Periodontal Research, **16**, 379-389.

Lally, E.T., Keiba, I.R., Demuth, D.R., Rosenbloom, J., Golub, E.E., Taichman, N.S. and Gibson, C.W. (1989). Identification and expression of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. Biochemical and Biophysical Research Communications, **159**, 256-262.

Lally, E.T., Keiba, I.R., Taichman, N.S., Rosenbloom, J., Gibson, C.W., Demuth, D.R., Harrison, G. and Golub, E.E. (1991). *Actinobacillus actinomycetemcomitans* leukotoxin is a calcium-binding protein. Journal of Periodontal Research, **26**, 268-271.

Larjava, H., Uitto, V-J., Eerola, E. and Haapasalo, M. (1987). Inhibition of gingival fibroblast growth by *Bacteroides gingivalis*. Infection and Immunity, **55**, 201-205.

Layman, L., Diedrich, D.L. (1986). Growth inhibition of effects of endotoxins from *Bacteriodes gingivalis* and *intermedium* on human gingival fibroblasts *in vitro*. Journal of Periodontology, **58**, 387-392.

Leadbetter, E.R., Holt, S.C. and Socransky, S.S. (1979). *Capnocytophaga*: New genus of Gram-negative gliding bacteria I. General characteristics, taxonomic considerations and significance. Archives of Microbiology, **122**, 9-16.

Liljemark, W.F., Bloomquist, C.G., Uhl, L.A., Schaffer, E.M., Wolfe, L.F., Pihlstrom, B.L. and Bandt, C.L. (1984). Distribution of oral *Haemophilus* species in dental plaque from a large adult population. Infection and Immunity, **46**, 778-786.

Liljenberg, B. and Lindhe, J. (1980). Juvenile periodontitis: some microbiological, histopathological and clinical characteristics. Journal of Clinical Periodontology, **7**, 748-61.

Lindhe, J. and Karring, T. (1989). The anatomy of the periodontium. In Textbook of Clinical Periodontology, 2nd ed, pp 19-69. Copenhagen: Munskgaard.

Lindhe, J. and Socransky, S.S. (1979). Chemotaxis and vascular permeability produced by human periodontopathic bacteria. Journal of Periodontal Research, **14**, 138-146.

Listgarten, M.A. (1988). The role of dental plaque in gingivitis and periodontitis. Journal of Clinical Periodontology, **15**, 485-487.

Listgarten, M.A., and Levin S. (1981). Positive correlation between the proportions of subgingival spirochetes and motile bacteria and susceptibility of human subjects to periodontal deterioration. Journal of Clinical Periodontology **8**, 122-138.

Löe, H., Theilade, E. and Jensen, S.B. (1965). Experimental gingivitis in man. Journal of Periodontology, **36**, 177-187.

Loesche, W.J., Giordano, J.R., Hujoel, P., Schwartz, J. and Smith, B.A. (1992). Metronidazole in periodontitis: reduced need for surgery. Journal Clinical Periodontology, 19, 103-112.

Majeed, K.N. and Macrae, I.C. (1994). Cytotoxic and haemagglutinating activities of motile aeromonas species. Journal of Medical Microbiology, **40**, 1-6.

Male, C. (1979). Immunoglobulin A1 protease production by *Haemophilus influenzae* and *Streptococcus penumoniae*. Infection and Immunity, **26**, 254-258.

Mandell, R.L. and Socransky, S.S. (1981). A selective medium for *Actinobacillus actinomycetemcomitans* and the incidence of the organism in juvenile periodontitis. Journal of Periodontology, **52**, 593-598.

Mandell, R.L., Ebersole, J.L. and Socransky, S.S. (1987). Clinical immunologic and microbiologic features of active disease sites in juvenile periodontitis. Journal of Clinical Periodontology, **14**, 534-540.

Mangan, D.F., Novak, M.J., Vora, S.A., Murad, J. and Krigen, P.S. (1989). Lectinlike interactions of *Fusobacterium nucleatum* with human neutrophils. Infection and immunity, **57**, 3601-3611.

Manson, J.D. and Lehner, T. (1974). Clinical features in juvenile periodontitis. Journal of Periodontology, **45**, 636-640.

Marsh, P.D. (1993). Antimicrobial strategies in the prevention of dental caries. Caries Research, **27** (supp) 72-76.

Mashimo, P.A., Yamamoto, Y., Nakamura, M. and Slots, J. (1983). Selective recovery of oral *Capnocytophaga* spp. with sheep blood agar containing bacitracin and polymyxin B. Journal of Clinical Microbiology, **17**, 187-191.

Mashimo, P.A., Yamamoto, Y., Slots, J., Par, K. and Genco, R.J. (1983A). The periodontal microflora of juvenile diabetics: culture, immunofluorescence and serum antibody studies. Journal of Periodontology, **54**, 420-430.

Mc Nabb, H., Mombelli, A. and Lang, N.P. (1992). Supragingival cleaning 3 times a week. The microbiological effects in moderately deep pockets. Journal of Clinical Periodontology, **19**, 348-356.

McArthur, W.P., Tsai, C.C., Baehni, P.C., Gencos, R.J. and Taichman, N.S. (1981). Leukotoxic effects of *Actinobacillus actinomycetemcomitans* modulation by serum components. Journal of Periodontal Research, **16**, 159-170.

McNabb, P.C. and Tomasi, T.B. (1981). Host defence mechanisms at mucosal surfaces. Annual Reviews of Microbiology, **35**, 477-496.

Meghji, S., Henderson, B., Nair, S. and Wilson, M. (1992). Inhibition of bone DNA and collagen production by surface-associated material from bacteria implicated in the pathology of periodontal disease. Journal of Periodontology, **63**, 736-742.

Meghji, S., Wilson, M., Barber, P. and Henderson, B. (1994). Bone resorbing activity of surface-associated material from *Actinobacillus actinomycetemcomitans* and *Eikenella corrodens*. Journal of Medical Microbiology, **41**, 197-203.

Meyer, D.H. and Fives-Taylor, P.M. (1993). Evidence that extracellular components function in adherence of *Actinobacillus actinomycetemcomitans* to epithelial cells. Infection and Immunity, **61**, 4933-4936.

Meyer, D.H., Sreenivasan, P.K. and Fives-Taylor, P.M. (1991). Evidence of invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans*. Infection and Immunity, **59**, 2719-2726.

Miky, F.H.M., Maltha, J.C., van Campen, G.J. (1990). Spirochetes in the early lesion of necrotizing ulcerative gingivitis, experimentally induced in

beagles. Oral Microbiology and Immunology, **5**, 86-89.

Miller, D.R., Lamsten, I.B. and Chasens, A.I. (1984). Role of polymorphonuclear leukocytes in periodontal health and disease. Journal of Clinical Periodontology, **11**, 1-15.

Mintz, K.P. and Fives-Taylor, P.M. (1994). Adhesion of *Actinobacillus actinomycetemcomitans* to a human oral cell line. Infection and Immunity, **62**, 3672-3678.

Miyasaki, K.T., Wilson, M.E. and Genco, R.J. (1986). Killing of *Actinobacillus actinomycetemcomitans* by human neutrophils Myeloperoxidase-Hydrogen Peroxide-Chloride system. Infection and Immunity, **53**, 161-165.

Miyasaki, K.T., Wilson, M.E., Brunetti, A.J. and Genco, R.J. (1986). Oxidative and nonoxidative killing of *Actinobacillus actinomycetemcomitans* by human neutrophils. Infection and Immunity, **53**, 154-160.

Miyasaki, K.T., Wilson, M.E., Zambon, J.J. and Genco, R.J. (1985). Influence of endogenous catalase activity on the sensitivity of the oral bacterium *Actinobacillus actinomycetemcomitans* and the oral *Haemophili* to the bactericidal properties of hydrogen peroxide. Archives of Oral Biology, **30**, 843-848.

Moore, W.E.C., Holdeman, L.V., Cato, E.P et al. (1985) Comparative bacteriology of juvenile periodontitis. Infection and Immunity, **48**, 507-519.

Moore, W.E.C., Holdeman, L.V., Cato, E.P., Smibert, R.M., Burmeisten, J.A. and Rauney, R.R. (1983). Bacteriology of moderate (chronic) periodontitis in mature adult humans. Infection and Immunity, **42**, 510-515.

Moore, W.E.C., Holdeman, L.V., Smibert, R.M., Hash, D.G., Burmeisten, J.A. and Ranney, R.R. (1982). Bacteriology of severe periodontitis in young adult humans. Infection and Immunity, **38**, 1137-1148.

Moore, W.E.C., Moore, L.H., Ranney, R.R., Smibert, R.M., Burmeister, J.A. and Schenkein, H.A. (1991). The microflora of periodontal sites showing active destructive progression. Journal of Clinical Periodontology, **18**, 729-739.

Mosmann, T. (1983). Rapid colorimetric assay from cellular growth and survival: application to proliferation and cytotoxic assays. Journal of Immunological Methods, **65**, 55-63.

Mulks, M.H. and Plaut, A. (1978). IgA protease production as a characteristic distinguishing pathogenic from harmless neisseriaceae. New England Journal of Medicine, **299**, 973-976.

Nakamura, M. and Slots, J. (1982). Aminopeptidase activity of *Capnocytophaga*. Journal of Periodontal Research, **17**, 597-603.

Newman, M.G. and Socransky, S.S. (1977). Predominant cultivable microbiota in periodontitis. Journal of Periodontal Research, **12**, 120-128.

Newman, M.G., Socransky, S.S., Savitt, E.D., Propas, D.A. and Crawford, A. (1976). Studies of the microbiology of periodontitis. Journal of Periodontology, **47**, 373-379.

Newman, M.G., Sutten, V.L., Pickett, M.J., Blachman, U., Greenwood, J.R., Grinekov, V. and Citron D. (1979). Detection, identification and comparison of *Capnocytophaga*, *Bacteroides ochraceus* and DF-1. Journal of Clinical Microbiology, **10**, 557-562.

Nisengard, R.J., Newman, M.G. and Zambon, J.J. (1994). Periodontal disease. In Oral Microbiology and Immunology, 2nd ed, pp 360-384. Saundres Company, Philadelphia

Nishihara, T., Ishihara, Y., Noguchi, T. and Koga, T. (1989). IL-1 induces bone resorption in organ culture. Journal of Immunology, **143**, 1881-1886.

Nyvad, B. and Kilian, M. (1987). Microbiology of the early colonization of human enamel and root

surfaces in vivo. Scandinavian Journal of Dental Research, **95**, 369-380.

Nyvad, B. and Kilian, M. (1990). Comparison of the initial Streptococcus microflora on dental enamel in caries-Active and in caries-inactive individuals. Caries Research, **24**, 267-272.

Ochiai, K., Kurita, T., Nishimura, K. and Ikeda, T. (1989). Immunoadjuvant effects of periodontitis-associated bacteria. Journal of Periodontal Research, **24**, 32-328.

Ohta, H., Hara, H., Fukui, K., Kurihara, H., Murayama, Y. and Kato, K. (1993). Association of *Actinobacillus actinomycetemcomitans* leukotoxin with nucleic acids on the bacterial cell surface. Infection and Immunity, **61**, 4878-4884.

Ohta, H., Kato, K., Fukui, K. and Gottschal, J.C. (1991). Microbial interactions and the development of periodontal disease. Journal of Periodontal Research, **26**, 255-257.

Ohta, H., Kato, K., Kokeguchi, S., Hara, H., Fukui, K. and Murayama, Y. (1991A). Nuclease-sensitive binding of an *Actinobacillus actinomycetemcomitans* leukotoxin to the bacterial cell surface. Infection and Immunity, **59**, 4599-4605.

Ohta, H., Kokeguchi, S., Fukui, K. and Kato, K. (1986). Leukotoxic activity in *Actinobacillus*(*Haemophilus*) *actinomycetemcomitans* isolated from periodontal disease patients. Microbiology and Immunology, **31**, 313-325.

Okuda, K. and Kato, T. (1987). Hemagglutination activity of lipopolysaccharides from subgingival plaque bacteria. Infection and Immunity, **55**, 3192-3196.

Okuda, K. and Takazoe, I. (1988). The role of *Bacteroides gingivalis* in periodontal disease. Advanced Dental Research, **2**, 260-268.

Okuda, K., Kato, T., Ishihara, K. and Naito, Y. (1991). Adherence to experimental pellicle of rough-type

lipopolysaccharides from subgingival plaque bacteria. Oral Microbiol Immunology, **6**, 241-245.

Okuda, K., Yamamoto, A., Naito, Y., Takazoe, I., Slots, J. and Genco, R.J. (1986). Purification and properties of hemagglutinin from culture supernatant of *Bacteroides gingivalis*. Infection and Immunity, **54**, 659-665.

Page, M.I. and King, E.O. (1966). *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. New England Journal of Medicine, **275**, 181-188.

Page, R.C. (1972). In: developmental aspects of oral biology (Slavkin, H.C. and Bavetta, L.A. eds). p 291-308. Academic Press, New York.

Page, R.C. (1986). Gingivitis. Journal of Clinical Periodontology, **13**, 345-355.

Page, R.C. and Schroeder, H.E. (1976). Pathogenesis of inflammatory periodontal disease. Journal of Laboratory Investigations, **33**, 234.

Page, R.C. and Schroeder, H.E. (1982). Periodontitis in man and other animals. Acomparative review. Basel: Karger.

Page, R.C., Altman, L.C., Ebersole, J.L., Vandesteen, G.E., Dahlberg, W.H., Williams, B.L. and Osterbeg, S.K. (1983). Rapidly progressive periodontitis: A distinct clinical condition. Journal of Periodontology, **54**, 197-209.

Papapanou, P.N., Sellen, A., Wennström, J.L. and Dahlén, G. (1993). An analysis of the subgingival microflora in randomly selected subjects. Oral Microbiology and Immunology, **8**, 24-29.

Parry, S.H. and Parter, P. (1978). Immunological aspects of cell membrane adhesion demonstrated by porcine enteropathogenic *Escherichia coli*. Immunology, **34**, 41-49.

Parry, S.H. and Porter, P. (1978). Immunological aspects of cell membrane adhesion demonstrated by porcine

enteropathgenic Escherichia coli. Immunology, **34**, 41-49.

Passo, S.A., Syed, S.A. and Silva jr, J. (1982). Neutrophil chemiluminescence in response to *Fusobacterium nucleatum*. Journal of Periodontal Research, **17**, 604-613.

Pavicic, M.J., van Winkelhoff, A.J., Douque, N.H., Steures, R.W. and de Graff, J. (1994). Microbiological and clinical effects of metronidazole and amoxicillin in *Actinobacillus actinomycetemcomitans* associated periodontitis. A 2 year evaluation. Journal of Clinical Periodontology, **21**, 107-112.

Pawlac and Hoag. (1984). Essential Book of Periodontics. Third Edition, pp 19-20. The C.V. Mosby Company.

Persson, S., Edlund, M.B., Claesson, R., Carlsson, J. (1990). The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. Oral Microbiology and Immunology, **5**, 195-201.

Petit, M.D.A., van Steenbergen, T.J.M., De Graff, J. and Van der Velden, U. (1993). Transmission of *Actinobacillus actinomycetemcomitans* in families of adult periodontitis patients. Journal of Periodontal Research, **28**, 335-345.

Plaut, A.G., Wistar, R.W.Jr., and Capra, J.O. (1974). Differential susceptibility of human IgA immunoglobulins to streptococcal IgA protease. Journal of Clinical Investigation, **54**, 1295-1300.

Poulsen, K., Theilade, E., Lally, E.T., Demuth, D.R. and Kilian, M. (1994). Population structure of *Actinobacillus actinomycetemcomitans* : a framework for studies of disease-associated properties. Microbiology, **140**, 2049-2060.

Progulske-Fox, A., Rao, V., Han, N., Lepine, G., Witlock, J. and Lantz, M. (1993). Molecular characterization of haemagglutinin genes of periodontopathic bacteria. Journal of Periodontal Research, **28**, 473-474.

Rabie, G., Lally, E.T. and Shenker, B.J. (1988). Immunosuppressive properties of *Actinobacillus actinomycetemcomitans* leukotoxin. Infection and Immunity, **56**, 122-127.

Robertson, P.B., Lantz, M., Marucha, P.T., Kornman, K.S., Trummel, C.L. and Holt, S.C. (1982). Collagenolytic activity associated with Bacteroides species and *Actinobacillus actinomycetemcomitans*. Journal of Periodontal Research, **17**, 275-283.

Robinson, P., Wakefield, D., Breit, S.N., Easter, J.F. and Penny R. (1984). Chemiluminescent response to pathogenic organisms: normal human polymorphonuclear leukocytes. Infection and Immunity, **43**, 744-752.

Rodenburg, J.P., Van Winkelhoff, A.J., Winkel, E.G., Goené, R.J., Abbas, F. and De Graff, J. (1990). Occurrence of *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in severe periodontitis in relation to age and treatment history. Journal of Clinical Periodontology, 17, 392-399.

Roitt, I. (1994). Essential Immunology, Eighth edition,Blackwell Scientific Publications, Oxford.

Rosan, B., Slots, J., Lamont, R.J., Listgarten, M.A. and Nelson, G.M. (1988). *Actinobacillus actinomycetemcomitans* fimbriae. Oral Microbiology and Immunology, **3**, 58-63.

Rozanis, J. and Slots, J. (1982). Collagenolytic activity of *Actinobacillus actinomycetemcomitans* and black-pigmented Bacteroides. Journal of Dental Research, **61**, 275 (Abstract 870).

Rozanis, J., Van Wart, H.E., Bond, M.D. and Slots, J. (1983). Further studies on collagenase of *Actinobacillus actinomycetemcomitans*. Journal of Dental Research, **62**, 300 (Abstract 1177).

Sandros, J., Papapanou, P. and Dahlén, G. (1993). *Porphyromonas gingivalis* invades oral epithelial cells in vitro. Journal of Periodontal Research, **28**, 219-226.

Sanz, M. and Newman, M.G. (1994). Dental plaque and calculus. In Oral Microbiology and Immunology, Second edition, pp 320-340. Saunders Company, Philadelphia.

Sasaki, S. (1979). Biological activity of lipopolysaccharides isolated from bacteria in human periodontal lesions. Bull Tokyo Dent Coll, **20**, 159-174.

Savitt, E.D., Socransky, S.S., Hammond, B.F. and Newman, M.G. (1975). Characterization of fusiform organisms isolated from periodontosis. Journal of Dental Research, **54A**, page 96, abstract no. 208.

Saxen, L. (1980). Juvenile periodontitis. Journal of Clinical Periodontology, **7**, 1-19.

Scannapieco, F.A., Kornman, K.S. and Coykendall, A.L. (1983). Observations of fimbriae and flagella in dispersed subgingival dental plaque and fresh bacterial isolates from periodontal disease. Journal of Periodontal Research, **18**, 620-633.

Scharmann, W., Jacob, F. and Portendorfen, J. (1976). The cytotoxic action of leucocidin from *Pseudomonas aeruginosa* on human polymorphonuclear leukocytes. Journal of General Microbiology, **93**, 303.

Schenkein, H.A. and Berry, C.R. (1991). Activation of complement by *Treponema denticola*. Journal of Dental Research, **70**, 107-110.

Schroeder, H.E. (1973). Transmigration and infiltration of leukocytes in human junctional epithelium. Helvetica Odontologica Acta, **17**, 6-15.

Scragg, M.A., Cannon, S.J. and Williams, D.M. (1994). Comparative cytotoxic effects of short-chain fatty acids produced by periodontal pathogens on two cultured fibroblast lines. Microbial Ecology in Health and Disease, **7**, 83-90.

Senior, B.W., Loomes, L.M. and Kerr, M.A. (1991). Microbial IgA proteases and virulence. Reviews in Medical Microbiology, **2**, 200-207.

Seow, W.K., Whitman, L.A., Bird, P.S. and Thong, Y.H. (1992). Modulation of human neutrophil adherence by oral bacteria. Australian Dental Journal, **37**, 121-125.

Seymour, G.J., Whyte, G.J. and Powell, R.N. (1985). Chemiluminescence in the assessment of polymorphonuclear leukocyte function in chronic inflammatory periodontal disease. Journal of Oral Pathology, **15**, 125-131.

Seymour, R.A. and Heasman, P.A. (1995). Tetracyclines in the management of periodontal diseases A review. Journal of Clinical Periodontology, **22**, 22-35.

Shenker, B.J., Kushner, M.E. and Tsai, C-C. (1982). Inhibition of fibroblast proliferation by *Actinobacillus actinomycetemcomitans*. Infection and Immunity, **38**, 986-992.

Shenker, B.J., McArthur, W.P. and Tsai, C-C. (1982). Immune suppression induced by *Actinobacillus actinomycetemcomitans*. I. Effects on human peripheral blood lymphocyte responses to mitogens antigens. Journal of Immunology, **128**, 148-154.

Shenker, B.J., Tsai, C-C. and Taichman, N.S. (1982). Suppression of lymphocyte responses by *Actinobacillus actinomycetemcomitans*. Journal of Periodontal Research, **17**, 462-465.

Shurin, S.B., Socransky, S.S., Sweeney, E. and Stossel, T.P. (1979). A neutrophil disorder induced by capnocytophaga, a dental microorganism. New England Journal of Medicine, **301**, 849-854.

Simpson, D.L., Berthold, P. and Taichman, N.S. (1988). Killing of human myelomonocytic leukaemia and lymphocytic cell lines by *Actinobacillus actinomycetemcomitans* leukotoxin. Infection and Immunity, **56**, 1162-1166.

Simpson, J.L., Leadbetter, E.R. and Holt, S.C. (1975). Isolation of anaerobic gliding bacteria from human dental plaque. Annual Meeting of American Society of microbiology, abstract, p 132.

Sims, W. (1970). Oral *Haemophili*. Journal of Medical Microbiology, **3**, 615-625.

Sjöström, K., Darveau, R., Page, R., Whitney, C. and Engel, D. (1992). Opsonic antibody activity against *Actinobacillus actinomycetemcomitans* in patients with rapidly progressive periodontitis. Infection and Immunity, **60**, 4819-4825.

Slots, J. (1976). The predominant cultivable organisms in juvenile periodontitis. Scandinavian Journal of Dental Research, **84**, 1-10.

Slots, J. (1981). Enzymatic characterisation of some oral and non-oral Gram-negative bacteria with the API ZYM system. Journal of Clinical Microbiology, **14**, 288-294.

Slots, J. (1982). Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. Journal of Clinical Microbiology, **15**, 606-609.

Slots, J. (1982A). Salient biochemical characters of *Actinobacillus actinomycetemcomitans*. Archives of Microbiology, **131**, 60-67.

Slots, J. and Genco, R.J. (1984). Microbial pathogenicity: black pigmented Bacteroides species, Capnocytophaga species and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonisation, survival and tissue destruction. Journal of Dental Research, **63**, 412-421.

Slots, J. and Rosling, B. (1983). Suppression of the periodontopathic microflora in localized juvenile periodontitis by systemic tetracycline. Journal of Clinical Periodontology, **10**, 465-486.

Slots, J., Evans, R.T., Lobbins, P.M. and Genco, R.J. (1980). *In vitro* antimicrobial susceptibility of *Actinobacllus actinomycetemcomitans*. Antimicrobial Agents and Chemotherapy, **18**, 9-12.

Slots, J., Reynolds, H.S. and Genco, R.J. (1980). *Actinobacillus actinomycetemcomitans* in human

periodontal disease: a cross-sectional microbiological investigation. Infection and Immunity, **29**, 1013-1020.

Slots, J., Zambon, J.J., Rosling, B.G., Reynolds, H.S., Christersson, L.A. and Genco, R.J. (1982). *Actinobacillus actinomycetemcomitans* in human periodontal disease. Journal of Periodontal Research, **17**, 447-448.

Smalley, J.W. and Birss, A.J. (1986). The cytotoxic effect of the outer membrane of *Bacteroides gingivalis* W50 on human gingival fibroblasts in culture (Abs. No. 24). Journal of Dental Research, **65**, 490.

Smalley, J.W., Birss, A.J. and Shuttleworth, C.A. (1988). The degradation of type 1 collagen and human plasma fibronectin by the trpysin-like enzyme and extracellular membrane vesicles of bacteroides gingivalis w50. Archives of Oral Biology, **33**, 323-329.

Smalley, J.W., Shuttleworth, C.A. and Birss, A.J. (1989). Collagenolytic activity of the extracellular vesicles of *Bacteroides gingivalis* w50 and an avirulent variant w50/BE1. Archives of Oral Biology, **34**, 579-583.

Smith, J.E., Anderson, J.G., Lewis, C.W. and Murad, Y.M. (1992). Cytotoxic fungal spores in the indoor atmosphere of the damp domestic environment. FEMS Microbiology Letters, **100**, 337-344.

Smith, S.R., Foyle, D.M., Needleman, I.G. and Pandya, N.V. (1994). The role of antibiotics in the treatment of periodontitis (Part I-systemic delivery). European Journal of Prosthodontic Restorative Dentestry, **3**, 79-86.

Sneath, P.H.A. and Johnson, R. (1973). Numerical taxonomy of *Haemophilus* and related bacteria. International Journal of Systematic Bacteriology, **23**, 405-418.

Socransky, S. S., Haffajee, A.D., Smith, G.L.F. and Dzink, J.L. (1987). Difficulties encountered in the

search for the etiologic agents of destructive periodontal diseases. Journal of Clinical Periodontology, **14**, 588-593.

Socransky, S.S and Haffajee, A.D. (1994). Evidence of bacterial etiology: a historical prospective. Periodontology 2000, **5**, 7-25.

Socransky, S.S. (1977). Microbiology of periodontal disease-present status and future considerations. Journal of Periodontology, **23**, 497-504.

Socransky, S.S. and Haffajee, A.D. (1991). Microbial mechanisms in the pathogenesis of destructive periodontal disease: a critical assessment. Journal of Periodontal Research, **26**, 195-212.

Socransky, S.S. and Haffajee, A.D. (1992). The bacterial etiology of destructive periodontal disease: current concepts. Journal of Periodontology **63**, 322-331.

Socransky, S.S. and Haffajee, A.D. (1993). Effect of therapy on periodontal infections. Journal of Periodontology, **64**, 754-759.

Socransky, S.S., Haffajee, A.D. (1990). Microbiological risk factors for destructive periodontal disease. In: Baden, J.D., ed. Risk Assessment in Dentistry. Chapel Hill: University of North Carolina Dental Ecology, 79-90.

Socransky, S.S., Holt, S.C., Leadbetter, E.R., Tanner, A.C.R., Savitt, E. and Hammond, B.F. (1979). *Capnocytophaga*: New genus of Gram-negative gliding bacteria. III. Physiological characterization. Archives of Microbiology, **122**, 29-33.

Spitznagel, J.jr., Kraig, E. and Kolodrubetz, D. (1991). Regulation of leukotoxin in leukotoxic and non-leukotoxic strains of *Actinobacillus actinomycetem-comitans*. Infection and Immunity, **59**, 1394-1401.

Stevens, P., Winston, D.J. and Van Dyke, K. (1978). In vitro evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: Utility in patients with severe

neutropenia and cellular deficiency states. Infection and Immunity, **22**, 41-51.

Stevens, R.H. and Hammond, B.F. (1982). Inhibition of fibroblast proliferation by extracts of *Capnocytophaga* species and *Actinobacillus actinomycetemcomitans*. Journal of Dental Research, **61**, 347, (Abst. 1515).

Stevens, R.H. and Hammond, B.F. (1988). The comparative cytotoxicity of periodontal bacteria. Journal of Periodontology, **59**, 741-749.

Stevens, R.H., Sela, M.N., McArthure, W.P., Nowotny, A. and Hammond, B.F. (1980). Biological and chemical characterization of endotoxin from *Capnocytophaga sputigena*. Infection and Immunity, **27**, 246-254.

Stokes, E.J. and Waterman, P.M. (1972). Antibiotic sensitivity tests by diffusion methods. Association of Clinical Pathologists, Broadshett 55.

Sutten, V.L. and Finegold, S.M. (1970). *Haemophilus aphrophilus* infections: clinical and bacteriologic studies. Annals New York Academy of Sciences, **174**, 468-487.

Sweet, S.P., MacFarlane, T.W. and Samaranayake, L.P. (1988). An epifluorescence technique for studying the adherence of oral bacteria to buccal epithelial cells. Microbial Ecology in Health and Disease, **1**, 221-225.

Taher, I.A.A. (1990). *Actinobacillus actinomycetemcomitans* and Periodontal Disease (Laboratory and Clinical studies). PhD. thesis, University of Glasgow, Scotland.

Taher, I.A.A. and MacFarlane, T.W. (1991). Adherence of *Actinobacillus actinomycetemcomitans* to buccal epithelial cells in vitro. Biomedical Letters, 46, 113-120.

Taichman, N.S. (1979). Interaction of inflammatory cells and oral microorganisms. VIII. Detection of leukotoxic activity of a plaque derived Gram-

negative microorganism. Infection and Immunity, **24**, 233-243.

Taichman, N.S. and Lindhe, J. (1989) Pathogenesis of plaque associated periodontal disease. In: Textbook of Clinical Periodontology, Second edition, pp 153-192. Copenhagen: Munskgaard.

Taichman, N.S. and Wilton, J.M.A. (1981). Leukotoxicity of an extract from *Actinobacillus actinomycetemcomitans* for gingival polymorphonuclear leukocytes. Inflammation, **5**, 1-12.

Taichman, N.S., Dean, R.T., and Sanderson C.J. (1980). Biochemical and morphological characterisation of the killing of human monocytes by a leukotoxin derived from *Actinobacillus actinomycetemcomitans*. Infection and Immunity, **28**, 258-268.

Taichman, N.S., Iwase, M., Korchak, H., Berthold, P. and Lally, E.T. (1991). Membranolytic activity of *Actinobacillus actinomycetemcomitans* leukotoxin. Journal of Periodontal Research, **26**, 258-260.

Taichman, N.S., Klass, J.E., Shenker, B.J., Macarak, E.J., Boehringer, H. and Tsai, C-C. (1984A). Suspected periodontopathic organisms alter in vitro proliferation of endothelial cells. Journal of Periodontal Research, **19**, 583-586.

Taichman, N.S., Shenker, B.J., Tsai, C-C., Glickman, L.T., Baehni, P.C., Stevens, R. and Hammond, B.F. (1984). Cytopathic effects of *Actinobacillus actinomycetemcomitans* on monkey blood leukocytes. Journal of Periodontal Research, **19**, 133-145.

Tanner, A.C.R., Dzink, J.L., Ebersole, J.L. and Socransky, S.S. (1987). *Wolinella recta*, *Campylobacter concisus*, *Bacteroides gracilis* and *Eikenella corrodens* from periodontal lesions. Journal of Periodontal Research, **22**, 327-330.

Tanner, A.C.R., Haffen, C., Brathall, G.T., Visconti, R.A. and Socransky, S.S. (1979). A study of the bacteria associated with advancing periodontitis in man. Journal of Clinical Periodontology, **6**, 278-307.

Tanner, A.C.R., Strzempko, M.N., Beisky, C.A. and McKinley, G.A. (1985). API ZYM and API An-Ident reactions of fastidious oral Gram-negative species. Journal of Clinical Microbiology, **22**, 333-335.

Tanner, A.C.R., Visconti, R.A., Socransky, S.S. and Holt, S.C. (1982). Classification and identification of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* by cluster analysis and deoxyribonucleic acid hybridizations. Journal of Periodontal Research, **17**, 585-596.

Taubman, M.A., Haffajee, A.D., Socransky, S.S., Smith, D.J. and Ebersole, J.L. (1992). Longitudinal monitoring of humoral antibody in subjects with destructive periodontal diseases. Journal of Periodontal Research, **27**, 511-521.

Tempro, P.J. and Slots, J. (1986). Selective medium for the isolation of *Haemophilus aphrophilus* from the human periodontium and other oral sites and the low proportion of the organisms in the oral flora. Journal of Clinical Microbiology, **23**, 777-882.

Theilade, E., Wright, W.H., Jensen, S.B. and Löe, H. (1966). Experimental gingivitis in man II. A longitudinal clinical and bacteriological investigation. Journal of Periodontal Research, **1**, 1-13.

Toda, K., Otsuka, M., Ishikawa, Y., Sato, M., Yamamato, Y. and Nakamura, R. (1984). Thiol-dependent collagenolytic activity in culture media of *Bacteroides gingivalis*. Journal of Periodontal Research, **19**, 372-.

Tolo, K. and Helgeland, K. (1991). Fc-binding components: a virulence factor in *Actinobacillus actinomycetemcomitans*? Oral Microbiology and Immunology, **6**, 373-377.

Tomasi, T.B.Jr. (1984). The secretory immune system. P 187-196. In D.P. Stites, J.D. Stoboo, H.H. Fudenberg and J.V. Wells (ed). Basic and Clinical

Immunology, 5th ed., Lange Medical Publications, Los Altos, Calif.

Tonetti, M.S. (1993). Etiology and pathogenesis. In the European Workshop on Periodontology: First Proceedings of the First European Workshop on Periodontology, Feb, 54-89.

Toshach, S. and Bain, G.O. (1958). Acquired aortic sinus aneurysm caused by *Haemophilus aphrophilus*. American Journal of Clinical Pathology, **30**, 328-335.

Tsai, C-C. and Taichman, N.S. (1986). Dynamics of infection by leukotoxic strains of *Actinobacillus actinomycetemcomitans* in juvenile periodontitis. Journal of Clinical Periodontology, **11**, 330-331.

Tsai, C-C., McArthur, W.P, Baehni, P.C., Evian, C., Genco, R.J. and Taichman, N.S. (1981). Serum neutralising activity against *Actinobacillus actinomycetemcomitans* leukotoxin in juvenile periodontitis. Journal of Clinical Periodontology, **8**, 338-348.

Tsai, C-C., McArthur, W.P., Baehni, P.C., Hammond, B.F. and Taichman, N.S. (1979). Extraction and partial characterization of a leukotoxin from a plaque-derived Gram-negative microorganism. Infection and Immunity, **25**, 427-439.

Tsai, C-C., Shenker, B.J., Dirienzo, J.M., Malamud, D. and Taichman, N.S. (1984). Extraction and isolation of a leukotoxin from *Actinobacillus actinomycetemcomitans* with polymyxin B. Infection and Immunity, **43**, 700-705.

Tsai, C-C., Stevens, R.H., Mahey, T.C. and Taichman, N.S. (1982). *Actinobacillus actinomycetemcomitans* (Aa) infection in children with periodontal lesions of the transitional dentition. Journal of Dental Research, **61**, Abstr. No. 1391, p 333.

Tuttle, R.S., Strubel, N.A., Mourod, J. and Mangan, D.F. (1992). A non-selective-like mechanism by which *Fusobacterium nucleatum* 10953 adheres to and

activates human lymphocytes. Oral Microbiology and Immunology, **7**, 78-83.

Van Dyke, T.E. and Vaikuntam, J. (1994). Neutrophil function and dysfunction in periodontal disease. Current Openion in Periodontology, 19-27.

Van Dyke, T.E., Bartholemew, E., Genco, R.J., Slots, J. and Levine, M.J. (1982). Inhibition of neutrophil chemotaxis by soluble bacterial products. Journal of Periodontology, **53**, 502-508.

Van Dyke, T.E., Levine, M.J. and Genco, R.J. (1982). Periodontal diseases and neutrophil abnormalities. In: Host-parasite Interactions in Periodontal Diseases, R.J. Genco and S.E. Mergenhagen, Eds, Washington D.C., American Society for Microbiology, p 235-245.

Van Dyke, T.E., Offenbachen, S., Kalman, J. and Arnold, R.R. (1988). Neutrophil defects and host-parasite interactions in the pathogenesis of localised juvenile periodontitis. Advanced Dental Research, **2**, 354-358.

van Steenbergen, T.J.M., Kastelein, P., Touw, J.J.A. and de Graaff, J. (1982). Virulence of black-pigmented *Bacteroides* strains from periodontal disease pockets and other sites in experimentally induced skin lesions in mice. Journal of Periodontal Research, **17**, 41-49.

van Winkelhoff, A.J., Tijhof, C.I. and de Graff, J. (1992). Microbiological and clinical results of metronidazole plus amoxicillin therapy in *Actinobacillus actinomycetemcomitans*-associated periodontitis. Journal of Periodontology, **63**, 52-57.

Vega, M.V., Maheswaran, K.S., Leininger, J.R. and Ames, T.R. (1987). Adaptation of a colorimetric microtitration assay for quantifying *Pasteurella haemolytica* A1 leukotoxin and antileukotoxin. American Journal of Veterinary Research, **48**, 1559-1564.

Visconti, A., Minervini, F., Lucivero, G. and Gambatesa, V. (1991). Cytotoxic and immunotoxic effects of Fusarium mycotoxins using a rapid colorimetric bioassay. Mycopathologia, **113**, 181-186.

Walker, C. and Gordon, J. (1990). The effect of clindamycin on the microbiota associated with refractory periodontitis. Journal of Periodontology, **61**, 692-698.

Warwood, N.M. (1981). *Capnocytophaga* bacteremia. Clinical Microbiology Newsletter, **3**, 4-5.

Weinberg, A. and Holt, S.C. (1990). Interaction of *Treponema denticola* TD-4, GM-1 and MS25 with human gingival fibroblasts. Infection and Immunity, **58**, 1720-1729.

Welch, W.D. (1980). Correlation between measurements of the luminol-dependent chemiluminescence response and bacterial susceptibility to phagocytosis. Infection and Immunity, **30**, 370-374.

Wennström, J.L. (1988). What is a clinically healthy periodontium? In: Periodontology Today, Int., Congr., Zürich, pp 1-5, Basel: Karger.

White, C.B., Lampe, R.M., Copeland, R.L. and Morrison, R.E. (1981). Soft tissue infection associated with *Haemophilus aphrophilus*. Pediatrics, **67**, 434-435.

Wikstrom, M., Revent, S., Johnsson, T. and Dahlén, G (1993). Microbial associations in periodontitis sites before and after treatment. Oral Microbiology and Immunology, **81**, 213-218.

Williams, B.L. and Hammond, B.F. (1979). *Capnocytophaga*:New genus of gram-negative gliding bacteria. IV. DNA base composition and sequence homology. Archives of Microbiology, **122**, 35-39.

Wilson, M., Kamin, S. and Harvey, W. (1985). Bone resorbing activity of purified capsule material from *Actinobacillus actinomycetemcomitans*. Journal of Periodontal Research, **20**, 484-491.

Winkler, J.R., Matarase, V., Hooven, C.I., Kramer, R.H. and Murray, P.A. (1988). An in vitro model to study bacterial invasion of periodontal tissues. Journal of Periodontology, **59**, 40-45.

Wolff, L.F., Aeppli, D.M. and Pihlstrom B et al. (1993). Natural distribution of 5 bacteria associated with periodontal disease. Journal of Clinical Periodontology, **20**, 699-706.

Yamazaki, y., Ebisu, S. and Okada, H. (1981). *Eikenella corrodens* adherence to buccal epithelial cells. Infection and Immunity, **31**, 21-27.

Yoneda, M., Maeda, K. and Aono, M. (1990). Suppression of bactericidal activity of human polymorphonuclear leukocytes by *Bacteroides gingivalis*. Infection and Immunity, **58**, 406-411.

Zafiropoulos, G.G., Flores-de-Jacoby, L., Hungerer, K.D.and Nisengard R.J. (1992). Humoral antibody responses in periodontal disease. Journal of Periodontology, **63**, 80-86.

Zambon, J.J. (1985). *Actinobacillus actinomycetemcomitans* in human periodontal disease. Journal of Clinical Periodontology, **12**, 1-20.

Zambon, J.J., Christersson, L.A. and Slots, J. (1983). *Actinobacillus actinomycetemcomitans* in human periodontal disease: prevalence in patient groups and distribution of biotypes and genotypes within families. Journal of Periodontology, **541**, 707-711.

Zambon, J.J., DeLuca, C., Slots, J. and Genco, R.J. (1983). Studies of leukotoxin from *Actinobacillus actinomycetemcomitans* using the promyelocytic HL60 cell line. Infection and Immunity, **40**, 205-212.

Zambon, J.J., Reynolds, H.S., and Slots, J. (1981). Black-pigmented Bacteroides spp. in the human oral cavity. Infection and Immunity, **32**, 198-203.

Zambon, J.J., Slots, J. and Genco, R.J. (1983). Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. Infection and Immunity, **41**, 19-27.

Zikan, J., Mestecky, J., Schrohenlohen, R.E., Tomana, M. and Kulhavy, R. (1972). Studies on human secretory immunoglobulin A. V Trypsin hydrolysis at elevated temperatures. Immuno-Chemistry, **9**, 1185-1193.