

Response of periodontopathogens to environmental changes

Thesis submitted by

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Doctor of Philosophy
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Abstract

Periodontal diseases are associated with inflammation, loss of gingival attachment and erosion of alveolar bone. More than 300 species of bacteria have been isolated from human subgingival plaque samples. Of these, certain species including *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* have been specifically implicated in the aetiology of one or more of the periodontal diseases. The microenvironment of the periodontal pocket is extremely complex and it is likely that there will be continual variation in the environmental conditions operating in this habitat. The aim of this thesis was to study the effect of environmental factors on the growth and virulence potential of periodontopathogenic bacteria.

The redox potential (E_h) of the environment was found to profoundly affect the growth and survival of *P. gingivalis* and *F. nucleatum*. Addition of the redox-modifying agent, methylene blue (MB), to suspensions of *P. gingivalis* and *F. nucleatum* caused an increase in the E_h of the medium and this was associated with killing of both organisms. In a small-scale clinical trial, application of MB to the periodontal pocket was associated with changes in the subgingival microflora which represented a shift towards a microflora more compatible with gingival health.

The effect of growth medium and incubation atmosphere on the acid (AcP) and alkaline (AlP) phosphatase activity of *A. actinomycetemcomitans* was studied. AcP activity was greater under anaerobic conditions compared to CO₂-enriched aerobic conditions.

The effect of IL-1 β and IL-6 on the growth of *P. gingivalis* and *A. actinomycetemcomitans* and the ability of these two organisms to degrade IL-1 β , IL-6 and IL-1ra was determined. Neither IL-1 β nor IL-6 affected the growth of either organism. However, *P. gingivalis* was shown to rapidly degrade these cytokines both in the absence and presence of serum. *A. actinomycetemcomitans* exhibited no cytokine-degrading activity.

The effect of a range of environmental conditions (growth in a CO₂-enriched aerobic atmosphere versus anaerobic growth, presence of serum or blood, biofilm versus planktonic mode of growth and iron depletion) on the surface protein and antigen profiles of *A. actinomycetemcomitans* was examined. Up-regulation and down-regulation of a number of proteins was observed when growth conditions were altered.

This study has shown that environmental conditions do affect the growth and virulence potential of periodontopathogenic bacteria and furthermore, that environmental modification may represent an alternative to traditional antimicrobial agents in eliminating these microorganisms from the periodontal pocket.

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Symbols and Abbreviations

Symbol/abbreviation	Description
AcP	acid phosphatase
AIP	alkaline phosphatase
α	alpha
RI proteinase	arginine I proteinase
BM broth	<i>Bacteroides</i> medium
β	beta
bpa	black-pigmented anaerobes
BHI	brain heart infusion agar
ECL	enhanced chemiluminescence
GCF	gingival crevicular fluid
HI	heat-inactivated
HRP	horseradish peroxidase
HGF	human gingival fibroblasts
IL-1	interleukin-1
IL-6	interleukin-6
IL-1ra	interleukin-1 receptor antagonist
IEF	isoelectric focusing
pI	isoelectric point
λ	lambda
LPS	lipopolysaccharide
LJP	localised juvenile periodontitis
MB	methylene blue
pO ₂	oxygen tension
PVDF	polyvinylidene difluoride
RABIT	Rapid Automated Impedance Technique
E _h	redox potential
SOD	superoxide dismutase
SAM	surface-associated material
WAB	Whitley Anaerobe broth
WIB	Whitley Impedance broth
WC	Wilkins-Chalgren agar

Acknowledgements

I am indebted to my supervisor Professor Michael Wilson for his continued support and inspiration throughout the course of my PhD and for the many opportunities he has given me along the way. I am also grateful to my second supervisor Dr. John Ward for his input into the latter part of my PhD and for the excellent training and facilities provided for me at UCL during my time there.

I would like to express particular thanks to Dr. Sean Nair, who always found time to answer questions and give advice.

I would like to acknowledge Dr. S. Poole at the National Institute for Biological Standards and Control for the generous supply of cytokines and antibodies, Dr. M. Rangarajan for purified *P. gingivalis* RI proteinase, Dr. Peter Tabona for the blood-grown *A. actinomycetemcomitans* SAM and Alun Kirby for the LJP sera.

A thankyou is also due to all my colleagues at the Eastman Dental Institute for their encouragement and friendship during my studies particularly Tracy Ward, Jon Pratten, Sam Packer, Zoey Jackson and Julia Banks.

Finally, I would like to thank my dear family and also Alexis for their love and support throughout my PhD.

Declaration

This thesis is the result of my own original investigations, except where otherwise stated. Determination of the bio-activity of IL-1 β following incubation with *P. gingivalis* culture supernatant was carried out by Dr. Kris Reddi of the Oral and Maxillofacial Surgery Department at the Eastman Dental Institute.

Publications as a result of this thesis

Papers

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Chapter 1

Introduction

1.1 Anatomy of the periodontium

The tissues that surround and support the teeth are known collectively as the periodontium and include the gingiva, alveolar bone, periodontal ligament and cementum.

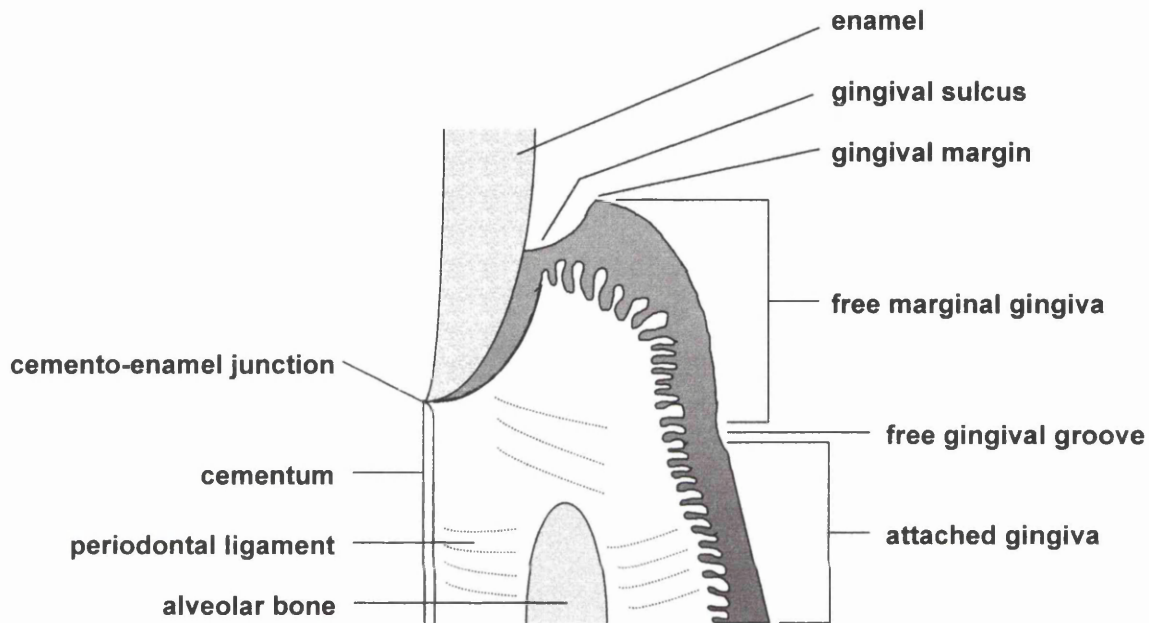


Figure 1.1 Periodontal structures

1.1.1 The gingiva

The gingiva is continuous with the oral mucosa and extends from the mucogingival line to the free marginal gingiva which surrounds the cervix of each tooth. The gingiva can be subdivided into three parts; free marginal gingiva, attached gingiva and interdental gingiva. The gingival tissue is made up of fibrous connective tissue (lamina propria) which is covered by the oral epithelium. Within this connective tissue, complex arrangements of collagen fibre bundles provide a firm attachment of the gingiva to the tooth root surface and the alveolar bone. The gingival tissue is highly vascularised and contains numerous cell types including plasma cells, fibroblasts, mast cells and lymphocytes.

1.1.1.1 The junctional epithelium

The junctional epithelium (JE) provides attachment of the free marginal gingiva to the tooth surface. The apical region of the JE is only a few cell layers thick while the coronal region adjacent to the sulcus may be 15 - 30 cell layers thick. The JE is constantly renewed and is quite permeable allowing the diffusion of bacterial products (Listgarten, 1986).

1.1.1.2 The gingival sulcus

The gingival sulcus is a narrow groove surrounding the tooth. In health the sulcus is approximately 0.5 mm in depth. It is lined by the sulcular epithelium which extends from the crest of the free marginal gingiva to the tooth surface.

1.1.2 Alveolar bone

The alveolar bone is made up of bony processes that project from the mandible and maxilla. These processes consist mainly of cancellous or spongy bone covered by a layer of harder cortical bone. The sockets which accommodate the roots of the teeth are lined with a layer of bone known as alveolar bone proper or cribiform plate. Alveolar bone is a vascular and innervated mineralised tissue. There is a constant turnover of alveolar bone (remodelling) which results from the processes of bone formation and bone resorption (Schwartz *et al.*, 1997).

1.1.2.1 Osteoblasts

The osteoblast is the cell responsible for bone formation. Osteoblasts are cuboidal cells which occur in continuous sheets. They have a high capacity for protein synthesis and possess a large Golgi apparatus and an extensive endoplasmic reticulum (Holtrop, 1991a). Osteoblasts synthesise a collagen-rich matrix (osteoid) which is subsequently mineralised to form mature bone. The organic matrix consists mainly of type I collagen. Other non-collagenous proteins such as osteonectin, osteopontin, osteocalcin and various proteoglycans are also present. Mineralisation of the matrix is also under the control of the osteoblasts. The activity of osteoblasts is tightly controlled by systemic hormones (e.g. parathyroid hormone, 1,25-dihydroxyvitamin D₃, oestrogen), growth factors (e.g. TGF- β , IGFs) and cytokines (e.g. IL-1 β) (Heersche and Aubin, 1991).

1.1.2.2 Osteoclasts

The osteoclast is the cell responsible for bone resorption. These cells are large in size (50 - 100 μm), multinucleated (2 - 10 nuclei) and highly motile. They normally occur singly or in low numbers at the resorption site (Holtrop, 1991b). The osteoclast attaches to the mineralised bone surface by forming a tight ring of adhesion (sealing zone). The area inside the sealing zone constitutes the bone resorbing compartment. Protons are extruded across the apical membrane (ruffled border) of the osteoclast into the bone resorbing compartment resulting in a decrease in local pH (Blair *et al.*, 1989). In addition the osteoclast secretes a number of enzymes including acid phosphatase, arylsulfatase, β -glucuronidase, cysteine proteinases and collagenase (matrix metalloproteinase 1, MMP-1) (Delaisse *et al.*, 1991; Delaisse and Vaes, 1992). The low pH causes dissolution of the mineral phase of the underlying bone, whilst the degradative enzymes break down the organic matrix.

The termination of bone resorption and initiation of bone formation occurs through a coupling mechanism. This coupling process ensures that the amount of bone removed during the resorption phase is equivalent to the amount laid down in the subsequent formation phase. Factors produced by osteoblasts during bone formation are incorporated into the extracellular matrix. During phases of bone resorption these factors are released and activated. These activated factors act to inhibit osteoclast activity and stimulate osteoblast activity. In addition osteoclasts and osteoblasts produce other factors which modulate both their own activity and the activity of the other cell type (Schwartz *et al.*, 1997).

Where bone resorption exceeds bone formation, for example in periodontal disease, there will be a net loss of bone.

1.1.3 The periodontal ligament

The periodontal ligament attaches the root of the tooth to the alveolar bone. It consists mainly of bundles of collagen fibres which are embedded in cementum on the tooth side and in the alveolar bone on the opposite side. Type I collagen accounts for about 80% of the periodontal ligament collagen. The periodontal ligament also contains blood and lymph vessels and numerous cell types

including fibroblasts (responsible for collagen synthesis), osteoblasts and osteoclasts.

1.1.4 Cementum

Cementum is a calcified tissue covering the root of the tooth. Cementoblasts located at the interface of the periodontal ligament with the cementum continually synthesise an organic matrix which is subsequently calcified to form cementum. The primary function of cementum is to attach the periodontal ligament fibres to the root surface.

1.2 Periodontal diseases

Periodontal diseases can be divided into conditions which affect only the gingiva (gingivitis) and conditions which involve other tissues of the periodontium, namely the periodontal ligament, cementum and alveolar bone. Periodontal diseases may be further classified according to the rate of progress of the disease (rapid, acute, chronic), the age group of the person affected (pre-pubertal, juvenile, adult), the distribution of the lesions (localised or generalised) or whether there are any predisposing factors (e.g. HIV infection, pregnancy, diabetes) (Ranney, 1993).

1.2.1 Gingivitis

Gingivitis begins with the accumulation of plaque along the gingival margin (Løe *et al.*, 1965; Theilade *et al.*, 1966). Bacterial products penetrate the gingival tissues leading to inflammation (chronic marginal gingivitis). Clinical manifestations of advanced gingivitis include bleeding on probing, erythema (redness) and oedema (swelling). If plaque is removed at this stage, periodontal health may be restored. Continued build-up of plaque may lead to periodontitis, although gingivitis may persist for many years without progression to periodontitis (Socransky *et al.*, 1984).

1.2.2 Chronic adult periodontitis

This is the most common form of periodontitis and affects a large proportion of the adult population to some degree (Brown and Løe 1993). The progression of the disease is generally slow and is preceded by precursor gingivitis. The

distribution of the disease is irregular with more severe destruction occurring in molar areas and anterior sections. There is evidence to suggest (Goodson *et al.*, 1982; Socransky *et al.*, 1984) that periodontitis occurs cyclically and that bursts of disease activity are followed by periods of quiescence and even healing. The disease is characterised by inflammation, loss of gingival attachment, tissue destruction and loss of alveolar bone. Alveolar bone loss results in tooth mobility and eventually tooth loss.

1.2.3 Juvenile periodontitis

Localised juvenile periodontitis (LJP) is a rare condition which begins during adolescence. The disease affects approximately 0.1% of the adolescent population (Blankenstein *et al.*, 1978; van der Velden, 1989), although considerably higher rates have been found in certain parts of the world and in different ethnic groups (Gjeramo *et al.*, 1984; Löe and Brown, 1991). LJP is characterised by an extremely rapid rate of bone loss which is restricted to the first permanent molars and incisor teeth. There is generally minimal gingival inflammation (Hall *et al.*, 1991) and the plaque associated with LJP is sparse. Often the first sign of disease is tooth mobility. A large proportion of LJP patients demonstrate depressed neutrophil chemotaxis with cells expressing a reduced number of cell surface receptors for chemotactic factors (Schenkein and van Dyke, 1994). There is strong evidence to suggest that *Actinobacillus actinomycetemcomitans* is the primary aetiological agent of LJP.

In some individuals a more generalised pattern of tissue destruction can be observed (generalised juvenile periodontitis).

1.3 Progression of periodontal disease

1.3.1 Development of plaque

The development of plaque involves a number of stages starting with initial attachment and colonisation of oral surfaces by pioneer species, through a relatively ordered succession of organisms, to a highly complex climax community comprised of up to 350 species. Immediately after a tooth surface is cleaned, a layer is formed over the enamel surface. This conditioning film is composed of glycoproteins, mucins and enzymes derived from the saliva, and is termed the acquired enamel pellicle. A pellicle will also form over the oral

mucosal surfaces, although the nature of this pellicle may be different from the acquired enamel pellicle. Bacteria adhere to the pellicle either non-specifically or specifically (via interactions between adhesins on the bacterial cell surface and receptors in the pellicle). The first species to attach are mainly Gram-positive facultative cocci including *Streptococcus sanguis*, *Streptococcus oralis*, *Streptococcus mitis* and *Actinomyces* spp. (Liljemark *et al.*, 1986, Nyvad and Kilian, 1987). These early colonisers provide the nascent surface for the attachment of secondary colonisers. Coaggregation reactions have been demonstrated between a number of oral bacteria (Kamaguchi *et al.*, 1994; Onagawa *et al.*, 1994; Amano *et al.*, 1997), and Kolenbrander and London (1993) proposed the following model for the colonisation of oral surfaces by bacteria, with *Fusobacterium nucleatum* acting as a bridge between early and late colonisers.

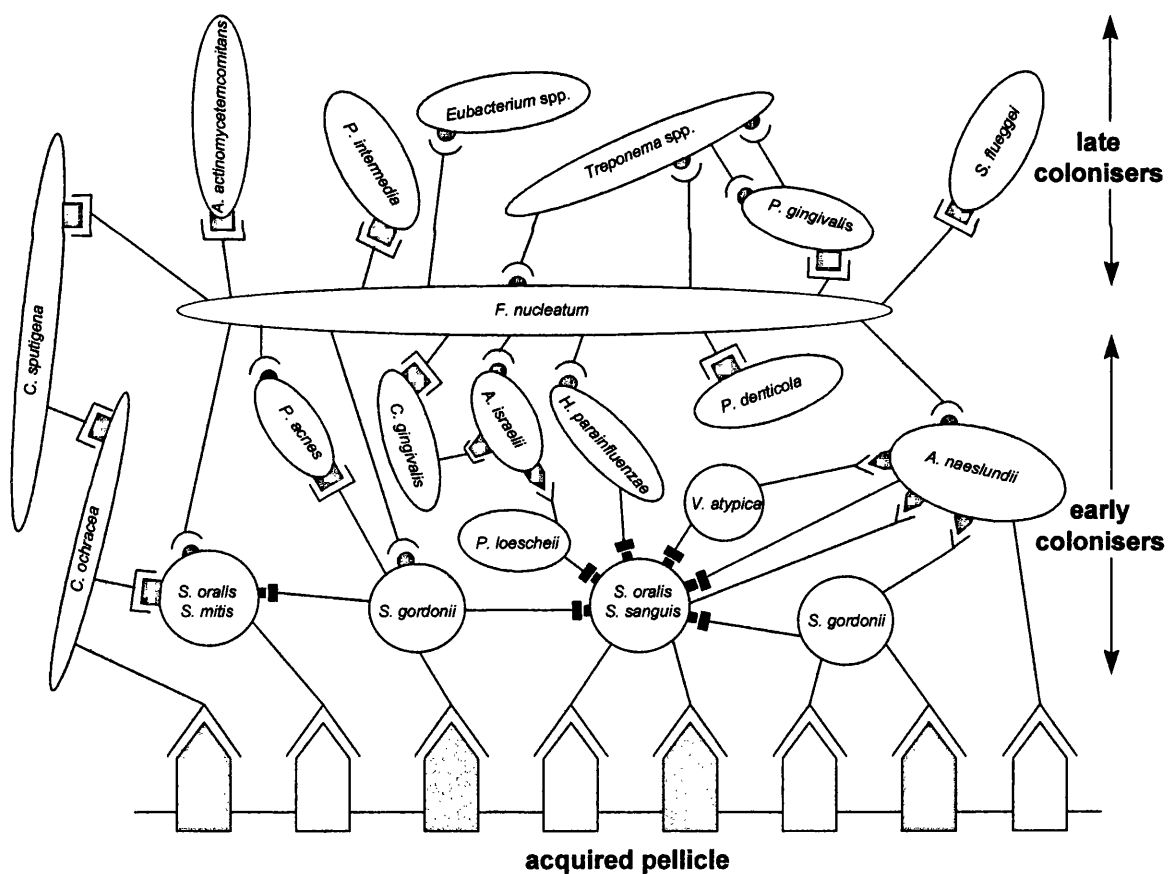


Figure 1.2 Diagrammatic representation of proposed bacterial accretion in dental plaque (Kolenbrander and London, 1993)

As the plaque increases in volume, conditions within the biofilm become more complex. Marsh and Martin (1992) proposed that as a result of diffusion limitations, a series of gradients will form across the plaque.

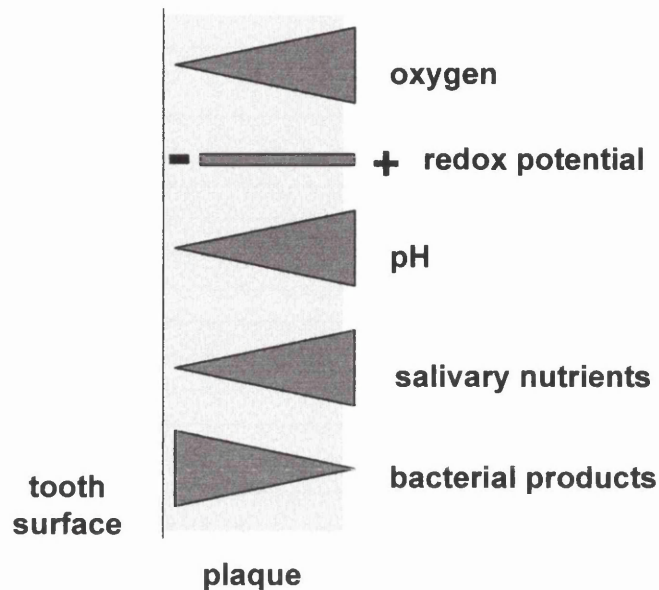


Figure 1.3 Model of dental plaque indicating the physicochemical gradients which form across the biofilm (Marsh and Martin, 1992)

At the surface of the plaque, which is bathed in saliva, conditions are aerobic and this is associated with a concomitantly high redox potential (E_h). Nutrients from the saliva are readily available. Acid produced as a result of the fermentation of dietary carbohydrates is rapidly neutralised by the saliva and thus the pH at the surface of mature plaque is close to neutral. Deep within the plaque however, oxygen becomes depleted and conditions become anaerobic. This, together with the production of bacterial metabolites in their reduced form, results in a decrease in E_h . Acid, which is unable to diffuse through the plaque, is trapped leading to a reduced pH near the tooth surface. Salivary nutrients become used up and there is a build-up of bacterial by-products. Depletion of nutrients may lead to the death of some species, however the by-products of metabolism of one species may provide a source of nutrients for another and complex nutritional interactions develop between species. Thus the plaque microflora becomes gradually more complex with different locations within the plaque having different microenvironments supporting different microbial communities.

Gingivitis, the precursor of periodontal disease, begins with the accumulation of plaque along the level of the gingival margin. As a result of an inflammatory response, there is an increase in the flow of gingival crevicular fluid (GCF). In addition to delivering components of the host defences, GCF provides a supply of proteins, glycoproteins and co-factors that may act as novel nutrients for periodontopathogenic bacteria. Gingivitis is also associated with bleeding. These changes in environmental conditions (i.e. an increase in the flow of GCF and the presence of blood) may lead to the selection of periodontopathogenic bacteria. Evidence to support this is provided by ter Steeg *et al.* (1987, 1988) who demonstrated that repeated passaging or continuous culture of subgingival plaque in the presence of human serum led to an enrichment of species implicated in periodontal disease such as *Bacteroides* spp., *F. nucleatum* and *Treponema denticola*. In addition, Loesche and Syed (1978) observed an increase in the levels of black-pigmented anaerobes from 0.01% to 0.2% of the total subgingival flora at sites of gingival inflammation which had progressed to the bleeding stage.

1.3.2 Formation of the periodontal pocket

Within a few days of undisturbed plaque formation, the gingival margin begins to show typical inflammatory changes including swelling and redness. Detachment of the JE and the increasing volume of plaque leads to a deepening of the gingival sulcus. This, together with oedema of the gingival tissues creates a small gingival pocket or pseudopocket (Holmstrup, 1996). The next stage in pocket formation is an apical proliferation and migration of the JE. The JE becomes attached to the root cementum and a true periodontal pocket is formed (Müller-Glauser and Schroeder, 1982). The epithelium which lines the periodontal pocket is referred to as the pocket epithelium. The pocket epithelium is invaded by neutrophils and is characterised by thickening with irregular proliferations of rete pegs and micro-ulceration of the epithelium (Saglie *et al.*, 1982). The periodontal pocket can be up to 12 mm in depth and provides a unique microenvironment for the proliferation of the subgingival microflora.

1.3.3 Microenvironment of the periodontal pocket

1.3.3.1 Anaerobiosis and E_h

Conditions within the periodontal pocket are highly anaerobic with a low oxygen tension (pO_2) and a low E_h . The E_h of the gingival crevice in health is in the region of +74 mV. In the periodontal pocket E_h values as low as -300 mV have been recorded (Marsh and Martin, 1992). The low E_h is due in part to the low pO_2 . The pO_2 of the anterior surface of the tongue when the mouth is closed is 16.4% (Eskow and Loesche, 1971). A study by Mettraux *et al.* (1984) demonstrated that the pO_2 in the periodontal pocket was 13.3 mm Hg (1.8% O_2) with deeper pockets having a lower pO_2 (11.6 mm Hg) than moderately deep pockets (15.0 mm Hg). The production of reducing agents such as H_2 and methyl mercaptan, and volatile fermentation products by the subgingival microflora will also contribute to the low E_h .

1.3.3.1.1 Measurement of E_h

E_h may be defined as the potential for electron transfer from a reductant (electron donor) to an oxidant (electron acceptor) and can be measured electrolytically. If an inert electrode is placed into a solution containing a redox system, it can either give up electrons to the solution or take electrons from it. The presence of the oxidised form of a substance in a solution will cause electrons to be drawn away from the electrode (electron suction) and the electrode will consequently become more positive. If the reduced form of a substance is present, electrons will be donated to the electrode (electron pressure) and the electrode will consequently become more negative. The E_h resulting from the two processes is a measure of the difference between the electron suction of the electron acceptor and the electron pressure of the electron donor (Jacob, 1970). The measuring electrode must be inert and act only as a conductor of electrons to or from the system. The measuring electrode is therefore constructed from a non-corrodable noble metal such as platinum, gold or iridium. The potential of the measuring electrode is compared against the constant potential of the reference electrode. The standard reference electrode is the hydrogen electrode which has an ascribed E_H (potential of the standard hydrogen electrode) of 0 mV. However this type of reference electrode is rarely used. Other types of reference electrode are the silver chloride

electrode, the calomel electrode and the sulphate electrode, each of which has its own constant potential.

When measuring E_h , there are some important factors to consider. E_h is affected by pH, and as the pH decreases, so the E_h increases. The relationship is not necessarily linear and varies for different redox systems. In addition, the introduction of even small amounts of oxygen into a redox system will have a significant effect on the observed E_h .

1.3.3.2 pH

Early studies by Kleinberg (1958) reported alkaline pH as high as 9.06 at inflamed sites. However this study did not take into account the effect of reducing agents which would most likely have been present. It has since been demonstrated that non-ionic reducing agents such as dithiothreitol are able to donate electrons to antimony electrodes, such as the one used by Kleinberg, resulting in pH values that are artificially high by as much as 1 - 1.5 units (Eggert *et al.*, 1991). Another study by Bickel and Cimasoni (1985) demonstrated a clear relationship between the pH of GCF and gingival index (GI). The mean pH of GCF collected from healthy sites was 6.90 compared to 7.79 and 8.66 for GCF collected from sites with a GI of 1 and 2 respectively. Again these measurements may have been artificially high as the study did not take into account the loss of CO_2 to the atmosphere following exposure of the GCF samples to air. Eggert *et al.* (1991) measured the pH *in situ* of healthy gingival crevices and periodontal pockets using glass micro-electrodes which are unaffected by the presence of reducing agents. The authors were unable to establish a relationship between crevicular pH and disease status. The mean crevicular pH was near 7.0 and ranged from 6.5 to 7.5 with a small proportion of individuals having at least one site with a pH between 7.5 and 7.8. However even a small shift in pH may be sufficient to alter the balance of the periodontal microflora. In mixed culture studies, an increase in pH from 7.0 to 7.5 led to an increase of *Porphyromonas gingivalis* from <1% of the total community to predominate the culture (Marsh and Martin, 1992). A shift towards a more alkaline pH in the periodontal pocket may be due in part to the degradation of proteins and urea.

1.3.3.3 Temperature

Temperatures in the oral cavity are approximately 1 - 2°C lower than core body temperature. In healthy subjects a natural gradient in temperature can be observed over the mandibular and maxillary arches with sulcular temperatures decreasing from posterior to anterior regions (Kung *et al.*, 1990). Temperatures at diseased sites are higher (0.96 - 1.76°C) than at healthy sites (Fedi and Killooy, 1992). In addition, a study by Meyerov *et al.* (1991), where pocket temperature was measured at 1 mm intervals, showed that pocket temperature increases with increasing depth.

1.3.3.4 Nutrients

In the periodontal pocket, the resident microflora are dependent on proteins and glycoproteins derived from GCF and host tissues as their primary source of nutrients. Collectively, the periodontal microflora elaborates a vast array of hydrolytic and proteolytic enzymes and there is evidence to suggest that these act in concert to breakdown host macromolecules.

Ter Steeg *et al.* (1987) found that a consortium of bacteria, derived from subgingival plaque, with different metabolic activities, demonstrated good growth in human serum (used to mimic GCF) whilst individual species grown in pure culture in serum exhibited only poor growth. Another example of such metabolic cooperation is provided by Bradshaw *et al.*, (1994) who demonstrated that addition of species with novel enzyme activities to a 5-member mixed culture, in which hog gastric mucin was the major carbon and energy source, led to an increase in the total viable count. This increase was made up of not only the numbers of the newly added species, but also an increase in the numbers of the existing community members.

Many other nutritional interactions have been shown to exist between different plaque bacteria (Grenier and Mayrand, 1986; Grenier, 1992a; Homer and Beighton, 1992) and some of these are illustrated in Fig. 1.4.

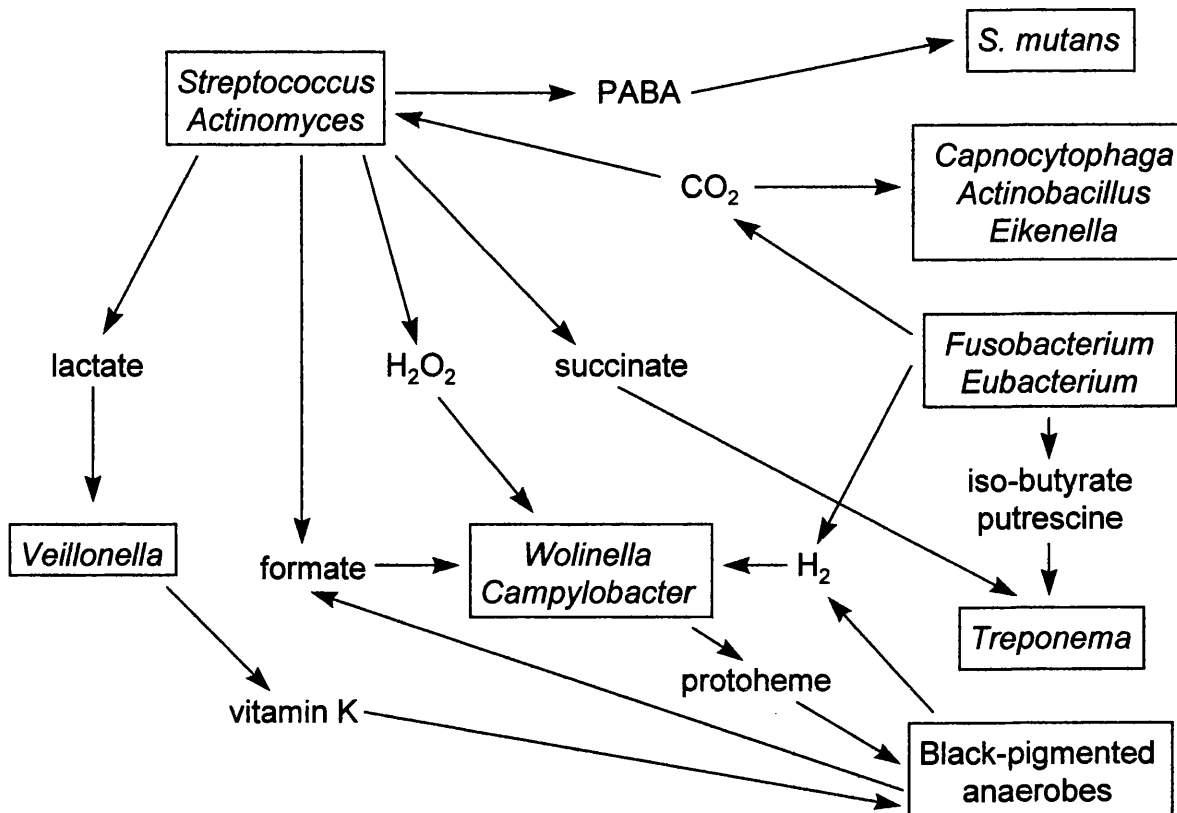


Figure 1.4 Nutritional interactions between members of the plaque microflora

1.3.3.5 Host defences

1.3.3.5.1 Gingival crevicular fluid

GCF is a serum exudate which is continually secreted from the gingival tissues into the sulcus through the sulcular epithelium. GCF has a cleansing and antimicrobial role. The flow of GCF is approximately 0.3 μl per tooth per hour at healthy sites. During inflammation the volume of fluid produced increases. Goodson (1989) reported GCF flow rates of 20 μl per hour at diseased sites which, with an estimated pocket volume of 0.5 μl , represented a GCF turnover of 40 times per hour. The mechanical cleansing action of the fluid flow will act to remove non-adherent bacterial cells and bacterial factors.

GCF contains a number of components of the immune system. Non-cellular factors present include IgG, IgM, IgA and complement. The predominant cellular component is the neutrophil, however lymphocytes (T and B-cells) and monocytes can also be detected.

A whole range of inflammatory mediators and cytokines including PGE₂ (Nelson *et al.*, 1992), IL-1 β and TNF- α (Heasman *et al.*, 1993) have been identified in

GCF which act to modulate the activity of host cells in response to the presence of bacteria and their components.

GCF contains a number of iron-sequestering proteins including transferrin and lactoferrin. Iron sequestration maintains the free iron concentration at very low levels. Free plasma iron has been estimated to be 10^{-18} moles per litre. This is far lower than the level ($0.1 - 0.4 \times 10^{-6}$ moles per litre) required for microbial growth (Weinberg, 1978). Other antimicrobial components of GCF include lysozyme and the protease inhibitors alpha-1-antitrypsin and alpha₂-macroglobulin.

1.3.3.6 Biofilm mode of life

Traditionally microbiologists have studied bacteria as homogenous suspensions of planktonic bacteria. However, in natural ecosystems, the majority of bacteria are attached to surfaces and form biofilms. Dental plaque is an excellent example of a biofilm.

One of the most notable features of biofilms is the large amount of extracellular matrix material. In supragingival plaque, the extracellular matrix consists mainly of polysaccharide material which is comprised largely of glucans and fructans. This extracellular polysaccharide (EPS) may play an important role in the adhesion of oral bacteria (Ellen and Burne, 1996).

Bacterial cells within a biofilm exhibit a number of different morphological and physiological properties compared to their planktonic counterparts. Indeed, adsorption to surfaces may be sufficient to induce change. Stal *et al.* (1989) showed that marine *Vibrio* species exhibit a change from a single polar flagellum to the production of numerous lateral flagella when located on a surface. Changes in O-antigen subunits of LPS in Gram-negative organisms have also been demonstrated in biofilm organisms (Cochrane *et al.*, 1988).

The general opinion is that surface-associated bacteria demonstrate increased metabolic activity, although usually only under low-nutrient conditions, compared to planktonic cells (Hamilton, 1987; Hamilton and Characklis, 1989; Jeffrey and Paul, 1986). Surface growth may provide an advantage by facilitating the capture of scarce nutrients. Costerton *et al.* (1987) proposed that nutrient trapping may occur at the surface of bacterial biofilms.

The virulence properties of biofilm bacteria may also differ from their planktonic counterparts. Jensen *et al.* (1992) showed that biofilms of *Pseudomonas aeruginosa* induced a much lower oxidative burst from polymorphonuclear leukocytes than the planktonic form of the organism. Another interesting finding is that of Hoppe (1984) who demonstrated the ability of attached bacteria to produce extracellular enzymes with a higher V_{max} and a lower K_m than the corresponding enzymes produced by planktonic cells.

Organisation of microbial populations into biofilms confers a number of advantages on the constituent organisms. For example access of components of the immune response system such as phagocytic cells, antibodies and complement is limited (Costerton *et al.*, 1987). Biofilms are inherently more resistant to antimicrobial agents than their planktonic counterparts (Wilson *et al.*, 1996a). This may be due, in part, to the protective role of the glycocalyx, although there is evidence to suggest that differences in the physiology and phenotype of biofilm bacteria in relation to their planktonic counterparts may also be important (Gilbert and Brown, 1995). The growth rate of an organism *in vivo* is likely to be much slower than in a nutrient-rich media *in vitro*. Slowly-growing cells have been shown to be more resistant to antimicrobial agents (Tuomanen *et al.*, 1986). Local differences in environmental conditions within the biofilm will lead to populations of organisms with physiologies and phenotypes unique to each particular subset of conditions.

1.4 Aetiology of periodontal disease

The microflora of the periodontal pocket is extremely complex and more than 300 species have been isolated from human subgingival plaque samples. Of these a number have been implicated in the aetiology of periodontal disease including *A. actinomycetemcomitans*, *P. gingivalis*, *Prevotella intermedia*, *F. nucleatum*, *Bacteroides forsythus*, *Eikenella corrodens*, *Peptostreptococcus micros* and *Eubacterium* species (Haffajee and Socransky, 1994). These putative periodontopathogens may be dependent on other non-pathogenic members of the microflora for essential nutrients, attachment sites, suppression of host defence mechanisms, generation of a favourable microenvironment or inhibition of competing microorganisms.

Darkground microscopy of subgingival plaque samples reveals a microflora dominated by rods and spirochaetes many of which are motile. The role of spirochaetes in periodontal disease is as yet unclear. Plaque from healthy sites exhibits few, if any, spirochaetes. However plaque from deep pockets contains large numbers of these organisms. The most extensively characterised periodontal spirochaete, *T. denticola*, produces a range of virulence factors which could potentially contribute to the disease process (Mikx and Keulers, 1992; Ohta *et al.*, 1986; Grenier, 1996).

Initiation of disease involves a number of different stages; transmission, adherence, colonisation, evasion of host defence mechanisms, invasion, tissue destruction and inhibition of tissue repair. Periodontopathogenic bacteria have been shown to produce a plethora of virulence factors which could potentially contribute to their ability to induce disease (Holt and Bramanti, 1991).

1.4.1 *Actinobacillus actinomycetemcomitans*

A. actinomycetemcomitans is a small Gram-negative, non-motile, saccharolytic, capnophilic coccobacillus. On initial isolation the colonies are small with a characteristic crossed-cigar configuration at the centre. *A. actinomycetemcomitans* is implicated in the aetiology of several forms of periodontal disease (Slots *et al.*, 1986; Haffajee and Socransky, 1994) but the strongest association of this organism with destructive periodontal disease is observed in LJP. *A. actinomycetemcomitans* is isolated with increased frequency and in higher numbers (Slots *et al.*, 1980a; Zambon *et al.*, 1983) from LJP lesions compared to plaque samples from healthy sites or diseased sites in other forms of periodontal disease. LJP patients demonstrate an enormously elevated antibody response to *A. actinomycetemcomitans* (Ebersole, 1990). Successful treatment of LJP patients results in elimination or a decrease in the levels of *A. actinomycetemcomitans*, whilst treatment failure is associated with failure to bring about a reduction in the levels of this organism (Haffajee *et al.*, 1984; Mandel *et al.*, 1986; Slots and Rosling, 1983). Furthermore, studies have also shown that levels of *A. actinomycetemcomitans* are higher at disease-active sites compared to inactive sites (Mandell *et al.*, 1987; Haffajee *et al.*, 1984). *A. actinomycetemcomitans* produces a vast array of potential virulence

factors which may contribute to the ability of the organism to induce disease (Wilson and Henderson, 1995).

1.4.1.1 Pathogenic strategies

The first stage in colonisation is attachment. *A. actinomycetemcomitans* produces large amounts of extracellular amorphous material which contributes to the ability of the organism to attach to epithelial cells *in vitro* (Meyer and Fives-Taylor, 1993). Fimbriae produced by certain strains of *A. actinomycetemcomitans* have also been shown to be important in mediating attachment to epithelial cells (Preus *et al.*, 1988) and saliva-coated hydroxyapatite (Rosan *et al.*, 1988). There is evidence to suggest that following attachment, *A. actinomycetemcomitans* is able to invade the periodontal tissues. *A. actinomycetemcomitans* has been detected in gingival tissue samples by immunofluorescence (Christersson *et al.*, 1987) and the organism has also been shown to invade a number of cell types *in vitro* (Meyer *et al.*, 1991; Blix *et al.*, 1991).

A. actinomycetemcomitans possesses a number of mechanisms by which it may interfere with host defences. The leukotoxin of *A. actinomycetemcomitans* forms pores in the membrane of target cells (Taichman *et al.*, 1991) causing lysis of neutrophils, monocytes and lymphocytes (Simpson *et al.*, 1988). *A. actinomycetemcomitans* produces immunoglobulin-degrading proteases (Gregory *et al.*, 1992, Jansen *et al.*, 1994) and Fc-binding proteins (Tolo and Helgeland, 1991). In addition the organism produces a 60 kDa protein that inhibits IgG and IgM synthesis by human peripheral blood lymphocytes (Shenker *et al.*, 1990). *In vivo* these features may lead to a reduction in opsonisation and phagocytosis of the organism. *A. actinomycetemcomitans* has been shown to be resistant to a number of the antibacterial components of neutrophil granules including H₂O₂ (Miyasaki *et al.*, 1984), defensins (Miyasaki *et al.*, 1990) proteinase 3, azurocidin, lysozyme and elastase (Miyasaki *et al.*, 1991). In addition, the organism appears to be able to inhibit the production of such compounds (Ashkenazi *et al.*, 1992). One of the first stages of the immune response is an infiltration of polymorphonucleocytes (PMNs) to the site. Van Dyke *et al.* (1982) reported a secreted compound from *A. actinomycetemcomitans* which was able to inhibit PMN chemotaxis.

A. actinomycetemcomitans possesses a number of virulence factors which could contribute to tissue destruction. Lipopolysaccharide (LPS) (Kiley and Holt, 1980) and lipid A-associated proteins (LAPs) (Reddi *et al.*, 1995a) have been shown to stimulate bone resorption. Another osteolytic agent, a 62 kDa protein isolated from *A. actinomycetemcomitans* surface-associated material (SAM), which possesses 95% sequence homology with *Escherichia coli* GroEL, has been shown to be a potent stimulator of bone resorption (Kirby *et al.*, 1995). *A. actinomycetemcomitans* produces a number of enzymes including acid and alkaline phosphatases (Fletcher *et al.*, 1995) and collagenase (Robertson *et al.*, 1982) which may contribute to tissue breakdown. LPS, LAPs and SAM from *A. actinomycetemcomitans* have been shown to stimulate the release of pro-inflammatory cytokines from monocytes and fibroblasts (Reddi *et al.*, 1995b, Reddi *et al.*, 1996a,b). IL-1 β , IL-6 and TNF- α are mediators of bone resorption (Schwartz *et al.*, 1997; Dziak, 1993; Tatakis, 1993; Ishimi *et al.*, 1990; Kurihara *et al.*, 1990) and IL-1 β and TNF- α are able to stimulate the release of MMPs from fibroblasts (Reynolds and Meikle, 1997).

In addition to its potential for inducing tissue damage, *A. actinomycetemcomitans* exhibits several mechanisms by which it may inhibit tissue repair. A number of studies have demonstrated the ability of extracts or components of *A. actinomycetemcomitans* to inhibit fibroblast proliferation (Kamin *et al.*, 1986; Meghji *et al.*, 1992a; White *et al.*, 1995). Gapstatin, an 8 kDa protein isolated from *A. actinomycetemcomitans* SAM appears to act by inhibiting fibroblasts in the G₂ phase of growth (Patel *et al.*, 1994). In addition there is evidence to suggest that *A. actinomycetemcomitans* may inhibit bone formation. SAM from the organism inhibited DNA and collagen synthesis by murine calvaria and osteoblasts (Meghji *et al.*, 1992b).

1.4.2 *Porphyromonas gingivalis*

P. gingivalis is an anaerobic Gram-negative, non-motile, asaccharolytic, short rod. On blood agar plates the organism produces dark olive-green to black colonies. There is a strong correlation of *P. gingivalis* with periodontal disease. The organism is absent or present in low numbers in most healthy individuals (Spiegel *et al.*, 1979; Duerden, 1980; White and Mayrand, 1981). In periodontal disease, *P. gingivalis* can be detected at 40 - 55% of sites (Savitt and

Socransky, 1984; Loesche *et al.*, 1985; Slots *et al.*, 1986). In addition, a proportional increase in the level of *P. gingivalis* in subgingival plaque has been correlated with severity of disease (Socransky *et al.*, 1991).

1.4.2.1 Pathogenic strategies

P. gingivalis produces a range of virulence factors (Cutler *et al.*, 1995, Haffajee and Socransky, 1994) which may contribute to the ability of the organism to colonise the tissues and initiate disease. Fimbriae produced by *P. gingivalis* have been shown to mediate attachment to oral epithelial cells and saliva-coated tooth surfaces (Slots and Gibbons, 1978; Okuda *et al.*, 1981). Coaggregation with other bacterial species may also facilitate colonisation (Kamaguchi *et al.*, 1994; Kinder and Holt, 1993, Amano *et al.*, 1997).

Once attached, the organism must obtain the appropriate nutrients in order to grow and multiply. *P. gingivalis* has an essential requirement for blood hemin (iron protoporphyrin IX). *P. gingivalis* is able to agglutinate (Mouton and Chandad, 1993) and lyse (Chu *et al.*, 1991) red blood cells thus liberating hemin and hemin-binding and iron transport systems have been described for this organism (Winston and Dyer, 1994; Genco, 1995; Bramanti and Holt, 1992; Smalley *et al.*, 1993a). *P. gingivalis* is a highly proteolytic organism and produces a number of proteolytic enzymes (Kuramitsu *et al.*, 1995) which can degrade proteins derived from host tissues and GCF providing the organism with small peptides and amino acids.

P. gingivalis exhibits a number of immune-evasion strategies. A capsule which is composed of polysaccharide material enables the organism to resist phagocytosis by PMNs (Sundqvist *et al.*, 1991; van Winklehoff *et al.*, 1993). *P. gingivalis* proteases have the capacity to reduce the bactericidal activity of human serum. Proteases from this organism have been shown to degrade IgG, IgA, IgM, the complement component C3 (Gregory *et al.*, 1992; Grenier, 1992b) and lactoferrin (Alugupalli and Kalfas, 1996). *P. gingivalis* can also inhibit neutrophil chemotaxis (Novak and Cohen, 1991) and volatile fatty acids produced by the organism as metabolic by-products inhibit T- and B-cell proliferation (Kurita-Ochiai, 1995).

The tissue damage observed in periodontal disease may be due in part to the products and components of *P. gingivalis*. SAM, LPS and LAPs from *P.*

gingivalis have been shown to induce bone resorption in a mouse calvarial assay (Reddi *et al.*, 1995a). Proteases may contribute to the soft tissue damage and these enzymes have also been shown to possess collagenolytic activity (Mayrand and Grenier, 1985; Smalley *et al.*, 1988; Kuramitsu *et al.*, 1995). *P. gingivalis* is able to stimulate cytokine release from a number of cell types (Reddi *et al.*, 1995b, 1996b). Induction of pro-inflammatory cytokine release by oral bacteria may indirectly contribute to tissue breakdown.

1.4.3 *Fusobacterium nucleatum*

F. nucleatum is a non-motile, anaerobic Gram-negative organism. Cells are rod shaped with long tapered ends. This species exhibits a high degree of heterogeneity and, on the basis of electrophoretic patterns of whole-cell proteins and DNA homology, Dzink *et al.* (1990) proposed that the species be divided into three different subspecies: *nucleatum*, *polymorphum* and *vincentii*.

F. nucleatum is one of the most commonly-occurring species in the healthy gingival crevice (Moore and Moore, 1994). A significant increase in the levels of this organism is observed at sites of gingival inflammation suggesting that *F. nucleatum* may play an important role in the initiation of periodontal disease (van Palenstein Helderman, 1975; Moore and Moore, 1994). In addition, increased levels of this organism have been demonstrated at disease-active sites compared to inactive sites (Dzink *et al.*, 1988).

1.4.3.1 Pathogenic strategies

F. nucleatum is able to attach to human oral epithelial cells and gingival fibroblasts (Xie *et al.*, 1991). Babu *et al.* (1995) suggested that fibronectin may play an important role in mediating attachment to host cells. *F. nucleatum* participates in a number of coaggregation reactions. The organism has been shown to coaggregate with oral streptococci (Takemoto *et al.*, 1993) and *P. gingivalis* (Kinder and Holt, 1993). It has been suggested that *F. nucleatum* may act as a bridge between the early and late colonisers of dental plaque (Kolenbrander and London, 1993). The formation of multigeneric aggregations may help maintain the presence of *F. nucleatum* and other periodontopathogens in the periodontal pocket.

F. nucleatum demonstrates a number of mechanisms by which it may evade immune responses. Grenier and Michaud (1994) demonstrated IgG Fc-binding activity in a number of oral bacteria including *F. nucleatum*. An immunosuppressive factor, FIP, has been purified from *F. nucleatum*. FIP inhibits T-cell activation by arresting cells in the G₁ phase of the cell cycle (Shenker and Datar, 1995; Demuth *et al.*, 1996).

Of particular importance to the periodontopathogenic potential of *F. nucleatum* would appear to be the production of toxic metabolites. Butyric and propionic acids produced by *F. nucleatum* have been shown to inhibit T- and B-cell proliferation (Kurita-Ochiai *et al.*, 1995) and human gingival fibroblast proliferation (Bartold *et al.*, 1991). In addition, hydrogen sulfide produced by *F. nucleatum* (Pianotti *et al.*, 1986) may interfere with opsonisation of the organism (Granlund-Edstedt *et al.*, 1993). LPS extracted from *F. nucleatum* demonstrated B lymphocyte mitogenicity and was found to inhibit cell growth and DNA synthesis of human gingival fibroblasts (Onue *et al.*, 1996). *F. nucleatum* also has the potential to modulate cytokine production by host cells and both particulate and soluble fractions of the organism were able to induce the release of TNF- α and IL-1 α from monocytes and human gingival fibroblasts (Rossano *et al.*, 1993).

1.4.4 Extracellular vesicles

A number of periodontopathogenic bacteria have been shown to produce extracellular vesicles (Mayrand and Grenier, 1989). These vesicles are a direct outgrowth or evagination of the outer membrane and possess a wide range of biological activities. Vesicles from *P. gingivalis* have been shown to possess haemagglutinating, haemin-binding and proteolytic activities (Smalley *et al.*, 1993b). In addition, vesicles from *P. gingivalis* contain large amounts of LPS. Vesicles from *A. actinomycetemcomitans* have been shown to possess leukotoxic (Berthold *et al.*, 1992) and bone resorbing (Mayrand and Grenier, 1989) activities.

These vesicles may act as a vehicle delivering potent virulence factors into the host tissues and may also act as scavengers of essential nutrients within the local environment of the bacterial cell.

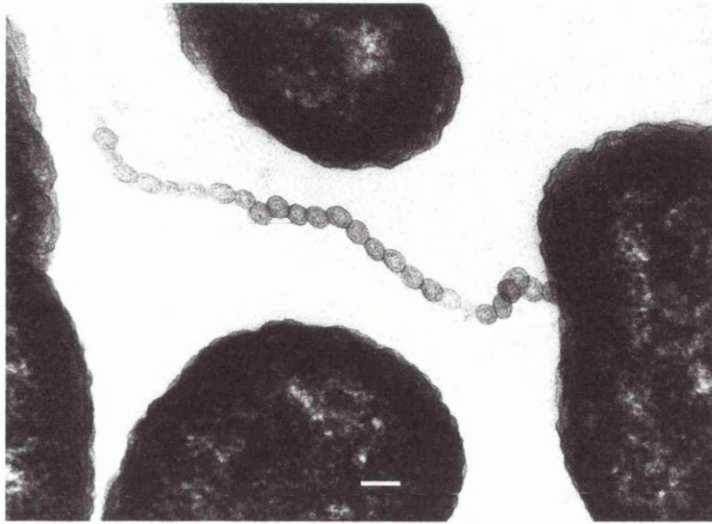


Figure 1.5 Electron micrograph of *A. actinomycetemcomitans* strain Y4 showing vesicles. Bar = 0.1 μm

1.5 Effect of environment on virulence

Although a whole armamentarium of potential virulence factors have been proposed for periodontopathogenic organisms (Holt and Bramanti, 1991), relatively few studies have looked at the effect of the environment on the expression of these factors. As stated by Kluver (1956) "... it is a general property of microbial life to change its enzymatic equipment, and in doing so to meet nature's challenges". Under any given set of environmental conditions, microorganisms express only that part of their genome which enables them to become structurally and functionally adapted to the prevailing conditions. Consequently an organism of a given phenotype is very much a product of its environment. Table 1.1 summarises some of the environmental conditions which may influence the growth and phenotype of a periodontopathogenic organism *in vivo*.

oxygen tension
redox potential
pH
temperature
osmolarity
ion concentration e.g. K^+ , Na^+ , Ca^{2+} , Mg^{2+}
nutrient source - host tissues
GCF
bacterial metabolites
material released from lysed bacterial cells
nutrient limitation e.g. iron
periods of starvation
contact with host cells
presence of host factors
interaction with other bacterial species
<i>positive</i> e.g. synergistic degradation of macromolecules
protection of anaerobic organisms from oxygen by aerobic species
exchange of genetic information
<i>negative</i> e.g. toxic bacterial metabolites
bacteriocins
competition for space, nutrients
biofilm and planktonic modes of growth
mechanical forces e.g. GCF flow
oral hygiene

Table 1.1 Factors influencing growth and phenotype of periodontopathogenic bacteria *in vivo*

In a study by Scannapieco *et al.* (1987) *A. actinomyctemecomitans* grown in an anaerobic atmosphere exhibited a different ultrastructure and surface protein profile compared to cells grown in a CO₂-enriched aerobic atmosphere. Differences in the surface protein profile of this organism were also demonstrated under conditions of iron limitation (Winston *et al.*, 1993). Spitznagel *et al.* (1995) showed that whilst iron depletion appeared to have no effect on the levels of leukotoxin produced by *A. actinomyctemecomitans*, production was affected by the presence of oxygen, and cells grown under anaerobic conditions produced more than three times the amount of leukotoxin

compared to cells grown under aerobic conditions. Smalley *et al.* (1994) showed that whilst *P. gingivalis* was able to bind hemin in an oxidising environment, the hemin-binding capacity was greatly enhanced under reducing conditions such as would be encountered in the periodontal pocket. When *P. gingivalis* was subjected to a 2°C increase in temperature from 37°C to 39°C, a sharp decrease in the production of fimbrillin and a concomitant increase in superoxide dismutase activity was observed (Amano *et al.*, 1994).

Such studies are essential in order to build a more complete picture of how the periodontopathogenic microflora may cause destruction of the periodontal tissues in the highly complex and continually changing microenvironment of the periodontal pocket *in vivo* and to identify potential therapeutic targets.

1.6 Pathology of periodontal diseases

The response of the host to the continued presence of bacteria and their products is to mount an immune response, which is intended to destroy the infectious agent, remove the debris and repair the damaged tissues.

In the early stages of the acute inflammatory response, a number of vasodilatory mediators including histamine, bradykinin and fibrin degradation products are released which results in an increase in vascular permeability. Activation of phospholipases leads to the degradation of membrane phospholipids to produce arachidonic acid. Arachidonic acid is, in turn, acted on by the enzymes lipoxygenase and cyclo-oxygenase to produce a variety of mediators including prostaglandins (e.g. PGE₂) and leukotrienes (e.g. LTB₄). The acute inflammatory response is characterised by an infiltration of neutrophils. LTB₄, the complement component C5a and small peptides released from bacteria act as chemoattractants for these cells. The primary role of the neutrophil is phagocytosis. Degranulation of neutrophils leads to the release of degradative enzymes including collagenase, elastase, β-glucuronidase and arylsulfatase which can contribute to localized tissue damage.

During the chronic phase of the inflammatory response, there is a mass infiltration of monocytes into the region which then differentiate into tissue macrophages. Chemoattractants for these cells include C5a and TGF-β. In addition to their phagocytic role, macrophages are an important source of

microbicidal reactive oxygen metabolites such as O_2^- , H_2O_2 and $\cdot OH$ which can also be toxic to host cells. Macrophages are also an important source of inflammatory mediators or cytokines.

1.6.1 Cytokines

Cytokines are soluble proteins or glycoproteins which act as intercellular signals modulating immunoinflammatory processes.

Cytokines are produced by a number of cell types including macrophages and fibroblasts, and may act in a paracrine (i.e. near to the producing cell) or autocrine (i.e. directly on the producing cell) manner. Cytokines may be produced constitutively or in response to a particular stimulus, for example complement factors and other cytokines. Bacteria have been shown to produce a whole range of molecules, in addition to LPS, which have the capacity to induce cytokine production (Henderson and Wilson 1995, Henderson and Wilson, 1996).

The cytokine network is extremely complex and it is not yet fully understood which cytokines are important in periodontal disease. However there is considerable evidence to suggest that the pro-inflammatory cytokines IL-1 and IL-6 are involved and elevated levels of these two cytokines have been demonstrated in GCF and periodontal tissues (Masada *et al.*, 1990; Reinhardt *et al.*, 1993; Bartold and Haynes, 1991; Takahashi *et al.*, 1994; Hou *et al.*, 1995; Stashenko *et al.*, 1991) of patients with periodontal disease.

1.6.1.1 Interleukin-1

IL-1 occurs in two forms (IL-1 α and IL-1 β) which recognize the same receptor and therefore possess similar biological activities. IL-1 is produced as a 31 kDa precursor which is cleaved to the mature 17.5 kDa form by pro-IL-1 converting enzyme (ICE). In the gingival tissues, macrophages are the main source of IL-1 (Matsuki *et al.*, 1991).

IL-1 plays a central role in the host response to bacterial invasion and has a wide range of target cells. The biological effects of this cytokine include the stimulation of fibroblast proliferation, T- and B-cell proliferation and induction of immunoglobulin secretion from mature B cells. IL-1 also stimulates the production of a number of cytokines (IL-1, IL-2, IFN- γ , IL-3, IL-4 and TGF- β) by

monocytes and neutrophils (Dinarello, 1994).

IL-1 possesses a number of biological activities which could potentially contribute to the tissue damage observed in periodontal disease. IL-1 is a potent mediator of bone resorption (Tatakis, 1993). The mechanism of IL-1 induced bone resorption is very complex and this cytokine has been shown to trigger a number of events which may ultimately lead to bone loss. Several studies have demonstrated the ability of IL-1 to induce fusion of osteoclast precursor cells to form multinucleate osteoclast cells (Pfeilschifter *et al.*, 1989; Kurihara and Roodman, 1990). IL-1 stimulates the production and release of PGE₂ by osteoblasts (Sato *et al.*, 1986; Tatakis *et al.*, 1988; Akatsu *et al.*, 1991) and fibroblasts (Newton and Covington, 1987; Richards and Rutherford, 1988) which in turn activates osteoclasts. In addition to its ability to initiate bone resorptive events, IL-1 can also inhibit bone formation (Stashenko *et al.*, 1987; Nguyen *et al.*, 1991). In a study by Canalis (1988), an IL-1 induced inhibition of collagen synthesis by cultures of rat calvariae was demonstrated. IL-1 may also contribute to soft tissue damage by stimulating the release of matrix metalloproteinases (MMPs) (Meikle *et al.*, 1989) and plasminogen activator (Mochan *et al.*, 1988) from human gingival fibroblasts.

1.6.1.2 Interleukin-6

IL-6 is produced by a wide variety of cell types including T- and B- cells, macrophages, fibroblasts, osteoblasts and osteoclasts. It is a glycoprotein with a molecular mass ranging from 20 - 30 kDa depending on the cellular source.

IL-6 is a multifunctional cytokine that regulates immune responses, hematopoiesis and acute phase reactions. One of the main functions of IL-6 is to stimulate the differentiation of B-cells into plasma cells which subsequently secrete large amounts of IgG, IgA and IgM (Narazaki and Kishimoto, 1994).

The exact role of IL-6 in the pathology of periodontal disease remains to be established, however there is growing evidence to suggest that IL-6 is an important mediator of bone resorption. In a study by Kurihara *et al.* (1990), IL-6 was shown to stimulate the formation of multinucleate osteoclast-like cells in human marrow cultures. These results are in accordance with Ishimi *et al.* (1990) who found that addition of IL-6 to fetal mouse calvaria stimulated calcium release in a dose dependent manner and this was associated with a

concomitant increase in osteoclast numbers.

1.6.1.3 Interleukin-1 receptor antagonist

Cytokine activities are regulated by inhibitors or antagonists. IL-1 receptor antagonist (IL-1ra) for example is structurally related to IL-1 and binds to the same receptors as IL-1, but does not trigger the intracellular signal transduction events which normally occur when IL-1 binds (Eisenberg *et al.*, 1990; Hannum *et al.*, 1990). Thus IL-1ra acts to suppress the effects of IL-1.

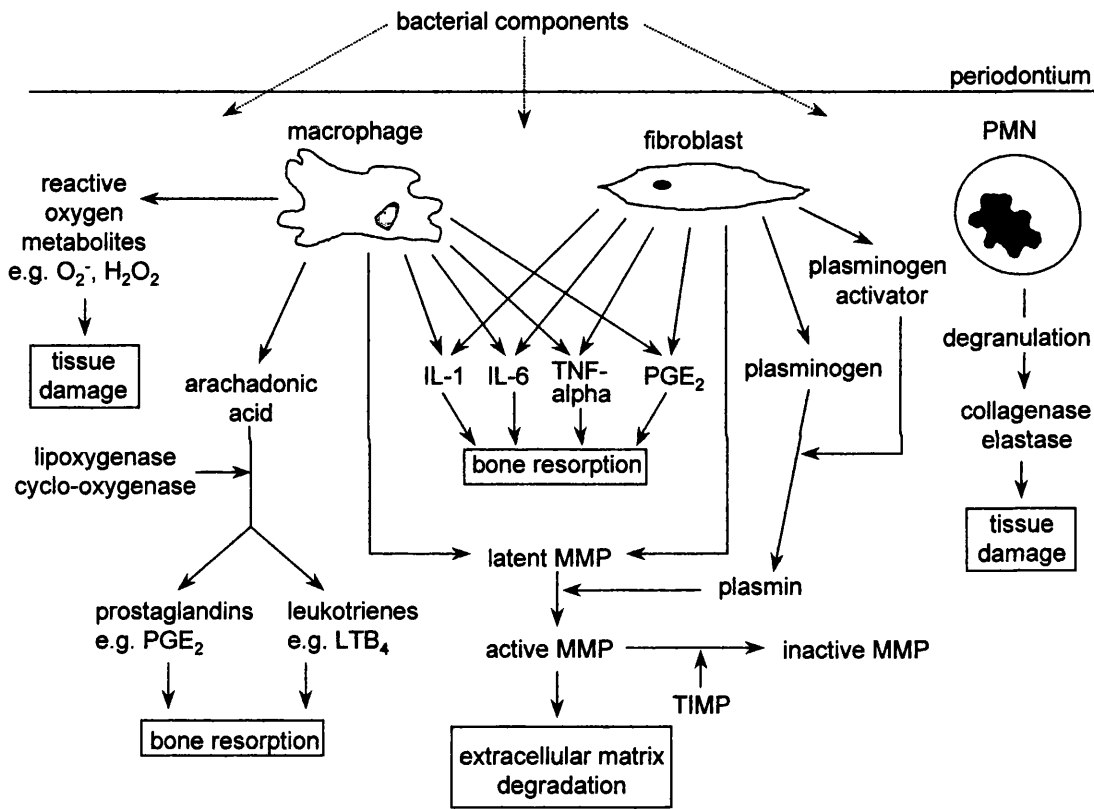


Figure 1.6 Contribution of host factors released in response to bacterial components in the pathology of periodontal disease

1.7 Treatment of periodontal diseases

1.7.1 Scaling and root planing

Scaling involves the mechanical removal of supra- and sub-gingival calculus. Root planing refers to the removal of embedded calculus and pathologically-altered cementum from the root surface. The aim of scaling and root planing is to generate a smooth tooth surface which is less conducive to the accumulation of plaque and a smooth root surface free of irritants such as calculus, plaque bacteria and endotoxin, thereby encouraging new epithelial attachment. The open-flap procedure is a surgical procedure whereby incisions are made in the gingival tissue creating a flap which can be folded back allowing improved access to the root surface for scaling and root planing.

1.7.2 Adjunctive therapies

In recent years, antibiotics and antimicrobial agents have been increasingly used as an adjunct to scaling and root planing (Gordon and Walker, 1993; Smith *et al.*, 1994; Needleman *et al.*, 1995; Seymour and Heasman, 1995). The two most widely used antibiotics are the tetracyclines (which include doxycycline and minocycline) and metronidazole. Tetracyclines have a broad spectrum of activity and are effective against many anaerobic and facultative bacteria and spirochaetes. Tetracyclines have been shown to be particularly effective in the treatment of LJP. The reason for this may be that *A. actinomycetemcomitans*, the main species implicated in the aetiology of LJP, is highly susceptible to tetracyclines (Slots *et al.*, 1980b). A useful property of the tetracyclines is that they become concentrated in GCF and levels 5 times higher than in serum can be detected following systemic administration (Ciancio *et al.*, 1980; Gordon *et al.*, 1981).

Metronidazole is only active at low E_h and is particularly effective against anaerobic organisms (Gordon and Walker, 1993). Metronidazole has been used in the treatment of periodontal disease and has been shown to reduce the proportions of anaerobic rods and spirochaetes in subgingival plaque (Loesche *et al.*, 1992).

Systemic administration of antibiotics in the routine treatment of periodontal diseases is not generally recommended due to the risk of side effects, and more recently attention has focused on the development of methods of local

application of antibiotics and antimicrobial agents (Needleman *et al.*, 1995). Slow or controlled release devices consist of an antibiotic or antimicrobial agent embedded in a matrix which is placed in the periodontal pocket. The drug is released gradually over a period of time as the matrix breaks down. Examples of controlled release devices include gels containing metronidazole (Elyzol) or minocycline (Dentomycin) and fibres containing tetracycline (Actisite). GCF antibiotic concentrations achieved are much higher than those obtained by systemic administration (Needleman *et al.*, 1995).

In addition to their antimicrobial effect, antibiotics and other antimicrobial agents can also reduce the activity of potentially damaging bacterial and host enzymes. Millward and Wilson (1990) demonstrated a reduction in the proteolytic activity of *P. gingivalis* in the presence of sub-inhibitory concentrations of chlorhexidine, whilst numerous studies have shown that tetracyclines inhibit tissue collagenase activity (Golub *et al.*, 1984; Ingman *et al.*, 1993).

1.7.3 Disadvantages of using antibiotics in the treatment of periodontal diseases

Numerous side effects have been reported following systemic antibiotic treatment of periodontal disease (Gordon and Walker, 1993; Slots and Rams, 1990). A common side effect is the disturbance of the gastrointestinal microflora leading to abdominal discomfort, nausea, vomiting and diarrhoea. Systemic antibiotic administration tends, therefore, to be restricted to certain conditions such as juvenile, rapidly progressive and refractory periodontitis. Locally-applied antibiotics are generally better tolerated.

However the main concern over the use of antibiotics in the treatment of periodontal disease is the development of resistance by bacteria in the oral cavity and elsewhere. The massive increase in the production and use of antibiotics in the last three decades has led to what might be described as a "crisis in antibiotic resistance" (Neu, 1992). Bacteria have become resistant to a wide range of antimicrobial agents as a result of chromosomal changes or the exchange of genetic material via plasmids and transposons. There is ample evidence to suggest that the periodontal microflora too, is developing resistance to a number of antibiotics. In a study by Lacroix and Walker (1995) tetracycline-resistant bacteria represented approximately 12% of the total viable count of

subgingival plaque samples, whilst Listgarten *et al.* (1993) demonstrated resistance to tetracycline, penicillin and metronidazole in a number of periodontopathogens including *A. actinomycetemcomitans*, *F. nucleatum* and *Prev. intermedia*. Long-term systemic therapy with tetracycline has been shown to increase the proportion of resistant strains present in the subgingival microflora (Kornman and Karl, 1982). Short-term therapy with tetracycline resulted in an increase in the minimum inhibitory concentration (MIC) to the agent in *Por. gingivalis* and *Prev. intermedia* (Abu-Fanas *et al.*, 1991). This supports the work of O'Connor *et al.* (1990) who demonstrated an increase in MIC to minocycline in *A. actinomycetemcomitans* following exposure to low concentrations of the antibiotic for several weeks.

1.7.4 Alternative approaches to the treatment of periodontal disease

1.7.4.1 Modification of the environment

The survival, growth and pathogenic potential of an organism in the periodontal pocket is dependent on environmental factors such as pH, E_h , pO_2 and the availability of nutrients. By modifying the conditions in the periodontal pocket, it may be possible to create an environment which is incompatible with the growth of periodontopathogenic bacteria.

The periodontal pocket has a low pO_2 and a low E_h which is essential for the survival of the anaerobic microflora. Several studies have looked at the effect of oxygen and oxygenating agents on gingivitis and periodontitis (Chasens, 1978). In 1937, Box reported that delivery of molecular oxygen into the gingival crevice resulted in an improvement in gingival health. However in a study by Hirsch *et al.* (1981), locally released molecular oxygen was found to have no significant effect on plaque formation and development of gingivitis. A number of oxygenating agents which release molecular oxygen have been investigated for their potential use in the treatment of gingivitis and periodontal disease. Merritt (1939, cited by Chasens, 1978) recommended the use of a toothpowder containing sodium perborate for the treatment of necrotising ulcerative gingivitis and observed no side-effects from its use. However Hirschfeld (1942) found that sodium perborate, when applied as a paste or mouthwash, caused chemical burns of the oral mucosa, hairy tongue, oedema of the lips and crevical sensitivity. Zinc perborate in the presence of moisture slowly and continuously

liberates active oxygen. Several studies indicate promising results with zinc peroxide in the treatment of oral infections (Knighton, 1940; Freeman, 1940). Monochlorosene in solution releases chlorine and oxygen. In a study by Weisman (1958) monochlorosene was shown to be a useful adjunct to oral hygiene, and greater reductions in levels of plaque were observed in the test group compared to the control group. No adverse reactions were reported. An *in vitro* study by Rosenthal (1958) however, raised doubts as to the bactericidal properties of monochlorosene. Sodium peroxyborate monohydrate (SPM) is claimed to have more than four times the oxygen concentration of sodium perborate. Wade *et al.* (1963) compared SPM with penicillin in the treatment of acute ulcerative gingivitis. SPM was found to be effective in 73% of cases compared to 83% effectiveness with penicillin. None of the patients treated with SPM demonstrated any side-effects. Urea peroxide in a glycerine gel has been shown to reduce the number of viable bacteria in saliva two hours after brushing and rinsing with the test product (Slanetz and Brown, 1946). Several studies have demonstrated that urea peroxide gel can reduce plaque levels (Shipman *et al.*, 1971; Shapiro *et al.*, 1973; Zinner *et al.*, 1970), although reductions in gingival inflammation were not necessarily greater in the test group compared to the control group. No side-effects were reported from the use of the product. Hydrogen peroxide (H_2O_2) is the most widely investigated oxygenating agent and a number of oral health care products containing H_2O_2 are currently available. In a review of the use of H_2O_2 in the treatment of gingivitis and periodontal disease (Marshall *et al.*, 1995), it was concluded that prolonged use of H_2O_2 can bring about a decrease in plaque and gingivitis indices. However for effective treatment of periodontal disease, H_2O_2 must be mechanically introduced into the periodontal pocket. Another oxygenating agent currently used in oral health care products is chlorine dioxide (ClO_2). ClO_2 has been shown to reduce dental plaque scores (Goultchin *et al.*, 1989) and oxidatively consume sulphur compounds which are responsible for oral malodour (Lynch *et al.*, 1997).

Certain compounds, while not liberating molecular oxygen, can raise the E_h of a system. An example of such an agent is methylene blue (MB). In a small-scale clinical trial (Wilson *et al.*, 1992), subgingival irrigation with MB was found to reduce the proportion of Gram-negative anaerobes and spirochaetes in

subgingival plaque samples. A reduction in GCF flow was also observed. A second clinical trial (Gibson *et al.*, 1994), where MB was administered both by subgingival irrigation and by incorporating the agent into a slow-release device was carried out and the microbiological findings of this trial are presented in Ch 3.

1.8 Aims of thesis

By understanding more about the effect of environmental conditions on the growth and virulence properties of periodontopathogenic bacteria and the interactions of these organisms with host molecules, it might be possible to design a treatment for periodontal disease based on the modification of the environment *in vivo* with the aim of eliminating periodontopathogenic bacteria from the periodontal pocket or attenuating their virulence.

The aims of this thesis were therefore:

- i) To investigate the effect of altering the E_h of the environment on the survival of *P. gingivalis* and *F. nucleatum* *in vitro* and to evaluate the efficacy of a redox-modifying agent in the treatment of periodontal disease by studying changes in the subgingival microflora.
- ii) To study the effect of growth medium and incubation atmosphere on the acid and alkaline phosphatase activities of *A. actinomycetemcomitans*.
- iii) To investigate the interactions of *A. actinomycetemcomitans* and *P. gingivalis* with cytokines.

Interactions to be studied;

- effect of IL-1 β and IL-6 on the growth of *A. actinomycetemcomitans* and *P. gingivalis*
 - degradation of IL-1 β , IL-6 and IL-1ra by *A. actinomycetemcomitans* and *P. gingivalis*
- iv) To examine the protein and antigen profiles of surface-associated material from *A. actinomycetemcomitans* grown under different conditions.

Growth conditions to be studied;

- growth in a CO₂-enriched aerobic atmosphere versus anaerobic growth
- biofilm versus planktonic mode of growth

- presence of serum
 - iron depletion
 - effect of nutrient-rich medium containing blood
- v) To begin to identify the surface-associated and exported proteins of *A. actinomycetemcomitans* using a plasmid-based *phoA* gene fusion system.

Chapter 2

General methods

2.1 Maintenance of bacterial strains

Bacterial strains were maintained by weekly sub-culture on Wilkins-Chalgren (WC) agar containing 5% (v/v) horse blood (Oxoid, Basingstoke, UK). Cultures were incubated anaerobically at 37°C. For long term storage a loopful of the organism was inoculated into 1 ml of WC broth containing 50% (v/v) glycerol and 2% (w/v) sorbitol in 5 ml cryovials (Nalgene, Rotherwas, UK). The vials were snap frozen in liquid nitrogen and placed at -70°C.

2.2 Culture media

2.2.1 *Bacteroides* medium (BM broth)

Per litre of distilled water:

Tryptone soya broth (10g), proteose peptone (10g), yeast extract (5g), NaCl (5g), cysteine-HCl (0.75g).

The pH of the medium was adjusted to 7.4 and then autoclaved at 121°C for 15 mins.

Immediately prior to use a hemin and menadione supplement was added to the medium to give final concentrations of 5 µg/ml and 0.5 µg/ml respectively.

2.2.1.1 Hemin and menadione supplement

50 mg of hemin was dissolved in 1 ml of 1 M NaOH, made up to 100 ml with dH₂O and autoclaved. When cool, 1 ml of a filter sterilised solution of menadione (5 mg/ml in ethanol) was then added to give a stock solution containing 0.5 mg/ml hemin and 0.05 mg/ml menadione. The stock solution was stored in the dark at 4°C.

2.2.2 Semi-defined medium for *A. actinomycetemcomitans*

The semi-defined medium for the growth of *A. actinomycetemcomitans* was based on that of Ohta *et al.* (1989).

Per litre of distilled water:

Na₂SO₄ (0.5g), KH₂PO₄ (0.2g), MgCl₂·6H₂O (0.4g), NaCl (1.2g), NH₄Cl (0.3g), KCl (0.3g), CaCl₂ (0.11g), yeast extract (2.0g), Tris (6.05g).

1 ml of a trace element solution was added per litre of medium. The pH of the medium was adjusted to 7.3 prior to autoclaving at 121°C for 15 mins. A separately autoclaved solution of glucose was then aseptically added to the medium to give a final concentration of 1.8 g/l.

2.2.2.1 Trace element solution

Per litre of distilled water:

EDTA (1.0g), FeSO₄·7H₂O (2.5g), ZnSO₄·7H₂O (0.2g), MnCl₂·4H₂O (0.5g), H₃BO₃ (0.05g), CoCl₂·6H₂O (0.15g), CuSO₄·5H₂O (0.15g), NiCl₂·6H₂O (0.025g), (NH₄)₆Mo₇O₂₄·4H₂O (0.1g), Na₂WO₄·2H₂O (0.05g), Na₂SeO₃·5H₂O (0.05g).

2.3 Buffers

2.3.1 Physiological saline

Per litre of distilled water:

NaCl (8.5g).

2.3.2 Phosphate buffered saline

Per litre of distilled water:

NaCl (8g), KCl (0.2g) Na₂HPO₄ (1.15g), KH₂PO₄ (0.2g).

2. 4 Viable counting technique

Serial 1:10 dilutions of the sample were prepared in WC broth and 20 µl of each dilution was then dropped onto the surface of WC agar plates in duplicate using specially calibrated 50 dropper pipettes (Bilbate Ltd., Daventry, UK). The drops were spread over the surface of the agar using a sterile glass spreader. Plates were incubated anaerobically at 37°C for 4 - 7 days and the colonies counted.

2.5 Determination of protein content of whole bacterial cells

Total protein content was determined by the enhanced alkaline copper protein assay of Lowry *et al.*, (1951) adapted for the assay of whole microorganisms. The method is based on the Biuret reaction of proteins with copper under alkaline conditions and the Folin-Ciocalteu phosphomolybdic-phosphotungstic acid

reduction to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic amino acids (primarily tyrosine and tryptophan). The reaction results in a blue colour which can be measured spectrophotometrically at 750 nm.

Reagent 1: 0.5% Na₂CO₃

Reagent 2: 1% sodium potassium tartrate containing 0.5% CuSO₄·5H₂O

Reagent 3: Immediately prior to use, 2 ml of reagent 2 was added to 50 ml reagent 1

Briefly 0.5 ml of 1.0 M NaOH was added to 0.5 ml of bacterial cell suspension in a glass test tube. Samples were boiled for 5 mins (to lyse the bacterial cells), allowed to cool and then 2.5 ml of Reagent 3 was added. After 10 mins at room temperature, 0.5 ml of 0.5x Folin-Ciocalteu reagent (Sigma, Poole, UK) was added and mixed immediately. Samples were left to stand for 30 mins to allow colour development and the absorbance at 750 nm was then measured. Bovine serum albumin (BSA, 0 - 200 µg/ml) was used as a standard.

2.6 Extraction of surface-associated material (SAM) from bacteria

Harvested bacteria were resuspended in cold, 0.85% sterile saline (1 g wet weight bacteria per 100 ml saline) and rotated gently at 4°C for 1 h. The suspension was centrifuged (3000 g, 20 mins) and the supernatant removed and kept at 4°C. The pellet was resuspended in fresh saline and the extraction procedure was repeated twice more. The supernatants were pooled, filtered through a 0.22 µm polytetrafluoroethylene (PTFE) filter, dialysed extensively against dH₂O for 5 days at 4°C using Spectrapor dialysis tubing with a 3.5 kDa cut-off (Medicell International Ltd., London, UK), and then lyophilised.

2.7 Determination of the protein content of SAM

The protein content of the SAM preparations was determined using the DC protein assay kit (Bio-Rad, Hemel Hempstead, UK). This assay is based on the method of Lowry *et al.*, (1951) but has been modified to save time and to minimise the volume of sample required. Briefly, 20 µl of each sample was dispensed into a 96 well microtitre plate. BSA standards ranging from 0.0 - 1.0 mg/ml were also included. 10 µl of reagent A was added and the plates were incubated at room temperature for 10 mins. 80 µl of reagent B was then added and the plates were

incubated at 37°C for 15 mins before measuring the absorbance at 650 nm using a Titertek plate reader.

2.8 Determination of the carbohydrate content of SAM

The carbohydrate content of the SAM preparations was determined using the method of Dubois *et al.* (1956). Briefly, 0.5 ml of 5% w/v phenol was added to 0.5 ml of sample in clean glass test tubes. Glucose standards ranging from 0 - 100 µg/ml were also included. Concentrated sulphuric acid (2.5 ml) was then added to each tube and vortexed immediately. Tubes were allowed to stand for 10 mins and were then placed in a water bath at 25 - 30°C for 15 mins. The absorbance of each sample was measured at 490 nm.

2.9 Determination of the endotoxin content of SAM

The endotoxin content of the SAM preparations was determined using the Pyrotell® *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Liverpool, UK). The principle of the assay is the ability of endotoxin to cause clotting of blood cells (amoebocytes) from the horseshoe crab, *Limulus polyphemus*.

10 µl of lysate was spotted onto the inside of the lid from a tissue culture grade 96 well microtitre plate (Sarstedt, Leicester, UK). An equal volume of ten-fold dilutions of the test sample (ranging from 20 pg - 20 µg of SAM per ml) was pipetted directly into the lysate. Endotoxin standards ranging from 0.0075 - 1.00 International Unit (IU)/ml were included as a means of validating the assay. The lid was covered and placed at 37°C for 1 h taking care not to agitate the lid and thus disturb gel formation. Following incubation, a small drop of 0.2% methylene blue in 70% ethanol was placed onto the surface of each spot. A negative result was indicated by the formation of a homogenous blue drop. A positive result (where gelation had occurred) was indicated by the exclusion of the dye from the spot resulting in the dye remaining on the surface (a blue star was often observed on the surface of positive spots). The assay was considered valid only if the lysate sensitivity was within one two-fold dilution of the label claim.

2.10 One-dimensional sodium-dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (1970). This is a discontinuous system consisting of 2 contiguous, but distinct gels; a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH and ionic strength. The stacking gel serves to concentrate the proteins whilst the resolving gel separates the proteins according to their molecular mass. When an electric current is applied, the proteins are driven into the gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel and a moving boundary region is rapidly formed with the highly mobile chloride ions at the front and the relatively slow glycinate ions at the rear. A localised high-voltage gradient forms between the leading and trailing ion fronts causing the SDS-polypeptide complexes to concentrate into a thin zone or stack. As proteins migrate into the resolving gel, molecular sieving operates causing the SDS-polypeptide complexes to separate on the basis of their molecular weight. SDS-PAGE was carried out using the Mini-PROTEAN[®] II cell (Bio-Rad).

Reagents required:

1.5 M Tris-HCl, pH 8.8

0.5 M Tris-HCl, pH 6.8

30% acrylamide stock solution (29.2% acrylamide, 0.8% bisacrylamide)

electrode buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3)

The composition of the gels were as follows:

Lower resolving gel

12 - 15% acrylamide, depending on pore size required

0.375 M Tris-HCl, pH 8.8

0.1% SDS

Polymerisation was induced by the addition of 100 μ l of freshly prepared 10% ammonium persulphate (APS) and 10 μ l of N,N,N',N',-tetramethylethylenediamide (TEMED) per 10 ml of gel solution.

Upper stacking gel

4% acrylamide

0.125 M Tris-HCl, pH 6.8

0.1% SDS

Polymerisation was induced by the addition of 50 μ l of 10% APS and 10 μ l of TEMED per 10 ml of gel solution.

Samples were mixed (1:1) with reducing sample buffer (Sigma) consisting of 0.06M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.25% bromophenol blue and then boiled for 5 mins prior to loading onto the gel. SDS-binding imparts a strong negative charge to the protein, dominating over its native charge. Thus, the charge to mass ratio becomes constant for most proteins and, under an electric field, the proteins separate according to their molecular mass.

Electrophoresis was carried out at a constant current of 30 mA per gel until stacking was complete, and then at 15 mA per gel until the bromophenol tracking dye had reached the bottom of the gel.

2.11 Two-dimensional electrophoresis

The method used for two-dimensional electrophoresis is based on that of O'Farrell (1975). In the first dimension proteins are separated across a pH gradient according to their isoelectric point (isoelectric focusing, IEF). The net charge on any protein, is the sum of positive and negative charges on amino acid residues as well as side groups such as carbohydrates, phosphates and sulphates, and is dependent on the pH of the surrounding environment. At one specific pH, a protein will have no net charge (i.e. the molecule has an equal number of positive and negative charges). This pH value is referred to as the isoelectric point (pI) for that protein. A pH gradient for IEF can be generated using carrier ampholytes incorporated into a gel matrix. Ampholytes are composed of isomers and homologues of aliphatic polyaminopolycarboxylic acids with pI values covering a pre-selected range. When an electric current is applied, ampholytes migrate through the gel and a pH gradient is established. A protein which is initially in a pH region below its pI will have a net positive charge and will move towards the cathode. Conversely, a protein which is in a pH region above its pI will have a net negative charge and will move towards the anode. A protein will continue to

migrate along the pH gradient until it reaches the pH where its net charge drops to zero at which point the protein is said to be 'focused'.

Proteins are then separated in the second dimension according to their molecular size by PAGE.

Two-dimensional electrophoresis was carried out using the PROTEAN® II xi cell (Bio-Rad).

2.11.1 First dimension

2.11.1.1 Casting of IEF tube gels

The composition of the IEF tube gel monomer was as follows:

5.5 g urea

1.5 ml 30% acrylamide/bisacrylamide stock solution

0.1 ml Bio-Lyte 5-7 ampholyte

0.4 ml Bio-Lyte 3-10 ampholyte

3 ml dH₂O

After the components had dissolved, 0.5 ml of a detergent solution (0.3 g CHAPS and 0.1 ml Nonidet P-40 dissolved in 0.9 ml dH₂O) was added and the total volume made up to 10 ml with dH₂O.

The monomer solution was then degassed thoroughly for 30 mins and polymerisation was induced by the addition of 10 µl of TEMED and 20 µl of 10% (w/v) APS. The monomer solution was dispensed into disposable glass test tubes and was drawn up into the capillary tubes (1.5 mm diameter) to a height of 13.5 cm by means of a syringe attached to the capillary tube by a short length of silicone tubing.

2.11.1.2 Sample preparation for IEF

SAM was dissolved in sample buffer (1% SDS, 0.232% dithiothreitol) to give a final protein concentration of 5 mg/ml. 100 µl of each SAM solution were boiled for 5 mins and allowed to cool. Once cool, 1/10 of the sample volume of iso-urea solution (0.1 g dithiothreitol, 0.2 g CHAPS, 5.4 g urea, 500 µl Bio-Lyte 3-10 ampholyte dissolved in 6.05 ml dH₂O) was added.

2.11.1.3 First dimension electrophoresis

40 μ l of each sample (i.e. 200 μ g of protein) was applied to the top of the tube gel using a Hamilton syringe. Samples were carefully overlaid with upper chamber running electrolyte (20 mM NaOH). The lower chamber electrolyte consisted of 10 mM H₃PO₄. IEF was carried out at 200 V constant voltage for 2 h, 500 V constant voltage for a further 2 h and finally 800 V constant voltage for 18 h. Once electrophoresis was complete, the tube gels were placed immediately at -20°C until required.

2.11.2 Second dimension electrophoresis

2.11.2.1 Casting of slab gels

The composition of the gels were as follows:

Lower resolving gel

13% acrylamide

0.4 M Tris-HCl, pH 8.8

Polymerisation was induced by the addition of 250 μ l of 10% APS (freshly prepared) and 25 μ l of TEMED per 100 ml of gel solution.

Upper stacking gel

4% acrylamide

0.125 M Tris-HCl, pH 6.8

0.1% SDS

Polymerisation was induced by the addition of 50 μ l of 10% APS and 10 μ l of TEMED per 10 ml of gel solution.

The electrode buffer consisted of 0.05 M Tris, 0.38 M glycine, 0.1% SDS, pH 8.3.

Tube gels were removed from -20°C and allowed to defrost. The tube gel was extruded onto a plastic sheet and 150 μ l of transfer buffer (0.07M Tris-HCl, pH 8.8, 2.9% SDS) was dispensed along the length of the gel. The tube gel was immediately applied to the top of the slab gel ensuring perfect contact between the two gels. Electrophoresis was carried out (35 mA constant current per gel, 3.5 h) until the dye front of the molecular weight markers had reached the bottom of the gel.

2.12 Staining of 1-D and 2-D gels

Gels were stained with Brilliant blue G - Colloidal (Sigma) or silver-stained using a commercially available kit (Pierce, Chester, UK).

2.13 Western immunoblotting

Following 1-D SDS-PAGE or 2-D electrophoresis, proteins were transferred on to Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Watford, UK) by electroblotting overnight in blotting buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol) at a constant voltage of 15 V using a TE Series Transphor Electrophoresis Unit (Hoefer Scientific Instruments) for the small 1-D gels or a Trans-Blot electrophoresis tank (Bio-Rad) for the large 2-D gels. Following electroblotting the membranes were washed in phosphate buffered saline (PBS) containing 1% Triton-X-100 for 5 mins and PBS containing 0.1% Triton-X-100 for 25 mins. The membranes were then transferred to blocking buffer (2% FCS or 5% skimmed milk powder in PBS-0.1% Triton-X-100) for 45 mins. The blocking buffer was removed and the primary antibody, diluted as appropriate in blocking buffer, was added and incubated at room temperature for 1 h. The membranes were then washed thoroughly in PBS-0.1% Triton-X-100 for 30 mins, changing the wash buffer at least 6 times. After washing, the secondary antibody, conjugated to horse-radish peroxidase (HRP) diluted 1:1000 in blocking buffer was added and incubated at room temperature for 1 h. The membranes were again washed and the peroxidase substrate 3,3'-diaminobenzidine tetrahydrochloride (0.67 mg/ml DAB in buffer containing 0.67 mg/ml urea hydrogen peroxide, Sigma FAST tablets) was added. The reaction was terminated by washing the membranes in dH₂O.

2.14 Determination of the molecular weight (MW) of proteins

The MW of proteins was determined by reference to a standard curve of the log of the MW of each protein standard plotted against its respective R_f value where R_f =

$$\frac{\text{distance between top of gel and leading edge of band or spot}}{\text{distance between top of gel and dye front}}$$

Chapter 3

Effect of methylene blue, a redox agent, on periodontopathogenic bacteria; *in vitro* and *in vivo* studies

3.1 Introduction

The characteristic lesion of periodontal disease is the periodontal pocket, formed by the detachment of the junctional epithelium from the tooth root surface. The microenvironment of the periodontal pocket is highly anaerobic, with a low oxygen tension (Mettraux *et al.*, 1983) and a low redox potential (E_h) (Kenney and Ash, 1969). Many anaerobic bacteria are highly sensitive to the presence of oxygen (Morris, 1980; Loesche, 1969) and are also dependent on a low E_h for growth (Gottschalk and Peinemann, 1992; Socransky *et al.*, 1964). The periodontal pocket therefore provides an ideal environment for these bacteria and a highly complex flora develops. A number of Gram-negative obligately anaerobic bacteria including *Por. gingivalis*, *Prev. intermedia*, *F. nucleatum* and various spirochaetes have been specifically implicated in the aetiology of chronic periodontitis (Socransky and Haffajee, 1992). The products of these organisms are thought to contribute to the tissue damage and bone loss characteristic of the disease (Holt and Bramanti, 1991). Treatment of periodontal disease involves the mechanical removal of subgingival plaque. Increasingly this procedure is supplemented by the application of antibiotics and antiseptics to the periodontal pocket (Slots and Rams, 1990). An alternative approach to controlling the microbial population would be to alter the environment to such an extent that organisms were no longer able to survive. Several studies have investigated the effect of exposing the anaerobic microflora of the periodontal pocket to molecular oxygen or oxygenating agents (Chasens, 1978). However, few have involved the addition of an oxidising agent to the periodontal pocket, which, without producing O_2 , would also cause an increase in E_h and hence oxidation of the pocket contents.

The E_h of the healthy gingival crevice is in the region of +74 mV (Kenney and Ash, 1969), whereas in the periodontal pocket E_h values as low as -300 mV have been recorded (Marsh and Martin, 1992). The E_h of the redox agent used in this study,

methylene blue (MB), when it is fully oxidised is +71 mV (Jacob, 1970). By adding MB to the periodontal pocket it may be possible to raise the E_h to a level which is incompatible with the survival of the anaerobic microflora.

3.2 Aims

The objectives of this study were to:

- i) determine whether MB, by altering the E_h , could affect the survival of the anaerobic periodontopathogens *P. gingivalis* and *F. nucleatum in vitro*
- ii) investigate whether the reducing agent dithiothreitol could negate the effect of MB
- iii) evaluate the use of MB in the treatment of chronic inflammatory periodontal disease

3.3 In vitro studies

3.3.1 Materials and methods

3.3.1.1 Effect of MB and dithiothreitol on the E_h of pre-reduced culture medium

Aliquots of sterile BM broth were incubated in an anaerobic jar for 24 h at 37°C. MB (The Boots Company Plc., Nottingham, UK) was added to give final concentrations ranging from 0.0 - 1.0 mg/ml. An equal volume of dH₂O was added in place of MB to controls. MB and dithiothreitol were added in combination to pre-reduced BM broth to give final concentrations of 1.0 mg/ml and 2.0 mg/ml respectively. Controls contained MB only, dithiothreitol only or neither. Samples were incubated in an anaerobic jar at 37°C and the E_h was determined at 0, 24 and 48 h using a combination redox cell with a platinum measuring electrode and a Ag/AgCl reference electrode (Russel pH Ltd., Auchtermuchty, UK) taking care to avoid agitation, which would have caused aeration of the sample. The pH of all samples was measured immediately after E_h determination.

3.3.1.2 Effect of MB on the survival of suspensions of *P. gingivalis* and *F. nucleatum*

Suspensions of *P. gingivalis* W50 and *F. nucleatum* NCTC 10562 were prepared in pre-reduced BM broth using bacteria harvested from 72 h WC agar plates. The suspensions were aliquoted into sterile test tubes and MB was added to give final

concentrations of 0.0 - 1.0 mg/ml. An equal volume of dH₂O was added in place of MB to controls. The samples were incubated in an anaerobic jar at 37°C. Viable counts and measurements of E_h and pH were carried out at 0, 24 and 48 h.

3.3.1.3 Effect of MB on the survival of suspensions of *P. gingivalis* and *F. nucleatum* in the presence of dithiothreitol

MB was added together with dithiothreitol to suspensions of *P. gingivalis* or *F. nucleatum* to give final concentrations of 1.0 mg/ml and 2.0 mg/ml respectively. Controls contained MB only, dithiothreitol only or neither. The samples were incubated in an anaerobic jar at 37°C, and viable counts and measurements of E_h and pH were carried out at 0, 24 and 48 h.

3.3.1.4 Effect of MB on the survival of 48 h broth cultures of *P. gingivalis* and *F. nucleatum*

Pre-reduced BM broth was inoculated with *P. gingivalis* or *F. nucleatum* and incubated anaerobically at 37°C for 48 h. Serial dilutions of this 48 h culture were prepared in pre-reduced BM broth and MB was added to give a final concentration of 1.0 mg/ml. An equal volume of dH₂O was added to controls in place of MB. Cultures were removed for viable counting and measurement of E_h and pH after 0, 24 and 48 h anaerobic incubation.

3.3.1.5 Oxidation of NADH by MB

100 µl of MB solution (prepared in dH₂O) was added to 900 µl of 0.2 mg/ml NADH (prepared in 0.05 M Tris-HCl, pH 7.0) in a cuvette to give final concentrations of MB of 0.0, 0.1, 1.0 and 10.0 µg/ml. Samples were incubated aerobically at 37°C and the absorbance at 340 nm was measured at t = 0, 15, 30, 45 and 60 mins against an appropriate blank.

The experiment was repeated under anaerobic conditions with MB at a final concentration of 10 µg/ml. All solutions were pre-reduced for 24 h beforehand and the experiment was carried out entirely in an anaerobic cabinet (Don Whitley Scientific, Shipley, UK). Cuvettes were carefully sealed using Blu-Tack before removing them from the anaerobic chamber for spectrophotometric measurements.

3.3.2 Results

3.3.2.1 Effect of MB and dithiothreitol on the E_h of pre-reduced culture medium

The E_h of pre-reduced BM broth was -288 ± 4 mV. While addition of water to pre-reduced broth raised the E_h to -207 ± 3 mV, addition of MB resulted in a greater increase of the E_h to -173 ± 7 mV, -159 ± 6 mV and -154 ± 3 mV for 0.01, 0.1 and 1.0 mg/ml MB respectively (Fig. 3.1). Following incubation in an anaerobic environment for 48 h, the E_h of broth containing 1.0 mg/ml MB was considerably higher (-171 ± 8 mV) than that of the dye-free control (-295 ± 7 mV), demonstrating the ability of MB to poise the E_h . MB at a concentration of 0.1 mg/ml also displayed a redox-poising capacity, although the effect was less than for 1.0 mg/ml MB.

MB had no significant effect on the pH of BM broth; at a concentration of 1.0 mg/ml, the pH was 6.59 ± 0.01 , whereas the pH of dye-free broth was 6.63 ± 0.09 . Following 48 h anaerobic incubation, the E_h of BM broth containing 2.0 mg/ml dithiothreitol was -386 ± 8 mV. The E_h of broth containing 1.0 mg/ml MB at this time was -154 ± 14 mV. However when MB at 1.0 mg/ml was added in combination with dithiothreitol at 2.0 mg/ml, the E_h of the broth after 48 h anaerobic incubation was similar (-349 ± 18 mV) to that of the overall controls (-306 ± 15 mV) containing neither MB or dithiothreitol (Fig. 3.2). Thus dithiothreitol was able to counteract the redox-poising ability of MB.

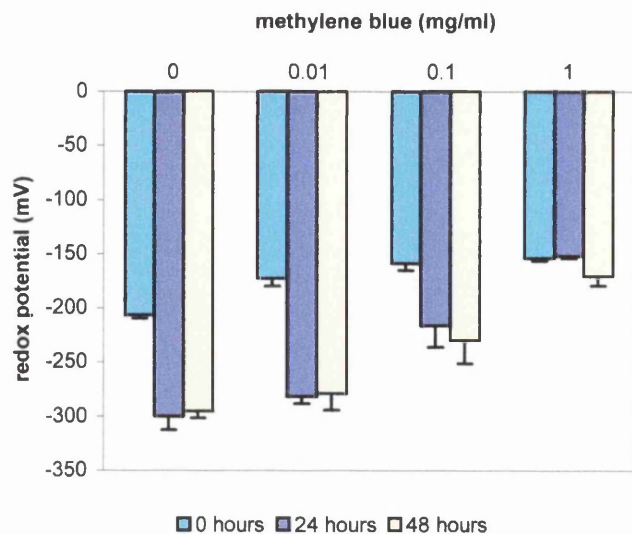


Figure 3.1 E_h of pre-reduced BM broth containing varying concentrations of MB following incubation in an anaerobic environment. The results are expressed as the mean \pm S.D. of 3 replicates.

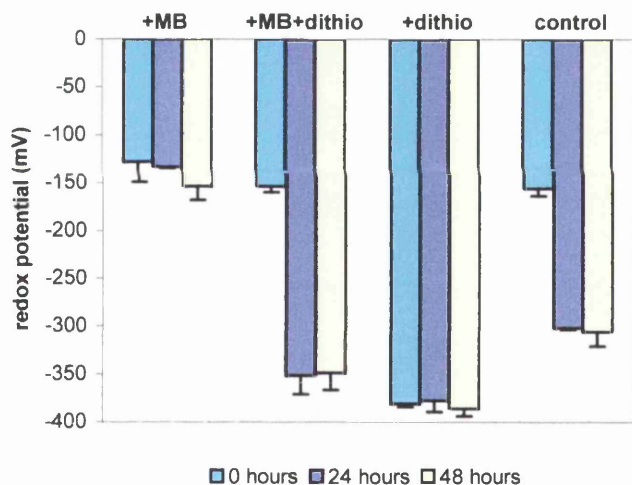


Figure 3.2 E_h of pre-reduced BM broth containing MB (1.0 mg/ml), dithiothreitol (2.0 mg/ml) or MB + dithiothreitol following incubation in an anaerobic environment. The results are expressed as the mean \pm S.D. of 3 replicates.

3.3.2.2 Effect of MB on the survival of suspensions of *P. gingivalis* and *F. nucleatum*

MB at a final concentration of 1.0 mg/ml poised the E_h of suspensions of *P. gingivalis* at a level which was approximately 200 mV higher than that of control dye-free suspensions. After 24 h anaerobic incubation, the E_h of suspensions containing 1.0 mg/ml MB was -150 mV compared to -359 mV for controls. This was accompanied by a reduction in the viable count of *P. gingivalis* from $28.75 \times 10^7 \pm 4.60 \times 10^7$ cfu/ml at $t = 0$ h to undetectable levels (< 500 cfu/ml) within 24 h (Fig. 3.3a). Lower concentrations of MB were unable to poise the E_h at a higher level and after 24 h anaerobic incubation the E_h of suspensions containing 0.01 and 0.1 mg/ml MB were -331 mV and -320 mV respectively. There were no significant differences in the viable counts of the *P. gingivalis* suspensions containing 0.01 and 0.1 mg/ml MB compared to the dye-free controls.

A similar pattern of results was obtained for suspensions of *F. nucleatum*. After 24 h anaerobic incubation the E_h of suspensions of *F. nucleatum* containing 1.0 mg/ml MB was -123 mV compared to -317 mV for the dye-free controls. A corresponding reduction in the viable count of the suspensions from $25.88 \times 10^6 \pm 1.25 \times 10^6$ cfu/ml at $t = 0$ h, to undetectable levels (< 500 cfu/ml) within 24 h was observed (Fig. 3.3b). MB at concentrations of 0.01 and 0.1 mg/ml was ineffective in reducing the viability of the *F. nucleatum* suspensions, and after 24 h anaerobic incubation the viable counts and E_h values were similar to that of the controls.

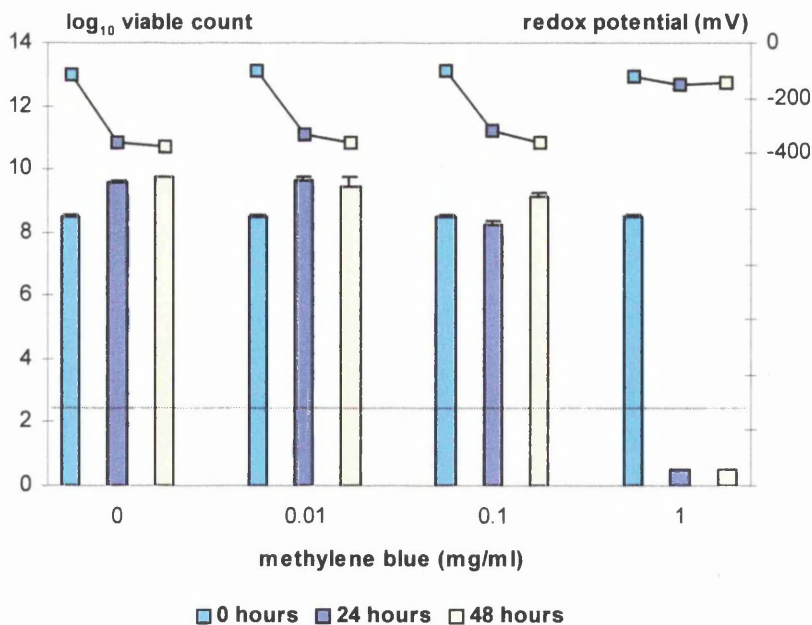


Figure 3.3a Effect of varying concentrations of MB on the survival of suspensions of *P. gingivalis* following incubation in an anaerobic environment. Bars represent the viable counts and boxes represent the E_h values. The horizontal dotted line indicates the lower limit of detection of the viable counting method used. The viable counts are expressed as the mean \pm S.D. of four replicates, while the E_h values are the mean of duplicate determinations.

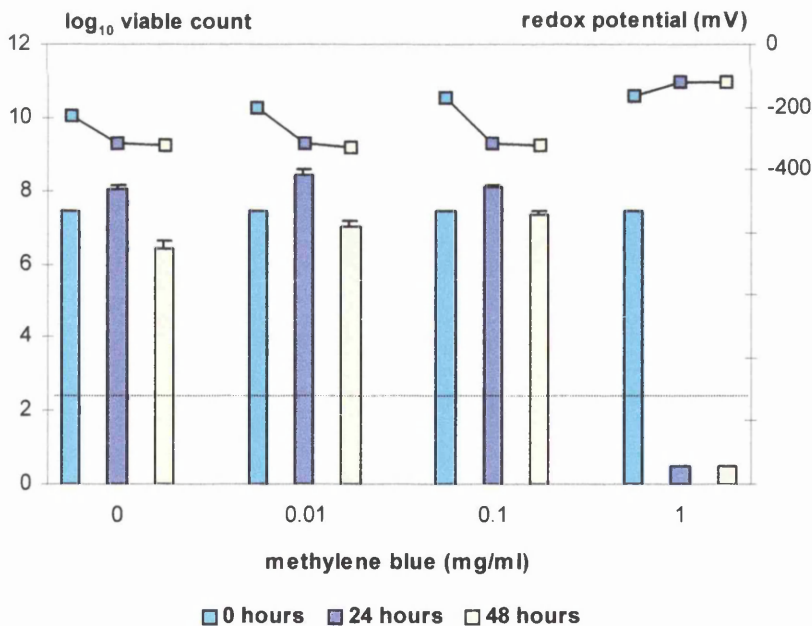


Figure 3.3b Effect of varying concentrations of MB on the survival of suspensions of *F. nucleatum*. Symbols are as in Fig. 3.3a. The viable counts are expressed as the mean \pm S.D. of four replicates, while the E_h values are the mean of duplicate determinations.

3.3.2.3 Effect of MB on the survival of suspensions of *P. gingivalis* and *F. nucleatum* in the presence of dithiothreitol

Dithiothreitol at a concentration of 2.0 mg/ml had no adverse effect on the viability of *P. gingivalis*. The viable count of suspensions containing dithiothreitol was $19.69 \times 10^8 \pm 2.37 \times 10^8$ after 24 h anaerobic incubation compared to the overall control which was $60.16 \times 10^8 \pm 2.81 \times 10^8$ at this time (Fig. 3.4a). Dithiothreitol caused an immediate decrease in the E_h of *P. gingivalis* suspensions to -367 mV at $t = 0$ h. As before, MB by itself at 1.0 mg/ml poised the E_h of *P. gingivalis* suspensions at a level approximately 200 mV higher than that of the overall controls, -142 mV compared to -348 mV. This was accompanied by complete killing of the *P. gingivalis* suspension which contained $47.50 \times 10^5 \pm 3.54 \times 10^5$ cfu/ml at $t = 0$ h. However, when MB and dithiothreitol were added in combination to suspensions of *P. gingivalis* to give final concentrations of 1.0 mg/ml and 2.0 mg/ml respectively, the E_h after 24 h anaerobic incubation had decreased to -327 mV which was similar to that of the overall control at this time (-348 mV). In the presence of dithiothreitol, MB at 1.0 mg/ml had no adverse effect on the survival of *P. gingivalis*, and indeed the organism was able to multiply.

Similar results were obtained for *F. nucleatum* (Fig. 3.4b) Dithiothreitol at 2.0 mg/ml counteracted the E_h poisoning effect of 1.0 mg/ml MB and the viable count and E_h values for these suspensions were similar ($10.25 \times 10^8 \pm 7.12 \times 10^8$, -328 mV) after 24 h anaerobic incubation to that of the overall controls ($33.10 \times 10^8 \pm 3.70 \times 10^8$, -344 mV).

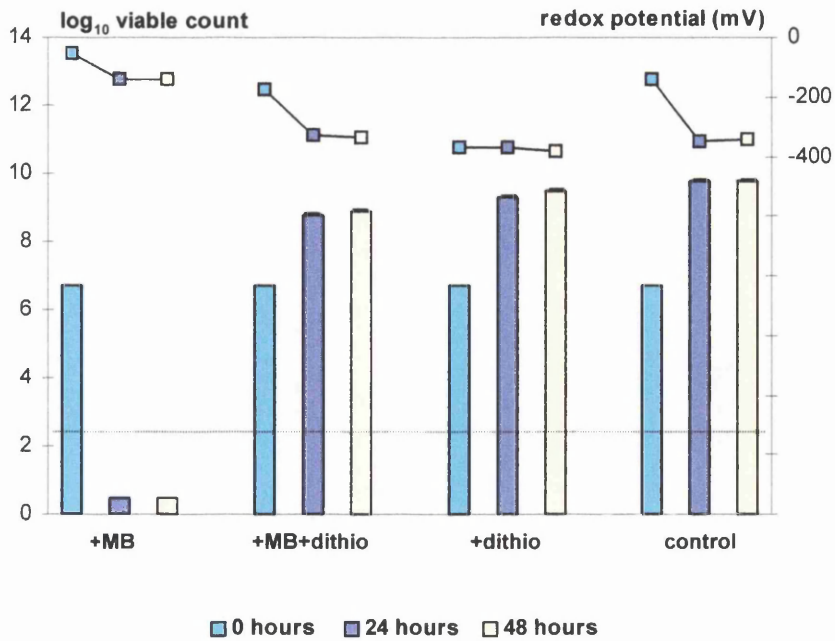


Figure 3.4a Effect of MB on the survival of *P. gingivalis* in the presence of dithiothreitol. Symbols are as in Fig 3.3a. The viable counts are expressed as the mean \pm S.D. of four replicates, while the E_h values are the mean of duplicate determinations.

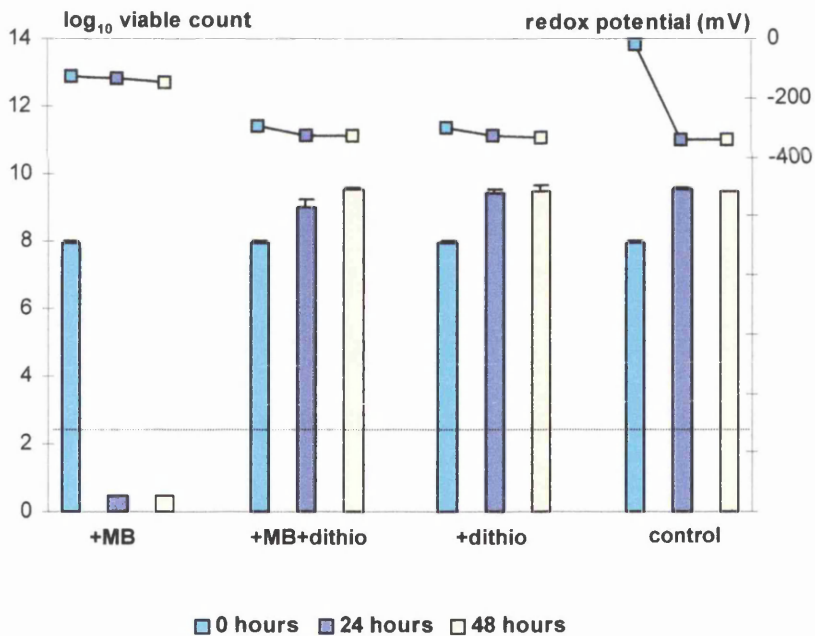


Figure 3.4b Effect of MB on the survival of *F. nucleatum* in the presence of dithiothreitol. Symbols are as in Fig 3.3a. The viable counts are expressed as the mean \pm S.D. of four replicates, while the E_h values are the mean of duplicate determinations.

3.3.2.4 Effect of MB on the survival of 48 h broth cultures of *P. gingivalis* and *F. nucleatum*

Fig. 3.5a shows the effect of MB at 1.0 mg/ml on serial dilutions of a 48 h broth culture of *P. gingivalis*. At this concentration MB had no significant effect on the viability of the undiluted culture of the organism, and the viable count and E_h values of MB containing cultures at $t = 48$ h were similar ($18.80 \times 10^7 \pm 2.82 \times 10^7$, -339 mV) to that of the dye-free controls ($39.35 \times 10^7 \pm 6.25 \times 10^7$, -363 mV).

However, when the culture was diluted 1/10 and 1/100, MB at 1.0 mg/ml caused a reduction in the viable count from $7.88 \times 10^8 \pm 2.14 \times 10^8$ and $7.88 \times 10^7 \pm 2.14 \times 10^7$ for the 1/10 dilution and the 1/100 dilution respectively, to undetectable levels within 48 h. The viable counts for the corresponding controls were $28.43 \times 10^7 \pm 4.90 \times 10^7$ and $88.83 \times 10^7 \pm 2.94 \times 10^7$. MB at 1.0 mg/ml was able to poise the E_h of the 1/10 and the 1/100 dilutions of the culture at a level significantly higher (-146 mV and -134 mV respectively) than that of the corresponding dye-free controls (-357 mV and -286 mV).

The effect of MB at 1.0 mg/ml on serial dilutions of a 48 h broth culture of *F. nucleatum* is shown in Fig. 3.5b. At this concentration MB was only effective against a 1/100 dilution of the culture. MB caused a reduction in the viable count of the 1/100 dilution from $19.4 \times 10^6 \pm 4.4 \times 10^6$ to undetectable levels after 48 h anaerobic incubation. The E_h of the 1/100 dilution containing MB after 48 h anaerobic incubation was -127 mV compared to -299 mV for the dye-free control. MB at 1.0 mg/ml was not effective against an undiluted culture of *F. nucleatum* and a 1/10 dilution of this culture. After 48 h anaerobic incubation, viable counts of the cultures were similar to that of the respective dye-free controls. The E_h values of these cultures after 48 h anaerobic incubation were also similar, -322 mV for the undiluted culture plus MB and -314 mV for the 1/10 dilution plus MB, compared to -296 mV and -286 mV for the respective controls. In these cultures MB was completely reduced. For the neat, undiluted culture, reduction of the dye occurred very rapidly and the E_h values at $t = 0$ h were the same as those of the controls.

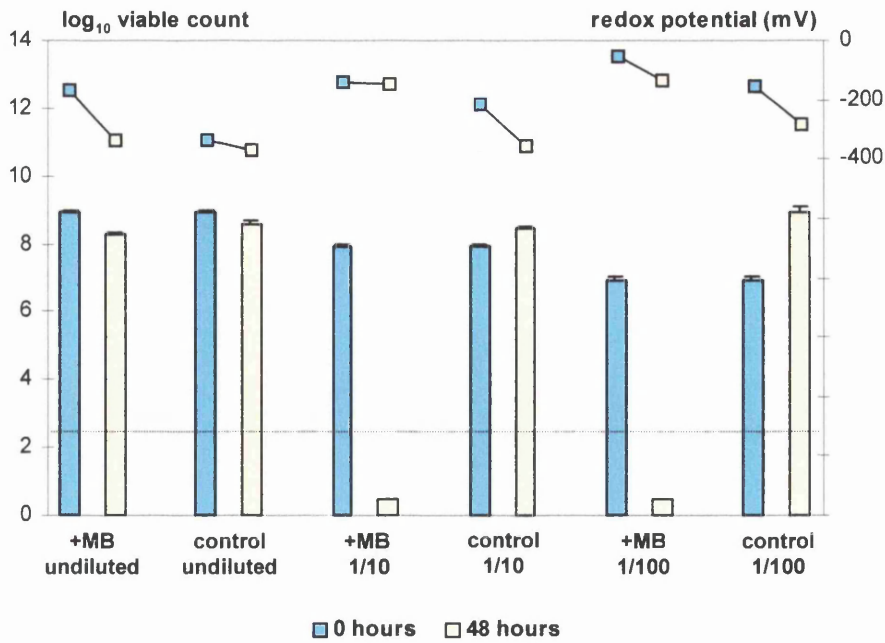


Figure 3.5a Effect of MB (1.0 mg/ml) on the survival of serial dilutions of a 48 h *P. gingivalis* culture. Symbols are as in Fig 3.3a. The viable counts are expressed as the mean \pm S.D. of four replicates, while the E_h values are the mean of duplicate determinations.

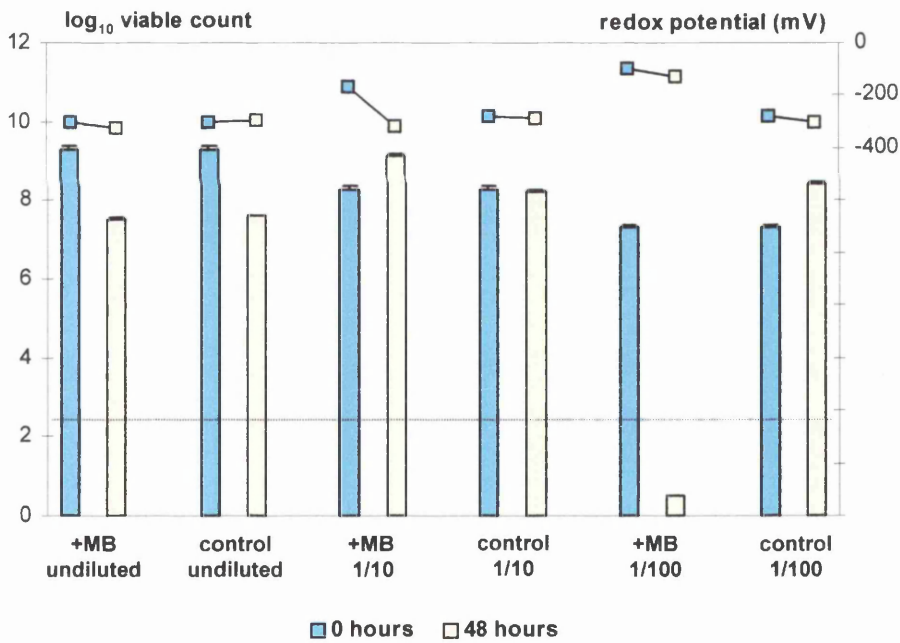


Figure 3.5b Effect of MB (1.0 mg/ml) on the survival of serial dilutions of a 48 h *F. nucleatum* culture. Symbols are as in Fig 3.3a. The viable counts are expressed as the mean \pm S.D. of four replicates, while the E_h values are the mean of duplicate determinations.

3.3.2.5 Oxidation of NADH by MB

In an aerobic environment, MB at 10 µg/ml caused oxidation of NADH, and a decrease in the absorbance at 340 nm with time was observed (Fig. 3.6a). Under anaerobic conditions, MB at 10 µg/ml caused no detectable oxidation of NADH (Fig. 3.6b). MB itself was rapidly reduced and samples were colourless within 15 mins of addition of the dye. Scanning spectrophotometry revealed that at the end of the incubation period, whilst a large peak at 663 nm (absorption maximum for MB) could be observed in the aerobically incubated samples, the corresponding peak in the anaerobically incubated samples was absent (data not shown).

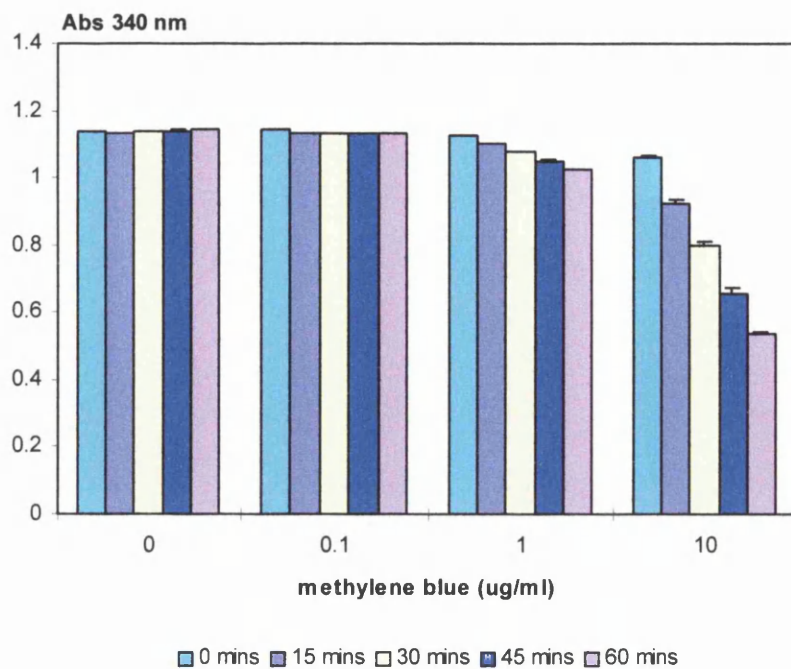


Figure 3.6a Oxidation of NADH by varying concentrations of MB under aerobic conditions. Absorbance values are expressed as the mean \pm S.D. of four replicates.

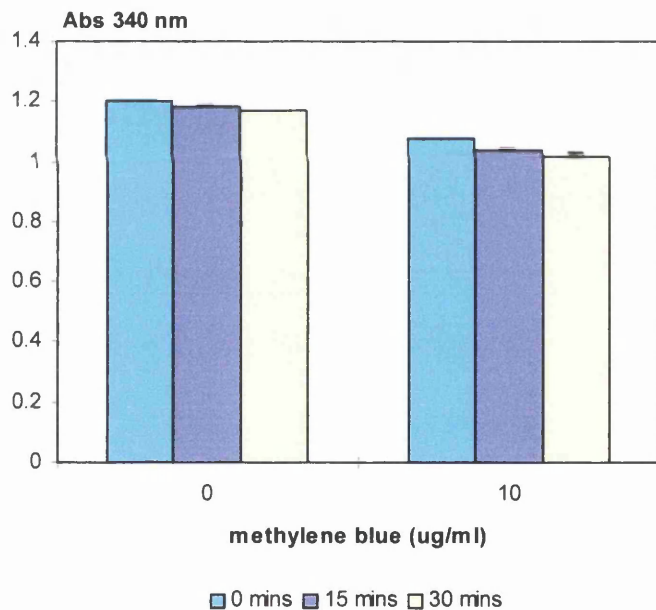


Figure 3.6b Oxidation of NADH by varying concentrations of MB under anaerobic conditions. Absorbance values are expressed as the mean \pm S.D. of four replicates.

3.4 *In vivo* studies - evaluation of the efficacy of MB in the treatment of chronic periodontitis

3.4.1 Materials and methods

A 3-month randomised single blind clinical trial was undertaken, using a quadrant split-mouth design in order to determine the efficacy of MB in the treatment of chronic periodontitis compared to traditional methods of subgingival root surface debridement.

3.4.1.1 Personnel involved in the clinical trial

M. Gibson - clinician

D. Mangat - clinician

G. Gagliano - darkground microscopy

J. Bulman - statistical analysis

J. Fletcher - microbiological monitoring

3.4.1.2 Patient selection

24 adult patients, 10 male and 14 female, aged 35 - 55 years (mean 42.7 ± 6.4) were admitted to the trial. Selection criteria were as follows:

1. The presence of at least one true interdental periodontal pocket ≥ 6 mm, with radiographic evidence of bone loss, in each of the 4 quadrants.
2. No history of any periodontal therapy in the preceding 6 months.
3. No use of antibiotics or oral antiseptics during the previous 6 months.
4. No systemic condition which may influence the course of the disease.
5. Ability to attend at regular intervals.

3.4.1.3 Treatment modalities

Following baseline recordings of clinical and microbiological parameters, one of four treatments was randomly assigned to each quadrant.

1. Irrigation with sterile water.
2. Irrigation with sterile 1% MB (Macarthy Medical, Romford, UK).
3. 33% (w/w) MB incorporated in a biocompatible and biodegradable collagen alginate vicryl composite controlled-release carrier (Johnson and Johnson Medical Biopolymers Ltd., Stirling, UK).

4. Subgingival debridement, under local anaesthesia, using a combination of ultrasonic (Cavitron) and conventional hand instrumentation.

Initial irrigation was carried out professionally, and subsequently on a daily basis by the patient, using a 2.5 ml syringe fitted with a 0.5 mm tip diameter blunt ended needle (Luer-Lock Hub, Stram Dental Inc., Illinois, USA) for a period of 4 weeks. The needle was pre-bent, the tip placed subgingivally and fluid injected to fill the pocket until just visible at the gingival margin. The sustained-release device which consisted of pre-sterilised strips, 10 mm x 2 mm, was packed into the pocket using a pushscaler until just visible at the gingival margin.

3.4.1.4 Efficacy variables

The following clinical and microbiological variables were measured on day 0 (baseline) and then at 1, 4, 8 and 12 weeks following treatment:

1. Plaque index (Silness and Løe, 1964).
2. Gingival index (Løe and Silness, 1963).
3. Probeable pocket depth.
4. Bleeding on probing using the criteria of the Papilla Bleeding Index (Saxer and Mühlemann, 1975, cited by Rateitschak *et al.*, 1985).
5. Bacteriological sampling.

3.4.1.5 Bacteriological sampling

Supragingival plaque was removed and 2 sterile endodontic paper points were introduced into each pocket until they had reached an apical limit. The paper points were left *in situ* for 15 secs and then transferred immediately to pre-reduced sterile saline. The sample was vortexed for 1 min and serial 10-fold dilutions were prepared in pre-reduced brain heart infusion broth (Oxoid). Duplicate aliquots of 20 µl of each dilution were spread over the surface of WC agar plates containing 10% (v/v) horse blood. One set of plates was incubated anaerobically at 37°C in order to determine the total number of obligate and facultative anaerobes (total anaerobic count). An additional set of WC plates was incubated in a CO₂-enriched atmosphere (generated by placing a lit candle inside an air-tight container) at 37°C in order to determine the total number of aerobes and facultative anaerobes (total

aerobic count). Plates were incubated for up to 14 days and the colonies were counted after 7 and 14 days using a low-power binocular microscope. Black-pigmented colonies obtained on plates incubated anaerobically were also counted and the colonies were then subcultured and tested for their ability to grow under aerobic conditions. Those that did not grow under aerobic conditions were designated black-pigmented anaerobes (bpa).

3.4.1.6 Statistical analysis

Aerobe/anaerobe log ratios were analysed using a one-way ANOVA supported, where appropriate by the Students *t*-test. The percentage bpa was analysed using the Kruskal-Wallis one-way ANOVA by rank for between-group comparisons and the Friedman two-way ANOVA by ranks for within-group comparisons.

3.4.2 Results

3.4.2.1 Effect of MB on the subgingival microflora of patients with chronic periodontitis

The baseline aerobe/anaerobe ratio of the 4 treatment groups ranged from 0.10 to 0.15 (Fig. 3.7). Sites subjected to subgingival debridement showed a significant increase in the aerobe/anaerobe ratio after 1 week and after 4 weeks the ratio had increased to more than 3 times the baseline value (0.37 compared to 0.11). Thereafter the ratio decreased, although the proportion of aerobic organisms at week 12 was still higher than at baseline.

Sites which received MB in the slow release form showed the greatest increase in the aerobe/anaerobe ratio. After 4 weeks the ratio had increased approximately 4-fold from 0.12 (baseline) to 0.41.

Sites irrigated with MB also showed a marked increase in the aerobe/anaerobe ratio and at week 4 the ratio was 3 times greater than the baseline level (0.33 compared to 0.11). After the cessation of MB irrigation (week 4), the ratio decreased, although at week 12 the ratio was still considerably higher (0.27) than at baseline.

The proportion of bpa at baseline ranged from 7 - 22% for the 4 treatment groups (Fig. 3.8). Sites subjected to subgingival debridement or water irrigation showed no significant decrease in the proportion of bpa during the course of the trial. In contrast, sites which received MB irrigation showed a significant reduction after 4 and 8 weeks. The proportion of bpa remained at a low level following cessation of treatment (week 4) and even after 12 weeks the %bpa was much lower than at baseline.

Sites receiving MB in the slow-release form showed a progressive decrease in the %bpa and by week 8 the difference was significantly lower than at baseline.

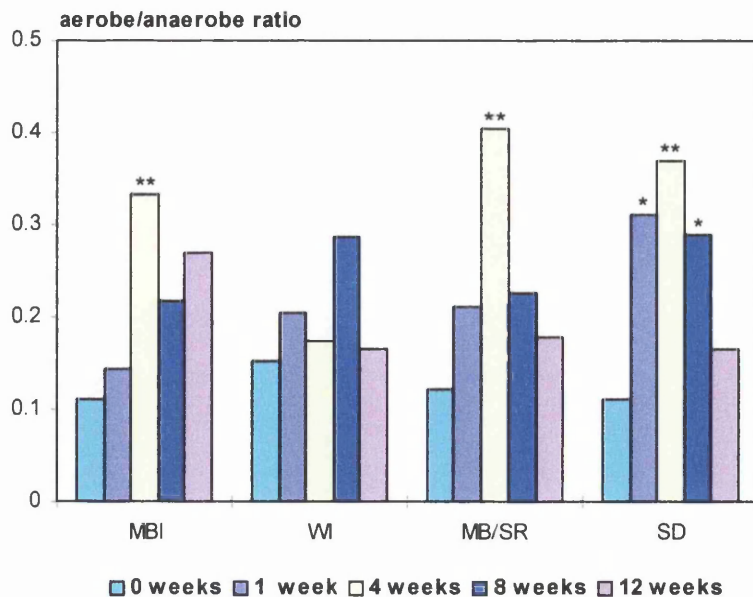


Figure 3.7 Effect of treatment modality on the relative proportions of bacteria in subgingival plaque samples able to grow aerobically and anaerobically. WI: sites irrigated with sterile distilled water; MBI: sites irrigated with a 1% aqueous solution of MB; MB/SR: sites receiving a MB slow release device; SD: sites subjected to subgingival debridement.
 * significantly different from baseline ($p < 0.05$)
 ** significantly different from baseline ($p < 0.01$)

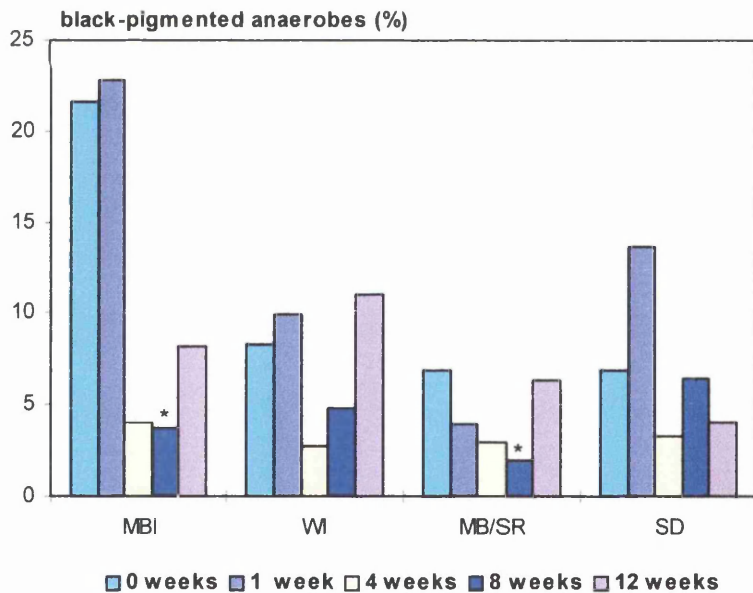


Figure 3.8 Effect of treatment modality on the proportions of black pigmented anaerobes (expressed as a percentage of the total anaerobic count) cultured from subgingival plaque samples.
 * significantly different from baseline ($p < 0.05$)

3.5 Discussion

It has long been known that both a low concentration of O₂ and a low E_h (Hungate, 1969; Aranki *et al.*, 1969) are important for the isolation and cultivation of strictly anaerobic bacteria. The presence of O₂, will by its very nature, lead to an increase in E_h. The question remains as to whether E_h, in the absence of O₂, has any effect on the growth of anaerobic bacteria. Socransky *et al.* (1964) showed that even in an O₂-free environment *Treponema microdentium* could only grow in a narrow E_h range of between -185 and -220 mV. Other workers suggest that it is the presence of O₂, causing an increased E_h, which is inhibitory to the growth of anaerobic bacteria. Walden and Hentges (1975) demonstrated growth of *Clostridium perfringens* under anaerobic conditions with an E_h of +325 mV maintained by the addition of potassium ferricyanide, whilst no growth occurred in aerated cultures with a low E_h suggesting that O₂ was the detrimental factor. Hanke and Katz (1943) however showed that it was possible to grow certain anaerobic bacteria in a continuous current of air if the E_h was maintained at a sufficiently low level. In these experiments the E_h was maintained electrolytically without the addition of any chemical agents.

In vivo it is likely that there will be complex interactions between different bacterial species. Bradshaw *et al.* (1996) demonstrated the survival and growth of strictly anaerobic species in aerated mixed planktonic cultures. In the presence of the aerobic species *Neisseria subflava*, the dissolved O₂ tension (dO₂) of the mixed culture was <5% of air saturation despite continuous aeration, and the E_h was -275 mV. In the absence of *N. subflava*, however, the dO₂ was 50 - 60% of air saturation and the E_h was +50 mV. Anaerobic species were able to survive and multiply even under these relatively aerobic conditions and it was suggested that clumps of bacteria resulting from specific coaggregations between different species (as proposed by Kolenbrander and London, 1993) afforded protection to anaerobic species against the toxic effects of O₂ and a high E_h. In a more recent study Bradshaw *et al.* (1998) showed that *F. nucleatum* was able to coaggregate both oxygen-tolerant and other obligately anaerobic species and moreover, was able to act as a bridge between otherwise non-coaggregating oxygen-tolerant-obligate anaerobe pairs to form three-species aggregates. When *F. nucleatum* was omitted from aerated mixed planktonic cultures, significant reductions in the numbers of black-pigmented anaerobes (*Por. gingivalis* and *Prev. intermedia*)

were observed, suggesting that the formation of metabolically organised aggregates is essential for the survival of obligately anaerobic organisms in an aerated environment.

Hungate (1969) stated that whilst agents such as potassium ferricyanide can be used to induce a high E_h independent of oxygen, and may interact with an E_h measuring electrode, this does not necessarily give an indication of whether the agent is capable of affecting the intracellular environment and hence cell metabolism. O'Brien *et al.* (1970) showed that another agent *N,N,N',N'*-tetramethylazofornamide (diamide) was capable of oxidizing flavin nucleotides (FMNH₂ and FADH₂) and also NADH and NADPH. If such an oxidizing agent were able to gain access to a bacterial cell it would be likely to exert a detrimental effect on biosynthetic pathways and energy generation.

The results of this study have shown that addition of MB to pre-reduced culture medium to give a final concentration of 1.0 mg/ml poised the E_h at a level some 170 mV higher than that of controls to which distilled water had been added in place of MB. This elevated E_h was maintained for at least 48 hours in the oxygen-free, highly reducing atmosphere of an anaerobic jar. When MB at a final concentration of 1.0 mg/ml was added to suspensions of *P. gingivalis* or *F. nucleatum*, the E_h was poised at a level higher than that of the controls and this was associated with complete killing of the organism. Addition of the reducing agent dithiothreitol to a suspension of *P. gingivalis* or *F. nucleatum* containing MB at 1.0 mg/ml enabled survival of the organism. This, together with the redox data, suggests that killing of *P. gingivalis* and *F. nucleatum* by MB is due to some redox-related mechanism.

Redox-modifying agents have a buffering capacity which is concentration-dependent (Jacob, 1970). Thus at 1.0 mg/ml MB was able to poise the E_h of suspensions of *P. gingivalis* and *F. nucleatum* at a higher level compared to controls. Lower concentrations of MB were unable to poise the E_h of *P. gingivalis* or *F. nucleatum* suspensions in the highly reducing conditions of an anaerobic atmosphere and the E_h decreased to control levels and the organism survived. In addition, the ability of a redox agent to poise the E_h will depend on the capacity of a system to counteract any change in E_h . Suspensions of *P. gingivalis* and *F. nucleatum* containing dithiothreitol had a large reducing capacity which was able to counteract the effect of the MB. Similarly, 48 h broth cultures of *P. gingivalis*

and *F. nucleatum* were unaffected by high concentrations of MB. This was most likely to have been due to a high reducing capacity as a result of the accumulation of large amounts of reduced metabolites during the 48 h growth period.

The current investigation has shown that under aerobic conditions, MB at 10 µg/ml was able to oxidise significant amounts of NADH. MB, in its oxidised form was continually regenerated due to the presence of oxygen. Under anaerobic conditions MB was rapidly consumed and converted to its reduced, colourless form. This would have resulted, presumably, in a concomitant oxidation of NADH, although the level of oxidation of NADH under anaerobic conditions was too low to detect spectrophotometrically. It was not possible to use MB concentrations higher than 10 µg/ml due to the interference of the intense blue colour of MB with the absorbance at 340 nm. However, if an excess of MB was added to NADH under anaerobic conditions (e.g. 1.0 mg/ml, the concentration required to kill suspensions of *P. gingivalis* and *F. nucleatum*) it might be expected that 100 times more NADH would have been oxidised. It is known that MB is taken up by viable bacterial cells (Tuite and Kelly, 1993). Thus MB may be acting intracellularly by consuming reducing power in the form of NADH (and NADPH) which is required for energy generation and biosynthesis. MB may also cause oxidation of components of electron transport chains.

Another possibility is that MB may exert its effect by oxidising enzymes which are only active in the reduced form. Sakurai *et al.* (1980) showed that the beta toxin from *C. perfringens* could be inactivated by oxidising agents and that activity could be restored by treatment with dithiothreitol. Toluidine blue O (a molecule which is structurally related to MB and which shares a similar E_h) has been shown to decrease the proteolytic activity of *P. gingivalis* (M. Bhatti, personal communication).

The results of this investigation have shown that MB poised the E_h of suspensions of *P. gingivalis* and *F. nucleatum* at a higher level compared to controls in a highly anaerobic environment and this was associated with the death of the organism. However, MB at 1.0 mg/ml had a limited E_h buffering capacity and was unable to poise the E_h of suspensions of *P. gingivalis* or *F. nucleatum* containing dithiothreitol and 48 h broth cultures of these organisms due to the large reducing capacity of these systems. The contents of the periodontal pocket would also have a large reducing capacity due to the accumulation of reduced bacterial

metabolites. It would therefore be necessary to add an excess of MB to the periodontal pocket to overcome this reducing capacity.

MB was the redox agent of choice in the current study because of its low toxicity for humans (it is used in the treatment of methaemoglobinaemia and urolithiasis) and because promising results had been obtained in a small-scale, short-term pilot study (Wilson *et al.*, 1992). This preliminary study demonstrated significant reductions in the proportion of anaerobes, Gram-negative anaerobes, spirochaetes and motile organisms, and corresponding increases in the proportions of facultative anaerobes and cocci at test sites (receiving MB irrigation) compared to control sites (receiving water irrigation). In addition the reduction in GCF flow was significantly greater at test sites compared to control sites. Although reductions in pocket depth and bleeding on probing were observed, differences between the two groups were not significant.

Similar results were observed for the current study. That is, whilst sites receiving MB (MBI and MB/SR) showed a greater shift towards a microflora associated with gingival health compared to sites receiving WI and SD, improvements in clinical indices, although significant, were not generally significantly different between treatment types.

At baseline, only a small proportion of subjects had gingival index scores of 0 or 1 (20 - 30%). This increased during the first 4 weeks to 45 - 60% with a decrease at week 12, although gingivitis scores were still relatively improved, compared to baseline, independent of treatment type. There was an overall decrease in mean probing depth at all sites and again this was independent of treatment type. Approximately 40% of all sites scored 0, 1 or 2 for bleeding at baseline implying that the majority of sites were markedly inflamed initially. By the end of the trial 50 - 70% of sites had lower bleeding scores. Differences between treatments were not statistically different.

There are a number of reasons why statistically significant differences between treatment groups were not observed for clinical measurements. It might be expected that there would be a delay between microbiological changes and clinical changes and as the trial was relatively short in duration, only early changes in clinical indices may have been detected. That the sustained-release device did not yield the beneficial clinical results expected on the basis of the microbiological data may have been due to a number of factors. The device may

have interfered with measurements of pocket depth and in addition, insertion of the device may have caused some gingival trauma. Despite attempts to match pockets at the outset, the MBI group had abnormally high initial numbers of bpa. Thus improvements in clinical indices may have been harder to achieve in this group compared to the other 3 groups.

The study showed improvements in all treatment groups including irrigation with sterile water (albeit to a lesser extent than treatment with MB or subgingival debridement). This is in accordance with other studies which have shown that irrigation itself may have some benefit (Aziz-Ghandour *et al.*, 1986; Sanders *et al.*, 1986; Vignarajah *et al.*, 1989; Schlagenhaut *et al.*, 1987, 1990). It is likely that the hydrodynamics of irrigation in combination with the syringe tip being inserted into the subgingival plaque will have a detrimental effect on the plaque.

Treatment of periodontal disease with MB would appear to have a number of advantages over subgingival debridement. At the end of the clinical trial a survey was carried out to assess adverse effects of each treatment modality. Eight patients receiving subgingival debridement reported hypersensitivity and pain. Hypersensitivity and pain were not reported by any of the patients receiving MBI, MB/SR or WI, although a small number of patients in these treatment groups did report some discomfort and tenderness. Another advantage of MB therapy is the time taken to administer the treatment. In this study the time taken for operator irrigation with MB, including isolation of the site with cotton wool rolls, did not exceed 1 min per tooth, whilst placement of the controlled release material took 2 - 3.5 mins depending on pocket depth and volume. This compares favourably with the time taken for adequate subgingival debridement which varied between 4 - 8 mins per tooth.

These results clearly demonstrate that, in terms of altering the microflora to one more compatible with periodontal health, treatment with MB (both as a subgingival irrigant and in the form of a slow-release device) is comparable if not better to the currently standard treatment of subgingival debridement. However, if MB were used as an adjunct, as opposed to an alternative to subgingival debridement, even greater improvements may be observed. In a more recent study by Ower *et al.* (1995), MB was used in combination with subgingival debridement. All experimental sites received subgingival debridement at day 0. Test sites received 32% MB (w/w) incorporated in a slow release device at days 0 and 28. Clinical

improvements were observed in both groups, although test sites showed consistently greater improvements, some of which were statistically significant. Significant between-group differences in relation to baseline levels were seen in bleeding index at days 7 and 56, in probeable pocket depth at day 56 and for the Perioscan BANA test (a positive result in which has been shown to correlate with the presence of one or more of the periodontopathogenic bacteria *P. gingivalis*, *T. denticola* and *B. forsythus*) at day 7. MB, when used as an adjunct to subgingival debridement, combines the advantages of removal from the root surface of plaque-retaining factors and factors which inhibit the regeneration of the periodontal ligament, with the advantages of an effective antimicrobial agent. Antibiotics and antiseptics are increasingly being used in the treatment of periodontal disease (Gordon and Walker, 1993; Smith *et al.*, 1994; Needleman *et al.*, 1995; Seymour and Heasman, 1995). Whilst many of the studies show promising results, there are disadvantages such as the emergence of resistant strains (Lacroix and Walker, 1995; Listgarten *et al.*, 1993) and side-effects (Gordon and Walker, 1993; Slots and Rams, 1990) which argues against the long-term use of such agents. The results of this study have shown that MB can produce equivalent clinical and bacteriological improvements. One major advantage of such environment-modifying agents is that resistance development in the target organism would be extremely unlikely as this would have to involve fundamental changes in the biochemistry and physiology of anaerobic bacteria. MB was chosen as the redox-modifying agent for this study because of its known safety for use in humans. However the E_h of MB in its fully oxidised state is only +71 mV. Agents with a higher E_h might be expected to exert a greater redox-modifying effect and work is ongoing at the Eastman Dental Institute to develop more potent redox-modifying agents for use in the treatment of periodontal disease.

Chapter 4

Effect of environmental factors on the phosphatase activity of *A. actinomycetemcomitans*

4.1 Introduction

Acid (AcP) and alkaline (AIP) phosphatases are a group of enzymes which are responsible for the hydrolysis of phosphate esters. Bacteria are relatively impermeable to phosphate esters that are not actively transported and substrates must be dephosphorylated before they can be taken up and metabolised by the cell. AcP and AIPases have been demonstrated in dental plaque (Lo Storto *et al.*, 1992; Bercy and Vreven, 1979; Friskopp and Hammarström, 1982) and in GCF (Frank and Cimasoni, 1972; Sueda and Cimasoni, 1968). A proportion of the phosphatase activity in GCF is of bacterial origin and cytohistochemical studies of dental plaque have demonstrated intramicrobial AcP and AIP activity.

Many oral bacteria including *Actinomyces* spp. (Howell and Fitzgerald, 1953) *Streptococcus mutans* (Greenman and Melville, 1978), *Capnocytophaga* spp. (Poirier and Holt, 1983a, b, c), *T. denticola* (Yotis *et al.*, 1993; Norton Hughes and Yotis, 1990) *A. actinomycetemcomitans* (Slots, 1982; Yotis, 1988) and *Bacteroides* spp. (Slots, 1981; Laughon *et al.*, 1982) have been shown to produce AcP and AIPases. In addition to a role in nutrition, AcP and AIPases may have a potential role in the pathogenesis of periodontal disease by dephosphorylating tissue phosphoproteins (Poirier and Holt, 1983b) and by indirectly contributing to plaque mineralisation (Bercy and Vreven, 1979).

4.2 Aim

The aim of this study was to determine the effect of growth medium and incubation atmosphere on the production of AcP and AIP by *A. actinomycetemcomitans*.

4.3 Materials and methods

4.3.1 Effect of growth medium on AcP and AIP production by *A. actinomycetemcomitans*

Three growth media were tested;

- i) a semi-defined medium (detailed in Ch 2), to represent nutrient-limited conditions
- ii) WC broth, to represent nutrient-rich conditions
- iii) heat-inactivated (56°C, 30 mins) foetal calf serum (FCS, Sigma), to mimic GCF

Media were inoculated with a dense, washed suspension of *A. actinomycetemcomitans* NCTC 9710 and cultures were incubated anaerobically at 37°C. Samples were removed at the early stationary phase of growth (24 - 26 h) and assayed for AcP and AIP activity using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate. The reaction mixture consisted of 0.45 ml 8 mM *p*-NPP and 0.45 ml of 0.2 M sodium acetate, pH 4.8 or 0.2 M glycine NaOH, pH 9.4. All reagents were prepared in saline containing 0.01% (w/v) cysteine-HCl (Yotis, 1988). To start the reaction 0.3 ml of whole culture was added and the samples were incubated anaerobically at 37°C for 3 h. Controls contained uninoculated media. The reaction was stopped by the addition of 0.3 ml of 2.5 M NaOH. Samples were centrifuged for 10 mins to pellet the cells and the absorbance at 420 nm of the supernatant was measured against an appropriate blank. The amount of *p*-nitrophenol (*p*-NP) liberated was determined by reference to a standard curve. A known volume of the whole culture was centrifuged and the pellet resuspended in dH₂O. The total protein content of the suspension was determined by the method of Lowry *et al.* (1951) adapted for the assay of whole microorganisms as described in Ch 2.

4.3.2 Effect of incubation atmosphere on AcP and AIP production by *A. actinomycetemcomitans*

Semi-defined medium was inoculated with *A. actinomycetemcomitans*. Cultures were incubated either anaerobically or in a CO₂-enriched aerobic atmosphere generated by placing a lit candle in with the cultures in a sealed jar. Samples were removed at the early stationary phase of growth (24 - 26 h) and assayed for AcP and AIP activity as described above. Reaction mixtures were incubated either anaerobically or in a CO₂-enriched aerobic atmosphere, according to the original growth conditions of the culture.

4.3.3 Effect of incubation atmosphere on the activity of *A. actinomycetemcomitans* and *E. coli* AcP

Samples removed from *A. actinomycetemcomitans* cultures grown in a CO₂-enriched aerobic atmosphere and purified AcP from *E. coli* (Sigma) were assayed for AcP activity under both CO₂-enriched aerobic conditions and anaerobic conditions. Purified *E. coli* AcP was dissolved in saline containing 0.01% (w/v) cysteine-HCl and was added to the reaction mixture to give a final concentration of 2.5 µg AcP/ ml.

4.4 Results

4.4.1 Effect of growth medium on AcP and AIP production by *A. actinomycetemcomitans*

Fig. 4.1 shows the AcP and AIP activities of *A. actinomycetemcomitans* grown in three different media incubated anaerobically. *A. actinomycetemcomitans* grown in FCS had the highest AcP activity, 100.6 ± 4.8 units compared to 82.3 ± 2.6 units for *A. actinomycetemcomitans* grown in WC broth. *A. actinomycetemcomitans* grown in the semi-defined medium demonstrated the lowest AcP activity, 70.7 ± 8.7 units. The highest level of AIP was obtained in WC broth, 27.8 ± 1.4 units. *A. actinomycetemcomitans* grown in the semi-defined medium and in FCS had similar AIP activities, 13.8 ± 1.0 and 12.4 ± 1.6 units respectively.

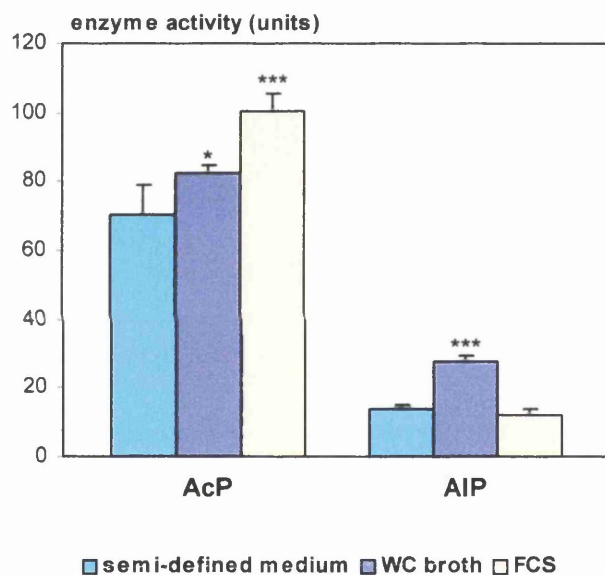


Figure 4.1 Effect of growth medium on AcP and AIP production by *A. actinomycetemcomitans*. Enzyme activities are expressed as the mean \pm S.D. of four replicates. One unit is equivalent to the hydrolysis of 1 nmole *p*-nitrophenyl phosphate/min/mg bacterial protein. Students t test; * $p < 0.05$, *** $p < 0.001$.

4.4.2 Effect of incubation atmosphere on AcP and AIP production by *A. actinomycetemcomitans*

Fig. 4.2 shows the AcP and AIP activities of *A. actinomycetemcomitans* grown in a semi-defined medium incubated in a CO₂-enriched aerobic atmosphere and an anaerobic atmosphere. *A. actinomycetemcomitans* grown anaerobically demonstrated significantly more AcP and AIP (70.7 ± 8.7 units and 13.8 ± 1.0 units respectively) than cultures which had been incubated in a CO₂-enriched aerobic atmosphere (50.7 ± 5.1 units and 7.1 ± 0.9 units respectively).

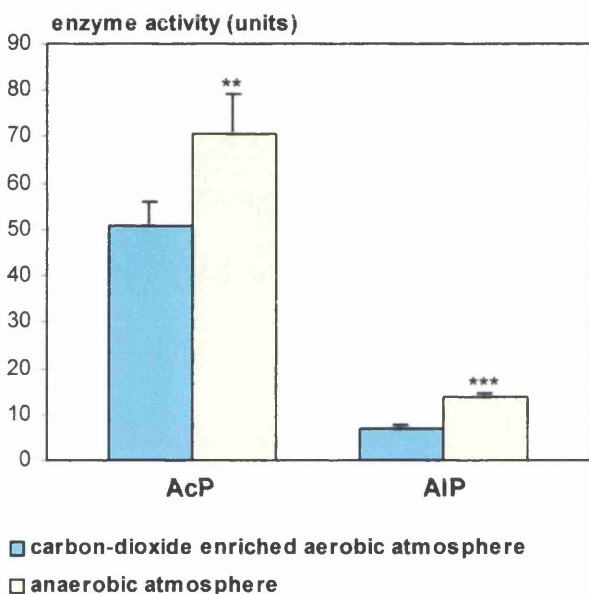


Figure 4.2 Effect of incubation atmosphere on AcP and AIP production by *A. actinomycetemcomitans* grown in a semi-defined medium. Enzyme activities are expressed as the mean \pm S.D. of four replicates. Units are as in Fig. 4.1. Students t test; **p < 0.01, ***p < 0.001.

4.4.3 Effect of incubation atmosphere on the activity of *A. actinomycetemcomitans* and *E. coli* Acp

The results shown in Fig. 4.2 do not indicate whether the differences observed were due to differences in the amount of enzyme produced or differences in the activity of the enzyme under different incubation atmospheres as reaction mixtures were incubated either anaerobically or in a CO₂-enriched aerobic atmosphere according to the original growth conditions of the culture.

Fig. 4.3 shows the effect of incubation atmosphere on the AcP activity of *A. actinomycetemcomitans* cultures and purified enzyme from *E. coli*. When cultures incubated in a CO₂-enriched aerobic atmosphere were assayed for AcP under anaerobic conditions (B) the level of activity was considerably greater compared to that observed when the same cultures were assayed for AcP under CO₂-enriched aerobic conditions (A). The AcP activity of CO₂-enriched aerobic cultures assayed anaerobically (B) was similar to that of anaerobic cultures assayed anaerobically (C). The activity of purified AcP from *E. coli* was greater under anaerobic conditions (E) than under CO₂-enriched aerobic conditions (D).

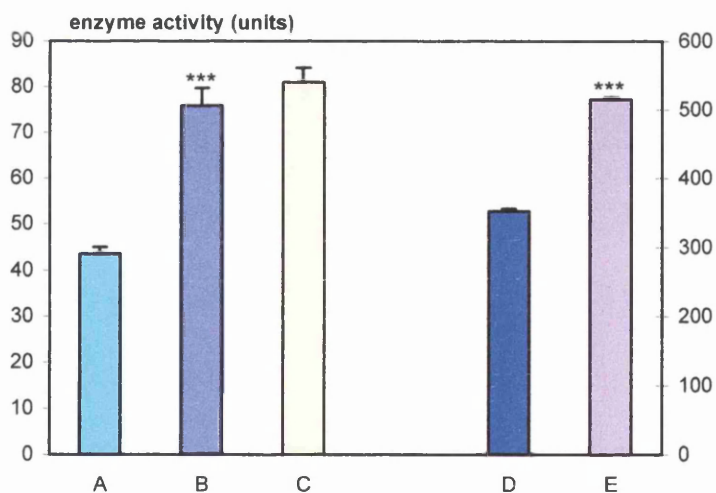


Figure 4.3 Effect of incubation atmosphere on the AcP activity of *A. actinomycetemcomitans* cultures and purified AcP from *E. coli*. (A), when grown in a CO₂-enriched aerobic atmosphere and assayed for AcP in the same environment; (B), when grown in a CO₂-enriched aerobic atmosphere and assayed for AcP anaerobically; (C), when grown anaerobically and assayed for AcP anaerobically; (D), activity of 2.5 µg/ml *E.coli* AcP assayed in a CO₂-enriched aerobic atmosphere; (E), activity of 2.5 µg/ml *E.coli* AcP assayed anaerobically. Enzyme activities are expressed as the mean ± S.D. of four replicates. Units are as in Fig. 4.1. Students t test; ***p <0.001.

4.5 Discussion

The level of AcP and AIP produced by *A. actinomycetemcomitans* grown in different media did not appear to be related to the level of free phosphate in the growth medium. The amount of free phosphate in each of the three media was 1.9, 5.9 and 3.0 mmoles per litre for the semi-defined medium, WC broth and FCS respectively. Whilst many bacterial phosphatases have been shown to be phosphate-repressible (Shah and Blobel, 1967; Torriani, 1990), others appear to be produced constitutively (Poirier and Holt 1983a; González *et al.*, 1989). Yotis *et al.* (1993) demonstrated that the uptake of certain phosphorylated compounds by *T. denticola* was dependent on their prior dephosphorylation. Choline and betaine have been shown to induce the production of an AcP by *P. aeruginosa* even in a non-phosphate limiting medium (Lucchesi *et al.*, 1989). Thus the presence or levels of a particular substrate in the different growth media may have been responsible for the differential stimulation of AcP and AIP production by *A. actinomycetemcomitans*.

The AcP and AIP activities of *A. actinomycetemcomitans* cultures which had been grown in a CO₂-enriched aerobic atmosphere were lower than that of cultures grown in an anaerobic environment. However these findings did not indicate whether the differences observed were due to differences in the amount of enzyme produced or differences in the activity of the enzyme under the different incubation atmospheres as reaction mixtures were incubated either anaerobically or in a CO₂-enriched aerobic atmosphere according to the original growth conditions of the culture. When cultures incubated in a CO₂-enriched aerobic atmosphere were assayed for AcP under anaerobic conditions the level of activity was considerably greater compared to that observed when the same cultures were assayed for AcP under CO₂-enriched aerobic conditions. The AcP activity of CO₂-enriched aerobic cultures assayed anaerobically was similar to that of anaerobic cultures assayed anaerobically. In addition the activity of purified AcP from *E. coli* was greater under anaerobic conditions than under CO₂-enriched aerobic conditions. A possible explanation for the greater activity of the phosphatase enzymes under anaerobic conditions is the presence of thiol groups. The protein tyrosine phosphatase of *Yersinia pestis* has been shown to possess an essential cysteine residue (Guan and Dixon, 1990) and it is possible that there are important thiol groups involved in

the phosphatase activities of *A. actinomycetemcomitans* and *E. coli*. Thus although the assay buffer contained the reducing agent cysteine-HCl, oxygen which was present in the CO₂-enriched aerobic atmosphere but not in the anaerobic atmosphere may have caused oxidation of such thiol groups leading to a reduction in the activity of these enzymes.

The AcP and AIP of *A. actinomycetemcomitans* may serve an important role in the nutrition of the organism in the periodontal pocket. Non-specific phosphatases could remove phosphate groups from non-transportable phosphate esters present in the environment, thereby allowing the hydrolysed component to be transported and utilised by the cell. Such non-specific phosphatase activity could supply a variety of nutrients to the organism depending on the organic moiety of the ester, in addition to phosphate.

In addition to a role in nutrition, the phosphatases of oral bacteria may also play a role in the pathogenesis of periodontal disease. AcP from *A. actinomycetemcomitans* was able to release inorganic phosphate from phosvitin (data not shown), a phosphoprotein which is similar in phosphoserine content to the phosphoproteins of dentin, enamel, cementum and bone (Shainkin and Perlmann, 1971; Glimcher, 1979). A study by Poirier and Holt (1983b) demonstrated similar activity in *Capnocytophaga ochracea*. Release of phosphatases from periodontopathogenic bacteria into the periodontal environment may lead to the hydrolysis of mineralised tissues whose physicochemical integrity are dependent on inter- and intra-molecular cross-linking between phosphoserine groups of phosphopeptides and calcium ions (Glimcher, 1979). Phosphatases may also play an indirect role in plaque mineralisation by removing pyrophosphate, a calcification inhibitor (Lucchesi *et al.*, 1989). This hypothesis is supported by the positive correlations found between AcP and AIP levels in saliva and calculus index (Bercy and Vreven, 1979).

This study has shown that *A. actinomycetemcomitans* produces significant amounts of AcP and AIP and that the production and activity of these enzymes is affected by growth medium and incubation atmosphere. The production of such enzymes by *A. actinomycetemcomitans* may facilitate the survival and growth of the organism in the nutritionally competitive environment of the periodontal pocket and may also contribute to the pathogenesis of periodontal disease.

Chapter 5

Interactions between periodontopathogenic bacteria and cytokines

5.1 Introduction

The immunoinflammatory response observed in periodontal disease is modulated mainly by cytokines which act as intercellular signalling molecules. Elevated levels of certain cytokines, for example the pro-inflammatory cytokines IL-1 β and IL-6, have been demonstrated in GCF and periodontal tissues of patients with periodontal disease (Masada *et al.*, 1990; Reinhardt *et al.*, 1993; Bartold and Haynes 1991; Takahashi, 1994; Hou *et al.*, 1995; Stashenko *et al.*, 1991). Cytokines may be produced constitutively or in response to a particular stimulus such as complement factors and other cytokines. Bacteria and their components are also able to stimulate the production of cytokines by host cells. Many studies concerning the stimulation of cytokine production by periodontopathogenic bacteria have focussed on LPS (Wilson, 1995), although more recently it has been shown that other cell wall constituents and secretory products can potently stimulate cytokine production (Henderson and Wilson, 1995; Henderson and Wilson, 1996).

In addition to the induction of cytokine production, there are a number of other ways in which bacteria may interact with cytokines *in vivo*. There is growing evidence to suggest that cytokines can promote bacterial growth (Porat *et al.*, 1991; Denis *et al.*, 1991) or that bacterial proteases can activate or inactivate cytokines (Theander *et al.*, 1998; Parmely *et al.*, 1990).

5.2 Aims

The objectives of this study were to:

- i) investigate whether IL-1 β and IL-6 affect the growth of *A. actinomycetemcomitans* and *P. gingivalis*
- ii) determine whether culture supernatants from *P. gingivalis* and *A. actinomycetemcomitans* could degrade IL-1 β , IL-6 and IL-1ra
- iii) determine whether biofilms of *P. gingivalis* could degrade IL-1 β , IL-6 and IL-

1ra, both in the presence and absence of serum

iv) investigate IL-1 β degradation by purified RI proteinase from *P. gingivalis*

5.3 Materials and methods

5.3.1 Effect of IL-1 β and IL-6 on the growth of *A. actinomycetemcomitans* and *P. gingivalis*

The effect of IL-1 β and IL-6 on the growth *A. actinomycetemcomitans* 670 and *P. gingivalis* W50 was determined by monitoring conductivity changes in cultures using the Rapid Automated Bacterial Impedance Technique (RABIT, Don Whitley Scientific). Bacteria harvested from plate cultures of the organisms were resuspended in pre-reduced Whitley Impedance Broth (WIB) in the case of *A. actinomycetemcomitans* or Whitley Anaerobe broth (WAB) (Don Whitley Scientific) in the case of *P. gingivalis*. The suspensions were used to inoculate tubes containing 2 ml of pre-reduced WIB or WAB as appropriate. IL-1 β or IL-6 was added to give final concentrations of 0, 1, 10, and 100 ng/ml in a total volume of 2.5 ml. Tubes were flushed with anaerobic gas for 30 secs, sealed with sterile bungs and inserted into the RABIT module pre-set at 37°C. Changes in conductivity (measured in μ Siemens, μ S) were recorded every 6 mins for up to 48 h.

5.3.2 Hydrolysis of IL-1 β , IL-6 and IL-1ra by culture supernatants of *P. gingivalis* and *A. actinomycetemcomitans*

P. gingivalis W50 and *A. actinomycetemcomitans* 670 were inoculated into pre-reduced BM broth. After 48 h anaerobic incubation at 37°C, cultures were centrifuged and the supernatants passed through a 0.22 μ m filter to provide a cell-free supernatant. IL-1 β , IL-6 or IL-1ra was added to the culture supernatants to give a final concentration of 2.5 μ g/ml. In the case of IL-6, the *P. gingivalis* culture supernatant was diluted 1 in 4 with BM broth prior to adding the cytokine. An aliquot was removed immediately and frozen at -70°C. This was designated as the t = 0h sample. The culture supernatants containing either IL-1 β , IL-6 or IL-1ra were then incubated anaerobically at 37°C. Further aliquots were removed at various time intervals and frozen immediately at -70°C

to prevent further hydrolysis. Controls contained heat-inactivated (100°C, 30 mins) culture supernatant plus cytokine, and cytokine-free supernatant.

5.3.3 Hydrolysis of IL-1 β , IL-6 and IL-1ra by biofilms of *P. gingivalis*

Biofilms of *P. gingivalis* were prepared as follows. Sterile nitrocellulose membranes (5 mm diameter) were placed on the surface of WC agar plates containing 5% (v/v) horse blood. The membranes were then inoculated with 10 μ l of a dense suspension of *P. gingivalis* and incubated anaerobically at 37°C for 48 h. The resulting biofilms were transferred to microtitre plates and IL-1 β , IL-6 or IL-1ra were added. The cytokines were prepared at a final concentration of 2.5 μ g/ml in BM broth. Biofilms were incubated with the cytokine solutions anaerobically at 37°C, and samples were removed at t = 0, 0.25, 0.5, 1, 2 and 4 h, centrifuged and the supernatant stored at -70°C prior to analysis. Heat-inactivated biofilm (121°C, 15 mins) plus cytokine, BM broth plus cytokine without biofilm and biofilm plus BM broth without cytokine were included as controls.

5.3.3.1 Hydrolysis of IL-1 β , IL-6 and IL-1ra by biofilms of *P. gingivalis* in the presence of serum

The experiments were repeated as before except that the cytokines were prepared in horse serum (Oxoid) instead of BM broth. Controls consisted of heat-inactivated biofilm plus cytokine, horse serum plus cytokine without biofilm, and biofilm plus serum without cytokine.

5.3.4 Hydrolysis of IL-1 β by purified RI proteinase

Purified *P. gingivalis* RI proteinase (Aduse-Opoku *et al.*, 1995) was diluted to 4 U/ml (final concentration) in assay buffer (0.1 M Tris-HCl, pH 8.0, 10 mM CaCl₂, 10 mM cysteine-HCl). IL-1 β was added to give a final concentration of 2.5 μ g/ml and the enzyme solutions were incubated anaerobically at 37°C. Controls contained heat-inactivated (100°C, 30 mins) RI proteinase plus IL-1 β . Samples were removed at t = 0, 0.25, 0.5, 1, 2 and 4 h. Proteolytic activity was stopped immediately at each sample time by the addition of an equal volume of formic acid. The samples were then dried on a SpeedVac concentrator (Savant,

Farmingdale, New York, USA) and stored at -70°C until required. To ensure complete inactivation of the RI proteinase prior to electrophoresis, each of the samples was resuspended in a solution of the proteinase inhibitor leupeptin at 1 mg/ml, incubated at room temperature for 1 h and then heated at 100°C for 3 mins before adding sample buffer.

5.3.5 SDS-PAGE and Western blotting

Samples were separated by SDS-PAGE by the method of Laemmli (1970) and Western blotting was carried out as described in Ch 2.

The primary antibodies used were sheep monoclonal anti IL-1 β , IL-6 or IL-1ra diluted 1:1000 in blocking buffer (2% FCS in PBS-0.1% Triton-X-100). Rabbit anti-sheep HRP conjugate (Dako, High Wycombe, UK) diluted 1:1000 in blocking buffer was used for the secondary antibody.

5.3.6 Assessment of bio-activity of IL-1 β following treatment with *P. gingivalis* culture supernatant

Induction of IL-6 release from human gingival fibroblasts (HGF) was used to assess the biological activity of IL-1 β following treatment with the *P. gingivalis* culture supernatants for various time intervals. HGF were plated in 24-well plates at a density of 30,000 cells per well in Dulbeccos modified Eagles medium (DMEM, Sigma) containing 2% FCS. The plates were then incubated at 37°C in 5% CO₂ overnight to allow the cells to adhere. The next day the cells were washed and the *P. gingivalis* culture supernatants containing IL-1 β were added. The culture supernatants were first diluted 1:125,000 in DMEM to give a theoretical IL-1 β concentration of 20 pg/ml (assuming no digestion had taken place). Controls consisted of IL-1 β at a concentration of 20 pg/ml and *P. gingivalis* supernatant with no cytokine added. Following overnight incubation, the media were removed from the HGF cells and assayed for IL-6 by ELISA as follows. Microtitre plates were coated with immuno-affinity purified polyclonal goat anti-IL-6 antibody at 1 μ g/ml, diluted in phosphate buffered saline (PBS), pH 7.2. Plates were incubated at 4°C overnight. The wells were decanted and washed with 0.01 M phosphate/0.05 M NaCl buffer containing 0.1% (v/v) Tween 20 (wash buffer, pH 7.2). IL-6 standards were added to wells over the dose

range 0 - 3 ng/ml and the samples to be tested added to the remaining wells. Plates were incubated for 2 h at room temperature and washed three times with wash buffer. Biotinylated affinity purified polyclonal goat anti-IL-6 antibody (100 μ l of a 14 μ g/ml solution) was added to each of the wells and incubated for 1 h at room temperature. The plates were washed three times and 100 μ l of a 1:5000 dilution of avidin-HRP (Dako) were added to each well. Plates were then incubated for 15 mins at room temperature before washing three times with wash buffer. Wells were developed with 100 μ l of 0.2 mg/ml orthophenylenediamine in 0.1 M citric acid-phosphate buffer pH 5.0 plus 0.4 μ l/ml 30% hydrogen peroxide. The reaction was terminated by the addition of 150 μ l of 1 M H₂SO₄, and the absorbance was measured at 492 nm on a Titertek plate reader.

This experiment was carried out by Dr K. Reddi.

5.4 Results

5.4.1 Effect of IL-1 β and IL-6 on the growth of *A. actinomycetemcomitans* and *P. gingivalis*

The effects of increasing concentrations of IL-1 β and IL-6 on the growth of *A. actinomycetemcomitans* and *P. gingivalis* are shown in Figs. 5.1a, b and Figs. 5.2a, b respectively. Concentrations of IL-1 β as high as 100 ng/ml had no effect on the rate of growth of *A. actinomycetemcomitans* (slope = 2.2 ± 0.1 μ S/min compared to 2.2 ± 0.1 μ S/min for cytokine-free controls). The final conductivity of the medium was similar for cultures containing 100 ng/ml IL-1 β and cytokine-free controls (1269.3 ± 17.7 μ S and 1279.3 ± 40.7 μ S respectively). IL-6 too, had no effect on the growth of the organism. The rate of growth of *A. actinomycetemcomitans* in the presence of IL-6 at 100ng/ml (slope = 2.1 ± 0.1 μ S/min) was the same as in the absence of the cytokine (slope = 2.1 ± 0.1 μ S/min). The final conductivity of cultures containing 100 ng/ml IL-6 was 1252.3 ± 12.7 μ S compared to 1282.3 ± 19.6 μ S for cytokine-free controls.

RABIT experiments to determine the effect of IL-1 β and IL-6 on the growth of *P. gingivalis* were repeated on three separate occasions in triplicate. For each experiment, large standard deviations were observed. IL-1 β and IL-6 at concentrations up to 100 ng/ml appeared to have no significant effect on the growth of *P. gingivalis*. The final conductivity of cultures containing 100 ng/ml IL-1 β was 4523.3 ± 162.5 μ S compared to 4686.8 ± 372.5 μ S for the cytokine-free controls. The rate of growth of *P. gingivalis* in the presence of IL-1 β at 100 ng/ml was similar (slope = 3.6 ± 0.2 μ S/min) to that in the absence of the cytokine (slope = 3.2 ± 0.7 μ S/min). The final conductivity of cultures containing 100 ng/ml IL-6 was 4836.9 ± 280.8 μ S compared to 4512.1 ± 414.2 for the cytokine-free controls. The rate of growth in the presence of this cytokine was similar (3.9 ± 0.1 μ S/min) to that in the absence of IL-6 (3.7 ± 0.2 μ S/min). Whilst lower concentrations of IL-6 appeared to exhibit an inhibitory effect on the growth of *P. gingivalis*, this effect was not statistically significant and was not observed in other experiments.

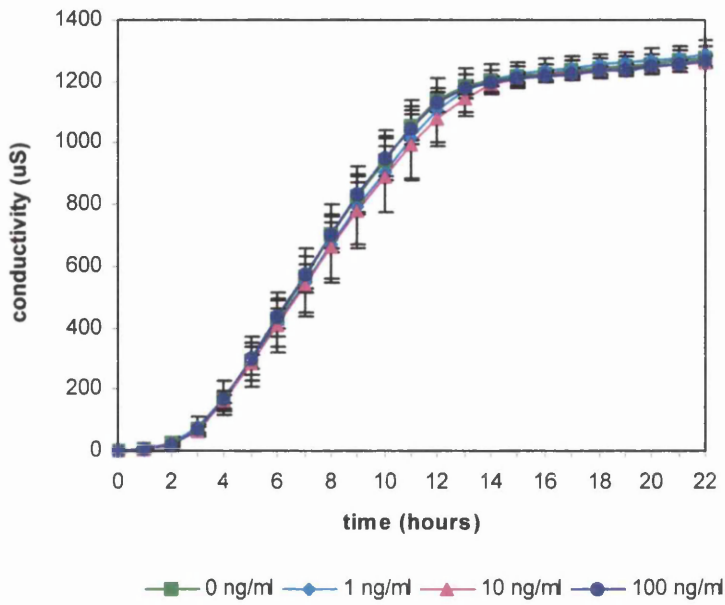


Figure 5.1a Growth of *A. actinomycetmcomitans* in the presence of IL-1β. Results are expressed as the mean ± S.D. of 3 replicates.

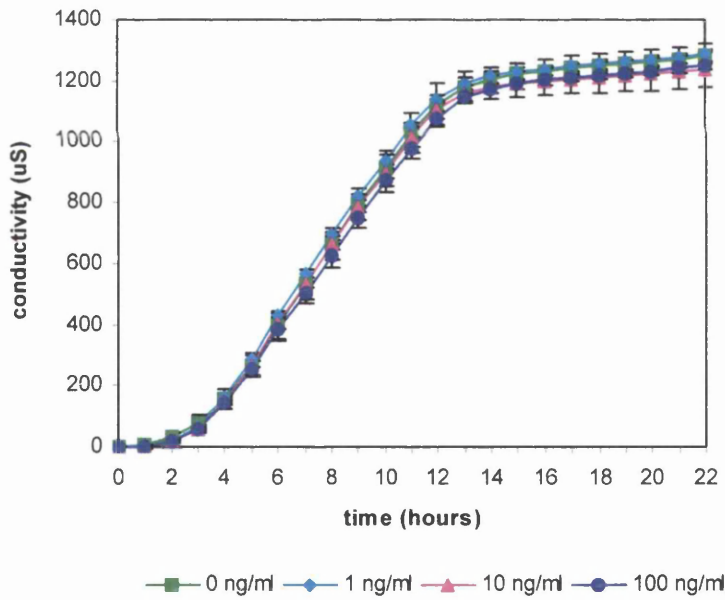


Figure 5.1b Growth of *A. actinomycetmcomitans* in the presence of IL-6. Results are expressed as the mean ± S.D. of 3 replicates.

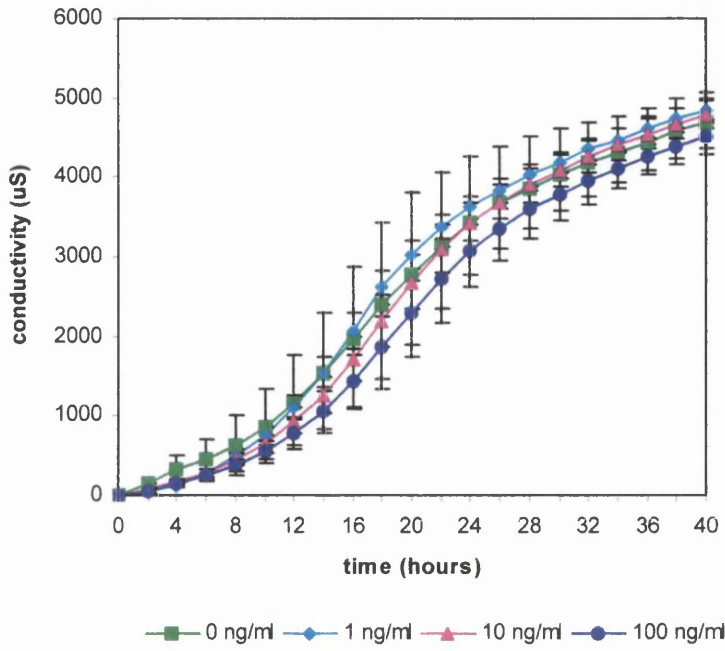


Figure 5.2a Growth of *P. gingivalis* in the presence of IL-1β. Results are expressed as the mean ± S.D. of 3 replicates.

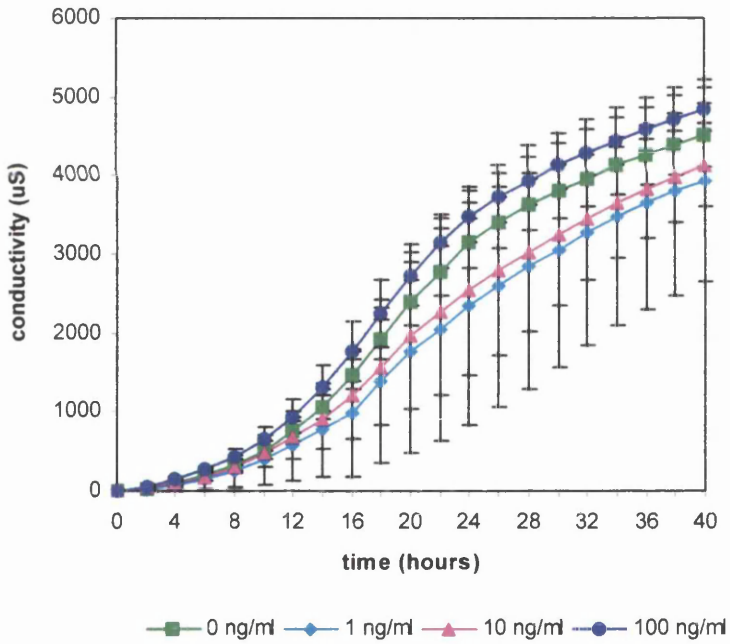


Figure 5.2b Growth of *P. gingivalis* in the presence of IL-6. Results are expressed as the mean ± S.D. of 3 replicates.

5.4.2 Hydrolysis of IL-1 β , IL-6 and IL-1ra by *P. gingivalis* and *A. actinomycetemcomitans*

Supernatants from cultures of *A. actinomycetemcomitans* were unable to hydrolyse IL-1 β , IL-6 or IL-1ra (Figs. 5.4a, b and c). In contrast, supernatants from cultures of *P. gingivalis* were able to hydrolyse all three cytokines as evidenced by the disappearance of bands with molecular masses corresponding to those of the intact cytokines with time, and the appearance of bands with lower molecular masses representing breakdown products (Figs. 5.3a, b and c). After 8 h anaerobic incubation, no IL-1 β was detectable (Fig. 5.3a) and only a small proportion of the IL-1ra remained (Fig. 5.3c). IL-6 appeared to be particularly sensitive to degradation by *P. gingivalis* and no IL-6 was detectable within minutes when incubated with undiluted culture supernatant (data not shown). Even when the *P. gingivalis* culture supernatant was diluted 1:4 with BM broth, only a small amount of IL-6 remained at t = 8h (Fig. 5.3b). No degradation was evident when the culture supernatants were boiled prior to addition of the cytokine (lane 2).

After 48 h anaerobic incubation, substantial biofilms of *P. gingivalis* were present on the surface of the membrane filters and these contained approximately 10^8 cfu/mm².

Biofilms of *P. gingivalis* were able to degrade IL-1 β , IL-6 and IL-1ra (Figs. 5.5a, b and c respectively) as indicated by the disappearance of the band corresponding to the cytokine with time and the appearance of lower molecular weight breakdown products. After 2 h incubation neither IL-1 β nor IL-1ra was detectable. Again IL-6 appeared to be particularly sensitive to hydrolysis by *P. gingivalis* biofilms and after only 0.5 h virtually no IL-6 remained. There was no evidence of cytokine degradation by the heat-inactivated biofilms (lane 1).

Degradation of IL-1 β , IL-6 and IL-1ra by biofilms of *P. gingivalis* also occurred in the presence of serum (Figs. 5.6a, b and c). However, degradation occurred at a slower rate than in the absence of serum. Only a small amount of IL-1 β and IL-1ra remained after 4 h. IL-6 was more rapidly degraded than the other cytokines and was undetectable after 2 h.

Fig. 5.7 shows the degradation of IL-1 β by purified *P. gingivalis* R1 proteinase at 4 U/ml. The rate of degradation was approximately double that of the *P.*

gingivalis culture supernatants and after 2 h only a small amount of IL-1 β remained.

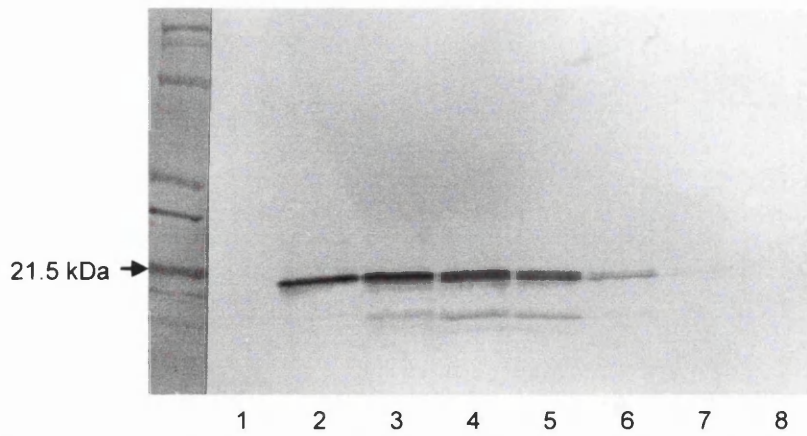


Figure 5.3a Effect of culture supernatant from *P. gingivalis* on IL-1 β . Lane 1, culture supernatant without cytokine; lane 2, heat-inactivated supernatant + cytokine; lane 3, supernatant + cytokine at t = 0h; lane 4, supernatant + cytokine at t = 1h; lane 5, supernatant + cytokine at t = 2h; lane 6, supernatant + cytokine at t = 4h; lane 7, supernatant + cytokine at t = 6h; lane 8, supernatant + cytokine at t = 8h.

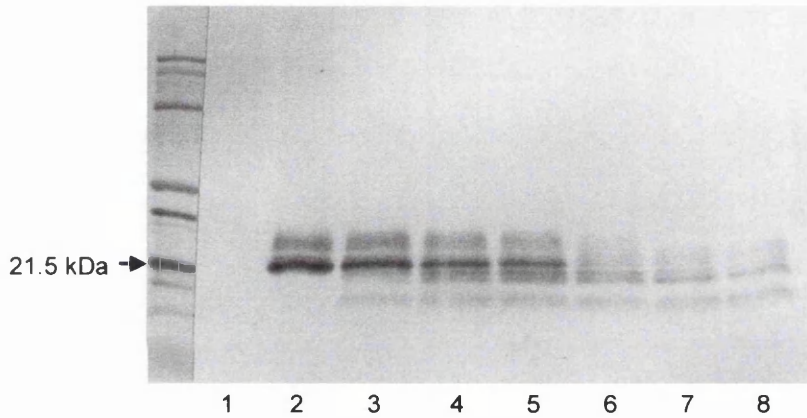


Figure 5.3b Effect of culture supernatant from *P. gingivalis* (diluted 1:4) on IL-6. Lanes are as in Fig. 5.3a.

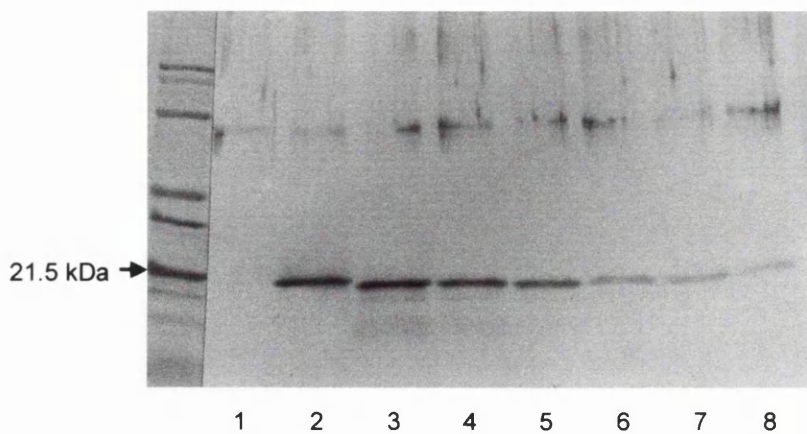


Figure 5.3c Effect of culture supernatant from *P. gingivalis* on IL-1ra. Lanes are as in Fig. 5.3a.

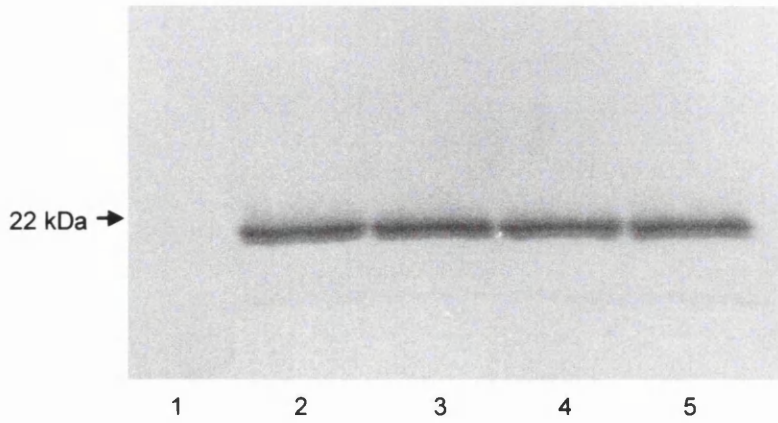


Figure 5.4a Effect of culture supernatant from *A. actinomycetemcomitans* on IL-1 β . Lane 1, culture supernatant without cytokine; lane 2, heat-inactivated culture supernatant + cytokine; lane 3, supernatant + cytokine at t = 0h; lane 4, supernatant + cytokine at t = 6h; lane 5, supernatant + cytokine at t = 24h.

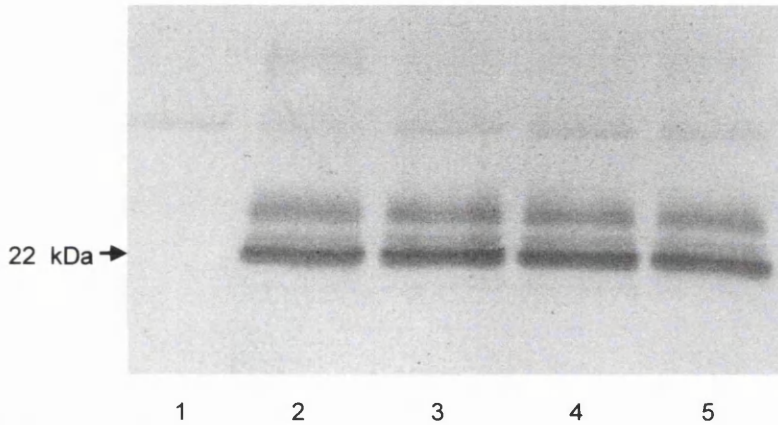


Figure 5.4b Effect of culture supernatant from *A. actinomycetemcomitans* on IL-6. Lanes are as in Fig. 5.4a.

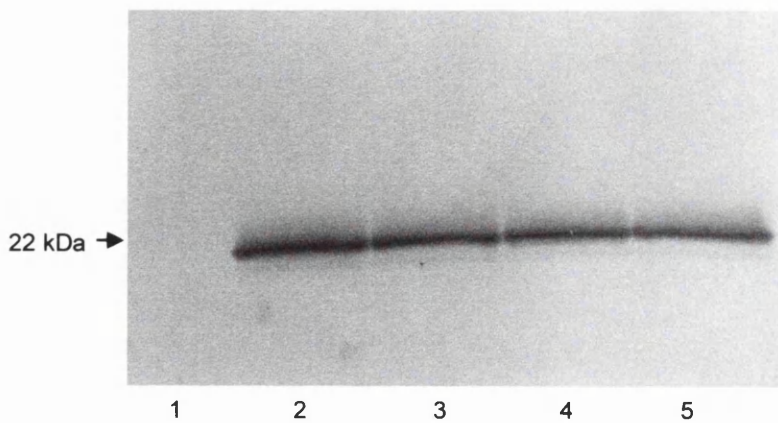


Figure 5.4c Effect of culture supernatant from *A. actinomycetemcomitans* on IL-1ra. Lanes are as in Fig. 5.4a.

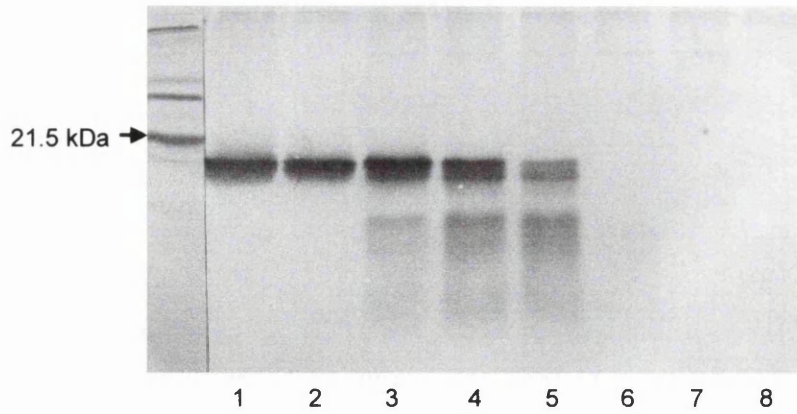


Figure 5.5a Effect of *P. gingivalis* biofilms on IL-1 β . Lane 1, heat-inactivated biofilm + cytokine; lane 2, biofilm + cytokine at t = 0h; lane 3, biofilm + cytokine at t = 0.25h; lane 4 biofilm + cytokine at t = 0.5h, lane 5, biofilm + cytokine t = 1h; lane 6, biofilm + cytokine t = 2h; lane 7, biofilm + cytokine t = 4h; lane 8, biofilm + BM broth.

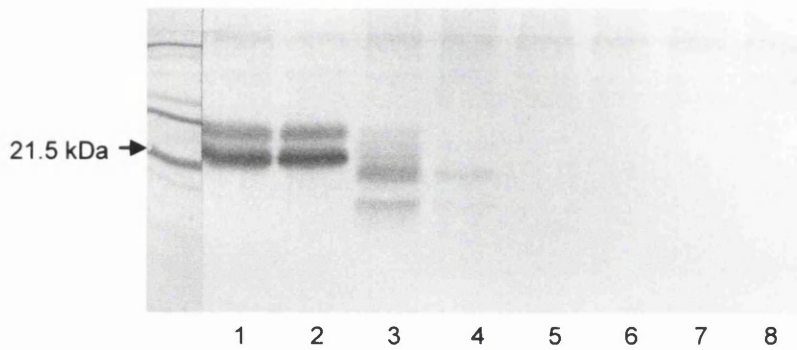


Figure 5.5b Effect of *P. gingivalis* biofilms on IL-6. Lanes are as in Fig. 5.5a.

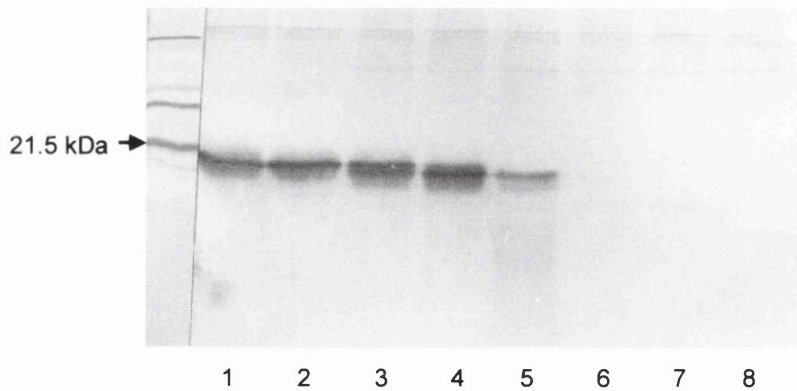


Figure 5.5c Effect of *P. gingivalis* biofilms on IL-1ra. Lanes are as in Fig. 5.5a.

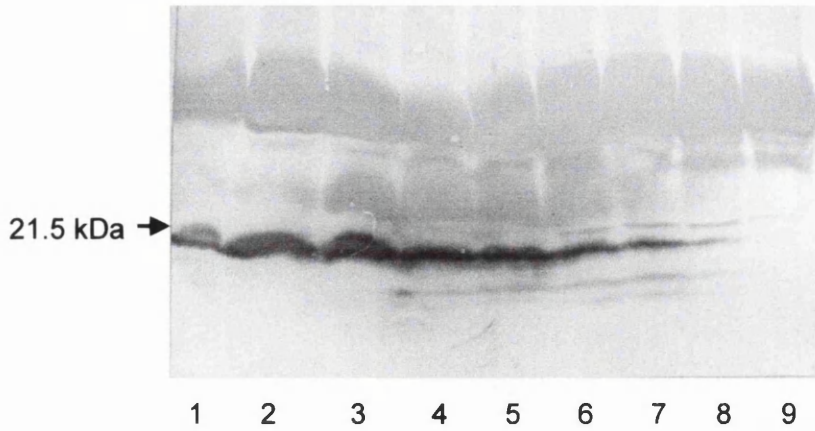


Figure 5.6a Effect of *P. gingivalis* biofilms on IL-1 β in the presence of horse serum. Lane 1, serum + cytokine; lane 2, heat-inactivated biofilm + cytokine; lane 3, biofilm + cytokine at t = 0h; lane 4, biofilm + cytokine at t = 0.25h; lane 5, biofilm + cytokine at t = 0.5h, lane 6, biofilm + cytokine t = 1h; lane 7, biofilm + cytokine t = 2h; lane 8, biofilm + cytokine t = 4h; lane 9, biofilm + serum.

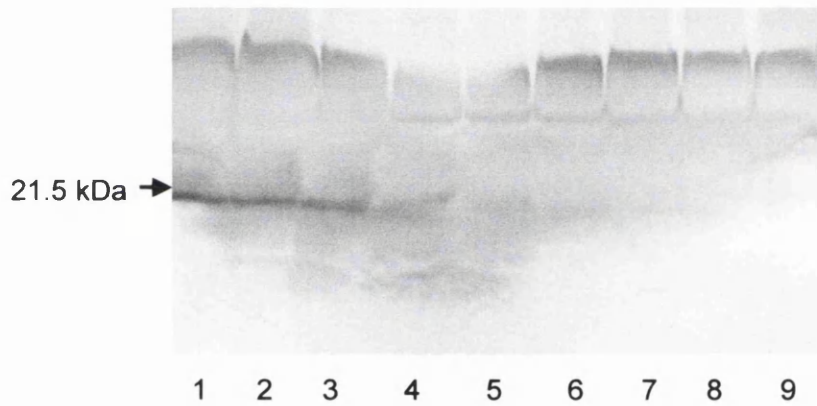


Figure 5.6b Effect of *P. gingivalis* biofilms on IL-6. Lanes are as in Fig. 5.6a.

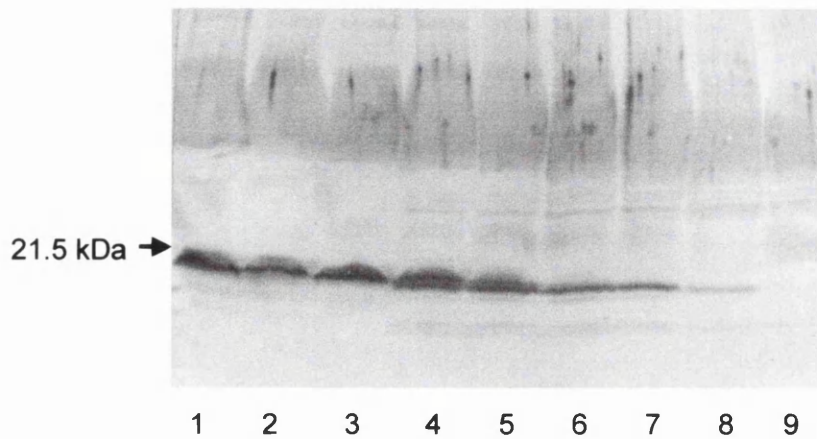


Figure 5.6c Effect of *P. gingivalis* biofilms on IL-1ra. Lanes are as in Fig. 5.6a.

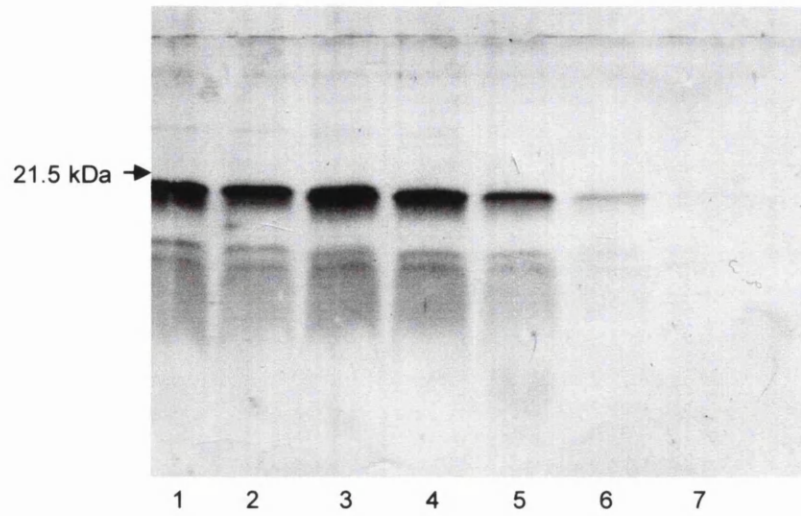


Figure 5.7 Effect of RI proteinase (purified from *P. gingivalis*) on IL-1 β . Lane 1, heat-inactivated RI + IL-1 β ; Lane 2, RI + IL-1 β at t = 0h; Lane 3, RI + IL-1 β at t = 0.25h; Lane 4, RI + IL-1 β at t = 0.5h; Lane 5, RI + IL-1 β at t = 1h; Lane 6, RI + IL-1 β at t = 2 h; Lane 7, RI + IL-1 β at t = 4h.

5.4.3 Bio-activity of IL-1 β following treatment with *P. gingivalis* culture supernatant

The biological activity of IL-1 β following incubation with *P. gingivalis* culture supernatant for various time intervals was determined by measuring its ability to stimulate the release of IL-6 from HGF (Fig. 5.8). The culture supernatants were diluted 1:125,000 which, if no cytokine degradation had taken place, would result in an IL-1 β concentration of 20 pg/ml. Using this dilution of the culture supernatants, the assay system used was sufficiently sensitive to detect the presence of biologically-active cytokine even if 99 % degradation had taken place. From Fig. 5.8 it can be seen that IL-1 β , after incubation with *P. gingivalis* culture supernatant for 4 h, was unable to stimulate the release of IL-6 from HGF. *P. gingivalis* culture supernatant with no cytokine added did not stimulate IL-6 release from HGF while IL-1 β at a concentration of 20 pg/ml stimulated the release of 820 pg/ml IL-6 following overnight incubation with the HGF.

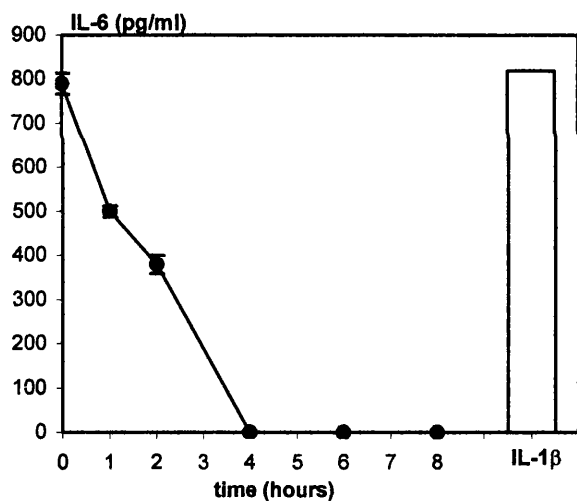


Figure 5.8 Effect of *P. gingivalis* culture supernatant on the biological activity of IL-1 β as assessed by its ability to stimulate IL-6 release from human gingival fibroblasts (HGF).

● = IL-6 release from HGF elicited by IL-1 β following incubation with *P. gingivalis* culture supernatant for various periods of time. The vertical bar represents the IL-6 release from HGF elicited by 20 pg/ml of IL-1 β . Incubation of *P. gingivalis* culture supernatant alone with HGF did not induce the release of IL-6. Results are expressed as the mean \pm S.D. of 6 replicate cultures.

5.5 Discussion

Studies by Porat *et al.* (1991) and Denis *et al.* (1991) demonstrated the ability of cytokines to stimulate the growth of virulent strains of *E. coli*. The RABIT, by measuring small changes in the conductivity of the culture medium, provides a highly sensitive means of detecting changes in bacterial growth patterns. However the results of the current study have shown that neither IL-1 β nor IL-6, at concentrations as high as 100 ng/ml, were able to stimulate the growth of *A. actinomycetemcomitans* or *P. gingivalis*. This does not necessarily mean that *in vivo* cytokines do not influence the growth of periodontopathogenic bacteria and it would certainly be of interest to repeat this study using freshly isolated strains of *A. actinomycetemcomitans* and to test a range of culture media (both of the studies mentioned above utilised tissue culture medium) and environmental conditions.

Biofilms and cell-free supernatants from planktonic cultures of the putative periodontopathogen *P. gingivalis* were able to rapidly degrade two key pro-inflammatory cytokines (IL-1 β and IL-6) as well as the anti-inflammatory cytokine IL-1ra. In addition purified *P. gingivalis* RI proteinase was able to degrade IL-1 β . IL-6 appeared to be more susceptible to proteolysis compared to IL-1 β and IL-6. The degradation of IL-1 β by culture supernatants of *P. gingivalis* was accompanied by loss of biological activity as demonstrated by its inability to induce IL-6 release from HGF.

In the periodontal pocket periodontopathogenic bacteria will be bathed in GCF, a serum-like exudate, which contains proteolytic inhibitors such as α -1-antitrypsin and α ₂-macroglobulin. An important finding of this study was that proteolytic degradation of cytokines by biofilms of *P. gingivalis* occurred in the presence of serum. The rate of cytokine degradation was slower and whilst this may have been due to the presence of protease inhibitors, it is more likely to be due to the presence of large amounts of alternative substrates (e.g. albumin). A study by Grenier (1996) demonstrated that *P. gingivalis* can in fact degrade host protease inhibitors such as α -1-antitrypsin, antichymotrypsin, α ₂-macroglobulin, antithrombin III, antiplasmin and cystatin C.

IL-1 and IL-6 play an important role in regulating the immune response to periodontopathogenic bacteria. IL-1 activates T- and B- cells and stimulates the

proliferation of these cells. IL-1 also induces the production of cytokines by monocytes and neutrophils (Dinarello, 1994). One of the main functions of IL-6 is to stimulate the differentiation of B-cells into antibody-secreting cells. In addition to their role in the inflammatory response, cytokines may also contribute directly to the pathology of periodontal disease. IL-1 is a potent mediator of bone resorption (Tatakis, 1993) and also induces connective tissue destruction via the release of metalloproteinases from target cells (Meikle *et al.*, 1989). IL-6 stimulates the formation of osteoclasts (Kurihara *et al.*, 1990; Ishimi *et al.*, 1990), the cells responsible for bone degradation. *In vivo*, the activities of cytokines are regulated by inhibitors and antagonists. IL-1ra binds to the same receptors on target cells as IL-1 but does not trigger the intracellular transduction processes which normally occur when IL-1 binds (Eisenberg *et al.*, 1990; Hannum *et al.*, 1990). Thus IL-1ra acts to suppress the effects of IL-1 on the cell. *In vivo*, cytokines, the cells producing them and their target cells form an interacting network which serves to control both the production and activities of these potent immunomodulators. Degradation of pro-inflammatory cytokines such as IL-1 β and IL-6 may lead to a decrease in the ability of the host to mount an effective response to the organism while degradation of the anti-inflammatory cytokine IL-1ra would interfere with a system which operates to control the potentially damaging activities of IL-1 β .

In summary IL-1 β and IL-6 did not affect the growth of the putative periodontopathogens *A. actinomycetemcomitans* and *P. gingivalis*. IL-1 β , IL-6 and IL-1ra were rapidly degraded by cell-free culture supernatants and biofilms of *P. gingivalis* and purified RI proteinase from this organism. Moreover proteolysis of cytokines occurred in the presence of serum. *In vivo* cytokine degradation by periodontopathogenic bacteria may lead to the disruption of the of the highly complex cytokine network responsible for maintaining periodontal health (Wilson *et al.*, 1998).

Chapter 6

Effect of environmental factors on the surface-associated proteins of *A. actinomycetemcomitans*

6.1 Introduction

Periodontal diseases are associated with inflammation, loss of gingival attachment and erosion of the alveolar bone. As a site progresses from health to disease, there will be considerable changes in the local environmental conditions. For example, as plaque increases in volume, oxygen becomes depleted, levels of CO₂ increase (a by-product of aerobic bacterial metabolism) and conditions rapidly become anaerobic. Periodontal inflammation is associated with an increase in the flow of GCF (Goodson, 1989) and bleeding. These highly complex biological fluids, namely GCF and blood, may provide novel nutrients for the growth of bacteria or may contain inhibitory factors such as the iron-sequestering molecules transferrin and lactoferrin. Studies on the virulence factors of pathogenic bacteria have traditionally been carried out on organisms grown on nutrient-rich media. However, *in vivo*, bacteria are likely to experience conditions of feast or famine (Wimpenny *et al.*, 1993).

In addition to temporal variations, there will also be spatial variations in environmental conditions. Marsh and Martin (1992) proposed that as a result of diffusion limitations, gradients in physicochemical factors will form across plaque biofilms. Thus an organism in one part of the plaque may experience very different conditions to an organism in another part of the biofilm.

Whilst the majority of the bacteria present in the periodontal pocket constitute part a biofilm, bacteria may also be present in the fluid phase both above the biofilm and in the interstices within it. There is evidence to suggest that these planktonic cells may differ in many respects from their biofilm counterparts (Wimpenny *et al.*, 1993).

Bacteria are highly adaptable and energetically-efficient organisms and, under a given set of environmental conditions, will only express that part of their genome which enables them to become structurally and functionally adapted to the prevailing conditions. Thus an organism of a given phenotype is very much a product of its environment. The bacteria involved in periodontal diseases exist

in a highly complex and continually-changing microenvironment to which they must rapidly adapt in order to survive.

Bacteria interact with the environment via molecules located on their surface and therefore the surface molecules of an organism are of particular interest. In addition, it is these molecules which will form the first point of contact with host cells and may subsequently cause damage to those cells or modulate their activity.

When cells of *A. actinomycetemcomitans* are examined by electron microscopy, an amorphous layer can be observed surrounding the bacterium (Holt *et al.*, 1980). This layer can be easily removed by gently rotating the bacterial cells in saline at 4°C. Cells from which the surface-associated material (SAM) has been removed appear denuded but remain largely intact with no discernible damage to the outer membrane (Wilson *et al.*, 1985). Analysis of *A. actinomycetemcomitans* SAM by two-dimensional PAGE reveals the presence of approximately 50 Coomassie blue-staining spots with molecular masses ranging from <14 to >66 kDa (Kirby *et al.*, 1995). A number of virulence properties have been attributed to SAM from *A. actinomycetemcomitans* including bone resorption (Wilson *et al.*, 1985; Kirby *et al.*, 1995; Reddi *et al.*, 1995a), stimulation of cytokine production (Reddi *et al.*, 1996a, b) and inhibition of osteoblast (White *et al.*, 1995) and fibroblast (Kamin *et al.*, 1986; Meghji *et al.*, 1992a) proliferation.

In addition to contributing to the direct pathogenic potential of the organism, SAM from *A. actinomycetemcomitans* may also play a role in attachment and evasion of host defence mechanisms. A study by Meyer and Fives-Taylor (1993) showed that washing cells of *A. actinomycetemcomitans* in PBS readily removed extracellular amorphous material and this was associated with a concomitant reduction in the ability of the organism to adhere to epithelial cells *in vitro*.

6.2 Aim

The aim of this study was to determine the effect of environmental conditions on the protein profiles of SAM from *A. actinomycetemcomitans*.

6.3 Materials and methods

6.3.1 Growth conditions

A. actinomycetemcomitans NCTC 9710 was grown under a range of different conditions on or in;

- 1) semi-defined agar incubated anaerobically/37°C (SAM 1)
- 2) semi-defined agar incubated in a CO₂-enriched aerobic atmosphere/37°C (SAM 2)
- 3) semi-defined broth incubated anaerobically/37°C (SAM 3)
- 4) semi-defined agar supplemented with 10% (v/v) horse serum incubated anaerobically/37°C (SAM 4)
- 5) semi-defined agar containing 100 µM 2,2'-dipyridyl (a ferrous iron chelator) incubated anaerobically/37°C (SAM 5)
- 6) Brain Heart Infusion (BHI) agar supplemented with 6% (v/v) horse blood incubated anaerobically/37°C (SAM 6).

Semi-defined medium was prepared as described in Ch 2 except that fructose was added in place of glucose to give a final concentration of 5 g/l. Sodium bicarbonate (4 g/l) was also added. To prepare the semi-defined agar, 12 g of agar technical no. 2 (Oxoid) was added per litre of medium.

For the iron-restricted medium, ferrous sulphate was omitted from the trace element solution. 2,2'-dipyridyl (Sigma) was dissolved in ethanol, filter-sterilised and added to the agar after autoclaving to give a final concentration of 100 µM. Cells used to inoculate the iron-restricted medium had been previously subcultured (1x) on the same medium in order to deplete the internal iron reserves.

250 agar plates or 6 litres of broth for each growth condition were inoculated with a dense suspension of *A. actinomycetemcomitans*. Plates and liquid cultures were incubated for 72 h under the appropriate conditions. Cells were harvested from the agar plates in ice-cold sterile saline. Suspensions were centrifuged (3000 g, 4°C, 20 mins) and the pellets stored at -70°C until required. Liquid cultures were first filtered through grade 1 filter paper (Whatman) to remove precipitated sodium bicarbonate. The filtrate was then centrifuged (3000 g, 4°C, 20 mins) and the pellet stored as before.

SAM was extracted from the cell pellets as described in Ch 2.

SAM 6 was prepared by Dr P. Tabona.

6.3.2 Determination of composition of SAM

The protein, carbohydrate (CHO) and LPS content of each of the SAMs was determined as described in Ch 2.

6.3.3 One-dimensional and two-dimensional gel electrophoresis

1-D and 2-D gel electrophoresis was carried out as described in Ch 2. Capillary tube gels were orientated on the slab gel with the acidic end (pH 3) adjacent to the molecular weight marker lane.

6.3.4 Preparation of proteins for sequencing

Three proteins (A, B and F) were selected for sequencing by mass spectrometry (carried out by Dr G. Talbo, University of Melbourne, Australia). Proteins were cut out directly from the 2-D gels and dried down on a SpeedVac concentrator (Savant) ready for sequencing.

Six proteins (A - F) were selected for sequencing by Edman degradation (carried out by Dr J. Gray, University of Newcastle Upon Tyne, UK, using a Beckman LF 3000 microsequencer). Proteins were first transferred from 2-D gels onto PVDF membrane by electroblotting as follows. After second dimension electrophoresis was complete, gels were rinsed in water containing 20 μ g/ml dithiothreitol (DTT water) and then soaked in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% (v/v) methanol, pH 11) for 25 mins. PVDF membrane was first pre-wetted in methanol for 2 - 3 secs, rinsed in DTT water and then soaked in transfer buffer. Electroblotting was carried out at 100 mA for 50 mins in pre-chilled transfer buffer in a Trans-Blot electrophoresis tank (Bio-Rad). After electroblotting was complete, the membrane was rinsed in DTT water several times and stained with Coomassie blue (1 mg/ml in 50% (v/v) methanol). The blot was destained in 50 % (v/v) methanol for 10 mins, rinsed several times with DTT water and air dried. Blots were stored at -20°C.

6.4 Results

6.4.1 Composition of SAM extracted from *A. actinomycetemcomitans* grown under different environmental conditions

	cell yield (dry weight) mg	SAM yield (dry weight) mg	% SAM	protein content of SAM (%)	CHO content of SAM (%)	LPS content IU/ μ g SAM
SAM 1	737.83	85.23	11.6	22	5.0	1.5 - 15
SAM 2	887.39	118.53	13.4	29	4.8	0.15 - 1.5
SAM 3	641.20	196.24	30.6	37	3.5	0.15 - 1.5
SAM 4	937.35	135.04	14.4	24	4.5	1.5 - 15
SAM 5	610.94	124.67	20.4	37	4.0	1.5 - 15
SAM 6	3135.00	435.00	13.9	34	3.5	1.5 - 15

Table 6.1
Table showing the yield and composition of the 6 SAMs

6.4.2 Surface protein profiles of *A. actinomycetemcomitans* grown under different environmental conditions - 1-D analysis

Fig. 6.1 shows the 1-D protein profiles of the SAM extracted from *A. actinomycetemcomitans* grown under different environmental conditions. A number of differences were observed.

Comparison of SAM 2 with SAM 1

- i) increased levels of a protein with a MW of 48 kDa
- ii) additional protein with a MW of 39 kDa

Comparison of SAM 3 with SAM 1

- i) additional protein with a MW of 72.5 kDa
- ii) increased levels of a protein with a MW of 70 kDa
- iii) significantly increased levels of a protein with a MW of 48 kDa
- iv) increased levels of a protein with a MW of 44 kDa
- v) additional protein with a MW of 39 kDa
- vi) decreased levels of a protein with a MW of 23 kDa
- vii) decreased levels of a protein with a MW of 17.5 kDa

Comparison of SAM 4 with SAM 1

- i) increased levels of a protein with a MW of 23 kDa

Comparison of SAM 5 with SAM 1

- i) increased levels of a protein with a MW of 48 kDa
- ii) additional protein with a MW of 39 kDa

Comparison of SAM 6 with SAM 1

- i) additional protein with a MW of 72.5 kDa
- ii) increased levels of a protein with a MW of 70 kDa
- iii) significantly increased levels of a protein with a MW of 48 kDa
- iv) increased levels of a protein with a MW of 44 kDa
- v) decreased levels of a protein with a MW of 23 kDa

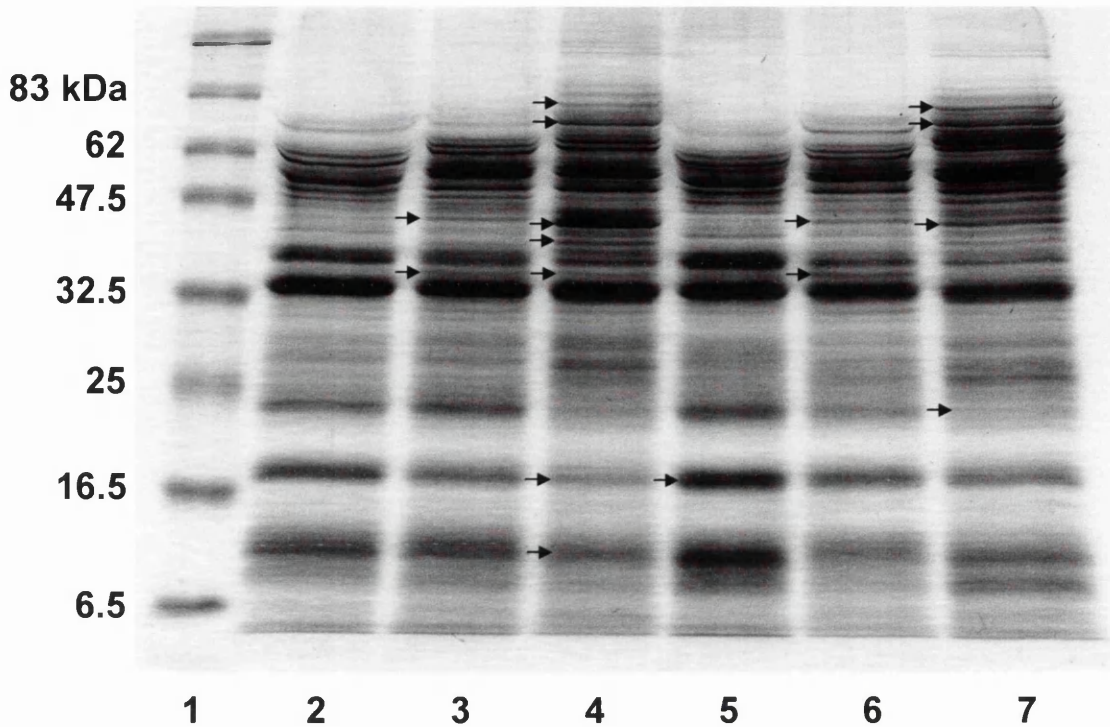


Figure 6.1 One-dimensional SDS-PAGE of SAM isolated from *A. actinomycetemcomitans* grown under different environmental conditions. 3 μg of protein from each SAM were separated on a 12% gel and stained with Brilliant blue G - Colloidal. Lane 1, molecular weight markers; Lane 2, SAM isolated from biofilm cells grown on semi-defined agar incubated anaerobically (SAM 1); Lane 3, SAM isolated from biofilm cells grown on semi-defined agar incubated in a CO_2 -enriched aerobic atmosphere (SAM 2); Lane 4, SAM isolated from planktonic cells grown in semi-defined broth incubated anaerobically (SAM 3); Lane 5, SAM isolated from biofilm cells grown on semi-defined agar supplemented with 10% (v/v) horse serum incubated anaerobically (SAM 4); Lane 6, SAM isolated from biofilm cells grown on semi-defined agar containing 100 μM 2,2'-dipyridyl incubated anaerobically (SAM 5); Lane 7, SAM isolated from biofilm cells grown on BHI agar supplemented with 6% (v/v) horse blood incubated anaerobically (SAM 6).

6.4.3 Surface protein profiles of *A. actinomycetemcomitans* grown under different environmental conditions - 2-D analysis

Three sets of 2-D gels were obtained, however for simplicity only one set is presented here (Figs. 6.2a-f). Approximately 120 proteins (or sub-units) were observed with MWs ranging from 10 to 70 kDa. Protein profiles for each SAM were compared with SAM 1 and the differences observed are described below. Whilst equal amounts of protein were loaded onto each gel, it is likely that slight variations in the fixation and staining of different gels will have occurred. Thus, when identifying changes in the level of a particular protein, only differences where the level of a protein had increased or decreased disproportionately relative to the other proteins within the profile were considered.

Comparison of SAM 2 with SAM 1

The protein profiles obtained for SAM 2 and SAM 1 were very similar and only a few differences could be discerned:

- i) increased levels of a series of proteins with a MW of 59 kDa
- ii) increased level of a protein with a MW 55 kDa
- iii) increased levels of a cluster of proteins with a MW of 54 kDa
- iv) a series of proteins with a MW of 37 kDa exhibited a different pattern (●●●) on the SAM 2 protein profile compared to the profile obtained for SAM 1 (●●●)
- v) increased levels of a protein with a MW of 33 kDa
- vi) decreased levels of a protein with a MW of 23 kDa

Comparison of SAM 3 with SAM 1

The protein profiles for SAM 3 and SAM 1 exhibited the same basic pattern, however a number of additional proteins (often present in only small amounts) could be observed. The main differences identified were:

- i) increased levels of a series of proteins with a MW of 48 kDa
- ii) increased levels of a protein with a MW of 46 kDa
- iii) decreased levels of a series of proteins with a MW of 44 kDa
- iv) absence of a protein with a MW of 46.5 kDa
- v) additional protein with a MW of 46.5 kDa
- vi) additional protein with a MW of 30 kDa

- vii) additional protein with a MW of 21 kDa
- viii) additional protein with a MW of 20.5 kDa
- ix) decreased levels of a protein with a MW of 20 kDa
- x) increased levels of a protein with a MW of 15.5 kDa

Comparison of SAM 4 with SAM 1

The protein profiles obtained for SAM 4 and SAM 1 were very similar, however a number of proteins present on the SAM 1 profile were absent or present at only low levels on the SAM 4 profile. Thus, the main differences identified were:

- i) decreased levels of a protein with a MW of 23.5 kDa
- ii) absence of a protein with MW of 20.75 and decreased levels of a protein with a MW of 21.5 kDa
- iii) decreased levels of a protein with a MW of 20 kDa
- iv) decreased levels of a protein with a MW of 10.5 kDa

Comparison of SAM 5 with SAM 1

The key differences observed were:

- i) increased levels of a protein with a MW of 70 kDa
- ii) increased levels of a series of proteins with a MW of 48 kDa
- iii) additional protein with a MW of 48 kDa
- iv) increased levels of a protein with a MW of 45 kDa
- v) additional protein with a MW of 38.5 kDa
- vi) additional protein with a MW of 37.8 kDa
- vii) additional cluster of proteins with an average MW of 35 kDa
- viii) additional protein with a MW of 21 kDa
- ix) additional protein with a MW of 11.8 kDa

Comparison of SAM 6 with SAM 1

The protein profiles obtained for SAM 6 and SAM 1 exhibited the same basic pattern, however numerous additional proteins (present in small amounts) could be observed on the SAM 6 profile. The protein profile for SAM 6 was in fact very similar to that obtained for SAM 3 and a number of the key differences identified between SAM 6 and SAM 1 were the same as those described for SAM 3. The major differences identified for SAM 6 were as follows:

- i) increased amounts of a protein with a MW of 56 kDa
- ii) increased amounts of a series of proteins with a MW of 48 kDa
- iii) increased amounts of a protein with a MW of 46 kDa
- iv) decreased levels of a series of proteins with a MW of 44 kDa
- v) additional protein with a MW of 30 kDa
- vi) two additional proteins with an average MW of 23 kDa
- vii) additional protein with a MW of 22 kDa
- viii) additional protein with a MW of 21 kDa
- ix) additional protein with a MW of 20.5 kDa
- x) additional pair of proteins with a MW of 17.7 kDa
- xi) additional protein with a MW of 15.5 kDa
- xii) increased levels of a protein with a MW of 15 kDa

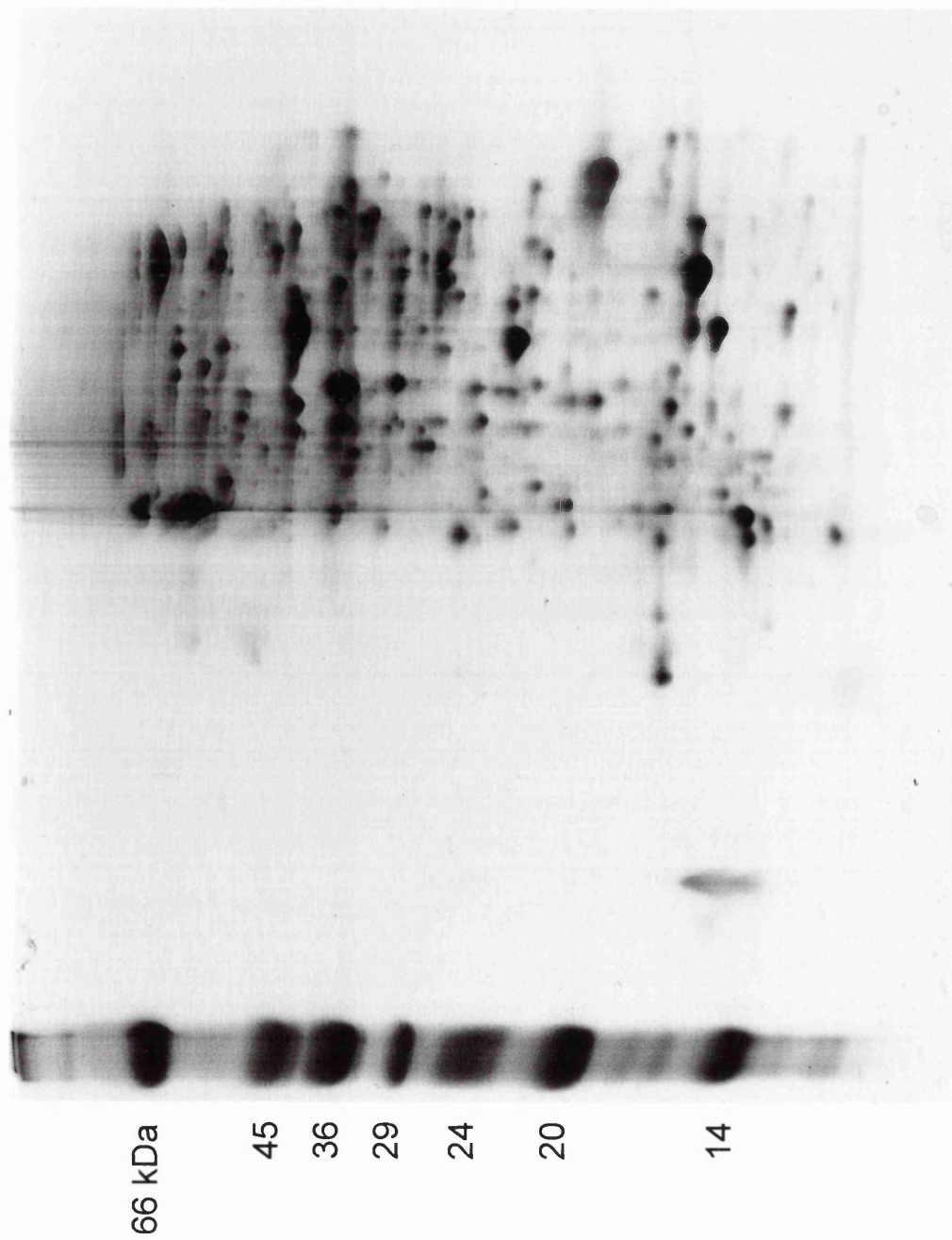


Figure 6.2a Two-dimensional SDS-PAGE of SAM isolated from biofilm cells of *A. actinomycesetemcomitans* grown on semi-defined agar incubated anaerobically. 200 μ g of protein were loaded. The gel was stained with Brilliant blue G - Colloidal. Molecular weight markers are indicated on the left-hand side of the gel.

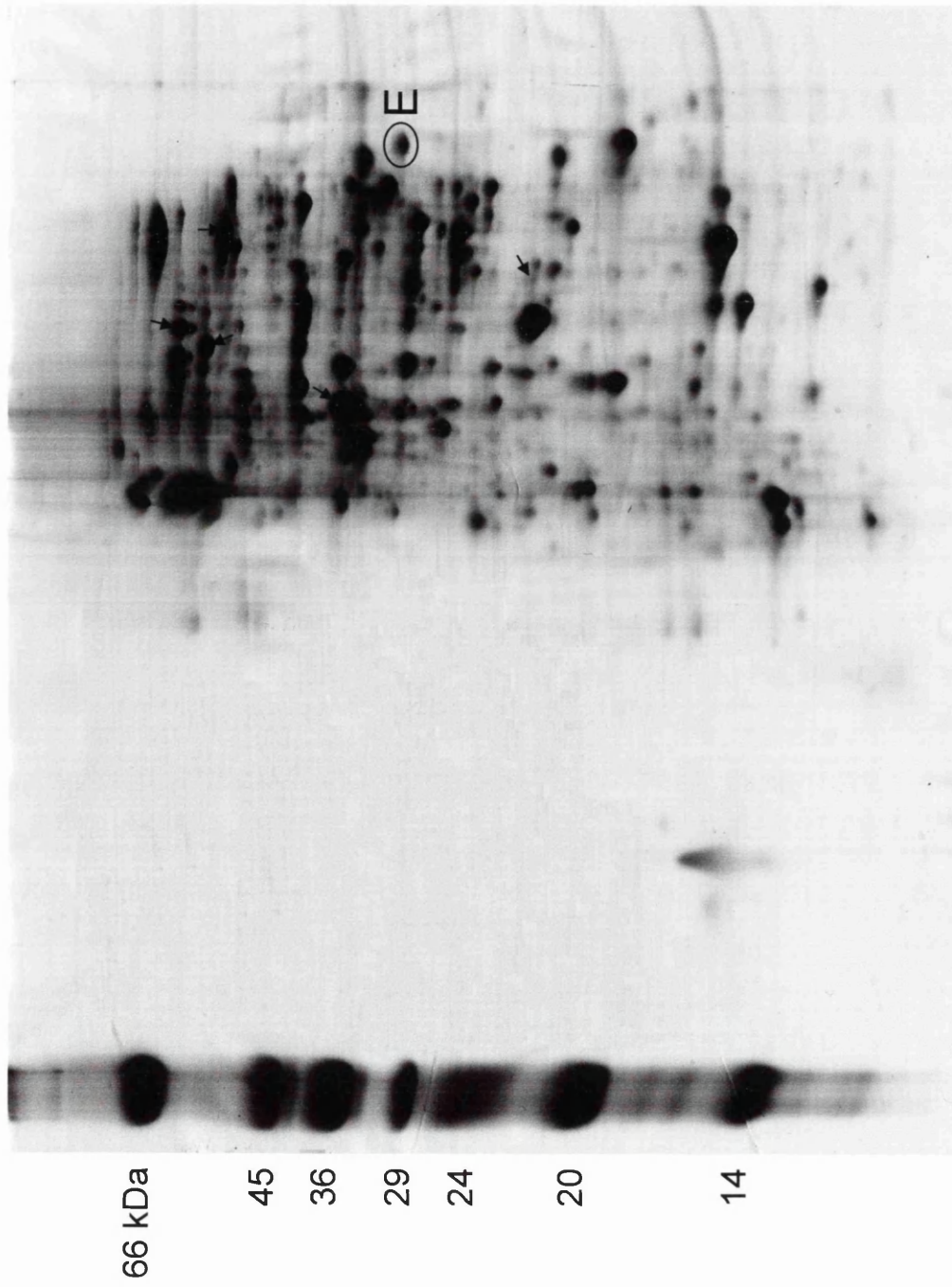


Figure 6.2b Two-dimensional SDS-PAGE of SAM isolated from biofilm cells of *Actinomyces cetemmitans* grown on semi-defined agar incubated in a CO₂-enriched aerobic atmosphere. 200 µg of protein were loaded. The gel was stained with Brilliant blue G - Colloidal. Molecular weight markers are indicated on the left-hand side of the gel.

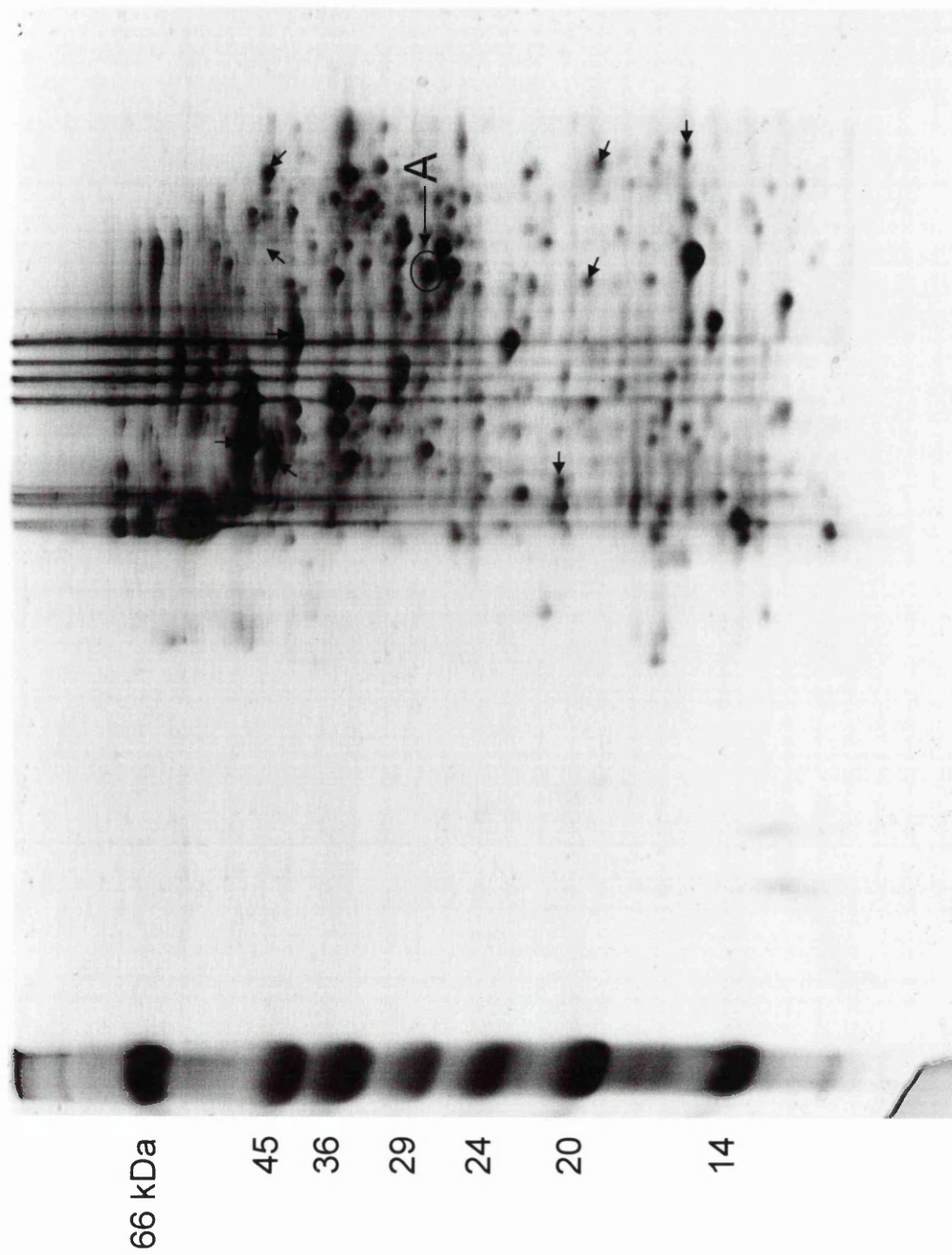


Figure 6.2c Two-dimensional SDS-PAGE of SAM isolated from planktonic cells of *A. actinomycetemcomitans* grown in semi-defined broth incubated anaerobically. 200 μ g of protein were loaded. The gel was stained with Brilliant blue G - Colloidal. Molecular weight markers are indicated on the left-hand side of the gel.

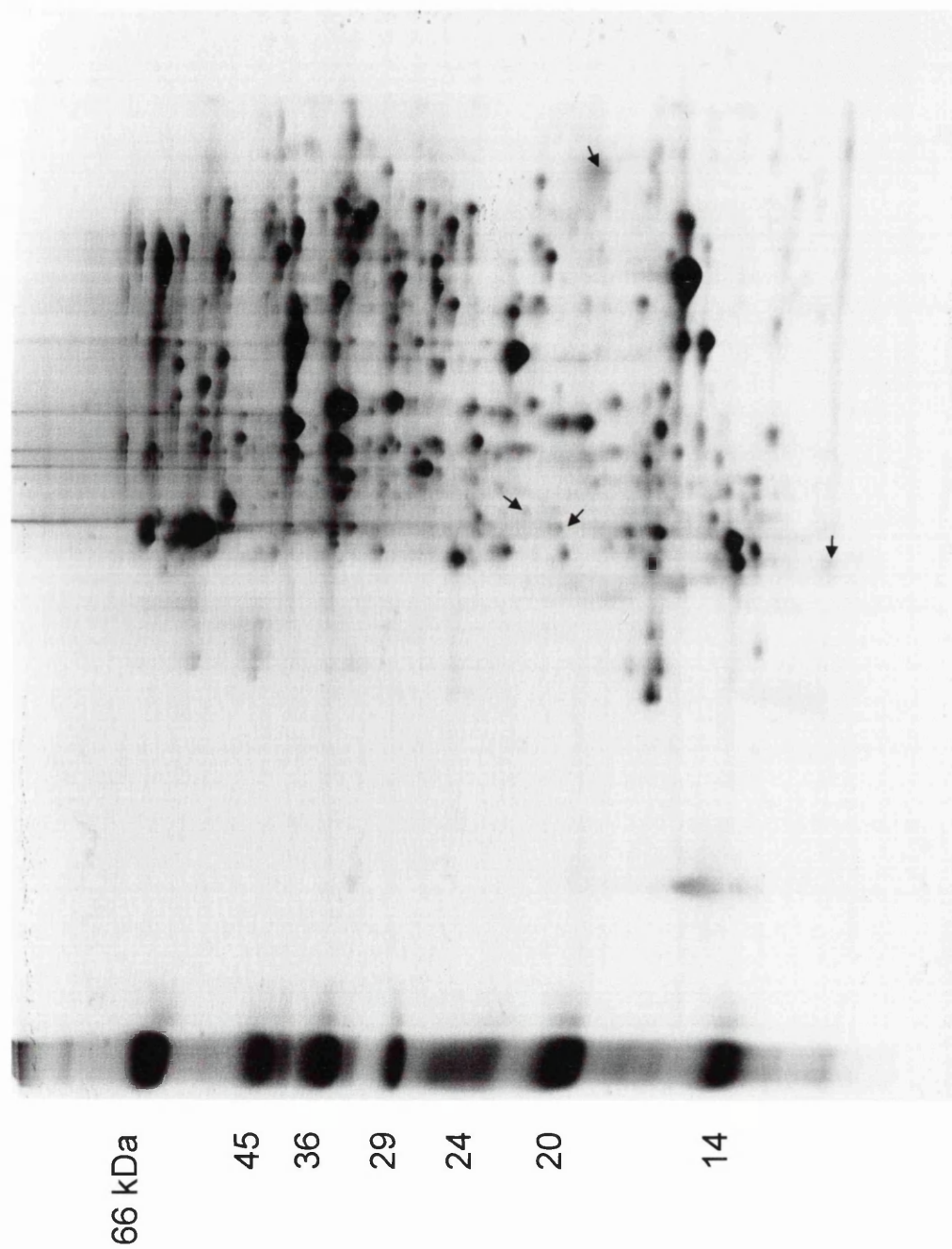


Figure 6.2d Two-dimensional SDS-PAGE of SAM isolated from biofilm cells of *A. actinomycetemcomitans* grown on semi-defined agar supplemented with 10% (v/v) horse serum incubated anaerobically. 200 μ g of protein were loaded. The gel was stained with Brilliant blue G - Colloidal. Molecular weight markers are indicated on the left-hand side of the gel.

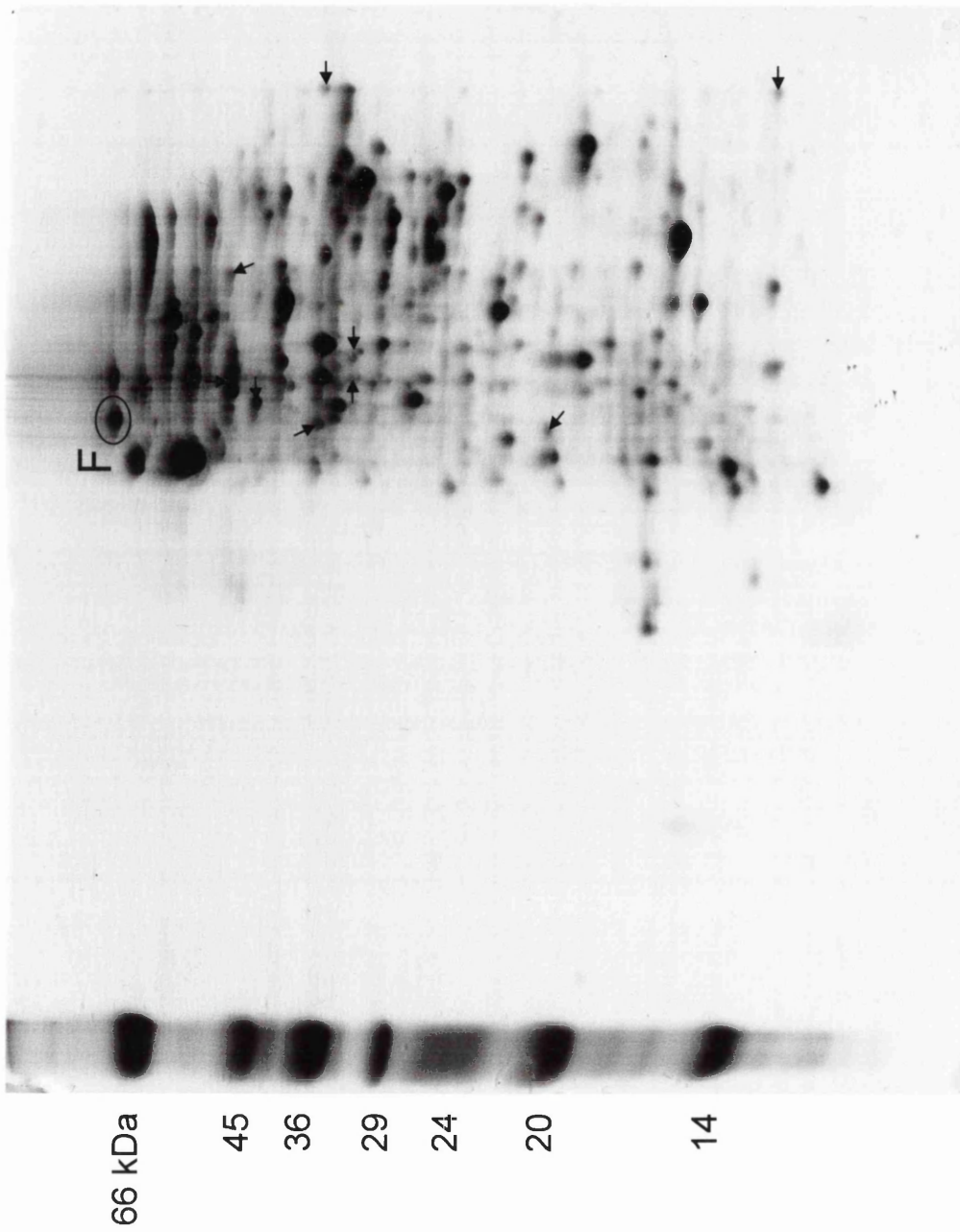


Figure 6.2e Two-dimensional SDS-PAGE of SAM isolated from biofilm cells of *A. actinomycetemcomitans* grown on semi-defined agar containing 100 μM 2,2'-dipyridyl incubated anaerobically. 200 μg of protein were loaded. The gel was stained with Brilliant blue G - Colloidal. Molecular weight markers are indicated on the left-hand side of the gel.

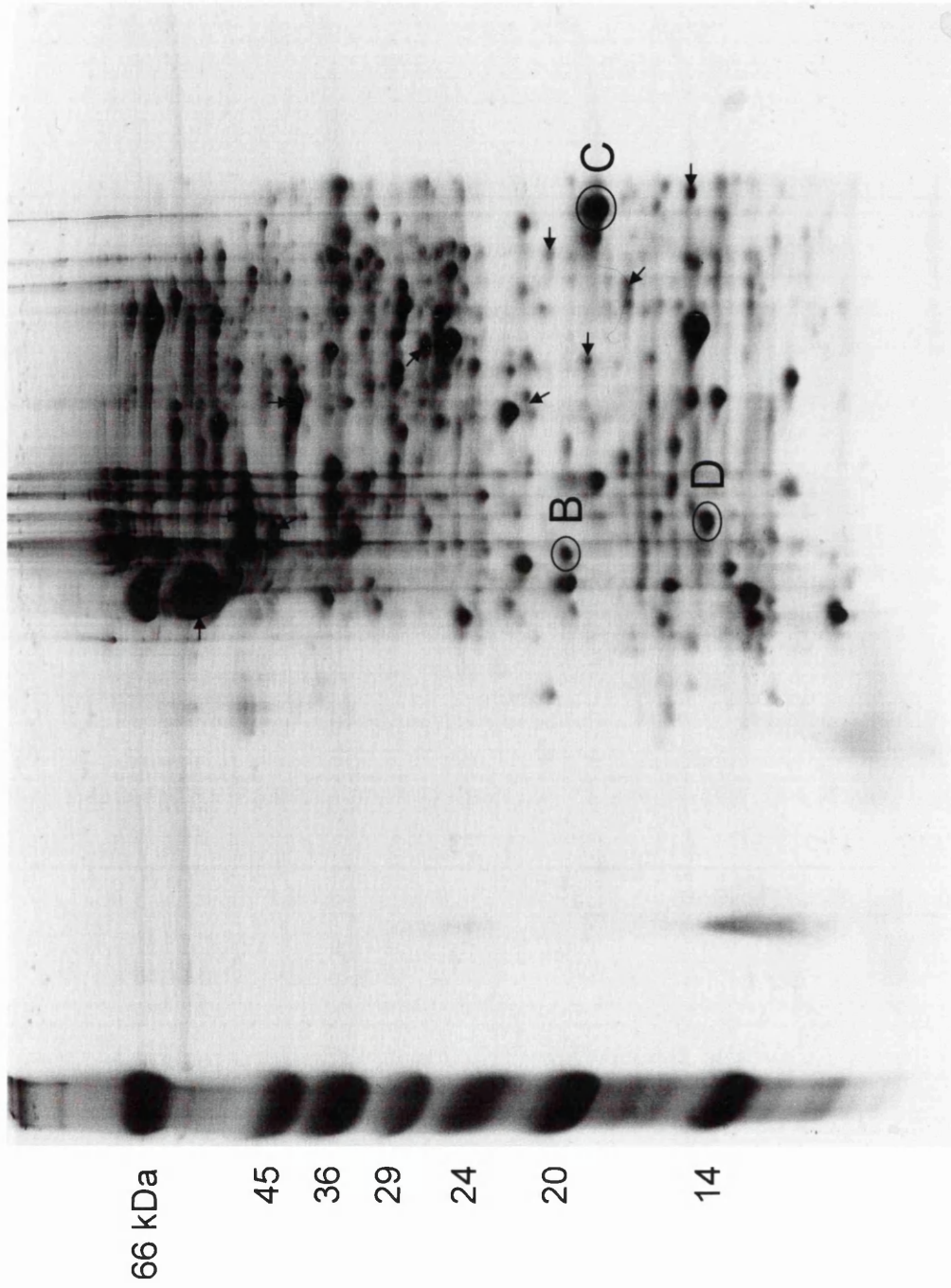


Figure 6.2f Two-dimensional SDS-PAGE of SAM isolated from biofilm cells of *Actinomyces cetemcomitans* grown on BHI agar supplemented with 6% (v/v) horse blood incubated anaerobically. 200 μ g of protein were loaded. The gel was stained with Brilliant blue G - Colloidal. Molecular weight markers are indicated on the left-hand side of the gel.

6.4.4 Identification of *A. actinomycescomitans* surface proteins

	MW (kDa)	N- terminal sequence and identification
A	30	V(Q?)-E-R-P-L-V-M-G-N-W?-K-L-N-G-D-S <i>H. influenzae</i> triose phosphate isomerase (100% identity in 13 aa overlap) <i>E. coli</i> triose phosphate isomerase (100% identity in 12 aa overlap)
B	21	S-K-V-T-L-T-G-T-P?-I-D-V-A-X-L <i>H. influenzae</i> probable thiol peroxidase (79% identity in 163 aa overlap) <i>E. coli</i> thiol peroxidase (64% identity in 162 aa overlap)
C	20	H-E-H-K-H-D-N-M-M-M-P-L-K-G-P <i>N. meningitidis</i> [Cu,Zn]-superoxide dismutase (82% identity in 191 aa overlap) <i>H. parainfluenzae</i> [Cu,Zn]-superoxide dismutase (83% identity in 191 aa overlap) <i>H. influenzae</i> [Cu,Zn]-superoxide dismutase (83% identity in 191 aa overlap) <i>A. pleuropneumoniae</i> [Cu,Zn]-superoxide dismutase (73% identity in 195 aa overlap) <i>H. ducreyi</i> [Cu,Zn]-superoxide dismutase (69% identity in 199 aa overlap) <i>A. actinomycescomitans</i> [Cu,Zn]-superoxide dismutase (100% identity in 87 aa overlap)
D	15	M-I-K-G-I-Q-I-T-Q-A-A-N-D-N-L <i>H. influenzae</i> YfiD hypothetical protein (100% identity in 15 aa overlap) <i>E. coli</i> YfiD protein (80% identity in 15 aa overlap)

Table 6.2
N-terminal sequence and identification of *A. actinomycescomitans* surface proteins

Homology searches were carried out on the N-terminal amino acid sequences using Fasta3 (www.ebi.ac.uk/searches/searches.html).

Protein A (triose phosphate isomerase) was present on the surface protein profiles of planktonic cells of *A. actinomycescomitans* grown in a semi-defined broth incubated anaerobically (SAM 3) and biofilm cells of the organism grown on a nutrient-rich agar containing horse blood incubated anaerobically (SAM 6). The protein was absent from SAM 1, 2, 4 and 5.

Protein B (thiol peroxidase) was present on the surface protein profiles obtained for SAM 3 and SAM 6 and was present at low levels on the surface protein

profile of biofilm cells of *A. actinomycetemcomitans* grown anaerobically under conditions of iron-restriction (SAM 5). Protein B was largely absent from SAM 1, 2 and 4.

Protein C ([Cu,Zn]-superoxide dismutase) was detected on the surface protein profiles of *A. actinomycetemcomitans* under all growth conditions tested. Significantly reduced levels of the protein were observed on the surface protein profiles of anaerobically incubated planktonic cells of *A. actinomycetemcomitans* grown in semi-defined broth (SAM 3) and anaerobically incubated biofilm cells of the organism grown on semi-defined medium containing horse serum (SAM 4).

Initially, when Fasta3 homology searches were carried out on the N-terminal sequences obtained for protein B and protein C no matches were found. When the sequences were searched against the *A. actinomycetemcomitans* genome database (www.genome.ou.edu.act.html), 100% matches were obtained with two different contiguous sequences within the database. The contiguous sequences were scanned for open reading frames and it was thus possible to determine the entire amino acid sequence for both protein B and protein C. Fasta3 searches were then carried out on the full length protein sequences.

The full length protein sequence obtained for protein C demonstrated a 22 amino acid leader sequence at the N-terminus prior to the start of the HEHKHD sequence obtained in the current study. The protein sequence was analysed using SignalP (www.cbs.dtu.dk/services/SignalP/), a programme which can identify signal sequences. SignalP confirmed the presence of 22 amino acid signal peptide at the N-terminal of the protein with a predicted cleavage site between position 22 and 23: ANA-HEH.

Protein D (YfiD protein) was present in significant amounts on the surface protein profile of anaerobically incubated biofilm cells of *A. actinomycetemcomitans* grown on a nutrient-rich agar containing horse blood (SAM 6). The protein was absent or present at very low levels under all other growth conditions tested.

Unfortunately no sequence data were obtained for proteins E and F.

6.5 Discussion

The aim of this investigation was to determine the effect of environmental conditions on the protein profile of *A. actinomycetemcomitans* SAM. Such studies may facilitate the identification of proteins which are 'switched on' *in vivo* as a site progresses from health to disease.

A. actinomycetemcomitans is able to grow both aerobically, provided that there is an increased level of CO₂, and anaerobically. This may be important *in vivo* enabling survival of the organism in early developing plaque where conditions may be relatively aerobic and in the periodontal pocket where conditions are likely to be highly anaerobic. Scannapieco *et al.* (1987) described an extracellular amorphous layer on the surface of *A. actinomycetemcomitans*, analogous to SAM, which could be removed by vortexing cells in PBS. Differences in the protein profile of this material extracted from aerobically grown biofilm cells and anaerobically grown biofilm cells were observed. Thus surface material removed from aerobically grown cells contained an additional protein with a MW of 115 kDa whilst two additional proteins with MWs of 17 and 52 kDa were observed in material extracted from anaerobically grown cells which were absent from aerobically grown cells. Another observation by Scannapieco *et al.* (1987) was that aerobically grown cells demonstrated abundant extracellular material while anaerobically grown cells were surrounded by much lower amounts of surface material. This correlates with the findings of the current study where a higher percentage yield of SAM was obtained from cells grown in a CO₂-enriched aerobic atmosphere (13.4%) compared to anaerobically grown cells (11.6%).

When the 2-D protein profile of SAM from biofilm cells of *A. actinomycetemcomitans* grown in a CO₂-enriched aerobic atmosphere (SAM 2) was compared with SAM from biofilm cells of the organism grown on the same semi-defined medium incubated anaerobically (SAM 1), a number of differences were observed. These differences related mainly to increases in the levels of a number of proteins, however one protein with a MW of 23 kDa was present at significantly reduced levels on the surface of cells grown in a CO₂-enriched aerobic atmosphere compared to cells incubated anaerobically. At least some of the proteins present in increased amounts on the surface of cells grown in a CO₂-enriched aerobic atmosphere may represent proteins required for

protection against reactive oxygen species. In a study by Amano *et al.* (1988) increased levels of superoxide dismutase and NADH oxidase were observed on exposure of aerotolerant species of black pigmented anaerobes to oxygen. There is evidence to suggest that *A. actinomycetemcomitans* can invade host tissues (Saglie *et al.*, 1987; Christersson *et al.*, 1987) and an ability to adapt to exposure to increased levels of oxygen and reactive oxygen species may facilitate the survival of the organism in this environment.

Within the periodontal pocket both biofilm and fluid-phase bacteria are likely to be present. Biofilm cells are known to differ from planktonic cells both morphologically and physiologically and in their virulence properties. A study by Meyer and Fives-Taylor (1994) demonstrated that fimbriae, present on the surface of biofilm cells of *A. actinomycetemcomitans*, were absent from liquid grown cells of the organism. When the protein profile of SAM extracted from planktonic cells of *A. actinomycetemcomitans* (SAM 3) was compared to that of biofilm cells of the organism grown on the same medium with agar added (SAM 1) a number of differences were observed. Most of the differences related to increased levels of particular proteins on the surface of planktonic cells. However one protein with a MW of 46.5 kDa was absent from the surface protein profile of planktonic cells and another protein with a MW of 20 kDa and a series of proteins with a MW of 44 kDa were present at reduced levels compared to the surface protein profile of biofilm cells. Whilst the 20 kDa protein has subsequently been identified as superoxide dismutase it is possible that the other proteins present at increased levels on the surface of biofilm cells may represent proteins involved in attachment and this certainly warrants further investigation.

Periodontal inflammation is associated with an increase in the flow of GCF and GCF flow rates of up to 20 μ l per hour have been recorded at disease-active sites (Goodson, 1989). GCF is a serum-derived exudate and in the current investigation horse serum was used to mimic GCF.

When the protein profile of SAM from biofilm cells of *A. actinomycetemcomitans* grown in the presence of 10% (v/v) horse serum (SAM 4) was compared with that of SAM from biofilm cells of the organism grown in the absence of serum (SAM 1), a number of differences were identified. In most cases the differences

observed represented reductions in the levels or loss of particular proteins from the protein profile.

In the periodontal pocket GCF may provide an important source of nutrients. The results of several *in vitro* studies have led to the suggestion that *in vivo*, degradation of salivary and GCF proteins and glycoproteins may be the result of the synergistic and concerted action of communities of bacteria (Bradshaw *et al.*, 1994; Homer and Beighton, 1992; ter Steeg *et al.*, 1987, 1988). Furthermore ter Steeg *et al.* (1987) showed that whilst human serum did not support the growth of *T. denticola* in pure culture, this organism flourished when it formed part of a consortium of bacterial species. Therefore the extent to which *A. actinomycetemcomitans* was able to use horse serum as a source of nutrients, particularly as the organism is not generally regarded as being highly proteolytic, may have been limited.

In addition to providing a potential source of nutrients, GCF may contain stimulatory or inhibitory factors. Enrichment studies involving the continuous culture of subgingival plaque in native human serum led to communities with a lower cell density, which were only able to utilise serum to a limited extent, compared to communities derived from continuous culture of subgingival plaque in heat-inactivated serum (ter Steeg *et al.*, 1988). It was proposed that the presence of inhibitory factors such as complement factor C3 in native serum may have resulted in the loss of key species from the consortium. Thus while growth of *A. actinomycetemcomitans* on semi-defined agar containing 10% (v/v) native horse serum was at least as good as that obtained on semi-defined agar without serum added, there may have been factors present which inhibited the production of certain surface-associated proteins. An alternative explanation might be that the serum did in fact provide some essential factor for the growth of *A. actinomycetemcomitans* and that the presence of serum resulted in the down-regulation of systems necessary for synthesising or scavenging this essential factor from non-serum supplemented semi-defined medium.

Iron sequestration represents an innate host defence mechanism and iron binding proteins such as transferrin, lactoferrin, haemoglobin and ferritin ensure that the level of free iron in host tissues is maintained at a level significantly lower than that required for bacterial growth. In order to survive the iron limiting environment encountered *in vivo*, pathogenic bacteria have evolved iron-

repressible mechanisms for the capture and uptake of iron. Systems for iron acquisition have also been described in oral bacteria (Winston and Dyer, 1994; Genco, 1995; Willemsem *et al.*, 1997).

In addition it has been shown that iron restriction can act as a trigger for the expression of virulence factors. For example toxin production by *Corynebacterium diphtheriae* was found to be maximal when cells were grown under iron limitation (Barksdale 1970). Similarly, levels of extracellular protease, elastase and exotoxin A of *P. aeruginosa* were greater under iron restricted conditions (Bjorn *et al.*, 1979).

Although ferrous sulphate was omitted from the trace element solution for the preparation of the iron-depleted medium, the contribution of elemental iron from the yeast extract added to the medium (2 g (w/v) per litre) would still have provided an excess of iron (final concentration 2.12 μM elemental iron - information provided by Oxoid). In an oxidised environment, iron will be present in its ferric (Fe^{3+}) state whilst under reduced conditions iron will exist in its ferrous (Fe^{2+}) state. As 2,2'-dipyridyl is a ferrous iron chelator it represented the most appropriate choice for the study of iron depletion under anaerobic conditions. Preliminary experiments were carried out to determine the maximum concentration of 2,2'-dipyridyl which did not inhibit growth of *A. actinomycetemcomitans* to any great extent. The concentration of 2,2'-dipyridyl selected to simulate iron restricted conditions (100 μM final concentration, data not shown) was very similar to that used in other studies concerning the effect of iron restriction on periodontopathogenic bacteria (Barua *et al.*, 1990; Spitznagel *et al.*, 1995).

When *A. actinomycetemcomitans* was grown under iron-depleted conditions, increases in the levels of certain proteins (with MWs of 70, 48 and 45 kDa) and a number of additional proteins (with MWs of 48, 48.5, 37.8, 35, 21 and 11.8 kDa) were observed on the protein profile of SAM extracted from the iron-starved cells (SAM 5). A number of other studies have demonstrated the induction of iron-repressible proteins by *A. actinomycetemcomitans* in response to iron limitation.

Winston *et al.*, (1993) demonstrated the presence of an additional 70 kDa protein in the outer membrane of *A. actinomycetemcomitans* grown under iron-restricted conditions. This protein was iron-repressible and on addition of iron in

the form of ferrous sulphate, levels of this protein decreased significantly. Spitznagel *et al.*, (1995) investigated the effect of iron on the production of leukotoxin by *A. actinomycetemcomitans*. Whilst iron-limitation did not appear to affect the levels of leukotoxin produced, it did induce the synthesis of two additional proteins with molecular weights of 30 and 70 kDa. In a more recent study Willemsem *et al.*, (1997) reported the cloning and sequencing of a gene encoding a periplasmic iron-regulated protein from *A. actinomycetemcomitans*. The 35 kDa protein designated AfuA demonstrated significant homology to the periplasmic iron transport proteins of *Haemophilus influenzae* (HitA, 80.4% identity) and *Neisseria meningitidis* (FbpA, 72.9% identity). Willemsem *et al.*, also demonstrated the presence of the 70 kDa protein in iron-starved cells of *A. actinomycetemcomitans*. At present, no role has been proposed for the 70 kDa protein of *A. actinomycetemcomitans* induced by iron limiting conditions. The 70 kDa protein (protein F) present at increased levels on the 2-D protein profile of SAM removed from iron-restricted cells (SAM 5) was therefore a prime candidate for identification. Unfortunately, attempts to obtain sequence information for protein F were unsuccessful.

SAM isolated from biofilm cells of *A. actinomycetemcomitans* grown on BHI agar supplemented with 6% (v/v) horse blood (SAM 6) demonstrated increased levels of certain proteins and a number of additional proteins compared to biofilm cells of the organism grown on a semi-defined medium (SAM 1). It is possible that some of these additional proteins may be derived from the blood itself, either as a result of binding of blood proteins to the bacterial cells or as a result of leaching of blood proteins from the agar during the harvesting procedure. Of particular interest however, is the significant increase in the levels of a protein with a MW of 56 kDa. A potent bone resorbing mediator present in *A. actinomycetemcomitans* SAM has been purified and demonstrates significant homology to the molecular chaperone GroEL from *E. coli* (Kirby *et al.*, 1995). Western blots of 2-D SDS-PAGE gels of *A. actinomycetemcomitans* SAM with a monoclonal antibody to the GroEL homologue (which neutralised bone resorbing activity) identified a protein with a MW of 62 kDa and a low pI. The position of the 56 kDa protein observed on the 2-D SDS-PAGE gels obtained in the current study corresponds exactly to that of the 62 kDa GroEL homologue identified on the 2-D SDS-PAGE gel obtained by Kirby *et al.* As significantly

increased levels of the 56 kDa protein were observed on the protein profile obtained for SAM extracted from biofilm cells of *A. actinomycescomitans* grown on BHI agar supplemented with horse blood, it might be postulated that nutrient rich conditions and bleeding may increase the bone resorbing potential of *A. actinomycescomitans in vivo*. Further work is necessary to confirm the identity of the 56 kDa protein as the *A. actinomycescomitans* GroEL homologue.

Four differentially expressed proteins were identified by N-terminal sequencing. Protein A was identified as triose phosphate isomerase, a key glycolytic enzyme. This finding does not represent the first demonstration of a glycolytic enzyme on the surface of a bacterium. In a study by Pancholi and Fischetti (1993) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified on the surface of group A streptococci. In addition to its GAPDH activity, the protein exhibited multiple binding activity to a number of mammalian proteins and ADP-ribosylating activity. More recently streptococcal surface GAPDH has been shown to mediate signal-transduction between streptococci and human pharyngeal cells (Pancholi and Vischetti, 1997) leading to the activation of intracellular pharyngeal protein tyrosine kinases. This group has also identified another key glycolytic enzyme, α -enolase, on the surface of group A streptococci. Like surface-associated GAPDH, surface-associated α -enolase appears to be multifunctional, and in addition to its glycolytic activity, was shown to be a strong plasmin(ogen) binding protein (Pancholi and Fischetti 1998). α -enolase has also been demonstrated in the cell wall of *Candida albicans* (Angiolella *et al.*, 1996). The function of surface-associated triose phosphate isomerase in *A. actinomycescomitans* remains to be determined.

The full length protein sequence obtained for protein B, determined by matching the N-terminal sequence obtained in the current study to an open reading frame in the *A. actinomycescomitans* genome database showed a strong homology to an *E. coli* thiol peroxidase described by Cha *et al.* (1995).

Peroxidases are enzymes which catalyse the reduction of alkyl hydroperoxides and hydrogen peroxide. The thiol peroxidase identified by Cha *et al.* (1995) termed p20, showed only distant homology to the thiol dependent hydroperoxide reductase (AhpC/AhpF) already described for *E. coli* and it was

suggested that p20 represented a new subtype of thiol-dependent antioxidant enzyme. p20 was dependent on the presence of a thiol reducing equivalent such as dithiothreitol for activity and *in vivo* the thioredoxin/ thioredoxin reductase/ NADH system acted as a thiol regenerating system. The MW of this thiol peroxidase was 20 kDa (hence p20) which is similar to that observed for protein B (21 kDa). The AhpC/AhpF thiol peroxidase enzyme of *E. coli* was shown to reside mainly in the cytoplasm, whilst p20 was localised mainly in the periplasmic space. Thus AhpC/AhpF may act to remove intracellular peroxides whilst p20 may destroy exogenous peroxides.

In a more recent report Cha *et al.* (1996) have shown significant homology of the thiol peroxidase p20 to a gene product of the ToxR regulon of *H. influenzae* and *Vibrio cholerae* and the coaggregation mediating adhesins of *S. sanguis*, *Streptococcus gordonii* and *Streptococcus parasanguis* and a new thiol peroxidase subfamily was proposed. This subfamily has been further characterised by Wan *et al.* (1997) and Zhou *et al.* (1997) who have termed the thiol peroxidase subfamily the scavengases. Two conserved cysteine residues are present at position 94 (Cha *et al.*, 1996) and 61 (Zhou *et al.*, 1997) of the scavengases and these have been shown to be essential for peroxidase activity. Cysteine residues are present at equivalent positions in the amino acid sequence obtained from the *A. actinomycetemcomitans* genome database, which showed a 100% match with the first 13 amino acids of the N-terminal sequence obtained for protein B, and thus it seems likely that another member of this thiol peroxidase subfamily, the scavengases, has been identified.

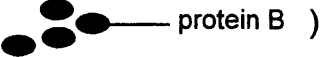
Protein C, the full length sequence of which was derived from the *A. actinomycetemcomitans* genome database, was identified as a [Cu,Zn]-superoxide dismutase. Superoxide dismutase (SOD) catalyses the dismutation of the highly reactive superoxide radical anion to H₂O₂ (which could in turn be acted on by peroxidase or catalase) and molecular oxygen. Two main classes of SOD have been identified in bacteria; manganese or iron-cofactored SOD ([Mn]-SOD and [Fe]-SOD respectively) and the more recently identified copper and zinc-cofactored SOD ([Cu,Zn]-SOD). [Mn]- and [Fe]-SODs are found in the bacterial cytosol whilst [Cu,Zn]-SOD exhibits a periplasmic location. [Cu,Zn]-SOD has been identified in an increasing range of bacteria including *H. influenzae* and *Haemophilus parainfluenzae* (Kroll *et al.*, 1991), *A.*

actinomycetemcomitans (Kroll *et al.*, 1995), *Salmonella* (Canvin *et al.*, 1996; Farrant *et al.*, 1997) and *N. meningitidis* (Wilks *et al.*, 1998).

Periplasmic [Cu,Zn]-SOD and thiol peroxidase may contribute to the pathogenicity of *A. actinomycetemcomitans in vivo* by providing protection against oxygen radical-mediated host defences.

Protein D (MW 14 kDa) demonstrated a strong homology to the *yfiD* gene product (YfiD) of *E. coli*. Expression of *yfiD* is regulated by FNR (Green *et al.*, 1998), an oxygen-responsive transcription regulator. FNR senses anaerobiosis through the acquisition of an oxygen-labile [4Fe-4S]²⁺ cluster that promotes dimerization of the protein and enhances site-specific DNA binding (Green *et al.*, 1996; Lazazzera *et al.*, 1996). FNR homologues have been identified in a number of bacteria including *C. ochracea*, *Capnocytophaga sputigena*, *Haemophilus aphrophilus*, *A. actinomycetemcomitans* (Hattori *et al.*, 1996) and *Actinobacillus pleuropneumoniae* (Green and Baldwin, 1997). *YfiD* expression in exponential cultures was 13 times higher under anaerobic conditions compared to aerobic conditions and a further 1.7-fold increase in the level of expression was observed in anaerobic stationary phase cultures compared to anaerobic exponential phase cultures (Green *et al.*, 1998).

The *yfiD* gene product (YfiD) is a 14 kDa protein of unknown function that shows strong homology to the C-terminal domain of pyruvate formate-lyase (PFL). Genes potentially encoding YfiD proteins have been identified in *Serratia liquefaciens*, *H. influenzae* and the bacteriophages T4 and T5 (Green *et al.*, 1998). PFL is enzymatically interconverted between its active and inactive form and this interconversion is dependent on oxygen levels. Enzymatic conversion of PFL to its active form occurs only under anaerobic conditions and results in the introduction of an organic free radical on a glycyl residue in the C-terminal portion of the protein. During the transition from anaerobic to aerobic growth the enzyme is converted back to its inactive form by enzymatic removal of the glycyl free radical (Sawers and Watson, 1998). The C-terminal 60 amino acids of the YfiD proteins identified so far exhibit approximately 80% homology with the C-terminal of PFL. The remaining N-terminal 67 amino acids of the YfiD proteins however lack any similarity to PFL suggesting that the YfiD proteins may represent a new class of oxygen-responsive glycyl radical enzymes.

The differential expression observed for proteins A - D was not always what might have been expected particularly with respect to the antioxidant enzymes thiol peroxidase and SOD. Protein B (thiol peroxidase) was absent from the surface of *A. actinomycetemcomitans* cells grown in a CO₂-enriched aerobic atmosphere (SAM 2) yet was present on the surface of cells of the organism incubated anaerobically (SAM 3, 5 and 6). Protein B was located within a cluster of four proteins with very similar MWs and pIs ( protein B)

The two middle proteins of this cluster were present in greater amounts on the surface protein profile of *A. actinomycetemcomitans* grown in a CO₂-enriched aerobic atmosphere compared to anaerobically grown cells (with the exception of cells grown in the presence of blood) and it possible that these spots represent the same enzyme with slightly different post-translational modifications. Other antioxidant enzymes such as catalase may have been present on the surface of *A. actinomycetemcomitans* which would affect the total antioxidant capacity of the organism. Further proteomic analysis of the surface of *A. actinomycetemcomitans* is needed to determine the full complement of surface proteins produced by the organism under different growth conditions.

This study has shown that environmental factors do affect the surface protein profile of *A. actinomycetemcomitans*. An ability to adapt to prevailing environmental conditions may facilitate the survival of the organism in the continually changing microenvironment of the periodontal pocket. In addition, certain environmental conditions such as the presence of blood may act as a trigger for the expression of a more virulent phenotype.

Chapter 7

Immune response to *A. actinomycetemcomitans* grown under different environmental conditions

7.1 Introduction

It is well established that patients with periodontal disease demonstrate elevated serum antibody responses to periodontopathogenic bacteria (Genco *et al.*, 1980). In addition there is evidence to suggest that the specificity of the serum antibodies reflects the known association between the form of the disease and the proposed aetiological agent(s) (Wilton *et al.*, 1991). Thus elevated levels of serum IgG antibodies to *A. actinomycetemcomitans* are observed in patients with LJP, whilst patients with adult onset rapidly progressive periodontitis demonstrate increased levels of serum IgG antibodies to *P. gingivalis* (Meghji *et al.*, 1995).

Sera from patients with LJP may provide a useful tool for the identification of molecules expressed by *A. actinomycetemcomitans* *in vivo* where conditions are likely to be very different from that encountered by the organism *in vitro*. In addition, screening of SAM isolated from *A. actinomycetemcomitans* grown under different environmental conditions with LJP sera may also provide information on the effect of the environment on the antigenic properties of the organism.

7.2 Aims

- i) to identify the major surface antigens of *A. actinomycetemcomitans*
- ii) to determine the effect of environmental conditions on the surface antigen profile of *A. actinomycetemcomitans*

7.3 Materials and methods

7.3.1 Preliminary screening of LJP and control sera

Four LJP sera (obtained from LJP patients attending the Eastman Dental Institute) and four control sera (obtained from healthy student dental nurses) were screened against one type of SAM (SAM 1) from *A. actinomycetemcomitans* by Western blotting as described in Ch 2.

6 µg of protein was loaded in each lane. The primary antibody was used at a dilution of 1:250 whilst the secondary antibody (peroxidase-conjugated goat anti-human IgG, Sigma) was used at a dilution of 1:1000. DAB was used as a substrate for peroxidase.

7.3.2 Immunoreaction of control and LJP sera with SAM from *A. actinomycetemcomitans* grown under different environmental conditions

The LJP serum giving the strongest response and the control serum giving the weakest response in the preliminary screening were selected for use in the immunoreaction experiments with SAM from *A. actinomycetemcomitans* grown under different environmental conditions.

6 µg of protein per lane and 200 µg of protein were loaded for 1-D and 2-D gel electrophoresis respectively.

Western blotting was carried out as described in Ch 2 except that PBS containing 0.1% Tween-20 was used in place of PBS-0.1% Triton-X-100. The primary antibody was used at a dilution of 1:250 whilst the secondary antibody (peroxidase-conjugated goat anti-human IgG) was used at a dilution of 1:1000. Enhanced chemiluminescence (ECL) was employed as the detection system using a commercially available kit (Amersham).

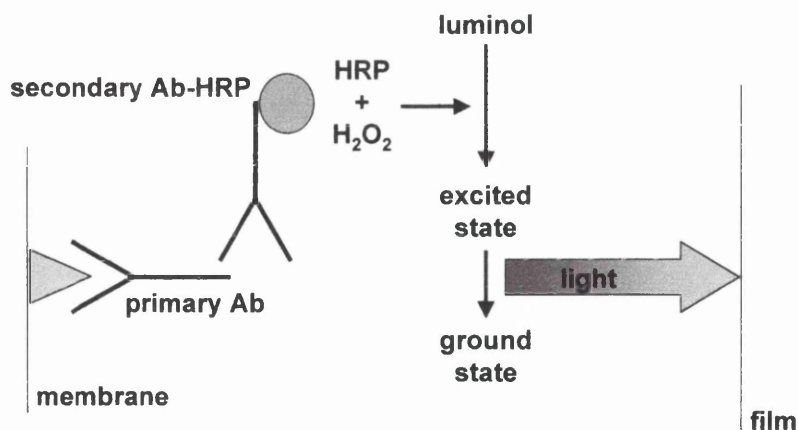


Figure 7.1 Principle of enhanced chemiluminescence (ECL) Western blotting

The principle of ECL is outlined in Fig. 7.1. Horseradish peroxidase (HRP) together with H₂O₂ catalyse the oxidation of luminol under alkaline conditions. Immediately following oxidation, the luminol is in an excited state which then decays to the ground state via a light-emitting pathway.

7.4 Results

7.4.1 Preliminary screening of LJP and control sera

Fig. 7.2 shows the immunoreaction of 4 control sera and 4 LJP sera with SAM from *A. actinomycetemcomitans*. Serum 1 gave the strongest reaction with *A. actinomycetemcomitans* SAM, whilst serum 7 showed the weakest reaction and thus these 2 sera were selected for use in subsequent experiments.

7.4.2 Surface protein and surface antigen profiles of *A. actinomycetemcomitans* grown under different environmental conditions - 1-D analysis

Fig. 7.3 shows the surface protein profiles of *A. actinomycetemcomitans* grown under different environmental conditions. Figs. 7.4 and 7.5 show the immunoreaction of SAM from *A. actinomycetemcomitans* grown under different environmental conditions with control and LJP serum respectively. In order to clarify the position of the bands on Figs. 7.3 - 7.5, each band (SAM 1 only) has been marked with a black or white (depending on the intensity of the band) line. To facilitate comparison between the 1-D SDS-PAGE gel and the Western blots with control and LJP sera, a number of bands with identical molecular mass have been tagged with a red line. The molecular masses of the major surface proteins and the major surface antigens of *A. actinomycetemcomitans* grown as a biofilm on a semi-defined medium incubated anaerobically can be seen in Table 7.1. Red circles correspond to bands on Figs. 7.3 - 7.5 which have been tagged with a red line.

The immunoreaction of the LJP serum with the *A. actinomycetemcomitans* SAM was far stronger than with the control serum. Even though the blot incubated with LJP serum was exposed to the light-sensitive film for a shorter period of time compared to the blot incubated with control serum, the image obtained was considerably more intense and in certain regions, particularly between 40 - 60 kDa, individual bands were quite difficult to discern.

A large proportion of bands recognised by the LJP serum were also recognised by the control serum, although as already discussed, the reaction was generally weaker. The blot with LJP serum demonstrated two additional low MW bands of 13.5 and 19 kDa which were not present on the blot with control serum. The

control serum showed a particularly strong response to the 8 and 14.5 kDa proteins present in *A. actinomycetemcomitans* SAM.

Proteins which are present in large amounts on the surface of *A. actinomycetemcomitans* may not necessarily induce a strong immune response. For example on Fig. 7.3 a band with a MW of 36 kDa dominated the protein profile of *A. actinomycetemcomitans* SAM. Whilst two bands with similar MWs (34.5 and 38kDa) to the 36 kDa protein could be observed on the blot with LJP serum, the intensity of these bands is proportionally less compared to the 1-D SDS-PAGE gel. Conversely a protein which is present in only small amounts may be highly immunogenic. Thus the very intense 8 kDa band obtained on the blot with LJP serum had only a weak counterpart on the 1-D SDS-PAGE gel.

The immunoreaction of each of the SAMs with serum from a patient with LJP was compared against SAM 1.

Comparison of SAM 2 with SAM 1

- i) additional band with a MW of 96 kDa
- ii) additional band with a MW of 74 kDa
- iii) increased amounts of a band with a MW of 32 kDa

Comparison of SAM 3 with SAM 1

The immunoreaction of SAM 3 with LJP serum was weak compared to SAM 1 and a number of bands were absent

Comparison of SAM 4 with SAM 1

- i) additional band with a MW of 104 kDa
- ii) absence of a band with a MW of 16 kDa

Comparison of SAM 5 with SAM 1

The immunoreaction of SAM 5 with LJP serum was weaker than with SAM 1 and a number of bands are present in lower amounts

- i) additional band with a MW of 74 kDa
- ii) additional band with a MW of 53 kDa
- iii) increased amounts of a band with a MW of 32 kDa

Comparison of SAM 6 with SAM 1

- i) additional band with a MW of 78 kDa
- ii) decreased amounts of a band with a MW of 68 kDa
- iii) decreased amounts of a band with a MW of 61 kDa
- iv) additional band with a MW of 53 kDa
- v) decreased amounts of a band with a MW 26 kDa
- vi) decreased amounts of a band with a MW 22.5 kDa
- vii) decreased amounts of a band with a MW 15 kDa
- viii) absence of a band with a MW of 13.5 kDa
- ix) decreased amounts of a band with a MW of 8 kDa

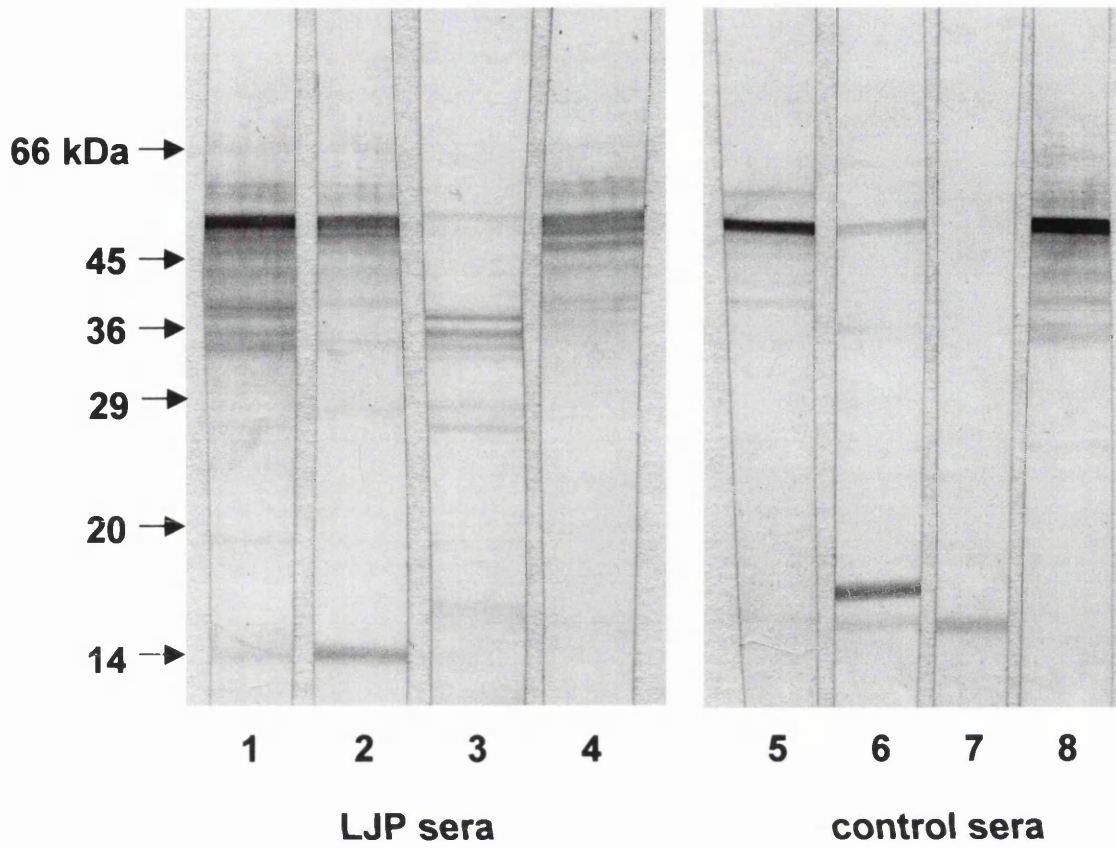


Figure 7.2 Preliminary screening of sera from 4 LJP patients (Lanes 1 – 4) and 4 control patients (Lanes 5 – 8) against SAM from *A. actinomycetemcomitans*. 6 μ g of protein were loaded in each lane.

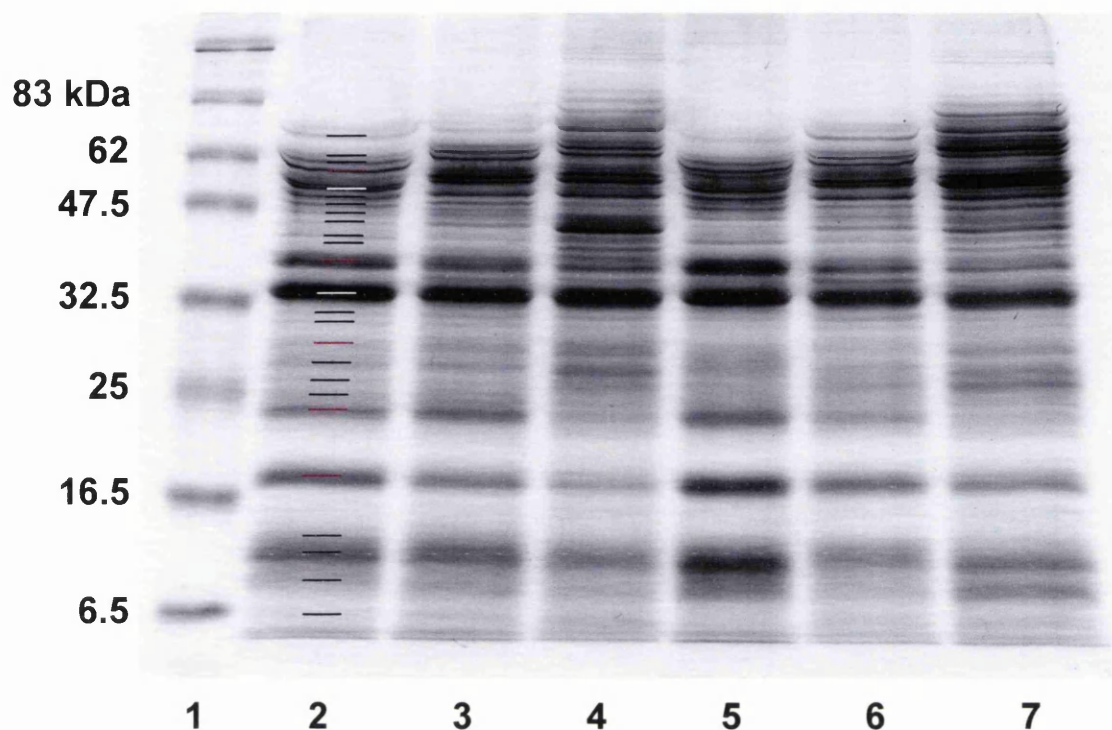


Figure 7.3 One-dimensional SDS-PAGE of SAM isolated from *A. actinomycetemcomitans* grown under different environmental conditions. 3 μg of protein from each SAM were separated on a 12% gel and stained with Brilliant blue G - Colloidal. Lane 1, molecular weight markers; Lane 2, SAM isolated from biofilm cells grown on semi-defined agar incubated anaerobically (SAM 1); Lane 3, SAM isolated from biofilm cells grown on semi-defined agar incubated in a CO_2 -enriched aerobic atmosphere (SAM 2); Lane 4, SAM isolated from planktonic cells grown in semi-defined broth incubated anaerobically (SAM 3); Lane 5, SAM isolated from biofilm cells grown on semi-defined agar supplemented with 10% (v/v) horse serum incubated anaerobically (SAM 4); Lane 6, SAM isolated from biofilm cells grown on semi-defined agar containing 100 μM 2,2'-dipyridyl incubated anaerobically (SAM 5); Lane 7, SAM isolated from biofilm cells grown on BHI agar supplemented with 6% (v/v) horse blood incubated anaerobically (SAM 6).

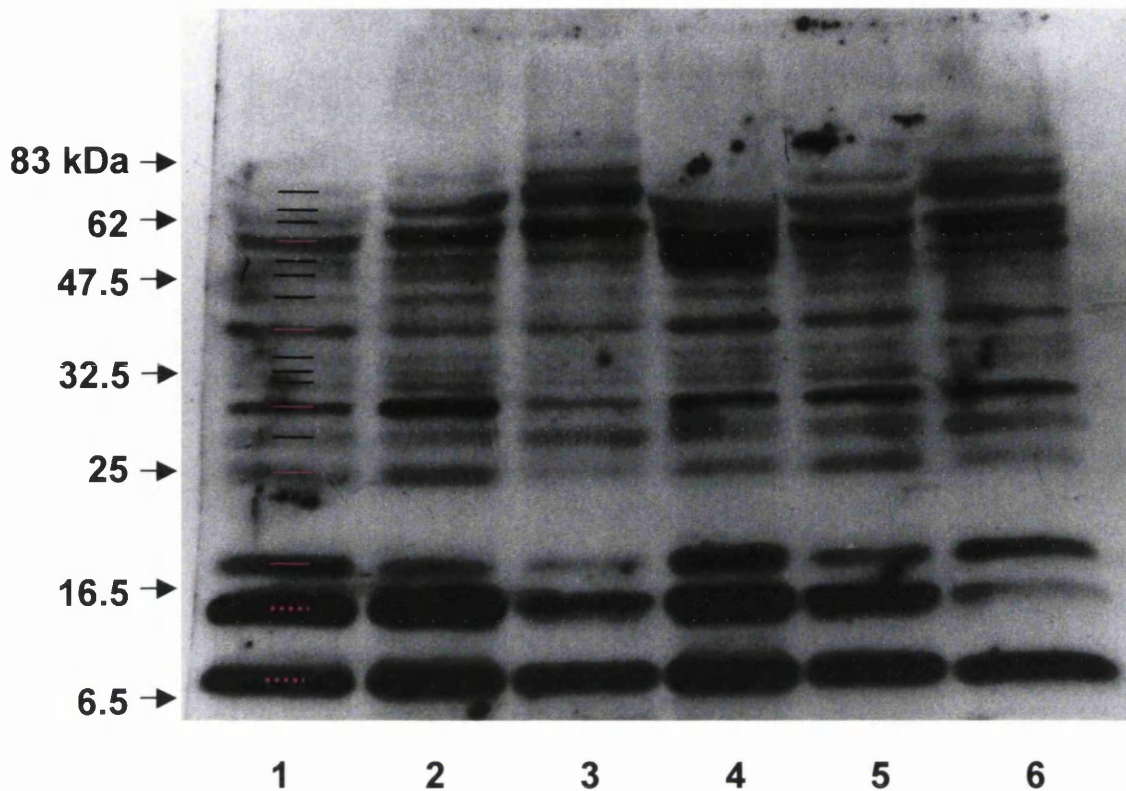


Figure 7.4 Immunoreaction of control serum with SAM isolated from *A. actinomycetemcomitans* grown under different environmental conditions. 6 μg of protein from each SAM were loaded. Lane 1, SAM isolated from biofilm cells grown on semi-defined agar incubated anaerobically (SAM 1); Lane 2, SAM isolated from biofilm cells grown on semi-defined agar incubated in a CO_2 -enriched aerobic atmosphere (SAM 2); Lane 3, SAM isolated from planktonic cells grown in semi-defined broth incubated anaerobically (SAM 3); Lane 4, SAM isolated from biofilm cells grown on semi-defined agar supplemented with 10% (v/v) horse serum incubated anaerobically (SAM 4); Lane 5, SAM isolated from biofilm cells grown on semi-defined agar containing 100 μM 2,2'-dipyridyl incubated anaerobically (SAM 5); Lane 6, SAM isolated from biofilm cells grown on BHI agar supplemented with 6% (v/v) horse blood incubated anaerobically (SAM 6). The positions of the molecular weight markers are indicated on the left-hand side of the blot.

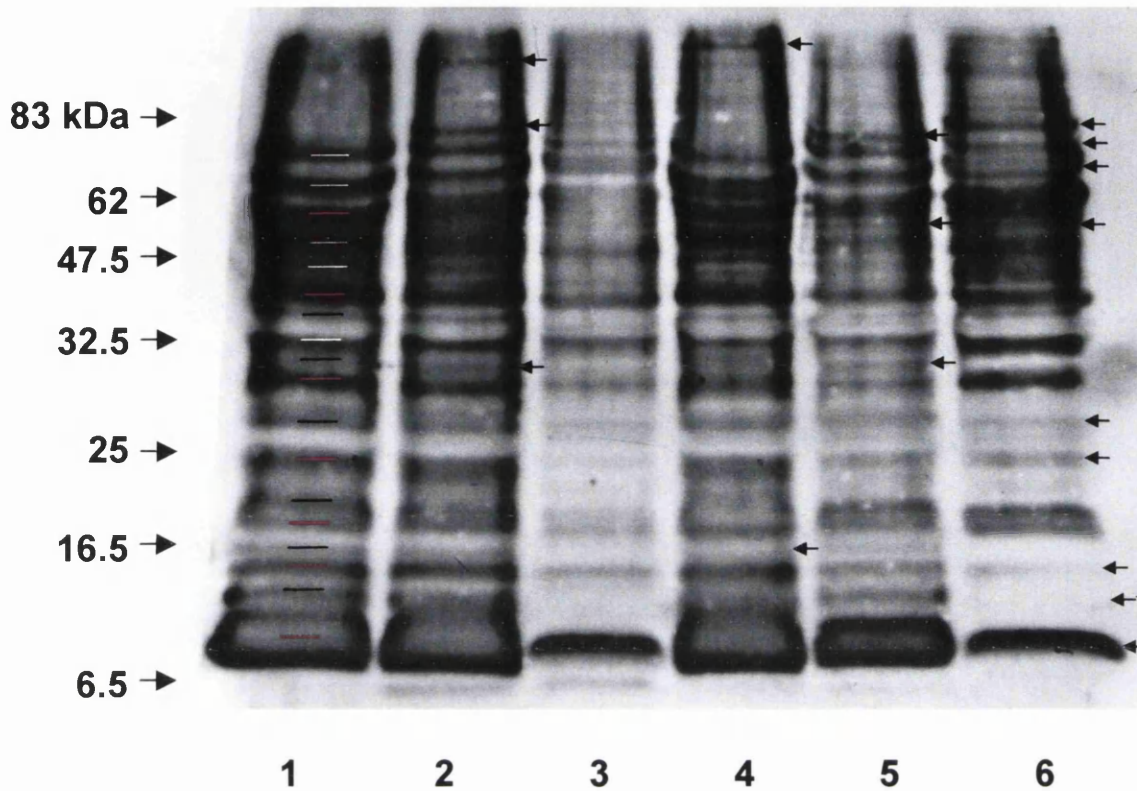


Figure 7.5 Immunoreaction of serum from an LJP patient with SAM isolated from *A. actinomycetemcomitans* grown under different environmental conditions. 6 μ g of protein from each SAM were loaded. Lane 1, SAM isolated from biofilm cells grown on semi-defined agar incubated anaerobically (SAM 1); Lane 2, SAM isolated from biofilm cells grown on semi-defined agar incubated in a CO₂-enriched aerobic atmosphere (SAM 2); Lane 3, SAM isolated from planktonic cells grown in semi-defined broth incubated anaerobically (SAM 3); Lane 4, SAM isolated from biofilm cells grown on semi-defined agar supplemented with 10% (v/v) horse serum incubated anaerobically (SAM 4); Lane 5, SAM isolated from biofilm cells grown on semi-defined agar containing 100 μ M 2,2'-dipyridyl incubated anaerobically (SAM 5); Lane 6, SAM isolated from biofilm cells grown on BHI agar supplemented with 6% (v/v) horse blood incubated anaerobically (SAM 6). The positions of the molecular weight markers are indicated on the left-hand side of the blot.

1-D SDS-PAGE gel of <i>A.a</i> SAM stained with Brilliant blue		1-D Western blot of <i>A.a</i> SAM + control serum		1-D Western blot of <i>A.a</i> SAM + LJP serum	
band no.	MW (kDa)	band no.	MW (kDa)	band no.	MW (kDa)
1	67	1	68	1	68
2	62	2	64	2	61
3	61	3	61	③	56
④	58	④	56	4	50
5	55	5	53	5	46
6	53	6	50	⑥	41
7	51	7	47	7	38
8	50	⑧	41	8	34.5
9	48	9	37	9	32
10	46	10	35.5	⑩	30
11	44	11	33.5	11	26
⑫	40.5	⑫	31	⑫	22.5
13	36	13	27.5	13	19
14	34	⑭	24	⑭	18
15	33	⑮	17	15	16
⑯	30	⑰	14.5	⑰	15
17	28	⑱	8	17	13.5
18	26			⑱	8
19	25				
⑳	23				
㉑	17.5				
22	14				
23	13.5				
24	12.5				
25	6.5				

Table 7.1

Table showing the molecular weights of the major surface proteins and major surface antigens of biofilm cells of *A. actinomycetemcomitans* grown on a semi-defined agar incubated anaerobically

7.4.3 Surface protein and surface antigen profiles of *A. actinomycetemcomitans* grown under different environmental conditions - 2-D analysis

Fig. 7.6 shows the surface protein profile of biofilm cells of *A. actinomycetemcomitans* grown on a semi-defined agar incubated anaerobically. Figs. 7.7a-f show the immunoreaction of SAM from *A. actinomycetemcomitans* grown under different environmental conditions with LJP serum. In order to facilitate correlation of the 2-D SDS-PAGE gel and the 2-D Western blots a number of proteins on the 2-D gel and their relative position on the 2-D blots have been marked with a red dot. Only proteins that were common to all 6 SAMs were selected as marker proteins.

The 56 kDa protein and the series of proteins with a MW of 44 kDa appeared to be among the immunodominant surface proteins of *A. actinomycetemcomitans* and these proteins were consistently present in the antigen profiles of SAM isolated from *A. actinomycetemcomitans* irrespective of growth conditions.

It is interesting to note that no IgG response was detected against the two major surface proteins with a MW of 37 kDa and that the IgG response to the 23.5 kDa surface protein was generally weak (except for SAM 6).

The 2-D Western blots of the six different SAMs were exposed to light sensitive film for varying lengths of time in an attempt to obtain an image with the greatest number of spots, whilst maintaining the background at a reasonably low level. Thus it is difficult to make direct comparisons between the blots as a spot which was present on one blot may have been absent from another simply because the background level was higher and therefore the blot was exposed to the film for a shorter period of time. However a number of notable features were observed. These have been indicated on the antigen profiles by means of an arrow and are listed below.

Key features of antigen profile of SAM 1

The MWs of the main antigenic determinants of SAM 1 were 67, 66, 56, 55 (series of 3 spots), 51 (2 spots), 48 (2 spots), 44.5, 44 (series of spots) 38, 36.5 (2 spots) and 14 kDa. A smear with a MW ranging from 30 - 100 kDa was observed on the right-hand side of the blot and another smear with a MW ranging from 44 - 50 kDa was observed on the left-hand side of the blot.

Key features of antigen profile of SAM 2

The MWs of the immunodominant molecules of SAM 2 were 66, 56, 53, 51, 48 (series of 3 spots), 47, 44.5, 44 (series of spots), 43, 38, 36.5, 29.5, 29, 28 and 27.5 kDa. The presence of a spot with a MW of 53 kDa corresponded with an increase in a cluster of proteins with a MW of 54 kDa on the 2-D SDS-PAGE gel of SAM 2 (Ch 6). An intense smear with a MW ranging from 27 - 100 kDa was observed on the right-hand side of the blot.

Key features of antigen profile of SAM3

The antigen profile of SAM 3 demonstrated a strong immunoreaction with a series of proteins with a MW of 48 kDa and two proteins with a MW of 46 kDa. This parallels the pattern observed on the corresponding 2-D SDS-PAGE gel (Ch 6) where increased amounts of the 48 and the 46 kDa proteins were observed. The MWs of the other immunodominant molecules of SAM 3 were 66, 56, 55 (series of spots), 51 (2 spots), 44 (series of spots), 42, 41, 38, 36.5 (2 spots), 28, 21, 14 and 13.5 kDa. A high MW smear of approximately 100 kDa, a smear ranging from 20 - 100 kDa and a smear with a MW of 38 - 44 kDa were obtained. In addition two regions with MWs ranging from 16 - 21 kDa and 13.5 - 15 kDa demonstrated a particularly strong immunoreaction with the LJP serum.

Key features of antigen profile of SAM 4

The MWs of the immunodominant molecules of SAM 4 were 56, 51, 44 (series of proteins), 38, 36.5 (2 separate spots), 28, 25, 18 (cluster of spots), 17, 16 and 15 (cluster of spots) kDa. Two smears running the entire length of the blot were observed on the right-hand side and another smear with a MW ranging from 38 - 50 kDa was observed on the left-hand side of the blot.

Key features of antigen profile of SAM 5

The background level obtained on the 2-D Western blot of SAM 5 was quite high and distinct spots were difficult to discern. However antigenic determinants with MWs of 65, 56, 51 (2 separate spots), 48, 44 (single well isolated spot plus a series of spots) 28, 27.5, 23.5 and 23 kDa were identified. In the low MW region of the blot a number of more clearly distinguishable spots with MWs of 18, 17.5 (2 separate spots), 17, 16.5, 14 (cluster of several spots) and 13.5 kDa

could be observed. Two smears with MWs of 18 and 13.5 kDa and a smear with a MW of 38 - 47 kDa were also obtained.

Key features of antigen profile of SAM 6

A strong immunoreaction was obtained with antigens with MWs of 66, 55 (series of spots), 51, 48 (series of spots), 46, 44 (series of spots), 38, 36.5, 36 (2 separate spots), 28, 27, 23.5, 23, 21, 19, 14.5 and 14 kDa. The strong reaction with the series of proteins with a MW of 48 kDa and the 46 kDa protein reflected the increased amounts of these proteins observed on the corresponding 2-D SDS-PAGE gel (Ch 6). In contrast the 56 kDa protein, which was present in large amounts on the 2-D SDS-PAGE gel, was represented by a proportionally weaker spot on the antigen profile of SAM 6. A high MW smear at approximately 100 kDa and two low MW smears at 17 and 14 kDa were observed.

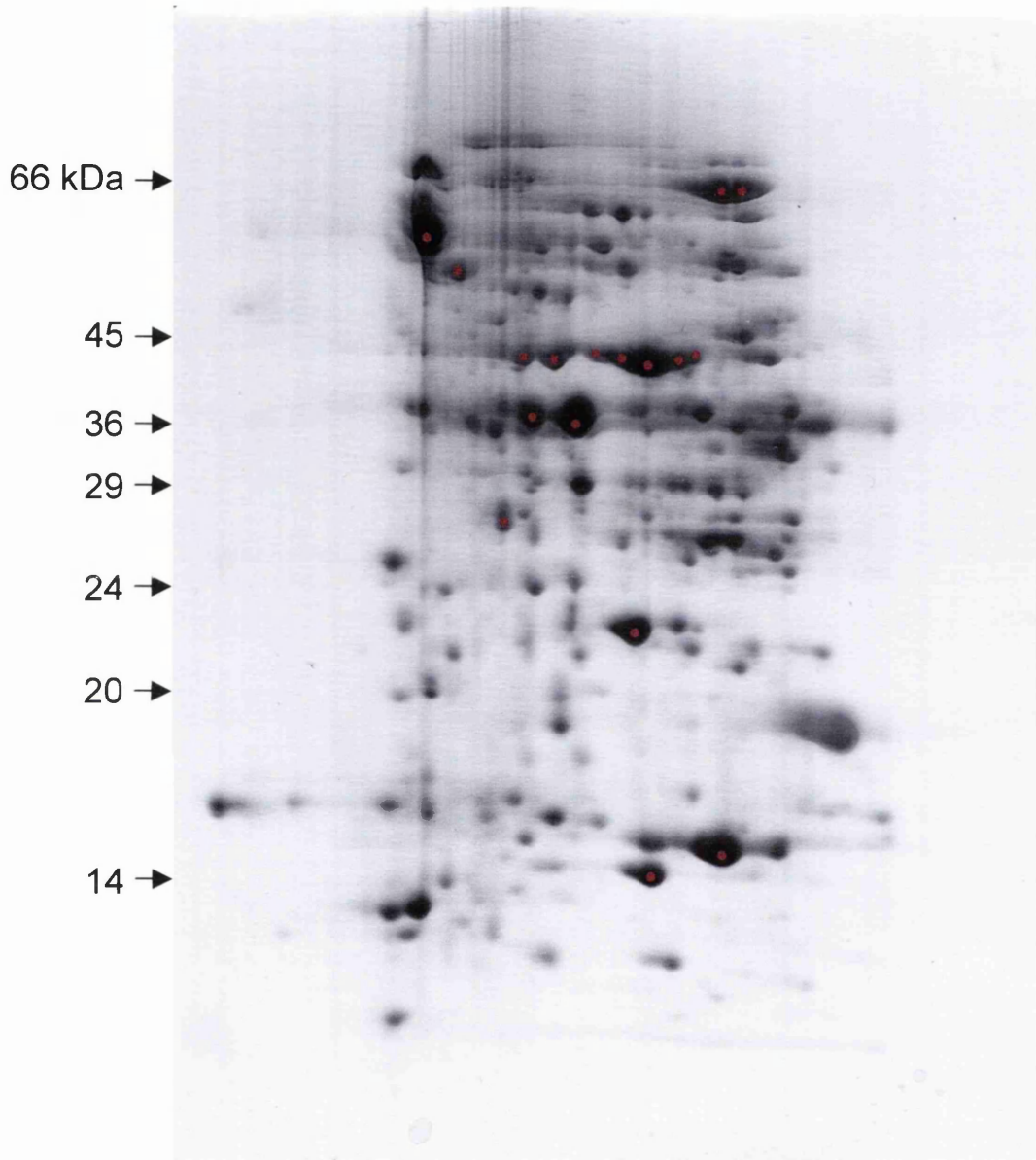


Figure 7.6 Two-dimensional SDS-PAGE of SAM isolated from biofilm cells of *A. actinomycetemcomitans* grown on semi-defined agar incubated anaerobically. 200 μg of protein were loaded. The gel was stained with Brilliant blue G - Colloidal. The positions of the molecular weight markers are indicated on the left-hand side of the gel.

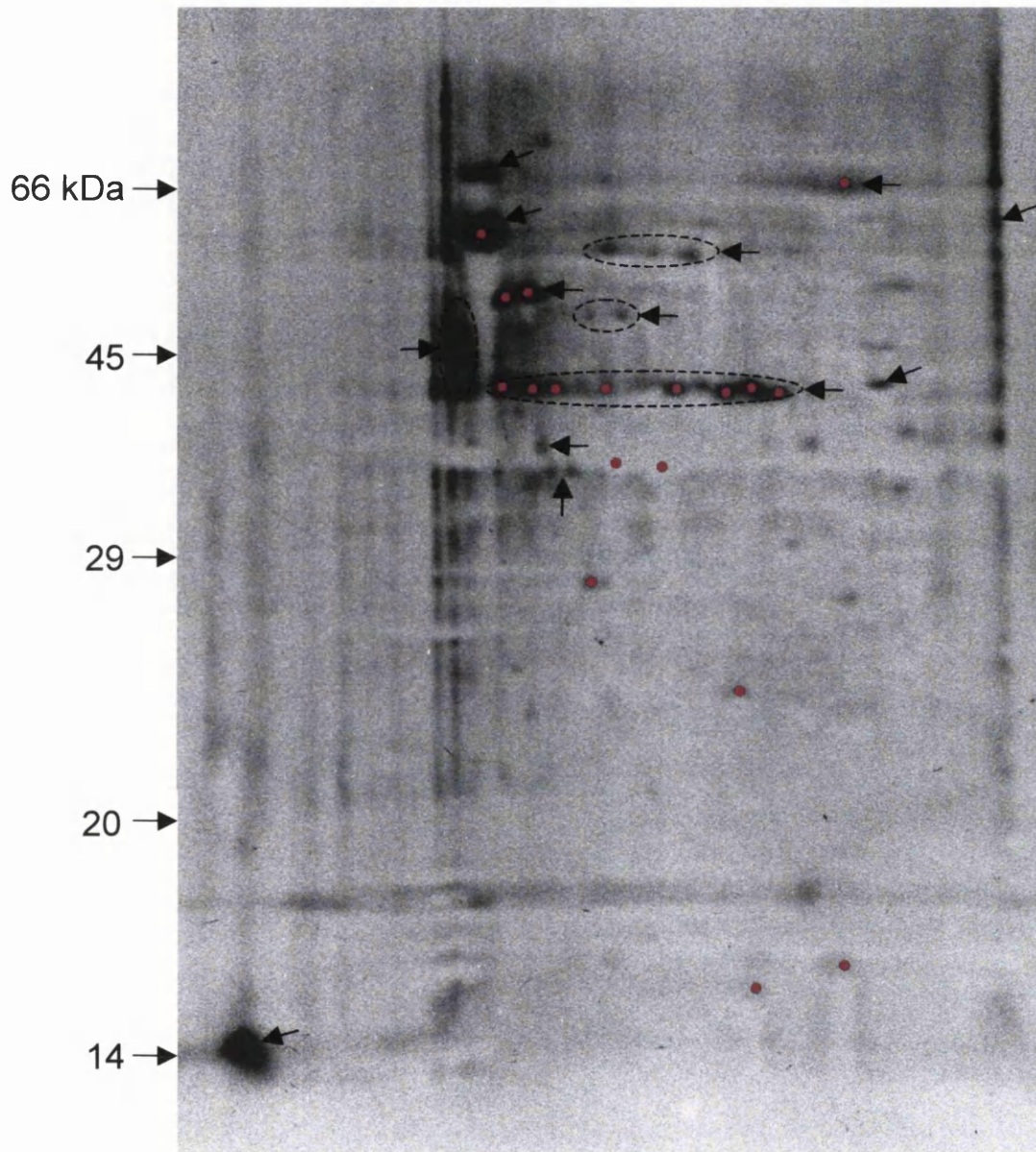


Figure 7.7a Two-dimensional Western blot of SAM isolated from biofilm cells of *A. actinomycetemcomitans* grown on a semi-defined agar incubated anaerobically (SAM 1). Serum from a patient with LJP was used as the primary antibody.

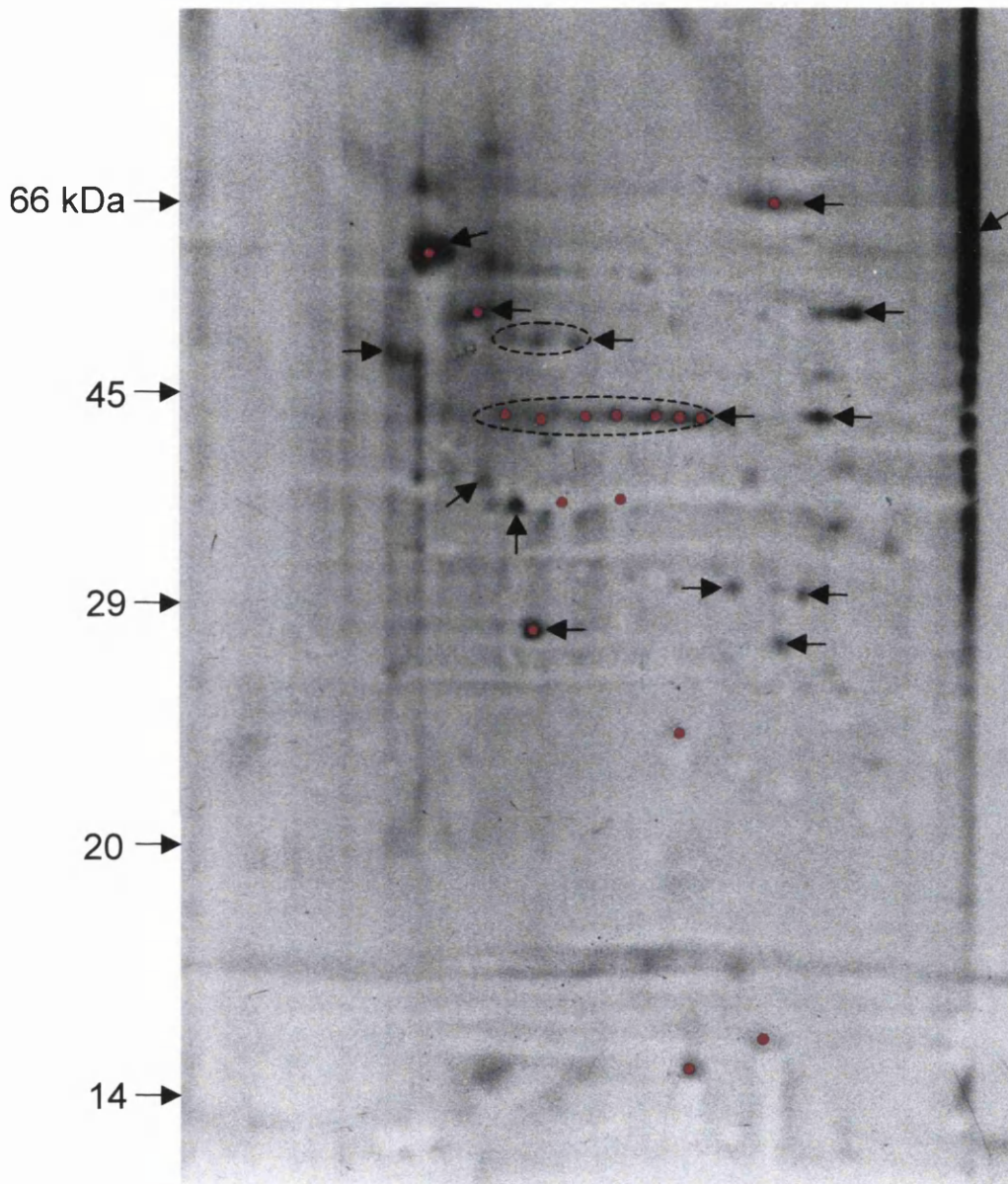


Figure 7.7b Two-dimensional Western blot of SAM isolated from biofilm cells of *A. actinomycetemcomitans* grown on a semi-defined agar incubated in a CO₂-enriched aerobic atmosphere (SAM 2). Serum from a patient with LJP was used as the primary antibody.

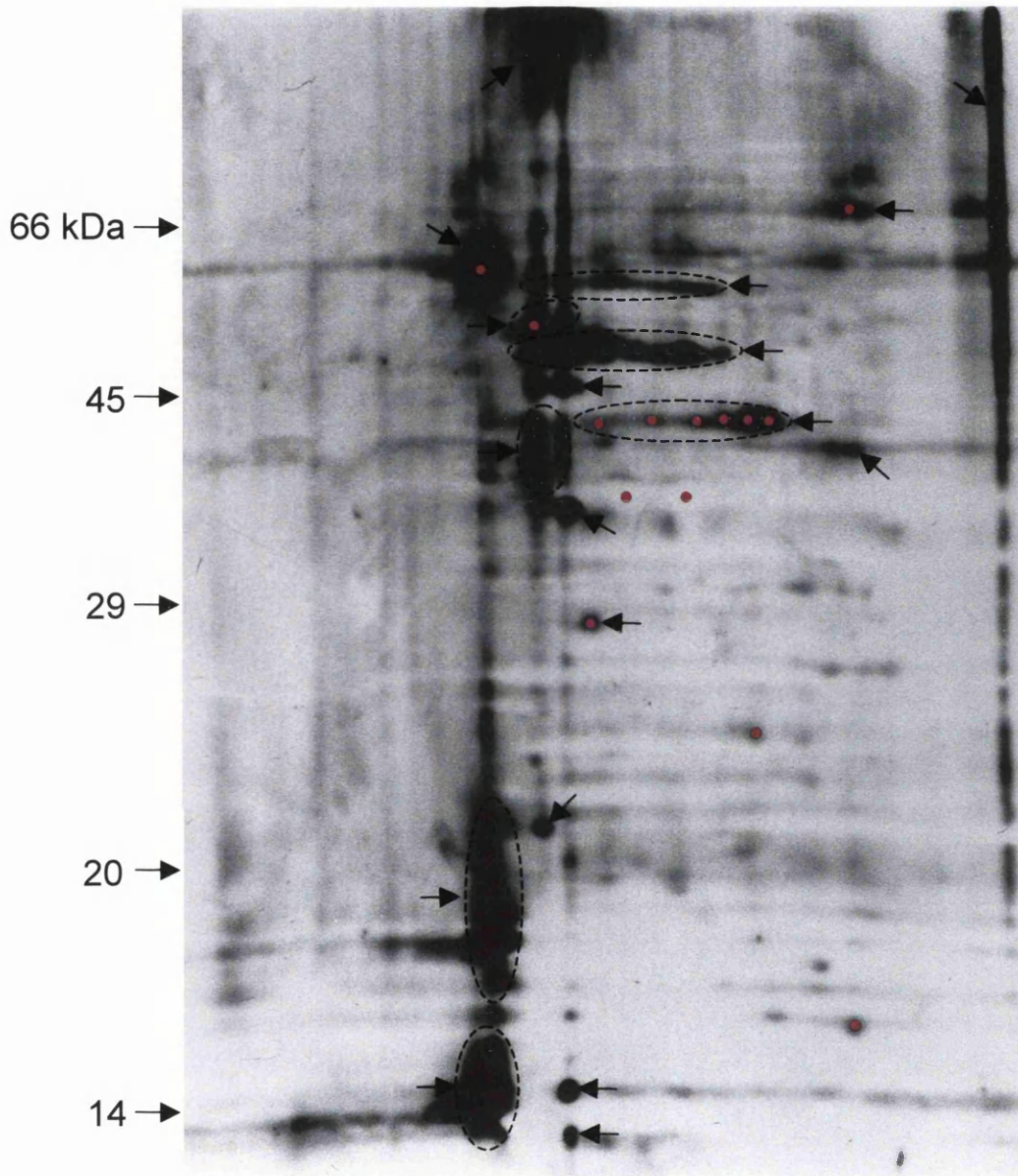


Figure 7.7c Two-dimensional Western blot of SAM isolated from planktonic cells of *A. actinomycetemcomitans* grown in a semi-defined broth incubated anaerobically (SAM 3). Serum from a patient with LJP was used as the primary antibody.

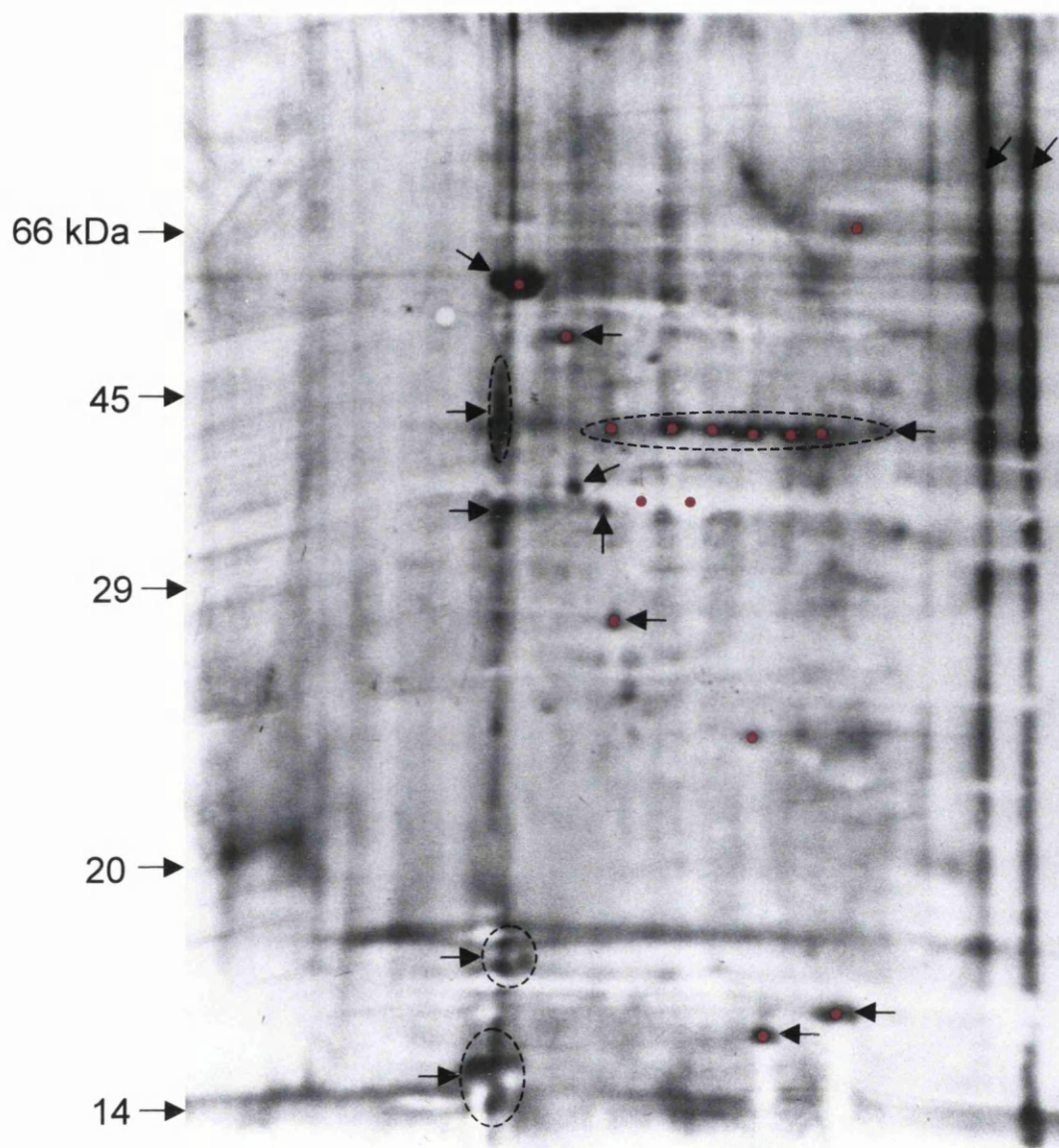


Figure 7.7d Two-dimensional Western blot of SAM isolated from biofilm cells of *A. actinomycetemcomitans* grown on a semi-defined agar supplemented with 10% (v/v) horse serum incubated anaerobically (SAM 4). Serum from a patient with LJP was used as the primary antibody.

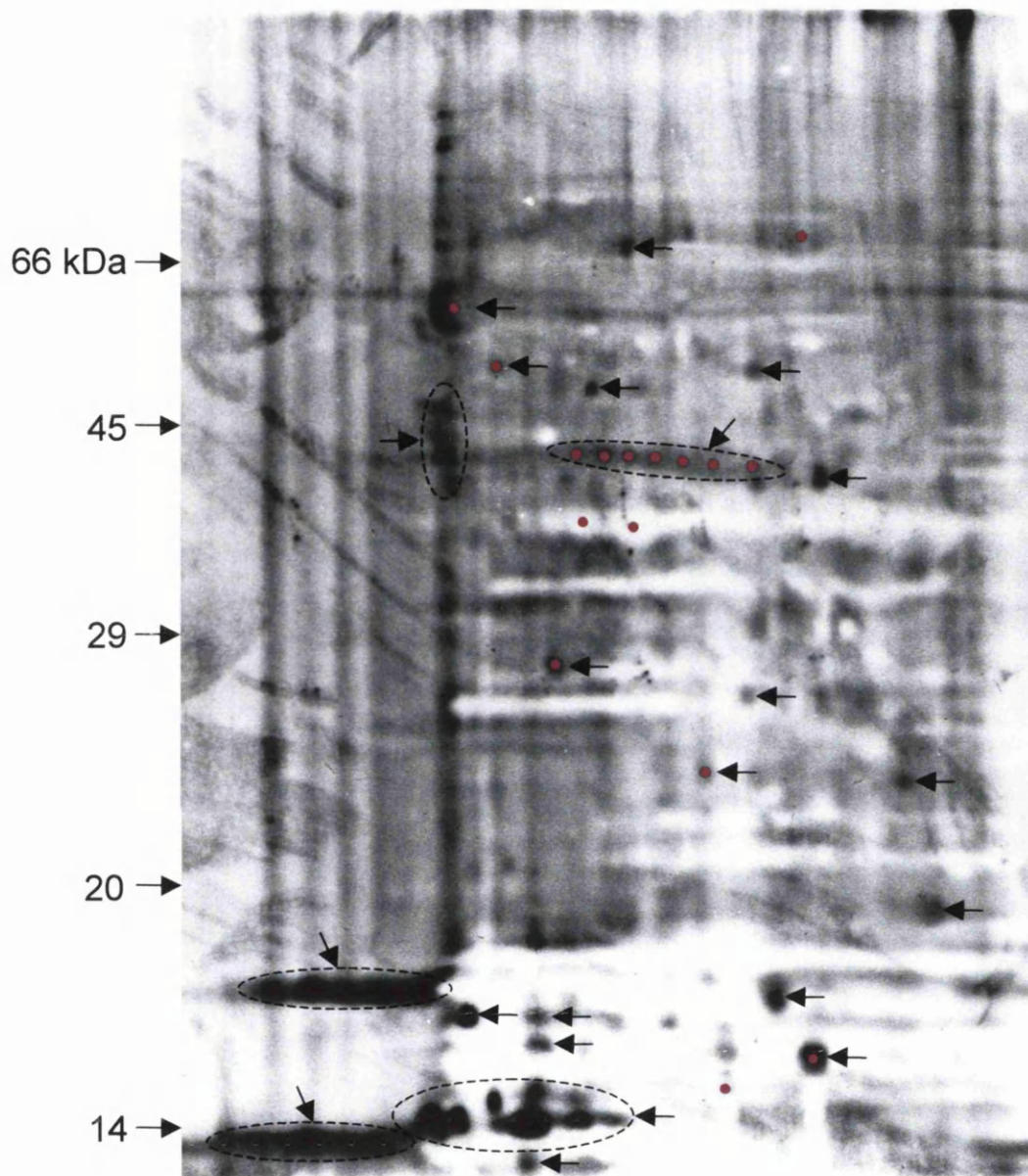


Figure 7.7e Two-dimensional Western blot of SAM isolated from biofilm cells of *A. actinomycetemcomitans* grown on a semi-defined agar containing 100 μM 2,2'-dipyridyl incubated anaerobically (SAM 5). Serum from a patient with LJP was used as the primary antibody.

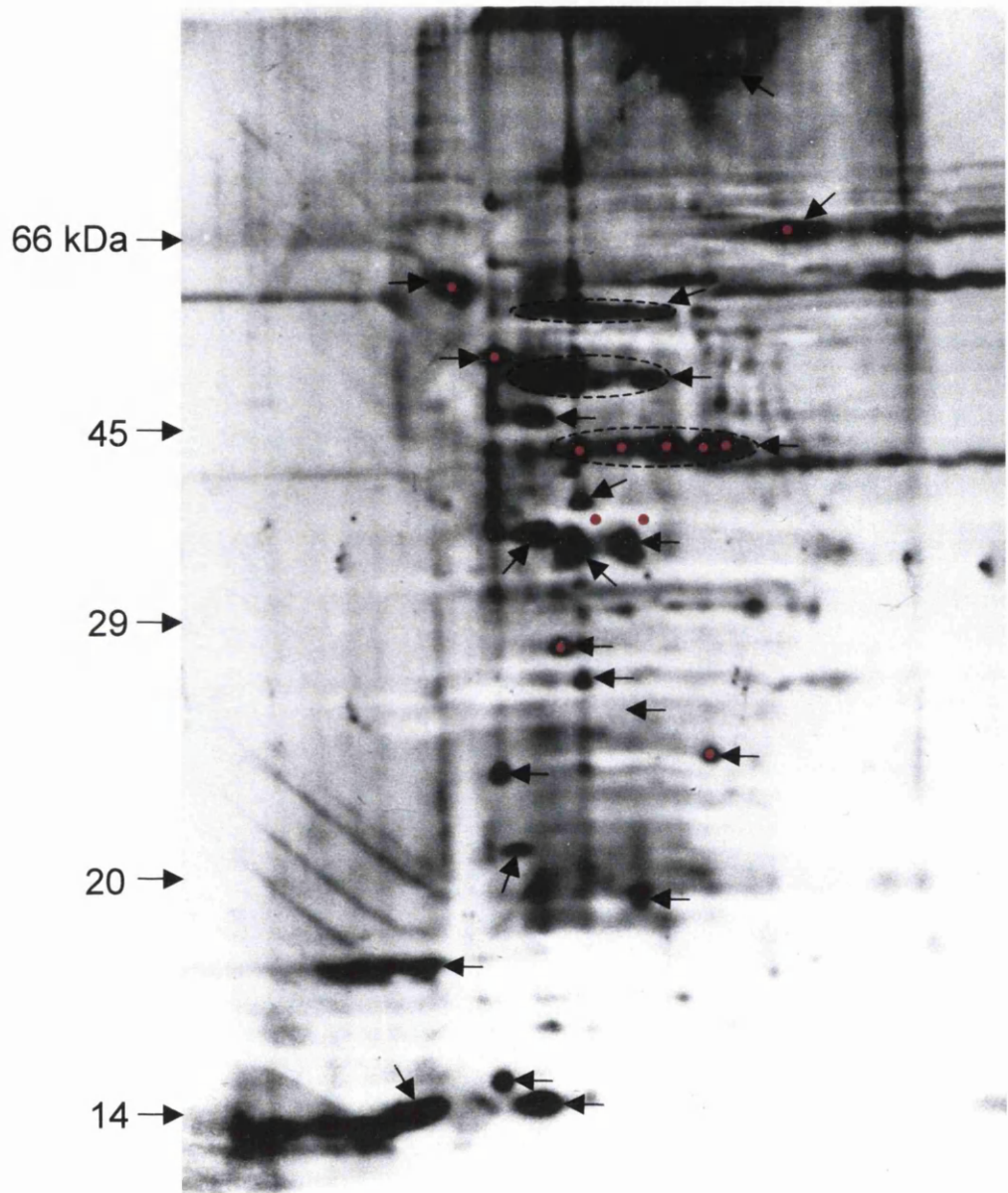


Figure 7.7f Two-dimensional Western blot of SAM isolated from biofilm cells of *A. actinomycetemcomitans* grown on BHI agar supplemented with 6% (v/v) horse blood incubated anaerobically (SAM 6). Serum from a patient with LJP was used as the primary antibody.

7.5 Discussion

A. actinomycetemcomitans elicits a significant immune response and numerous studies have shown considerably elevated serum antibody levels against this microorganism in patients with LJP compared to periodontally healthy controls (Sims *et al.*, 1991; Cole *et al.*, 1995). These antibodies appear to be protective for the host and serum neutralising activity has been demonstrated against a number of *A. actinomycetemcomitans* virulence factors (Tsai *et al.*, 1981; Meghji *et al.*, 1993; White *et al.*, 1995).

Several antigenic proteins have been described for *A. actinomycetemcomitans* (Ebersole *et al.*, 1995; Saito *et al.*, 1993; Meghji *et al.*, 1995; Bolstad *et al.*, 1990, Sims *et al.*, 1991). In addition, the organism produces other non-proteinaceous immunogenic molecules such as capsular polysaccharide (McArthur *et al.*, 1996) and LPS (Page *et al.*, 1991). Distinct smears were observed on the 2-D antigen profiles and whilst some of these smears may represent poorly separated proteins, it is possible that others may represent an immunoreaction with capsular polysaccharide and/or LPS. Corresponding smears were often not detected on the Brilliant blue stained 2-D SDS-PAGE gels thus supporting the possibility that some smears represented immunoreactions with non-proteinaceous material.

A large number of the *A. actinomycetemcomitans* surface antigens recognised by the LJP serum were also identified by the control serum, although the immunoreaction with LJP serum was significantly greater than with control serum. This is in accordance with other studies (Flemmig *et al.*, 1996; Cole *et al.*, 1995) and it is probable that these antigens share cross-reactive epitopes with other microorganisms. Strong immunoreactions with antigens common to other bacteria may mask antibody responses to *A. actinomycetemcomitans* surface antigens. Immunoabsorption of the LJP serum with, for example an *E. coli* lysate, may facilitate the identification of antigenic determinants unique to *A. actinomycetemcomitans*. However, two antigens with MWs of 13.5 kDa and 16 kDa were observed on 1-D blots with LJP serum which were not detected with control serum and these antigens certainly warrant further investigation.

The 56 kDa protein, putatively identified in Ch 6 as the *A. actinomycetemcomitans* GroEL homologue (Kirby *et al.*, 1995), was among the immunodominant surface proteins of *A. actinomycetemcomitans*. Monoclonal

antibodies to the *A. actinomycetemcomitans* GroEL homologue have been shown to inhibit the bone resorbing activity of SAM from the organism (Kirby *et al.*, 1995). In addition high-titre antisera from patients with periodontal disease have been shown to inhibit *A. actinomycetemcomitans* SAM-induced bone resorption (Meghji *et al.*, 1993). *In vivo*, antibodies to the *A. actinomycetemcomitans* GroEL homologue may act to limit the bone resorbing capacity of this organism.

In general the surface antigen profiles obtained on the 1-D and 2-D immunoblots reflected the surface protein profiles observed on the 1-D and 2-D SDS-PAGE gels. However, in some cases proteins which were present in large amounts on the surface of *A. actinomycetemcomitans* were absent from the 2-D surface antigen profiles. Conversely certain proteins present only in small amounts on the surface of *A. actinomycetemcomitans* reacted strongly with the LJP serum and produced an intense spot on the 2-D surface antigen profile. Thus it would appear that surface proteins of *A. actinomycetemcomitans* vary in their ability to induce an immune response. An alternative explanation may be that the intensity of the spot obtained on the surface antigen profiles actually reflects the amount of the protein expressed under environmental conditions encountered *in vivo*.

The control serum demonstrated a particularly strong immunoreaction with a 14.5 kDa antigen compared to the LJP serum. It might be postulated that these antibodies could play a protective role in preventing colonisation and infection with *A. actinomycetemcomitans* and it would certainly be of interest to identify this antigen.

Whilst a considerable amount of information is available on the antigenic properties of *A. actinomycetemcomitans*, no study as yet has looked at the effect of environmental factors on the antigen profile of this organism. An immunoblot of *A. actinomycetemcomitans* with serum from a patient infected with this organism identifies proteins (and other immunogenic molecules) which are expressed by the organism *in vivo*. Within different regions of the oral cavity, during different stages of disease, and even at different locations within plaque, environmental conditions are likely to be quite different. Consequently a host immune response will be elicited against a whole range of phenotypic (and

presumably antigenic) variants of a particular organism, in this case *A. actinomycetemcomitans*.

It is clear from the results obtained in the current investigation that the nature of the growth environment did affect the surface antigen profile of *A. actinomycetemcomitans*. Changes in the surface antigen profile in response to environmental factors might be expected to reflect those observed in the surface protein profiles (Ch 6). In most instances this was indeed the case. For example planktonic cells of *A. actinomycetemcomitans* grown in a semi-defined broth incubated anaerobically demonstrated an increased amount of a series of proteins with a MW of 48 kDa and this was paralleled with an increased immunoreaction to this series of proteins as observed on the 2-D surface antigen profile. However in some cases the reverse was true. Thus whilst anaerobic growth of *A. actinomycetemcomitans* on BHI agar containing 6% (v/v) horse blood resulted in an increased amount of a 56 kDa protein, the corresponding spot on the surface antigen profile was relatively faint. A possible explanation for this is that the protein produced under these growth conditions may have had slightly different antigenic properties resulting in a weak immunoreaction with the LJP serum.

In summary, the current study has identified a number of immunogenic molecules on the surface of *A. actinomycetemcomitans*. Whilst a large proportion of these antigens were also recognised by serum from a periodontally-healthy individual, the immunoreaction with serum from a patient with LJP was considerably greater. Finally it has been shown that environmental conditions do affect the antigenic properties of the surface of *A. actinomycetemcomitans*.

Chapter 8

Identification of *A. actinomycetemcomitans* exported proteins

8.1 Introduction

A. actinomycetemcomitans has been strongly implicated in the aetiology of LJP, a disease characterised by the rapid destruction of the tooth-supporting tissues. This organism produces a plethora of virulence factors that may contribute to its periodontopathogenic potential (Wilson and Henderson, 1995).

Of particular interest are the exported proteins of the organism, whether they are located on the surface of the bacterium, or are secreted into the surrounding environment, as it is these molecules which may come into direct contact with host cells *in vivo* and may cause damage to those cells or modulate their activity. Many exported proteins are translated as a precursor polypeptide carrying a signal sequence which directs the protein to the cell membrane and facilitates its secretion across it. The signal sequence is then cleaved from the protein by a signal peptidase.

In this study a plasmid-based *phoA* gene fusion system was used in an attempt to identify exported proteins of *A. actinomycetemcomitans*. The *phoA* gene encodes alkaline phosphatase (AIP) which is only active following export across the inner membrane into the periplasmic space. The vector contains a truncated form of *phoA* which lacks the promoter and signal sequence required for expression and export of AIP. Genomic libraries of the organism of interest are constructed in the truncated *phoA* vector and screened on media containing a chromogenic AIP substrate. Positive clones (which appear blue) produce AIP fusion proteins. As the *phoA* vector lacks a promoter and signal sequence, these exported AIP fusion proteins must be derived from *phoA* vector into which a functional promoter and signal sequence from the donating bacterial species have been inserted upstream of the truncated *phoA* sequence. In addition to a promoter and signal sequence, it is likely that at least part of the gene encoding an exported protein will be present. This system has been successfully used to identify a number of exported proteins in *Streptococcus pneumoniae* (Pearce *et al.*, 1993).

Whilst clones producing AIP fusion proteins may only contain a segment of a gene encoding an exported protein, sequence data obtained from these clones can be used to search the *A. actinomycetemcomitans* genome database (www.genome.ou.edu/act.html) to identify the full length gene sequence and, to design probes to screen other libraries, such as a λ library, which would be more likely to contain the full gene sequence.

8.2 Aim

The aim of this investigation was to identify exported proteins of *A. actinomycetemcomitans* using a plasmid-based *phoA* gene fusion system.

8.3 Materials and methods

The strategy for cloning exported proteins from *A. actinomycetemcomitans* is summarised in Fig. 8.1.

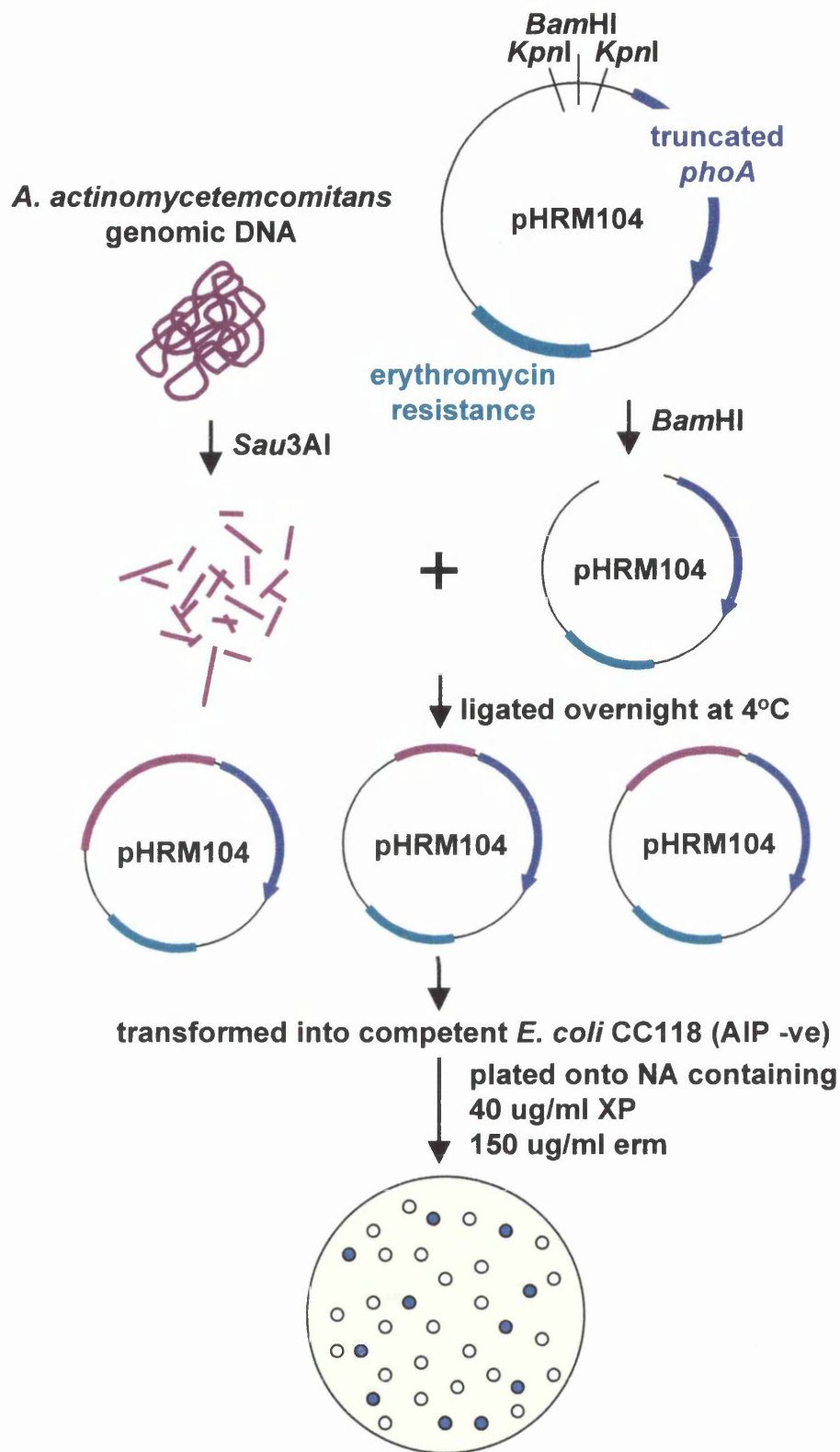


Figure 8.1 Diagram to show the construction of the *phoA* library. DNA extracted from *A. actinomycetemcomitans* was partially digested with *Sau3AI* and ligated with the *phoA* vector, pHRM104, which had been linearised with *BamHI*. The ligation reaction was used to transform competent *E. coli* CC118 (AIP negative). Cells were plated out onto nutrient agar (NA) containing 150 µg/ml erythromycin (erm) and 40 µg/ml of the chromogenic AIP substrate 5-bromo-4-chloro-3-inolyl phosphate (XP). Positive clones producing AIP fusion proteins were identified by their blue phenotype.

8.3.1 Extraction of genomic DNA

The growth from 12 streak plates of *A. actinomycetemcomitans* was harvested into TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The suspension was centrifuged (3000 g, 4°C, 15 mins) and the pellet was resuspended in 5 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl, pH 7.5). Two loopfuls of lysozyme plus two loopfuls of proteinase k were added and the cell suspension was incubated at 56°C for 5 mins until lysis had begun to occur. The sample was transferred onto ice and 0.1 vols of 10% (w/v) SDS were added followed by 0.3 vols of 6 M NaCl. An equal volume of phenol-chloroform (1:1) was then added, mixed by gentle inversion and centrifuged at 3000 g, 4°C for 15 mins. The upper aqueous layer was removed and transferred to a fresh tube and extracted twice with an equal volume of phenol-chloroform and then twice with an equal volume of chloroform. After the final extraction, the upper aqueous layer was removed and 0.1 vols of 5 M NaCl and 2.5 vols of 100% ethanol were added. The sample was placed at -70°C for 1.5 h and then centrifuged at 10,000 g, 4°C for 30 mins. The DNA pellet was washed in 70% ethanol and allowed to air dry. The DNA pellet was then resuspended in 0.75 ml sterile dH₂O and stored at -70°C until required.

8.3.2 Construction of *phoA* library

Genomic DNA extracted from *A. actinomycetemcomitans* was partially digested with *Sau3A*I (or *Bsp143*I, an isoschizomer of *Sau3A*I). The reaction mixture (which contained 50 µl genomic DNA, 10 µl 10x concentrated Universal buffer, 30 µl TE buffer and 10 µl *Sau3A*I), was incubated at 37°C for 3 h. Vector DNA was extracted from 200 ml of overnight cultures of *E. coli* JM107 containing the vector, pHRM104, using a Midi plasmid preparation kit (Qiagen Ltd., Crawley, UK). In order to linearise the vector, pHRM104 was incubated with *Bam*HI at 37°C for 2 h. The *Sau3A*I digested *A. actinomycetemcomitans* DNA was then ligated together with the linearised pHRM104 at 4°C overnight. The ligation reaction contained 40 µl *Sau3A*I fragments, 20 µl linearised pHRM104, 6 µl 10x ligase buffer and 2 µl T4 DNA ligase. Aliquots were removed at each stage and analysed by agarose gel electrophoresis to confirm that restriction digests and ligation reactions had taken place satisfactorily.

8.3.3 Preparation of competent *E. coli* CC118 cells

A generous loopful of *E. coli* CC118 was used to inoculate 100 ml of nutrient broth (NB) containing 20 mM MgSO₄. Cultures were incubated at 37°C with rapid shaking for approximately 3 - 4 h until the cultures were just past the mid-logarithmic phase of growth (absorbance at 600 nm = 0.600). Cultures were then transferred to pre-chilled centrifuge tubes and centrifuged at 4°C for 5 mins. Cells were resuspended in approximately 50 ml of ice-cold 15 mM CaCl₂ and centrifuged again at 4°C for 5 mins. The cells were then resuspended in 3 ml of ice-cold 75 mM CaCl₂ containing 15% glycerol and dispensed in 300 µl aliquots into 3 ml pre-chilled tubes. Tubes were incubated on ice for a further 10 mins and were then transferred immediately to -70°C until required.

8.3.4 Transformation of *E. coli* CC118

An aliquot of competent *E. coli* CC118 was slowly defrosted and 29 µl of the ligation reaction was added. The cells were incubated on ice for 45 mins and then heat-shocked at 37°C for 10 mins. The transformed cells were transferred to 5 ml of NB and incubated at 37°C with shaking for 1.5 h. The cells were plated onto NA containing 150 µg/ml erythromycin (erm) and 40 µg/ml 5-bromo-4-chloro-3-inolyl phosphate (XP) and incubated overnight at 37°C. AIP positive colonies were picked and subcultured onto fresh erm/XP plates. For long term storage a loopful of each clone was resuspended in 1 ml of NB containing 20% glycerol. Vials were placed at -70°C.

8.3.5 Plasmid preparation

Plasmid DNA was extracted from overnight cultures of each clone using a Qiaprep spin plasmid kit (Qiagen).

8.3.6 Determination of insert size

The *Bam*HI cloning site of pHRM104 is flanked on each side by a *Kpn*I restriction site (Fig. 8.3). Thus in order to determine the size of the insert, recombinant plasmid DNA was digested with *Kpn*I to excise the *A. actinomycetemcomitans* DNA fragment. The digestion reaction (which contained 10 µl plasmid DNA, 2 µl 10x concentrated Universal buffer, 6 µl TE

buffer and 2 μ l *Kpn*I) was incubated at 37°C for 2 h. The reaction was stopped by the addition of 10 μ l of gel loading buffer (Amersham Pharmacia Biotech, Little Chalfont, UK).

All restriction enzymes and their corresponding buffers were obtained from Promega (Southampton, UK).

8.3.7 Agarose gel electrophoresis

DNA fragments were resolved by electrophoresis on 0.8% or 1% horizontal agarose gels (containing 0.5 μ g/ml ethidium bromide) in 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3). DNA from bacteriophage λ digested with *Pst*I was loaded as a size standard. Gels were run at 5 V/cm for 1 - 3 h and bands were visualised under ultraviolet light.

8.3.8 Sequencing of AIP positive clones

Sequencing was carried out using the dideoxy-chain termination method (Sanger *et al.*, 1977) using a T7 sequencing kit (Amersham Pharmacia Biotech) according to the manufacturers instructions. The primer used for sequencing (5' CGGTTTTCCAGAACAGG 3') binds to bases 51 - 67 at the beginning of the truncated *phoA* gene and reads across the fusion junction into the *A. actinomycetemcomitans* insert. [α -³⁵S]dATP α S (Amersham Pharmacia Biotech) was used to label sequencing reactions.

Sequencing reactions were separated on 8% acrylamide gels containing 23.4% urea in 1x TBE buffer using a Model S2 sequencing gel system (Life Technologies, Paisley, UK). TBE buffer (1x) was used for the running buffer. Electrophoresis was carried out at a constant power of 40 W for approximately 3 h until the bromophenol blue marker dye had reached the bottom of the gel. When electrophoresis was complete, the gel was soaked for 20 mins in fixing buffer containing 10% acetic acid and 10% methanol. The gel was then transferred to a supporting sheet of filter paper, covered with plastic wrap and dried on a vacuum gel dryer. After drying, the plastic wrap was removed and an X-ray film was placed on the surface of the gel in a film cassette. The film was left in contact with the gel for 1 - 2 days at room temperature and then developed.

8.4 Results

8.4.1 Insert sizes

80 clones producing AIP fusion proteins were isolated. These fusion proteins are derived from vector DNA into which a promoter, signal sequence and probably at least part of a gene encoding for an exported protein derived from *A. actinomycetemcomitans* have been inserted upstream from the truncated *phoA* gene (Fig. 8.2).

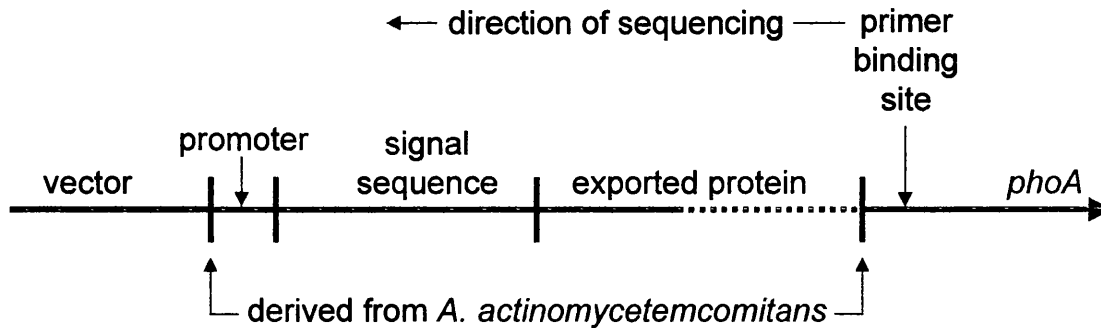


Figure 8.2 Diagram to show the fusion junction between *A. actinomycetemcomitans* insert and truncated *phoA* gene

Fig. 8.3 shows the inserts, excised by digestion with *KpnI*, for 6 of the AIP positive clones. Insert sizes ranged from 0.2 - 4.0 kb (Table 8.1).

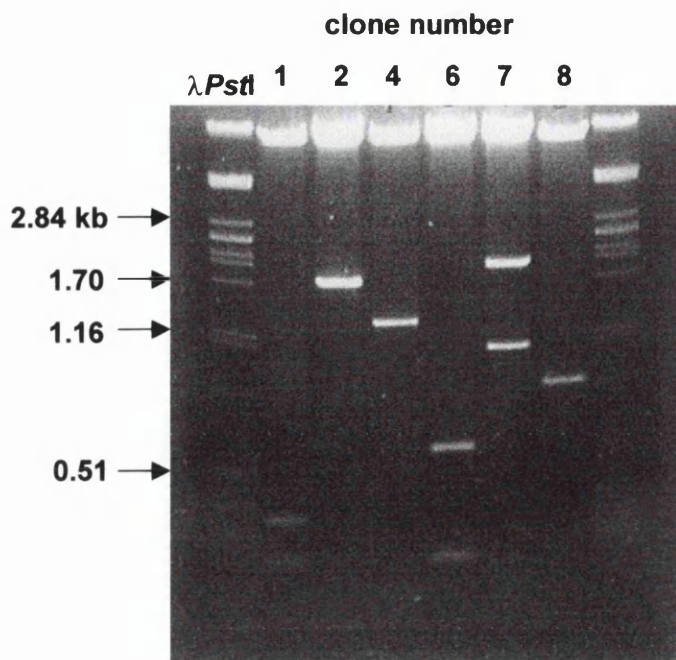


Figure 8.3 *Kpn*I digests of plasmid DNA from AIP positive clones run on a 0.8% agarose gel

clone number	insert size (kb)	clone number	insert size (kb)
1	0.50	18	1.00
2	1.70	19	0.70
4	1.40	20	0.68
6	0.83	21	0.70
7	2.90	22	1.80
8	0.80	23	0.28
9	0.22	24	0.36
10	0.40	25	1.80
11	1.32	26	0.36
12	1.85	27	2.50
13	1.32	28	0.50
14	2.20	29	0.70
15	1.55	30	0.32
16	4.00	31	2.60
17	0.68	32	0.78

Table 8.1 Insert sizes of 32 of the AIP positive clones

8.4.2 Sequence data

Sequencing was attempted for six clones producing AIP fusion proteins. Reasonable lengths of DNA sequence with minimal ambiguities were obtained for four clones. The DNA sequences obtained for clones 6, 10, 33 and 74 were searched for open reading frames in frame with the aspartic acid residue (D) at the insert-*phoA* fusion junction using the DNASIS software package. The amino acid sequences obtained for each clone were then searched against the *A. actinomycetemcomitans* genome database. Near 100% matches were obtained for the four clones with different contiguous sequences within the genome database. It was thus possible to determine the full length amino acid sequence for three of the exported proteins (proteins 6, 10 and 74). In the case of clone 33 the matching contiguous sequence contained only the first part of the gene encoding the exported protein. Homology searches were carried out on the full length protein sequences of proteins 6, 10 and 74 and the partial amino acid sequence of protein 33 using Fasta3. (www.ebi.ac.uk/searches/searches.html). The amino acid sequences derived from the *A. actinomycetemcomitans* genome database were also analysed for the presence of a signal sequence using SignalP (www.cbs.dtu.dk/services/SignalP/). Signal sequences were identified for proteins 6, 33 and 74 and the predicted cleavage sites are indicated in Table 8.2 by means of a vertical line.

clone 6

.....*MLNYEGVIMKKS^DKLVA- LAAILSCAAIPTTVMA|ESNVQQTMEKSNMSEAKKDLHEIY
 MKKSKLWALLAAILSCAAIPTTVMA ESNVQQTMEKSNMSEAKKD
 LAGGCFWGIEAYMERIYGVEDAISGYANGKSDKTNYQKIGITDHAETVKVTYDRNKVSLDK
 LLKYYFQVIDPTSVNKQGNDRGRQYRTGIYYQDTADKSIIEKEITALQGKYKNKIQIEVEPLH
 NYIVAEYHQDYLLKNPNGYCHIDLEQANYVIIDEKDYPKPSDAELKAKLTPLQYSVTQKNK
 TEHSFSNEYWDNFKPGIYVDVTTGEPLFSSKDKFESGCGWPSFTKPITKEVVTYQDDHSF
 NMLRTEVISRSGKAHLGHVFDDGPKDKGGLRYCINSAAVKFIPLEDMVKENYGYLTNAVKE

Full length protein sequence showed homology to:

H. influenzae hypothetical protein (73% identity in 356 aa overlap)

H. pylori peptide methionine sulfoxide reductase (65% identity in 333 aa overlap)

clone 10

.....*MAMNNILGLFAHSPLKPLQKHSNKVTECCCELLIPFFEATFNTDWWKAEQISLDISAHER
 DWWKAEQIRLDISAHER
 QADALKREIRLKLPRGLFMPIDRTDLELVTQQDKLANYTKDIAGRMIGSSVKYSGGNEKRI
 QRDALKREIRLKLPRGLFMPID
 YELCTSQFGRYRTSP

Full length protein sequence showed homology to:

H. influenzae hypothetical protein (81% identity in 108 aa overlap)

clone 33

.....*MFKLLMIPLLVLTA^DCGTA|SYQPDQDEEQLNQLIVSQLKTKGSSRV- end of
 MFKLL I IPLLVLTA^DCGT - TA SYQRD contiguous
 sequence

Extended aa sequence showed homology to:

H. influenzae probable lipoprotein (48% identity in 42 aa overlap)

clone 74

.....*MKKHLKSPHFFCLTATIL|ALAGCTPTIQLDTPKEGITINMNVVDHNINVKIDEKTKVT
 TILALA GCTPTIQLDTPKEGITINMNVVD
 AGENSAVEANKTPAEK

Full length protein sequence showed homology to:

E. coli hypothetical 6.8 kDa protein (52% identity in 42 aa overlap)

Table 8.2 Amino acid sequence data for 4 of the AIP positive clones and their alignment with amino acid sequences derived from the *A. actinomycetemcomitans* genome database.

Purple residues represent sequence information obtained in the current study; D represents the fusion junction with the truncated *phoA* gene; green residues represent information derived from the *A. actinomycetemcomitans* genome database; * denotes start of protein; ← indicates direction of sequencing; | indicates predicted signal peptide cleavage site.

8.5 Discussion

Eighty clones producing AIP fusion proteins were isolated. From Table 8.1 it is apparent that a number of clones may carry the same insert. Further restriction digests of these clones, with a range of restriction enzymes, are necessary to ensure that identical clones are eliminated.

From Table 8.2 it is clear that only a segment of each gene encoding an exported protein has been inserted upstream of the truncated *phoA* gene, and therefore the four *phoA* clones characterised so far will not be expressing a complete, functional *A. actinomycetemcomitans* exported protein. However using the information available in the *A. actinomycetemcomitans* genome database it was possible, for three of the clones (6, 10 and 74), to determine the entire sequence of the exported protein and the gene that encodes it. When Fasta3 searches were carried out on the three full length protein sequences, two proteins (10 and 74) showed homology to hypothetical, as yet uncharacterised proteins from *H. influenzae* and *E. coli*. The next step would be to design primers (using the DNA sequence information derived from the *A. actinomycetemcomitans* genome database) targeted at each end of the genes encoding the exported proteins and generate PCR products using *A. actinomycetemcomitans* genomic DNA as a template. The PCR products could then be cloned directly into a high level expression vector such as pET. Certain pET vectors allow the fusion of a histidine tag to the N- or C-terminus of the protein enabling rapid affinity purification. The biological activities of the exported proteins could then be determined. Protein 6 demonstrated homology to a peptide methionine sulfoxide reductase (MsrA) from *Helicobacter pylori*. Methionine (Met) is one of the most readily oxidised amino acid constituents of proteins. The oxidation product, Met sulfoxide, can be converted back to Met by MsrA. Met oxidation may lead to loss of functional activity, although functional activation by Met oxidation has also been observed (Vogt, 1995). Mutants of *E. coli* (Moskovitz *et al.*, 1995) and *Saccharomyces cerevisiae* (Moskovitz *et al.*, 1997) deficient in MsrA were shown to be more sensitive to oxidative stress, suggesting that MsrA plays an important role in the repair of oxidative damage in these organisms. In addition MsrA has been shown to be essential for the maintenance of functional adhesins in *S. pneumoniae*, *Neisseria gonorrhoeae* and *E. coli* (Wizemann *et al.*, 1996).

Signal sequences were identified for three of the amino acid sequences derived from the *A. actinomycetemcomitans* genome database (proteins 6, 33 and 74). These sequences demonstrated features characteristic of prokaryotic signal sequences, namely a basic N-terminal region, a central hydrophobic core and a polar C-terminal region (von Heijne, 1990). Protein 10 however, did not exhibit a signal sequence. In order for AIP to cleave XP and produce a blue (positive) colony, it must first be exported across the cytoplasmic membrane into the periplasmic space where it is converted to its active form. As the *phoA* vector lacks a promoter and a signal sequence, positive clones must be producing fusion proteins derived from *phoA* vector into which a functional promoter and signal sequence from the donating bacterial species (in this instance *A. actinomycetemcomitans*) have been inserted upstream from the truncated *phoA* gene. An alternative explanation is that a gene (or part of a gene) encoding a transmembrane protein has been inserted upstream of the truncated *phoA* gene. AIP may have been translocated across the cytoplasmic membrane by a membrane spanning domain of the transmembrane protein to which it is fused, and may thus be located in the periplasmic space anchored to the outer surface of the cytoplasmic membrane.

The preliminary findings of the current investigation indicate that the plasmid-based *phoA* gene fusion system may provide a valuable tool for the identification and cloning of secreted and surface proteins of *A. actinomycetemcomitans*. In addition to sequencing remaining clones, future work may include;

- i) identification of clones with large fragments of *A. actinomycetemcomitans* exported proteins fused to AIP. This could be achieved by separating proteins from whole cell lysates of clones by SDS-PAGE and then carrying out Western blots using anti-AIP as the primary antibody.
- ii) use of sequence information from AIP positive clones to design probes to screen other *A. actinomycetemcomitans* libraries (e.g. pUC18 library, λ library) which are more likely to contain the entire gene sequence.
- iii) use of full length gene sequence information derived from the *A. actinomycetemcomitans* genome database (pinpointed by the sequence data obtained from AIP positive clones) to design primers and generate

PCR products (using *A. actinomycetemcomitans* genomic DNA as a template) which can then be cloned directly into a high level expression vector enabling further characterisation of the biological activities of the exported proteins.

- iv) an investigation of the effect of environmental conditions on the production of individual exported proteins.

Chapter 9

Summary and future work

Periodontal diseases are associated with inflammation and erosion of the tooth supporting alveolar bone. Loss of gingival attachment results in the formation of a periodontal pocket. The microflora of the periodontal pocket is extremely complex and more than 300 bacterial species have been isolated from human subgingival plaque samples. Of these, certain species including *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* have been specifically implicated in the aetiology of various forms of periodontal disease (Haffajee and Socransky, 1994) and a plethora of virulence factors have been attributed to these putative periodontopathogens (Holt and Bramanti, 1991; Wilson and Henderson, 1995; Cutler *et al.*, 1995; Bolstad *et al.*, 1996). Within the periodontal pocket there exists a highly dynamic ecosystem and the resident microorganisms are likely to be exposed to continual fluctuations in environmental conditions. In addition to physical factors such as temperature, pH, E_h and pO_2 , the presence of host cells and host molecules and other bacterial species will all operate to influence the phenotypic properties of a bacterial cell *in vivo*. The aim of this thesis therefore was to study the response of periodontopathogenic bacteria to environmental changes.

The periodontal pocket is highly anaerobic with a low E_h (Marsh and Martin, 1992; Kenney and Ash, 1969) and a low pO_2 (Mettraux *et al.*, 1984). It is not clear whether it the low E_h *per se* or the low pO_2 (resulting in a concomitantly low E_h) which is important for the survival of anaerobic microorganisms *in vivo*. Walden and Hentges (1975) demonstrated growth of *C. perfringens* under anaerobic conditions with an E_h of +325 mV, whilst no growth occurred in aerated cultures with a low E_h . Conversely, Hanke and Katz (1943) demonstrated growth of certain anaerobic bacteria in a continuous current of air provided that the E_h was maintained at a sufficiently low level.

The results of the current study have shown that addition of the redox-modifying agent, MB, to suspensions of *P. gingivalis* and *F. nucleatum* resulted in an increase in the E_h of the system (in the absence of oxygen) and this was

associated with killing of the organisms. That the effect could be negated by addition of the reducing agent dithiothreitol, implied that the bactericidal activity of MB was redox-mediated. It was proposed that MB may exert its effect by consuming intracellular reducing power in the form of NADH (or NADPH), by causing oxidation of components of electron transport chains or by inactivating enzymes which are active only in the reduced form. This study has shown that MB can indeed oxidise NADH, although the effects of MB on other aspects of cellular physiology and enzyme activity remain to be elucidated.

The efficacy of MB in the treatment of periodontal disease was evaluated in a small-scale clinical trial (Gibson *et al.*, 1994). The microbiological data from this clinical trial (presented in Ch 3) together with data from two other studies (Wilson *et al.*, 1992; Ower *et al.*, 1995), showed that treatment with MB resulted in a decreased proportion of black-pigmented anaerobic organisms and an increased proportion of aerobic organisms, changes which are more compatible with a healthy gingival microflora. Concomitant improvements in clinical parameters were also observed.

The emergence of antibiotic resistance is a growing problem and has also been documented in the subgingival microflora (Lacroix and Walker, 1995; Listgarten *et al.*, 1993). Redox-modifying agents such as MB may provide an effective alternative to antibiotics and antiseptics in the treatment of periodontal disease.

In Ch 4 it was observed that the levels of AcP and AIP produced by *A. actinomycetemcomitans* grown in different media did not appear to be affected by the level of inorganic phosphate in the medium. Bacteria are relatively impermeable to phosphate esters and substrates must be dephosphorylated before they can be taken up by the cell. Therefore it was proposed that the level of AcP and AIP produced in the different media may have been related to the levels of a particular phosphorylated substrate.

In addition to up-regulation or down-regulation of *de novo* protein synthesis, changes in environmental conditions may also affect the activity of pre-formed, cell-associated or extracellular virulence factors. AcP produced by *A. actinomycetemcomitans* grown in a CO₂-enriched aerobic atmosphere and purified AcP from *E. coli* were found to be more active when assayed for activity under anaerobic conditions compared to CO₂-enriched aerobic conditions. A

possible explanation for this is the oxidation of thiol groups within the AcP molecule in the presence of oxygen. *In vivo* it is likely that the activity of pre-formed enzymes and other virulence factors which have already been released from bacterial cells into the external milieu will be affected by changes in, for example, temperature, pH, E_h , pO_2 or the presence of host factors.

The presence of host molecules constitutes an important part of the environment of a periodontopathogenic bacterium *in vivo*. A number of studies have demonstrated the ability of cytokines to stimulate the growth of bacteria (Porat *et al.*, 1991; Denis *et al.*, 1991). As increased levels of a number of cytokines have been observed in the GCF and gingival tissues of patients with periodontal disease (Masada *et al.*, 1990; Reinhardt *et al.*, 1993; Barthold and Haynes, 1991; Takahashi *et al.*, 1994; Hou *et al.*, 1995; Stashenko *et al.*, 1991), it was decided to investigate the effect of these immunomodulatory molecules on the growth of two periodontopathogenic bacteria. The findings of this study (presented in Ch 5) showed that neither IL-1 β nor IL-6 affected the growth of *A. actinomycetemcomitans* or *P. gingivalis*. However, biofilms and supernatants from planktonic cultures of *P. gingivalis* were shown to rapidly degrade IL-1 β , IL-6 and IL-1ra and purified RI proteinase from the organism was able to degrade IL-1 β . Degradation of IL-1 β by *P. gingivalis* was associated with loss of biological activity. These results represent the first demonstration of cytokine-degrading activity by an oral organism. In the periodontal pocket, the subgingival microflora will be bathed in GCF, a serum-derived exudate. Serum and GCF contain protease inhibitors such as α -1-antitrypsin and α ₂-macroglobulin, and an important finding of the current investigation was that cytokine degradation by biofilms of *P. gingivalis* occurred even in the presence of serum. Degradation of cytokines by periodontopathogenic bacteria *in vivo* may result in a perturbation of the highly complex cytokine network responsible for maintaining periodontal health (Wilson *et al.*, 1998).

Bacteria interact with the surrounding environment via molecules located on their surface. Surface molecules are involved in attachment, coaggregation reactions and scavenging of essential nutrients. In addition, molecules located

on the surface of an organism represent the first point of contact with the host cell surface. Upon contact these molecules may cause damage to the host cell or may modulate its activity. *A. actinomycetemcomitans* has been shown to produce a loosely-adherent SAM which can be easily removed by gently rotating the cells in saline at 4°C. A number of virulence properties have been attributed to *A. actinomycetemcomitans* SAM including a potential role in attachment (Meyer and Fives-Taylor, 1993), stimulation of bone resorption (Wilson *et al.*, 1985; Kirby *et al.*, 1995; Reddi *et al.*, 1995a), inhibition of osteoblast (White *et al.*, 1995) and fibroblast (Kamin *et al.*, 1986; Meghji *et al.*, 1992a) proliferation and stimulation of cytokine production (Reddi *et al.*, 1996a, b).

The aim of the current investigation was to study the effect of environmental conditions on the protein profile of *A. actinomycetemcomitans* SAM. The environmental parameters studied were; growth in an CO₂-enriched aerobic atmosphere compared to growth in an anaerobic environment, planktonic versus biofilm mode of growth, growth in the presence of serum (to mimic GCF) or blood and growth under iron-restricted conditions. A number of differences in the surface protein profiles of *A. actinomycetemcomitans* grown under different environmental conditions were observed and these have been described (Ch 6). Four differentially expressed surface proteins were identified namely the glycolytic enzyme triose phosphate isomerase, the antioxidant enzymes thiol peroxidase and superoxide dismutase, and an as yet uncharacterised protein known as YfiD. The further characterisation of proteins which are differentially expressed by *A. actinomycetemcomitans* under different growth conditions may provide essential information as to how the organism might be able to adapt and survive under changing environmental conditions *in vivo* and may lead to the identification of environmentally-triggered virulence factors. Continuation of the proteomic analysis of the surface of *A. actinomycetemcomitans* is therefore a priority for future work.

Patients with LJP demonstrate significantly elevated serum antibody responses to *A. actinomycetemcomitans* compared to periodontally healthy individuals (Sims *et al.*, 1991; Cole *et al.*, 1995). Western immunoblots of *A. actinomycetemcomitans* SAM with serum from a patient with LJP and serum

from a periodontally healthy individual are presented in Ch 7. A large proportion of the *A. actinomycetemcomitans* surface proteins recognised by the LJP serum were also identified by the control serum, although the immunoreaction with the LJP serum was considerably greater than with the control serum. It is probable that these antigens share cross-reactive epitopes with other microorganisms. The surface proteins of *A. actinomycetemcomitans* appear to vary in their ability to elicit an immune response. Thus certain proteins, present in large amounts on the surface protein profiles, reacted weakly with the LJP serum and were absent from the surface antigen profiles or produced only weak spots, whilst other proteins which were present in only small amounts on the surface protein profiles, reacted strongly with the LJP serum and produced intense spots on the surface antigen profile. With regard to the effect of environmental conditions on the surface antigen profile of *A. actinomycetemcomitans*, changes in the surface antigen profile generally reflected changes observed in the surface protein profile, although this was not always the case. As a result of continual variations in environmental conditions, it is likely that *in vivo* an immune response will be elicited against a range of phenotypic variants of *A. actinomycetemcomitans*.

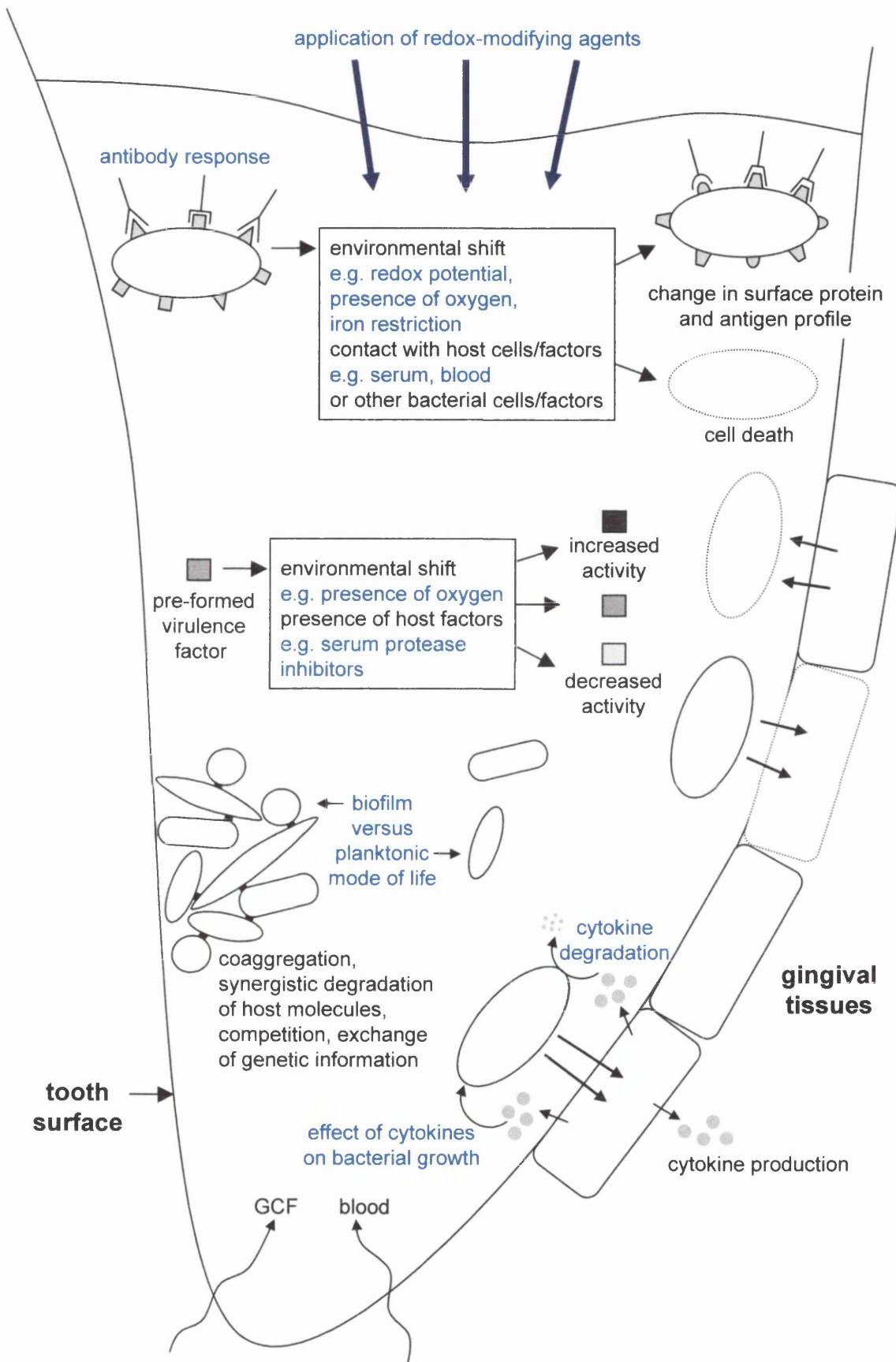


Figure 9.1 The dynamic microenvironment of the periodontal pocket. Aspects investigated in this thesis are highlighted in blue.

Fig. 9.1 illustrates the environmental influences that may operate in the periodontal pocket *in vivo*. Aspects of the periodontal microenvironment and their effect on periodontopathogenic bacteria *in vitro* that have been investigated in this thesis are highlighted. The results of these investigations have shown that environmental conditions do affect the growth and virulence potential of periodontopathogenic bacteria and furthermore, that environmental modification may represent an alternative to the use of antibiotics and antiseptics in the treatment of periodontal disease.

Future work

The use of redox-modifying agents would appear to represent a promising alternative to the use of antibiotics and antiseptics in the treatment of periodontal disease. More work is needed to evaluate new redox agents with greater redox-modifying potential and to establish the mechanisms by which such redox-modifying agents exert their effect.

With respect to the AcP and AIP activities of *A. actinomycetemcomitans*, a study of the substrate specificity of these enzymes would be of considerable interest and might provide further information on their potential role *in vivo*. Preliminary studies have shown that AcP from *A. actinomycetemcomitans* was able to remove phosphate groups from tyrosine residues (data not shown). Given the significance of tyrosine phosphatases and tyrosine kinases in controlling fundamental cellular activities in both eukaryotic and prokaryotic cells, this finding certainly warrants further investigation.

Periodontopathogenic bacteria have been shown to potently stimulate cytokine production by mammalian cells *in vitro* (Wilson *et al.*, 1996b). The results of the current investigation have demonstrated that *P. gingivalis* is able to rapidly degrade cytokines. A possible area for future work and an important point to consider is what might be the net effect of these two opposing activities on the cytokine network *in vivo*?

In this study the protein profiles of SAM isolated from *A. actinomycetemcomitans* grown under different environmental conditions have been determined. It would also be of considerable interest to compare the relative virulence potential of each of the six SAMs. This could be achieved by

assaying for the ability of each SAM to stimulate cytokine release from host cells, inhibit host cell proliferation or induce bone resorption.

There are many aspects of the host environment and their effect on periodontopathogenic bacteria which remain to be investigated, for example the effect of contact of bacteria with host cells. This would require the exposure of bacteria to mammalian cells, for example epithelial cells, grown in tissue culture. Obtaining a sufficient quantity of bacterial extract (e.g. SAM) for analysis by 2-D gel electrophoresis would be technically difficult, time consuming and expensive. An alternative approach (which would require a smaller quantity of bacterial cells) might be to compare the mRNA profiles of exposed and non-exposed bacterial cells using RNA arbitrarily primed polymerase chain reaction (RAP-PCR). Indeed this approach would be useful for investigating the effect of any environmental variable on bacterial gene expression.

To date, only a small proportion of the constituents of *A. actinomycetemcomitans* SAM have been fully characterised. These include the anti-proliferative 8 kDa protein termed gapstatin (White *et al.*, 1995; White *et al.*, 1998), a 2 kDa peptide which induces IL-6 transcription in fibroblasts (Reddi *et al.*, 1996a) and the 62 kDa bone resorbing GroEL homologue (Kirby *et al.*, 1995). The 2-D protein profiles obtained in the current study demonstrate that the SAM of *A. actinomycetemcomitans* contains at least 120 proteins or peptides. Using 2-D electrophoresis and mass spectrometry, Link *et al.* (1997) have identified some 300 major proteome components of *H. influenzae*. An ideal aim for the future would be to establish a collaborative project with another group with facilities for protein sequencing and to continue with the proteomic analysis of *A. actinomycetemcomitans* SAM.

Another potential reservoir of information concerning the surface (and exported) proteins of *A. actinomycetemcomitans* is the *phoA* library. Sequencing of the remaining clones is a priority for future work.

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The Effect of a Redox Agent, Methylene Blue, on the Survival of *Porphyromonas gingivalis* In Vitro

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Abstract. Methylene blue, at a concentration of 1.0 mg/ml, was able to raise the redox potential (E_h) of pre-reduced culture medium from -288 mV to -154 mV and to poise the medium at an E_h of -171 mV during 48-h incubation in an anaerobic atmosphere. Addition of the dye to suspensions of the anaerobic periodontopathogen, *Porphyromonas gingivalis*, to give a final concentration of 1.0 mg/ml raised their E_h to -125 mV. During a 24-h period of anaerobic incubation, the dye maintained the E_h at a level (-150 mV) that was more than 200 mV higher than control, dye-free suspensions, and this was accompanied by at least a 6-log reduction in the viable count. The bactericidal effect of the methylene blue could be counteracted by the reducing agent dithiothreitol at a concentration of 2.0 mg/ml, which lowered the E_h to a level (-333 mV) similar to that of controls. These results have demonstrated that the presence of 1.0 mg/ml methylene blue, in its oxidized form, in an environment is inimical to the survival of *P. gingivalis* and that this is related, directly or indirectly, to its ability to raise the E_h of the environment. Topical application of methylene blue, or other redox agents, may be an effective means of eliminating this organism from the periodontal pockets of patients with chronic periodontitis.

Accumulation of dental plaque below the level of the gingival margin results in chronic periodontitis, which is characterized by loss of gingival attachment, resulting in the formation of a periodontal pocket. The microflora of the periodontal pocket are extremely complex, and some 300–400 species have been isolated from human subgingival plaque samples [10]. Of these, several Gram-negative obligate anaerobes, including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Wolinella recta*, *Fusobacterium nucleatum*, and various spirochaetes, are regarded as being important in the etiology of the disease [10, 15]. The ability of such organisms to survive in the periodontal pocket is dependent, in part, on the existence of a low redox potential (E_h), and E_h values as low as -300 mV have been recorded in this microenvironment [7, 9].

Current methods of treating chronic periodontitis involve the mechanical removal of subgingival plaque. However, there is increasing interest in supplementing this procedure with the application of antibiotics and antiseptics to the periodontal pocket. An alternative approach to targeting the bacteria with antimicrobials would be to alter their environ-

ment to such an extent that they would no longer be able to survive. Raising the E_h of the periodontal pocket may be one way of achieving this. The purpose of this study was to determine the effect in vitro of methylene blue, an agent capable of altering the E_h of a system, on the survival of the periodontal pathogen *Porphyromonas gingivalis*.

Materials and Methods

Bacterial strains and media. *Porphyromonas gingivalis* W50 was maintained by weekly sub-culturing on Wilkins-Chalgren (WC) agar (Oxoid Ltd., Basingstoke, UK) supplemented with 10% (w/v) horse blood. The plates were incubated anaerobically at 37°C with the GasPak System (Oxoid Ltd). In some experiments *P. gingivalis* was grown in a liquid medium (BM) consisting of tryptone soya broth (10 g), proteose peptone (10 g), yeast extract (5 g), glucose (5 g), NaCl (5 g), and L-cysteine (0.75 g) per liter of distilled water. The pH was adjusted to 7.4, and the medium was autoclaved at 121°C for 15 min. Immediately prior to use, hemin and menadione were added to give final concentrations of 5 µg/ml and 0.5 µg/ml respectively.

Effect of methylene blue and dithiothreitol on the E_h of pre-reduced culture medium. Aliquots of sterile BM broth were incu-

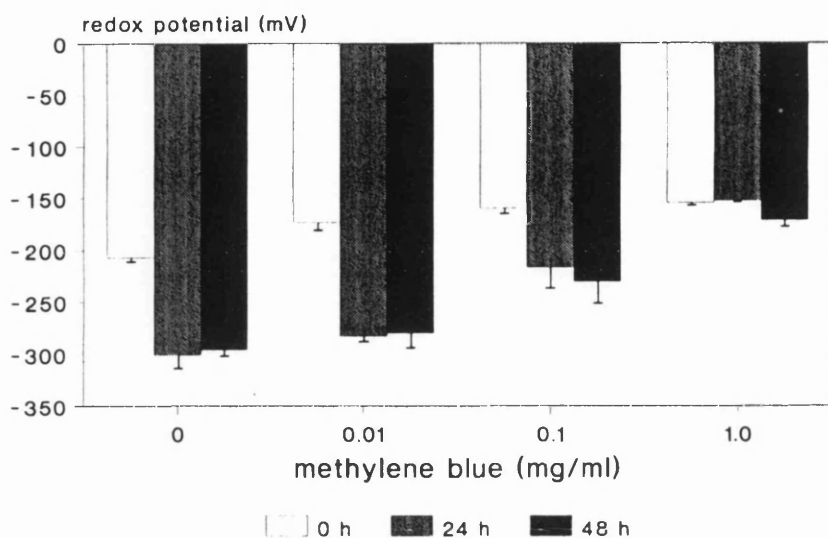


Fig. 1. Redox potentials of pre-reduced BM broth in the presence of various concentrations of methylene blue after incubation in an anaerobic jar for 48 h. The results are expressed as the mean \pm standard deviation of three replicates.

bated in an anaerobic jar for 24 h at 37°C, and then methylene blue (MB) was added to give final concentrations of 0.01, 0.1, and 1.0 mg/ml. Distilled water in place of MB was used in controls. The E_h of each was then determined with a combination redox cell with a platinum electrode (Russell pH Ltd., Auchtermuchty, UK), taking care to avoid agitation, which would have caused aeration and subsequent erroneous values for the E_h . Replicate series of the above were incubated in an anaerobic jar, and the E_h of each solution was determined after 24 h and 48 h.

MB and/or dithiothreitol was added to aliquots of pre-reduced sterile BM broth to give solutions containing: 1.0 mg/ml MB, 2.0 mg/ml dithiothreitol, 1.0 mg/ml MB + 2.0 mg/ml dithiothreitol. Distilled water was added to controls. The E_h of each was determined as described above. Further series of the above solutions were incubated in an anaerobic jar, and their E_h was determined after 24 h and 48 h.

Effect of methylene blue on survival of *P. gingivalis* suspensions.

A suspension of *P. gingivalis* was prepared in pre-reduced BM broth with cells harvested from WC agar plates. MB was added to aliquots of this suspension to give final concentrations of 0.01 to 1.0 mg/ml. Distilled water was added to controls. The samples were incubated at 37°C in an anaerobic jar, survivors were enumerated after 24 h and 48 h, and the minimum bactericidal concentration (MBC) was determined. Replicate suspensions were used for E_h determination, measurements being taken immediately after removal from the anaerobic jar.

Effect of dithiothreitol on survival of *P. gingivalis* in the presence of methylene blue.

MB and dithiothreitol were added together to suspensions of *P. gingivalis* to give final concentrations of 1.0 mg/ml and 2.0 mg/ml respectively. MB, dithiothreitol, or water was added separately to suspensions as controls. The suspensions were then incubated anaerobically at 37°C and viable counts carried out at 24 h and 48 h. Replicate suspensions were used for E_h determination.

Effect of methylene blue on broth cultures of *P. gingivalis*. MB was added to aliquots of 48-h cultures of *P. gingivalis* in BM broth to give a final concentration of 1.0 mg/ml. Distilled water was added to controls. Samples were taken for viable counting and

E_h determination after 0, 24, and 48 h of anaerobic incubation. The experiment was then repeated on 1 in 10 serial dilutions of the *P. gingivalis* cultures diluted in pre-reduced BM broth.

Results

Effect of methylene blue on the E_h of pre-reduced culture media.

The E_h of pre-reduced BM broth was -288 ± 4 mV (mean \pm standard deviation). While addition of water to pre-reduced broth raised the E_h to -207 ± 3 mV, MB had a greater effect, resulting in an E_h of between -173 ± 7 mV and -154 ± 3 mV (Fig. 1). After incubation in an anaerobic jar for 48 h, the E_h of the solution containing 1.0 mg/ml MB was considerably higher (-171 ± 8 mV) than that of the dye-free BM (-295 ± 7 mV), demonstrating the ability of the dye to poison the E_h . MB at a concentration of 0.1 mg/ml also displayed a poisoning effect, although not so great as in the case of the higher MB concentration. MB had no significant effect on the pH of the solutions; at a concentration of 1.0 mg/ml the pH was 6.59 ± 0.01 , whereas the pH of the dye-free broths was 6.63 ± 0.09 .

Addition of dithiothreitol to BM broth to give a final concentration of 2.0 mg/ml resulted in an E_h of -386 ± 8 mV after 48 h (Fig. 2). When MB and dithiothreitol were added together to the broths, the E_h after 48 h of anaerobic incubation (-349 ± 18 mV) was similar to that of dye-free (-306 ± 15 mV) or dithiothreitol-containing broths. Hence, dithiothreitol was able to counteract the redox-poisoning ability of MB.

Effect of methylene blue on the survival of *P. gingivalis*. 1.0 mg/ml MB reduced the viable count of a suspension containing $2.88 \times 10^8 \pm 0.46 \times 10^8$ cfu/

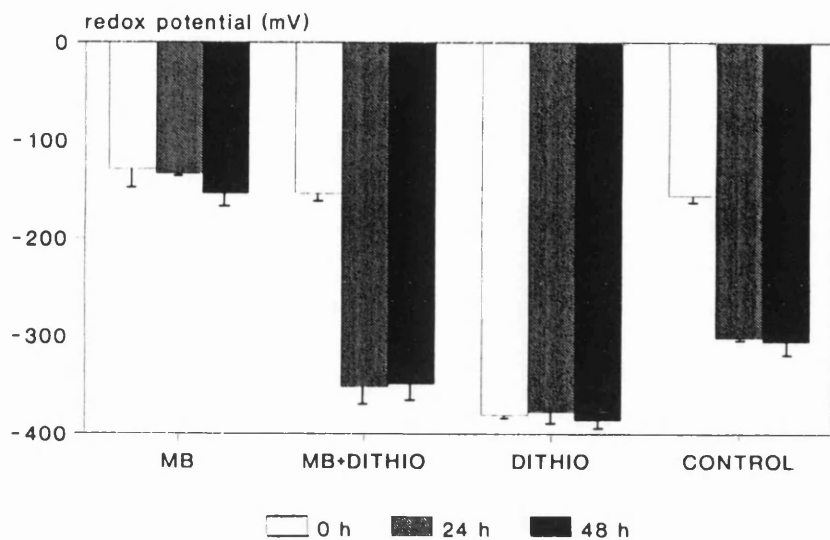


Fig. 2. Redox potentials of pre-reduced BM broth in the presence of 1.0 mg/ml methylene blue (MB), 2.0 mg/ml dithiothreitol (DITHIO), and a mixture of both after incubation in an anaerobic jar for 48 h. The results are expressed as the mean \pm standard deviation of three replicates.

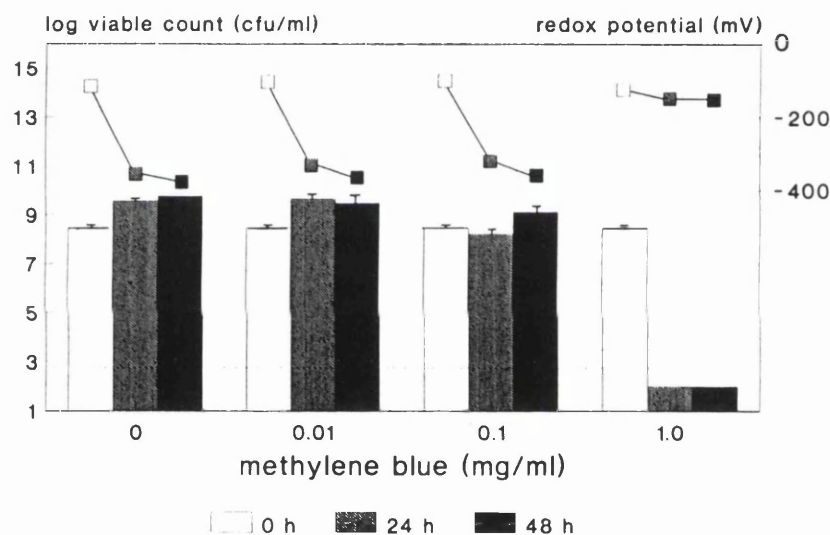


Fig. 3. Effect of methylene blue on the survival of suspensions of *P. gingivalis* during incubation in an anaerobic jar for 48 h. The horizontal broken line represents the lower limit of detection of the viable counting method used. Bars and lines represent viable counts and E_h values respectively. The viable counts are expressed as the mean \pm standard deviation of four replicates, while the E_h values are the mean of duplicate determinations.

ml of *P. gingivalis* to undetectable levels (< 500 cfu/ml) within 24 h (Fig. 3). Despite the fact that incubation was carried out anaerobically, the E_h of the suspensions containing 1.0 mg/ml of MB was maintained at a value not lower than -150 mV over the 48 h incubation period, whereas in dye-free controls the E_h decreased to -359 mV within 24 h and was -377 mV after 48 h (Fig. 3). No substantial decreases in viability were observed at lower concentrations of MB and, after 48-h incubation, the E_h of these suspensions were similar to those of dye-free controls.

Effect of dithiothreitol on survival of *P. gingivalis* in the presence of methylene blue. Dithiothreitol at a concentration of 2.0 mg/ml had no adverse effect on the viability of *P. gingivalis*, the viable count being $3.08 \times 10^9 \pm 0.14 \times 10^9$ cfu/ml after 48 h, while the

corresponding count of dithiothreitol-free suspensions was $5.55 \times 10^9 \pm 0.82 \times 10^9$ cfu/ml. From Fig. 4 it can be seen that 1.0 mg/ml of MB caused the death of $4.75 \times 10^6 \pm 0.35 \times 10^6$ cfu/ml within 24 h. Dithiothreitol (2.0 mg/ml) in the presence of 1.0 mg/ml of MB allowed survival of *P. gingivalis* and, in fact, permitted growth of the organism to numbers similar to that of the controls without MB. In the MB-containing suspensions the E_h reached only -142 mV after 48 h, whereas in the dye-free controls it decreased to -343 mV after 48 h. The E_h of dithiothreitol-containing suspensions was even lower, reaching -385 mV after 48 h. Addition of dithiothreitol and MB to the bacterial suspensions resulted in E_h values much lower than those of the MB-containing suspensions. These were similar to the controls that contained neither MB nor dithiothreitol and reached -333 mV after 48 h.

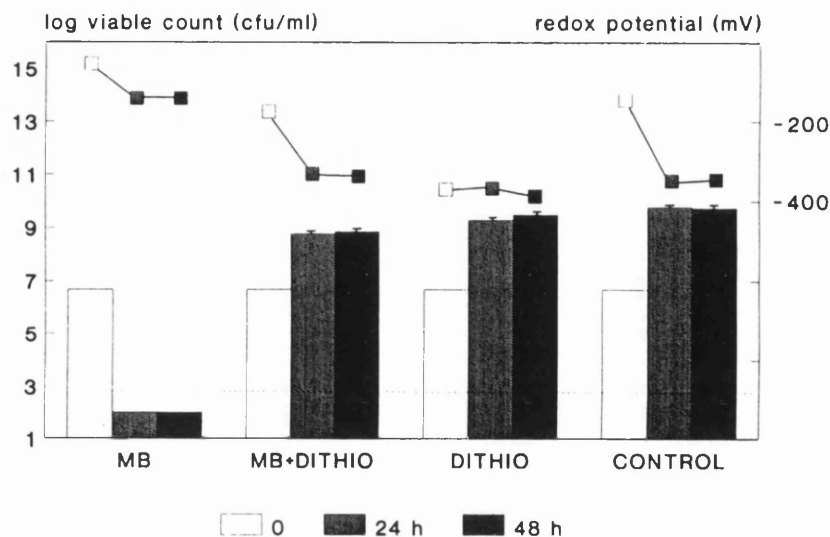


Fig. 4. Effect of methylene blue (MB) in the presence of dithiothreitol (DITHIO) on the survival of suspensions of *P. gingivalis*. Symbols are as in Fig. 3. The viable counts are expressed as the mean \pm standard deviation of four replicates, while the E_h values are the mean of duplicate determinations.

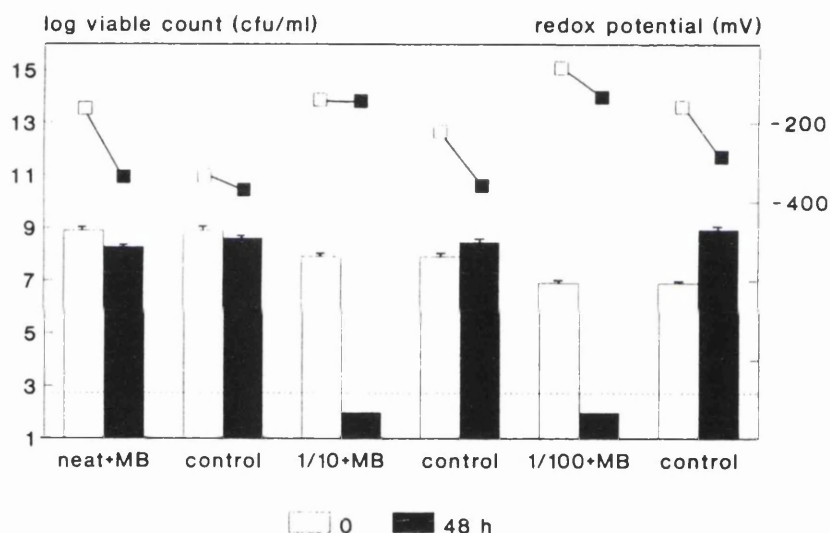


Fig. 5. Effect of methylene blue on the survival of 48-h cultures of *P. gingivalis* (neat = undiluted culture, 1/10 = 10-fold dilution of culture, 1/100 = 100-fold dilution of culture). Symbols are as in Fig. 3. The viable counts are expressed as the mean \pm standard deviation of four replicates, while the E_h values are the mean of duplicate determinations.

Effect of methylene blue on broth cultures of *P. gingivalis*. The effect of MB on the survival of *P. gingivalis* cultures diluted to different extents is shown in Fig. 5. When the original culture was diluted 1 in 10, no *P. gingivalis* were detected in the dye-containing cultures, while the viable count in the dye-free controls was $28.43 \times 10^7 \pm 4.90 \times 10^7$ cfu/ml. There were also corresponding differences in the E_h of the cultures. Hence, the E_h of the dye-containing cultures was -146 mV while that of the dye-free controls was -357 mV. A similar pattern was observed when the initial culture was diluted 1 in 100. *P. gingivalis* did not survive in the MB-containing cultures, while high counts ($8.89 \times 10^8 \pm 2.94 \times 10^8$ cfu/ml) were obtained in the dye-free controls. The respective E_h values were -134 mV and -286 mV after 48 h. In the case of the undiluted cultures, dramatic reductions in the viable count were not detected. After 48 h the viable count of the

dye-containing undiluted cultures ($18.80 \times 10^7 \pm 2.82 \times 10^7$ cfu/ml) was similar to that of the controls ($39.35 \times 10^7 \pm 6.25 \times 10^7$ cfu/ml). Correspondingly, the E_h of the dye-containing cultures was only slightly higher (-339 mV) than that of the controls (-371 mV; Fig. 5).

Discussion

It has long been recognized that E_h is an important ecological determinant for anaerobic bacteria [3, 6, 16]. For example, several investigations have demonstrated that such organisms require a low E_h for growth and survival [2, 5, 8] and that there is a limiting E_h above which growth cannot be initiated [12]. Although there are few reported studies concerned specifically with oral anaerobes, those that have been published show that the E_h of the environment does affect the growth and survival of these

organisms. Hence, Socransky et al. [17] found that *Treponema microdentium* could grow only over the narrow E_h range of -185 to -220 mV, while Aranki et al. [1] showed that the number of anaerobes recovered from the gingival crevice was greater at an E_h of -300 mV than at -150 mV.

On the other hand, some investigators have suggested that E_h per se may not be a major growth-determining factor. Hence, Walden and Hentges [18] have demonstrated the ability of several anaerobes to survive at a high E_h ($+325$ mV) induced by the presence of potassium ferricyanide. However, the nature of the redox agent would appear to be important, since it has also been shown that while growth of *Clostridium acetobutylicum* was unaffected at an E_h of $+350$ mV when the poisoning agent was ferricyanide, growth was inhibited by poisoning the E_h at $+80$ mV with tetramethylazofornamide (diamide) [12]. This may be related to the ability of the diamide to oxidize a number of electron donors, hence consuming the "reducing power" essential for biosynthesis and growth [13].

The present investigation has shown that addition of MB to pre-reduced culture medium to give a final concentration of 1.0 mg/ml resulted in an increase in the E_h of the medium and that this raised E_h could be maintained for at least 48 h in the powerfully reducing atmosphere present in an anaerobic jar. A similar effect on E_h was observed when the dye was added to suspensions of *P. gingivalis*, and this was accompanied by death of all of the bacteria present. The observed killing of *P. gingivalis* by MB may have occurred by a redox-related mechanism or by some other, as yet unrecognized, effect on the organism. The ability of the reducing agent dithiothreitol to reverse the dye-induced killing of *P. gingivalis* is suggestive of a redox-mediated phenomenon. This is supported by the redox data on the bacteria-free broths and the bacterial suspensions. Hence, in both cases dithiothreitol was able to overcome the E_h -raising effect of the MB and lower the E_h to values comparable to those found in the dye-free broths, which, in the case of the bacterial suspensions, resulted in survival of the organism.

With regard to the possibility of a redox-independent toxicity, MB appears to be only weakly antibacterial. For example, under aerobic conditions, it did not inhibit the growth of 13 different Gram-negative species at 1.0 mg/ml, the concentration used in the current investigation, although it was bacteriostatic to several Gram-positive species at this concentration [4]. There appear to be no publications concerned with the possible mechanism of such toxicity.

As well as being able to alter the E_h of a system, redox-modifying agents also have a poisoning or buffering capacity which is concentration dependent. In this investigation, the redox-poisoning capacity of MB was demonstrated by its ability to prevent a lowering of the E_h of suspensions of *P. gingivalis* and uninoculated media when these were incubated in an anaerobic jar for 48 h. Furthermore, the poisoning effect of the MB was dose-related, concentrations lower than 0.1 mg/ml being unable to counteract the reducing power of an anaerobic jar. However, the capacity of the most effective redox-poisoning solutions (1.0 mg/ml of MB) could be overcome by extremely powerful reductants such as 48-h cultures of *P. gingivalis*. Although addition of MB to such cultures caused an initial rise in E_h , this was not maintained during the 48-h period of incubation, and the viability of these cultures was not dramatically affected. The concentration of bacteria in these cultures was similar to that in the suspensions used previously, the major difference being the considerably greater reducing capacity which would have accumulated during the 48-h growth period in the case of the former [16]. The absence of appreciable killing under these circumstances supports the hypothesis that the killing observed in other experiments was a redox-related phenomenon, since a similar degree of killing ought to have been detectable on addition of MB to these cultures if some other mechanism of toxicity was involved. MB would appear to be toxic to *P. gingivalis* only in the oxidized form.

While the results of this investigation are consistent with a hypothesis linking environmental E_h with the death of *P. gingivalis*, interpretation of the meaning of E_h measurements can be difficult [11, 12, 16]. Hence, the MB-induced rise in E_h of the cultures per se may not have been the prime cause of the killing of *P. gingivalis*. This may be more directly attributable to the oxidation of electron donors (e.g., NADH and NADPH) by MB, resulting in the consumption of reducing power which is thereby diverted from its essential role in energy generation and biosynthesis. The E_h of the cultures may have been an indication of the likelihood of such reactions taking place or may have merely signified the presence of an oxidizing agent with the ability to form a redox couple capable of interacting with the E_h -measuring electrode. The latter may not necessarily be related to intracellular oxidizing capability [6]. In the sense described, MB would be exerting a cidal effect on anaerobic bacteria in much the same way as oxygen, although the latter has, in addition, other means of adversely affecting such organisms [11]. The ability of MB to raise the E_h of an environment resulting in

death of the incumbent anaerobic microflora, possibly by oxidation of essential electron donors and/or enzymes active only in the reduced form, may be useful in treating diseases such as chronic periodontitis. In the lesion of this disease, the periodontal pocket, a highly reducing environment exists in which the E_h can be as low as -300 mV [7, 9]. This enables the survival of rich and diverse anaerobic microflora, which includes organisms such as *P. gingivalis*, which are thought to be responsible for the tissue destruction accompanying the disease [14]. The results of this investigation have demonstrated that MB can not only raise the E_h of an anaerobic environment but can also maintain it at this higher level, and this is accompanied by the death of large numbers of the anaerobic bacteria (*P. gingivalis*) present. It may, therefore, be useful in eliminating this organism, and possibly other anaerobes, from the periodontal pockets of patients with chronic periodontitis. Indeed, a small-scale clinical trial has shown that MB decreased the proportion of Gram-negative anaerobes, spirochaetes, and other anaerobic bacteria in the subgingival plaque of patients with chronic periodontitis [19]. Work is continuing on the development of other redox agents that may be applicable to the treatment of this disease in humans.

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The effect of methylene blue, a redox agent, on the viability of *Fusobacterium nucleatum*

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Key words: methylene blue, redox potential, *Fusobacterium nucleatum*

Abstract

The aim of this study was to determine whether methylene blue, by raising the redox potential (E_h), could affect the survival of the anaerobic periodontopathogen *Fusobacterium nucleatum*. Methylene blue (MB), a redox dye with an E_h of +71 mV in its fully oxidized state, at a final concentration of 1.0 mg/ml was able to poise the redox potential of a suspension of *F. nucleatum* at a level some 200 mV higher than that of dye-free controls in an anaerobic environment. After 24 h anaerobic incubation, the viable count of the *F. nucleatum* suspension containing MB had decreased from $25.9 \times 10^6 \pm 1.3 \times 10^6$ cfu/ml to undetectable levels. MB at 1.0 mg/ml in the presence of 2.0 mg/ml dithiothreitol did not affect the survival of *F. nucleatum* and the E_h of suspensions containing both MB and dithiothreitol were similar to those of the controls after 24 h anaerobic incubation. These results have shown that, when added in excess of the reducing capacity of a system, MB can cause death of the anaerobic periodontopathogen *F. nucleatum*. Topical application of MB or other redox agents may provide an effective way of eliminating this and possibly other anaerobic bacteria from the periodontal pockets of patients with chronic periodontitis.

Introduction

The characteristic lesion of chronic periodontitis is the periodontal pocket formed by the detachment of the junctional epithelium from the tooth root surface. The microenvironment of the periodontal pocket is highly anaerobic, with a low oxygen tension (Mettraux *et al.*, 1984) and a low redox potential (E_h) (Kenney and Ash, 1969). Many anaerobic bacteria are highly sensitive to the presence of oxygen (Morris, 1980; Loesche, 1969) and are also dependent on a low E_h for growth (Gottschalk and Peinemann, 1992; Socransky *et al.*, 1964). The periodontal pocket therefore provides an ideal environment for these bacteria and a highly complex flora develops.

A number of Gram-negative obligately anaerobic bacteria including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Campylobacter rectus* and various spirochaetes have been specifically implicated in the aetiology of chronic periodontitis (Socransky and Haffajee, 1992). The products of these organisms are thought to contribute to the tissue damage and bone loss characteristic of the disease (Slots and Genco, 1984). Treatment of periodontal disease involves the mechanical removal of subgingival plaque. Increasingly this procedure is supplemented by the application of antibiotics and antiseptics to the periodontal pocket (Slots and Rams, 1990).

An alternative approach to controlling the microbial population would be to alter the environment to such an extent that organisms were no longer able to survive. Several studies have investigated the effect of exposing the anaerobic microflora of the periodontal pocket to molecular oxygen or oxygenating agents (Chasens, 1978). However, few have involved the addition of an oxidizing agent to the periodontal pocket, which, without producing O₂, would also cause an increase in E_h and oxidation of the pocket contents.

The E_h of the healthy gingival crevice is in the region of +74 mV (Kenney and Ash, 1969), whereas in the periodontal pocket, E_h values as low as -300 mV have been recorded (Marsh and Martin, 1992). The E_h of the redox agent used in this study, MB, when it is fully oxidized is +71 mV (Jacob, 1970). By adding MB to the periodontal pocket it may be possible to raise the E_h to a level which is inhibitory to the survival of the anaerobic microflora.

The aim of this study was to determine whether MB, by raising the E_h of its environment, could affect the survival *in vitro* of the anaerobic periodontopathogen *Fusobacterium nucleatum*.

Materials and methods

Bacterial strains and media

Fusobacterium nucleatum NCTC 10562 was maintained by weekly sub-culture on Wilkins-Chalgren (WC) anaerobe agar supplemented with 5% horse blood (Oxoid Ltd, Great Britain). The plates were incubated anaerobically at 37°C using the Oxoid GasPak system. The liquid medium used in experiments (BM broth) consisted of tryptone soya broth (10 g), proteose peptone (10 g), yeast extract (5 g), NaCl (5 g) and L-cysteine (0.75 g) per litre of distilled water. The pH was adjusted to 7.4 before autoclaving at 121°C for 15 min. Immediately prior to use a haemin and menadione supplement was added to give final concentrations of 5 µg/ml and 0.5 µg/ml, respectively. BM broth was pre-reduced in an anaerobic jar for 24 h at 37°C. All chemicals were obtained from Sigma Chemical Company Ltd, Great Britain, unless otherwise stated.

Effect of methylene blue on the survival of a suspension of *F. nucleatum*

A suspension of the organism was prepared in pre-reduced BM broth using bacteria harvested from 72 h WC agar plates. The suspension was aliquoted into sterile test tubes and methylene blue (Boots Company PLC, Great Britain) was added to give final concentrations of 0.0–1.0 mg/ml. An equal volume of distilled water was added in place of MB to controls. The samples were incubated in an anaerobic jar at 37°C. Viable counts and measurements of E_h were carried out at 0, 24 and 48 h. A combination redox cell with a platinum electrode and a Ag/AgCl reference electrode was used for measuring

E_h (Russel pH Ltd, U.K.). E_h measurements were taken immediately after removal of the suspensions from the anaerobic environment taking care to minimise aeration when inserting the redox probe. The pH of all samples was measured immediately after E_h determination.

Effect of methylene blue on the survival of a suspension of *F. nucleatum* in the presence of dithiothreitol

MB was added together with dithiothreitol to a suspension of *F. nucleatum* to give final concentrations of 1.0 mg/ml and 2.0 mg/ml, respectively. Controls contained MB only, dithiothreitol only or neither. The samples were incubated in an anaerobic jar at 37°C and viable counts and measurements of E_h were carried out at 0, 24 and 48 h as described above.

Effect of methylene blue on the survival of a 48 h broth culture of *F. nucleatum*

Pre-reduced BM broth was inoculated with *F. nucleatum* and incubated anaerobically at 37°C for 48 h. Serial dilutions of this 48 h culture were prepared in pre-reduced BM broth and MB was added to give a final concentration of 1.0 mg/ml. Distilled water was added to controls in place of MB. Cultures were removed for viable counting and measurement of E_h after 0, 24 and 48 h anaerobic incubation.

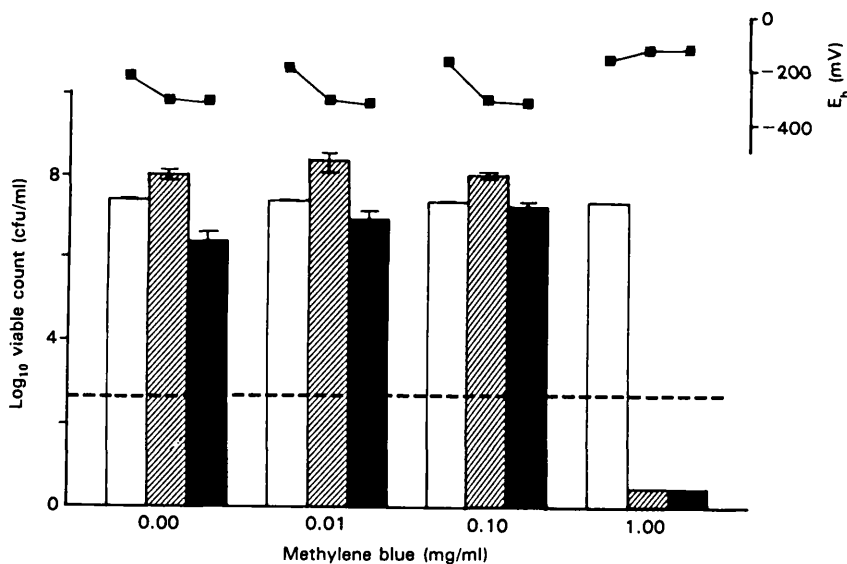


Figure 1 Effect of methylene blue on the survival of a suspension of *F. nucleatum* in an anaerobic environment. Histogram represents viable counts at 0 h (open column), 24 h (hatched column) and 48 h (solid column). ■, E_h values. The horizontal broken line indicates the lower limit of the viable counting method used. The viable counts are expressed as the mean and standard deviation of four replicates while the E_h values are the mean of duplicate determinations.

Results

MB at a final concentration of 1.0 mg/ml poised the E_h of suspensions of *F. nucleatum* at a level which was approximately 200 mV higher than that of control dye-free suspensions. After 24 h anaerobic incubation the E_h of suspensions containing 1.0 mg/ml MB was -123 mV compared to -317 mV for controls. This was accompanied by a reduction in the viable count of *F. nucleatum* from $25.9 \times 10^6 \pm 1.3 \times 10^6$ cfu/ml at 0 h to undetectable levels (< 500 cfu/ml) within 24 h (Figure 1). Lower concentrations of MB were unable to poise the E_h at a higher level and after 24 h anaerobic incubation the E_h of suspensions containing 0.01 and 0.1 mg/ml MB were -316 mV and -315 mV, respectively. There were no significant differences in the viability of these suspensions when compared with their respective controls.

Dithiothreitol at a concentration of 2.0 mg/ml had no adverse effect on the viability of *F. nucleatum*. The viable count of suspensions containing dithiothreitol was $25.3 \times 10^8 \pm 8.1 \times 10^8$ after 24 h anaerobic incubation compared with the overall control which was $33.1 \times 10^8 \pm 3.7 \times 10^8$ at this time (Figure 2). Dithiothreitol caused an immediate decrease in the E_h of the *F. nucleatum* suspension to -300 mV at 0 h. As before, MB by itself at 1.0 mg/ml poised the E_h of *F. nucleatum* suspensions at a level approximately 200 mV higher than that of the overall controls, -135 mV compared to -344 mV. This was accompanied by complete killing of the

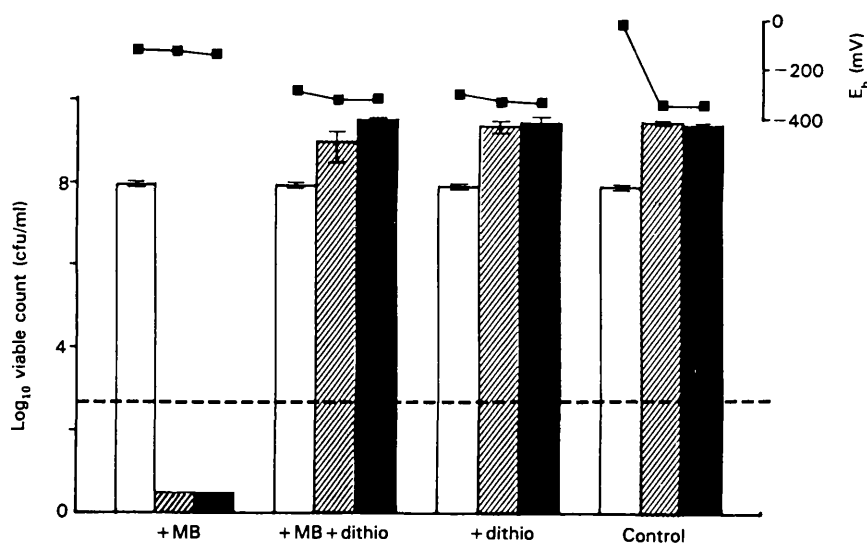


Figure 2 Effect of methylene blue (MB) in the presence of dithiothreitol (dithio) on the survival of a suspension of *F. nucleatum*. Symbols are as in Figure 1. The viable counts are expressed as the mean and standard deviation of four replicates while the E_h values are the mean of duplicate determinations.

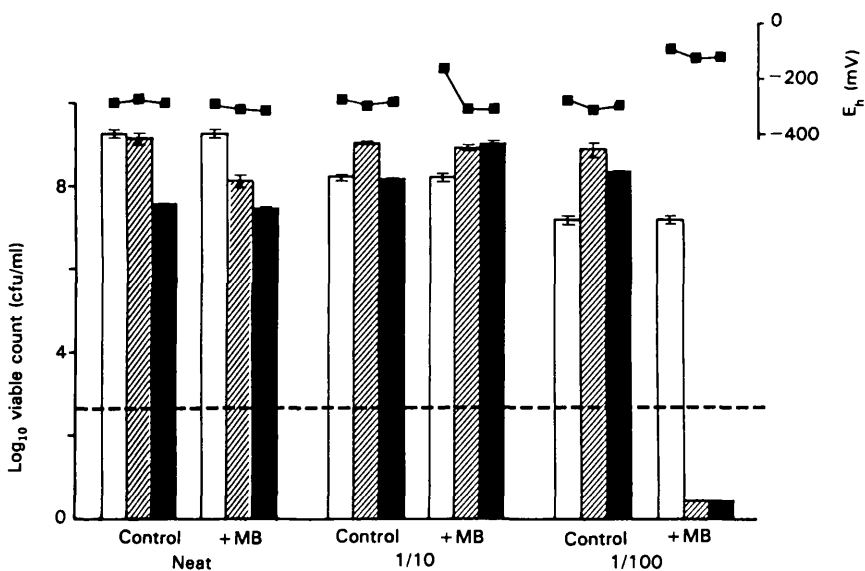


Figure 3 Effect of methylene blue on a 48 h broth culture of *F. nucleatum* (neat indicates undiluted culture; 1/10, 10-fold dilution of culture; 1/100, 100-fold dilution of culture). Symbols are as in Figure 1. The viable counts are expressed as the mean and standard deviation of four replicates while the E_h values are the mean of duplicate determinations.

F. nucleatum suspension which contained $8.8 \times 10^7 \pm 1.3 \times 10^7$ cfu/ml at 0 h. However, when MB and dithiothreitol were added together to suspensions of *F. nucleatum* to give final concentrations of 1.0 mg/ml and 2.0 mg/ml, respectively, the E_h after 24 h anaerobic incubation decreased to -328 mV which was similar to that of the overall control at this time (-344 mV). In the presence of dithiothreitol, MB at 1.0 mg/ml had no adverse effect on the survival of *F. nucleatum* and, indeed, the organism was able to multiply.

Figure 3 shows the effect of MB at 1.0 mg/ml on serial dilutions of a 48 h broth culture of *F. nucleatum*. At this concentration MB was only effective against a 1/100 dilution of the culture. MB caused a reduction in the viable count of the 1/100 dilution from $19.4 \times 10^6 \pm 4.4 \times 10^6$ to undetectable levels after 24 h anaerobic incubation. The E_h of the 1/100 dilution containing MB was -129 mV compared with -311 mV for the dye-free control. MB at 1.0 mg/ml was not effective against an undiluted culture of *F. nucleatum* and a 1/10 dilution of this culture. Viable counts of the cultures were similar to those of the respective dye-free controls after 24 and 48 h anaerobic incubation. The E_h values of these cultures after 24 h anaerobic incubation were also similar, -315 mV for the undiluted culture plus MB and -308 mV for the 1/10 dilution plus MB, compared with -285 mV and -302 mV for the respective controls. In these cultures MB was completely reduced. For the neat, undiluted culture, the reduction of the dye occurred very rapidly and the E_h values at 0 h were the same as those of the controls.

Discussion

It has long been known that both a low concentration of O_2 and a low E_h (Hungate, 1969; Aranki *et al.*, 1969) are important for the isolation and cultivation of strictly anaerobic bacteria. The presence of O_2 , will by its very nature, lead to an increase in E_h . The question remains as to whether E_h , in the absence of O_2 , has any effect on the growth of anaerobic bacteria. Socransky *et al.* (1964) showed that even in an O_2 -free environment *T. microdentium* could only grow in a narrow E_h range of between -185 and -220 mV. Other workers suggest that it is the presence of O_2 , causing an increased E_h , which is inhibitory to the growth of anaerobic bacteria. Walden and Hentges (1975) demonstrated growth of *C. perfringens* under anaerobic conditions with an E_h of $+325$ mV maintained by the addition of potassium ferricyanide, whilst no growth occurred in aerated cultures with a low E_h suggesting that O_2 was the detrimental factor. However, Hanke and Katz (1943) showed that it was possible to grow certain anaerobic bacteria in a continuous current of air when the E_h was maintained at a sufficiently low level. In these experiments the E_h was maintained electrolytically without the addition of any chemical agents.

Hungate (1969) stated that whilst agents such as potassium ferricyanide can be used to induce a high redox potential independent of oxygen, and may interact with an E_h measuring electrode, this does not necessarily give an indication of whether the agent is capable of affecting the intracellular environment and hence cell metabolism. O'Brien *et al.* (1970) showed that another agent *N,N,N',N'*-tetramethylazofornamide (diamide) was capable of oxidizing flavin nucleotides (FMNH₂ and FADH₂) and also NADH and NADPH. If such an oxidizing agent were able to gain access to a bacterial cell it would be likely to exert a detrimental effect on biosynthetic pathways and energy generation.

Previous experiments in our laboratory have shown that the addition of MB to pre-reduced culture medium to give a final concentration of 1.0 mg/ml poised the E_h some 170 mV higher than that of controls to which distilled water had been added in place of MB (Fletcher and Wilson, 1993). This elevated E_h was maintained for at least 48 h in the oxygen free, highly reducing atmosphere of an anaerobic jar. This current investigation has shown that when MB was added to suspensions of *F. nucleatum*, the E_h was poised at a level higher than that of the controls and this was associated with complete killing of the organism. Addition of the reducing agent dithiothreitol to a suspension of *F. nucleatum* containing MB at 1.0 mg/ml ensured survival of the organism. This, together with the redox data, suggests that killing of *F. nucleatum* by MB is due to some redox-related mechanism.

Redox modifying agents have a buffering capacity which is concentration-dependent (Jacob, 1970). Thus at 1.0 mg/ml MB was able to poise the E_h of suspensions of *F. nucleatum* at a higher level compared with controls. Lower

concentrations of MB were unable to poise the E_h of a suspension of *F. nucleatum* in the highly reducing conditions of an anaerobic atmosphere and the E_h decreased to control levels and the organism survived. In addition, the ability of a redox agent to poise the E_h will depend on the capacity of a system to counteract any change in E_h . Suspensions of *F. nucleatum* containing dithiothreitol had a large reducing capacity which was able to counteract the effect of the methylene blue. Similarly a 48 h culture of *F. nucleatum* was unaffected by high concentrations of MB. This was most likely to have been due to a large reducing capacity as a result of the accumulation of large amounts of reduced metabolites during the 48 h growth period.

MB is known to be taken up by viable bacterial cells (Tuite and Kelly, 1993) and we have also shown that MB is capable of oxidizing NADH and NADPH (unpublished data). Thus MB may be acting intracellularly by consuming reducing power in the form of NADH and NADPH which is required for energy generation and biosynthesis. MB may also cause oxidation of components of electron transport chains. Sakuri *et al.* (1980) showed that the beta toxin from *C. perfringens* could be inactivated by oxidizing agents and that activity could be restored by treatment with dithiothreitol. It is possible that MB may be capable of oxidizing enzymes which are only active in the reduced form.

The results of this investigation have shown that MB poised the E_h of a suspension of *F. nucleatum* at a higher level compared with controls in a highly anaerobic environment and this was associated with the death of the organism. MB at 1.0 mg/ml had a limited E_h buffering capacity and was unable to poise the E_h of suspensions of *F. nucleatum* containing dithiothreitol and 48 h broth cultures of the organism due to the large reducing capacity of these systems. The contents of the periodontal pocket would also have a large reducing capacity due to the accumulation of reduced bacterial metabolites. It would therefore be necessary to add an excess of MB to the periodontal pocket to overcome this reducing capacity. A small scale clinical trial has shown MB to be effective in decreasing the proportion of Gram-negative anaerobes, spirochaetes and other anaerobic bacteria in the subgingival plaque of patients with chronic periodontitis (Wilson *et al.*, 1992), suggesting that this approach has potential for the treatment of this disease.

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Accepted 16 December 1994

Evaluation of the efficacy of a redox agent in the treatment of chronic periodontitis

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Gibson MT, Mangat D, Gagliano G, Wilson M, Fletcher J, Bulman J, Newman HN: Evaluation of the efficacy of a redox agent in the treatment of chronic periodontitis. *J Clin Periodontol* 1994; 21: 690-700. © Munksgaard, 1994.

Abstract. A redox dye, methylene blue, was compared with subgingival root surface debridement and sterile water in the treatment of adult periodontitis. Plaque and gingival indices, bleeding on probing, and microbiological samples were obtained at baseline, and at 1, 4, 8 and 12 weeks following treatment. All subjects had matched pockets in each of the 4 quadrants, of 5 mm or more. One treatment consisted of 0.1% methylene blue gel irrigated professionally at 0, 1 and 4 weeks, and by subjects at days in between up to 4 weeks, at chosen sites within a randomly selected quadrant (split-mouth design). A 2nd treatment was sterile water irrigation as above. A 3rd quadrant received subgingival debridement, and sites in the 4th received methylene blue incorporated into a slow-release device of a biodegradable collagen alginate vicryl composite. All sites showed improvements in clinical and microbiological parameters. However, no statistically significant differences between treatment types were found for clinical measurements. Although plaque index tended to increase after week 1, gingival index was reduced, as was the papilla bleeding index. Probing depth reductions were approximately 1.2 mm for all treatments. Microbiological variables showed an increase in cocci and a decrease in motile organisms for all groups, the latter reaching statistical significance for subgingival debridement. The reductions in spirochaetes were significant for subgingival debridement and methylene blue by slow-release. Culture demonstrated an increase in the aerobe:anaerobe ratio for all groups, which was statistically significant initially (weeks 1 and 4) for subgingival debridement. Methylene blue was also effective statistically in improving this ratio, both by irrigation and slow-release (week 4). Methylene blue also significantly reduced the numbers of black-pigmented anaerobes during the trial period, both by irrigation and slow-release, which sterile water and subgingival debridement failed to do. No serious adverse experiences were seen, however, significantly greater morbidity was associated with subgingival debridement. These results clearly demonstrate that in altering the microflora to one that is more compatible with periodontal health, methylene blue treatment is comparable, or even better, than the currently standard treatment of subgingival debridement, and is better tolerated.

Key words: redox potential; chronic periodontitis; methylene blue

Accepted for publication 2 December 1993

It is well-established that chronic periodontitis is the result of the accumulation of subgingival plaque. Traditionally, treatment has been aimed at surgical and more recently, primarily non-surgical therapy and has consisted of thorough debridement of the root surfaces, together with pocket elimination or reduction (Lindhe & Nyman 1975,

Pihlstrom et al. 1981, 1983, Hill et al. 1981, Lindhe et al. 1982a, 1984, Isidor et al. 1984a, Westfelt et al. 1985, Isidor & Karring 1986, Ramfjord et al. 1987). Mechanical therapy is time-consuming, operator-dependent, demanding of patient compliance, and not universally successful. Chemical plaque control appears to hold promise as an

adjunct to routine oral hygiene, by overcoming some of the problems of mechanical methods. In chronic periodontitis, the subgingival microflora is diverse and dominated by a range of obligate anaerobes, some of which have been specifically implicated in the aetiology of chronic periodontitis. These include *Porphyromonas gingivalis*, *Pre-*

motella intermedia, *Fusobacterium nucleatum*, *Campylobacter rectus*, *Eubacterium* spp. and spirochaetes (Slots & Listgarten 1988, Moore 1987). Since oxygen is toxic to such organisms, early therapeutic measures included attempts to increase available oxygen within the pocket by applying gaseous oxygen (Dunlop 1913). More recently, Ivanov et al. (1979) Gotsko et al. (1980) and Sumachev (1983) have recommended hyperbaric oxygen. Shannon et al. (1988), however, showed that neither hyperbaric nor monobaric oxygenation improved periodontal wound healing. Application of hydrogen peroxide has been evaluated both in vitro and in vivo. Keyes et al. (1978a,b) and Keyes (1982) reported that a daily application of baking soda, hydrogen peroxide and salt had a similar effect to scaling and root planing. These findings have not been confirmed (Greenwell et al. 1985, Wennstrom et al. 1987).

Since the growth of anaerobes in an ecosystem is also dependent on a low redox potential (Smith & Williams 1984, Finegold 1989), an alternative approach to their control could involve alteration of the redox potential (Eh) of the subgingival region. Redox potentials as low as -300 mV exist in periodontal pockets, enabling the survival of anaerobic bacteria (Kenney & Ash 1969). Furthermore, these workers have shown that the Eh (+70 mV) of the healthy gingival crevice, with its low proportions of anaerobes, is significantly higher than that of the periodontal pocket (-48 mV). By raising the redox potential of the periodontal pocket, it may be possible to create an environment incompatible with growth of anaerobic periodontal pathogens, thereby enabling control of these organisms.

A variety of substances, e.g., redox dyes and transition metal ions, can raise the redox potential of an ecosystem without producing molecular oxygen. A recent pilot clinical study using one of these substances, methylene blue, applied by subgingival irrigation, demonstrated shifts towards a subgingival flora more compatible with periodontal health, and a reduction in inflammation, as assessed by gingival crevicular fluid flow, over a 28-day period (Wilson et al. 1992). The purpose of the present study was to investigate the effects of methylene blue applied to periodontal pockets by irrigation or in a sustained release carrier, in patients with chronic periodontitis.

Material and Methods

Patient selection

24 adult patients, 10 male and 14 female, aged 35-55 years (mean 42.7 ± 6.43) were admitted to the trial. The criteria for selection were as follows.

- (1) The presence of at least one true interdental periodontal pocket ≥ 6 mm, with radiographic evidence of bone loss, in each of the 4 quadrants.
- (2) No history of any periodontal therapy in the preceding 6 months.
- (3) No use of antibiotics or oral antiseptics during the previous 6 months.
- (4) No systemic condition which might influence the course of the disease.
- (5) Ability to attend at regular intervals.

Clinical trial design

A 3-month randomised single blind controlled study was undertaken, using a quadrant split-mouth design. The nature and design of the study were explained to the subjects and voluntary written consent obtained for their participation. Sites were selected from probing depth charts, and adjacent teeth were not used because of potential interference of two different treatments. The study was approved by the local Joint Research and Ethics Committee. 6 weeks prior to entry, subjects were educated as to the nature of chronic inflammatory periodontal disease and the advantages and disadvantages of existing treatments. Oral hygiene instruction was given (MG), emphasizing the need for efficient plaque removal, specifically using a miniscrub technique with a small-headed brush (Sensodyne 3.5, Stafford Miller, UK) and in flossing and the use of a fluoride toothpaste. A 2nd appointment 1 to 2 weeks later was made to re-inforce oral hygiene. At this visit, all teeth received a supragingival scale and polish (MG). Since some sites were to receive irrigation, subjects were also carefully instructed in subgingival irrigation at sites other than test or control or immediately adjacent sites, at this second visit, as described by Hardy et al. (1982). A 2.5 ml syringe fitted with a 0.5 mm tip diameter blunt-ended needle with a side aperture (Luer-Lock Hub, Stram Dental Inc., Illinois, USA) was used. The needle was pre-bent, and the tip placed subgingivally and fluid slowly injected to fill the

pocket until just visible at the gingival margin.

A separate investigator (DM), using a random table, assigned one of four different treatments to each quadrant as follows.

- (1) Sterile water irrigation.
- (2) Irrigation with 1% methylene blue (Macarthy Medical, Romford, Essex).
- (3) 33% (w/w) methylene blue incorporated in a biocompatible and biodegradable collagen alginate vicryl composite controlled-release carrier (Johnson and Johnson Medical Biopolymers Ltd., Stirling, Scotland).
- (4) Subgingival debridement, under local anaesthesia, using a combination of ultrasonic (Cavitron), and conventional hand instrumentation.

On entry to the trial, the teeth to be treated were shown to each subject and the presence and position of periodontal pockets demonstrated. Initial irrigation was carried out by the investigator (DM) after baseline recordings, and further instruction given as to which fluid should be placed in which site. Individual labelled bottles, one for each day of each irrigant, were given to the subjects. After root debridement in one quadrant, irrigation was carried out, and, in the fourth quadrant, the sustained-release device was placed. These pre-sterilized strips, 10 mm \times 2 mm, were gently packed into the pocket, without producing subject discomfort, using a pushscaler, until just visible at the gingival margin.

Efficacy variables

Recordings of the efficacy variables were made at days 0, 7, 28, 56 and 84, and in the following order, by the same investigator (MG).

- (1) Plaque index (Silness & Loe 1964).
- (2) Gingival index (Loe & Silness 1963).
- (3) Bacteriological sampling.
- (4) Probeable pocket depth
- (5) Bleeding on probing using the criteria of the Papilla Bleeding Index (Mühlemann 1977). The University of North Carolina Periodontal Probe (Hufriedy, USA) with 0.5 mm tip diameter was used for pocket measurements.

Bacteriological sampling was performed as follows: supragingival plaque was removed, and two sterile endodontic paper points introduced into each pocket until they had reached an apical limit. These were left in situ for 15 s and

immediately transferred to 0.2 ml sterile pre-reduced normal (0.85%) saline. Samples were used for both darkfield examination and cultural analysis. Darkfield examination was undertaken within 10 minutes of collection. The proportions of spirochaetes, motile organisms, rods and cocci were then determined, counting 200 organisms per sample (GG).

A series of 10-fold dilutions of each plaque suspension was prepared in pre-reduced brain-heart infusion broth (Oxoid Ltd., Basingstoke, UK). Duplicate aliquots of 0.02 ml of each dilution were spread on Wilkins-Chalgren agar (Oxoid Ltd.) supplemented with 10% defibrinated horse blood (WC) – for estimation of the total number of obligate and facultative anaerobes (total anaerobic count) and black-pigmented anaerobes. These plates were incubated under anaerobic conditions (Gas Pak, Oxoid Ltd.) at 37°C. An additional set of WC plates was incubated in a candle jar at 37°C to determine total aerobes and facultative anaerobes (total aerobic count). Plates were incubated for up to 14 days and colonies counted after 7 and 14 days using a low-power binocular microscope. Black-pigmented colonies on the plates incubated anaerobically were also counted, then sub-cultured and tested for their ability to grow under aerobic conditions. Those that could not were designated black-pigmented anaerobes.

The clinical examiner (MG) was tested for consistency until satisfactory for all clinical variables prior to commencement of the study. Duplicate measurements of non-participating subjects with similar probing depths were made 30 min apart ($\kappa > 0.9$ for all measurements). Similarly, the examiners for darkfield (GG) and cultural examination (JF) were also trained for consistency in pilot studies. Duplicate counts were made for each until high levels of correlation were obtained prior to the study.

Subjects were informed that any adverse experiences should be reported for documentation on a questionnaire.

Statistical analysis

Since the majority of subjects presented with just one eligible site per quadrant, it was on this basis that analysis was undertaken. Thus for each treatment, analysis refers to one site per subject, and therefore in this context the term

“site” and “subject” may be used interchangeably. To have attempted to include a second site in those few quadrants where they occurred would have introduced an unquantifiable bias to the result.

Clinical data

For PI, GI, PD and PBI, when computing the change in the variable under review within a given group over time, the Sign Test was used. In this test, the number showing an improvement over the time period was compared with the number showing deterioration. Those showing no change were excluded from the analysis. As the samples were small, binomial probability analysis was used to determine whether the proportion of subjects showing an improvement differed significantly from 0.5, which would be the expected probability if there were no difference in the proportions improving and deteriorating.

For comparisons between groups at given time intervals, the proportions improving and deteriorating were again compared, but since in these instances the samples were independent, a z -test of the differences between the proportions was employed.

Bacteriological data

3 main analyses were undertaken.

(a) *Darkfield microscopy*. The proportion of subjects showing improve-

ment or deterioration from day 0 in terms of (i) spirochaetes (ii) cocci (iii) motiles and (iv) others was determined as above.

(b) *Aerobe/anaerobe log ratios* were analyzed using one-way ANOVA supported, where appropriate, by t -tests.

(c) *% of black pigmented anaerobes* were analyzed using the Kruskal-Wallis one-way ANOVA by rank for between-group comparisons, and the Friedman two-way ANOVA by ranks for within-group comparisons.

Results

A total of 96 sites in 24 patients were included in the final analysis. 1 patient did not receive the sterile water irrigation, since the pre-selected tooth was removed from the study for treatment of a combined periodontal-endodontic lesion in the six weeks prior to the baseline measurement.

Clinical variables

Plaque index

At baseline, approximately 60% of all subjects had plaque scores of 0 or 1 (Fig. 1). Being a subjective measurement, and not normally distributed, analysis was based on the proportion of subjects scoring less than 2 for determining between group differences. The majority of subjects therefore had low

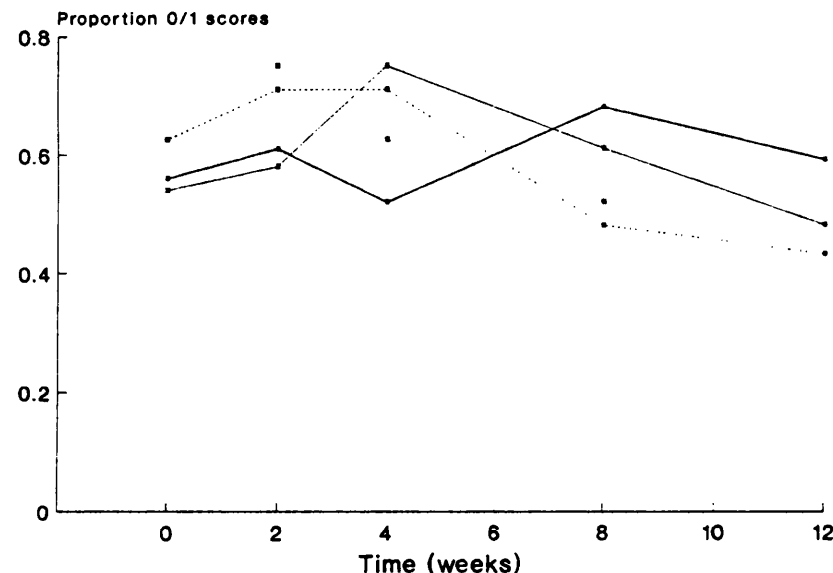


Fig. 1. Effect of treatment modality on the proportions of sites with a plaque index of 0 or 1 (—: sites irrigated with a 1% aqueous solution of methylene blue; —: sites irrigated with sterile distilled water; ···: sites receiving a methylene blue slow release device; - - -: sites subjected to subgingival debridement).

plaque scores at the beginning of the trial. No statistical differences were observed at any time point for each treatment. Fluctuations were observed, however, with a trend towards an increased proportion of subjects with low plaque scores (0/1) after 1 week with a general decrease in the proportion of subjects with low plaque scores at week 12.

The results may also be expressed in terms of the proportion of the total numbers of subjects who showed improvement, no change or deterioration at each time point. All sites, independent of treatment type, showed an improvement in plaque score from baseline at one week. Thereafter, more sites showed increased plaque scores than

decreased, although MBI and MB/SR groups sustained lower plaque levels until week 8. On a subject basis however, none of these differences were statistically significant.

Gingival index

The results for gingival index show a similar pattern (Fig. 2). However a low proportion (20–30%) of subjects had gingival index scores of 0 or 1 at baseline (that is, 70–80% had higher plaques (2 or 3) at baseline). This increased in the first month to 45–60%, with a reduction at week 12, although gingivitis scores were still relatively improved independent of treatment type. Regarding proportions of subjects showing an improvement, no change or deterioration, no one treatment type was significantly different. However, few subjects showed deterioration in gingival index at any time. Whilst sites initially showed improvement even with sterile water irrigation, this was not sustained. The most sustained improvement was observed for gingival index following subgingival debridement.

Probing depth

For all sites, independent of treatment type, there was an overall mean reduction in probing depth (Fig. 3). The largest decrease appeared to be for subgingival debridement (7.4 mm to 6.2 mm), although differences between groups were small. The proportions of sites improving, deteriorating or unchanging at each time point were similar in all 4 groups.

Papilla bleeding index

Analysis was based on the proportion of subjects scoring less than 3. Approximately 40% of all sites scored 0, 1 or 2 for bleeding at baseline (Fig. 4), implying that the majority of sites were markedly inflamed initially. By the end of the trial, 50–70% of sites had lower bleeding scores. Differences between treatments were again not statistically significant. The methylene blue irrigation and slow-release groups showed a trend towards improvement at the last time point.

Bacteriological variables

Dark-ground microscopy

Cocci. The baseline median for all groups was 4–6% (Fig. 5), but this rapidly increased at week 1 in all groups. The elevation in cocci was

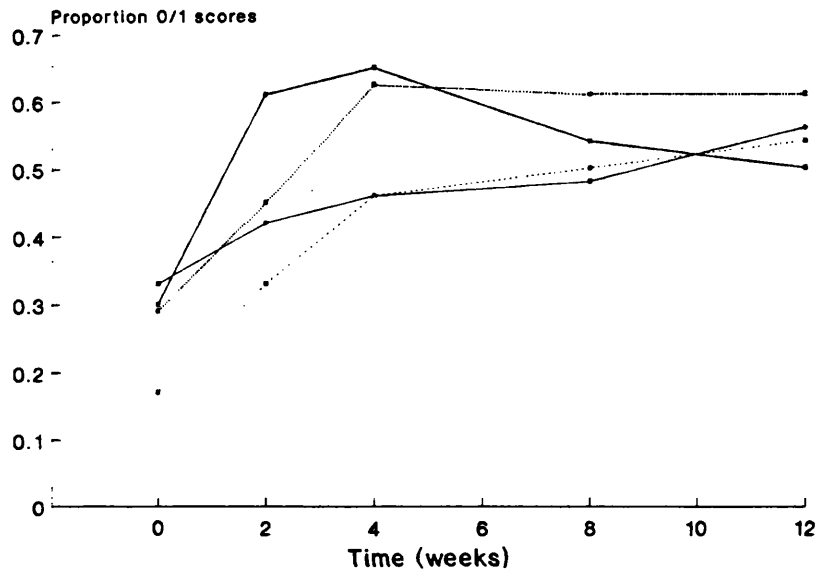


Fig. 2. Effect of treatment modality on the proportions of sites with a gingival index of 0 or 1 (—: sites irrigated with a 1% aqueous solution of methylene blue; —: sites irrigated with sterile distilled water; ···: sites receiving a methylene blue slow release device; ---: sites subjected to subgingival debridement).

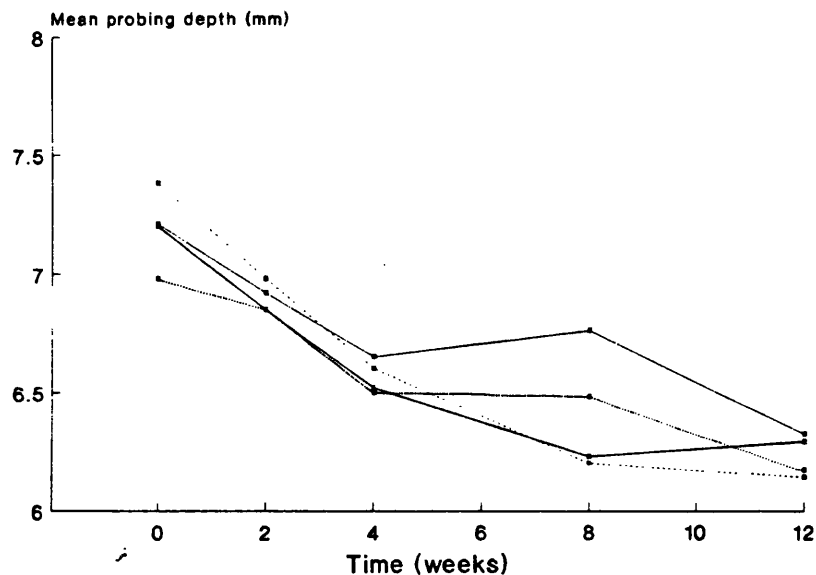


Fig. 3. Effect of treatment modality on the mean probing depth (—: sites irrigated with a 1% aqueous solution of methylene blue; —: sites irrigated with sterile distilled water; ···: sites receiving a methylene blue slow release device; ---: sites subjected to subgingival debridement).

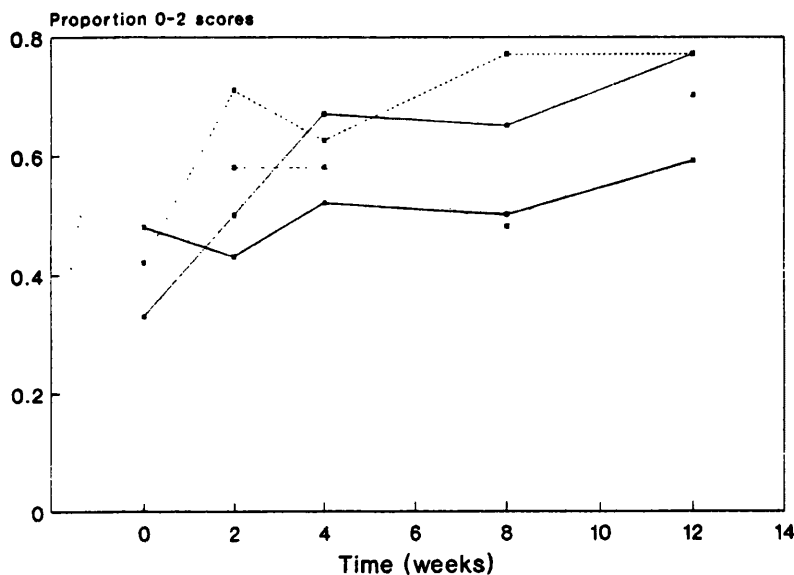


Fig. 4. Effect of treatment modality on the proportions of sites with a papillary bleeding index score of 0-2 (—: sites irrigated with a 1% aqueous solution of methylene blue; —: sites irrigated with sterile distilled water; ···: sites receiving a methylene blue slow release device; ---: sites subjected to subgingival debridement).

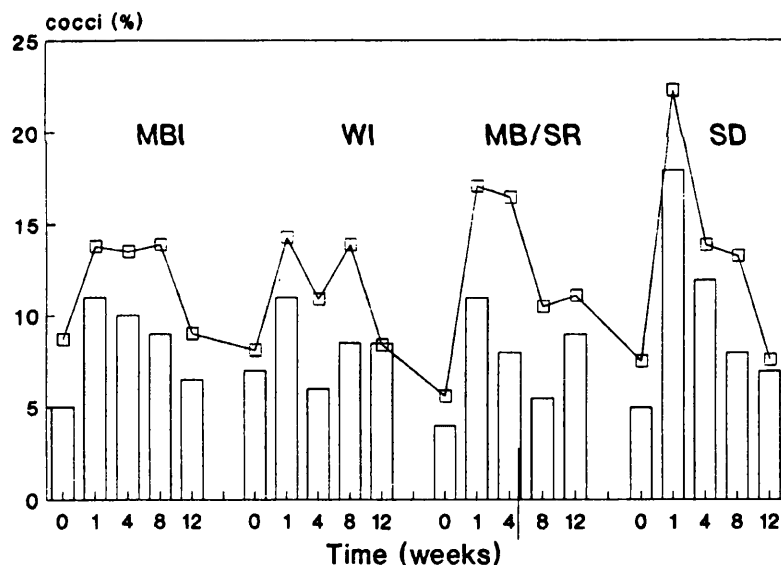


Fig. 5. Effect of treatment modality on the proportions of cocci in samples of subgingival plaque taken from the sites (MBI: sites irrigated with a 1% aqueous solution of methylene blue; WI: sites irrigated with sterile distilled water; MB/SR: sites receiving a methylene blue slow release device; SD: sites subjected to subgingival debridement). Vertical bars denote the median counts and open squares denote the mean counts.

highest (18%) for the subgingival debridement group. Early increases in cocci were not sustained, although the subgingival debridement group at 8 weeks (0.80) and the slow-release methylene blue group at 8 (0.78) and 12 weeks (0.82) showed significant improvements compared with baseline in

terms of the proportion of sites improving versus deteriorating. Regardless of treatment type, the proportion of improving sites was always greater than the proportion of deteriorating sites.

Motile organisms. Marked reductions in motile organisms for all treatments occurred after baseline (Fig. 6). Al-

though these were not sustained after treatments were discontinued (4 weeks for WI, MBI and MB/SR groups and 1 weeks for SD), the proportions of motiles at the end of the trial period were less than at the outset. Expressed in terms of improving/deteriorating sites the proportion showing improvement (i.e., a decrease) was always greater than that showing deterioration for all treatment types. For the methylene blue slow-release group, the differences were statistically significant at weeks 4 (0.79) and 12 (0.78). In methylene blue irrigated sites, the proportion showing improvement was always greater than that showing deterioration except at week 12. However, on none of these occasions did the differences reach statistical significance. A similar pattern was found for the sites that received subgingival debridement except that the differences were statistically significant at weeks 1 (0.79) and 4 (0.83). The proportion of sites showing an improvement after irrigation with water was also always greater than that showing deterioration. This was statistically significant only after week 1 (0.77).

Spirochaetes. Both the methylene blue groups showed gradual reductions of spirochaetes (Fig. 7). Whilst initial reductions observed for water irrigation were not sustained after cessation of irrigation at week 4, with levels returning close to pre-treatment counts at week 12. Marked reduction in spirochaetes was observed one week after subgingival debridement which was not sustained, although counts remained below those at the commencement of therapy.

Sites receiving the methylene blue by slow release were the only ones in which the proportion of sites showing improvement was consistently statistically significantly greater than the proportion showing deterioration. The only other treatment which showed a significantly greater proportion of improving sites was subgingival debridement at week 8 (0.77). With methylene blue irrigation, the proportion of improving sites was greater than the proportion of deteriorating sites, except for week 4 when they were equal.

Culture

Ratio of aerobes to anaerobes. The aerobe/anaerobe ratios of the 4 groups at baseline ranged from 0.1 to 0.15 (Fig. 8). The sites treated by subgingival debridement showed a statistically significant increase in the ratio after 1

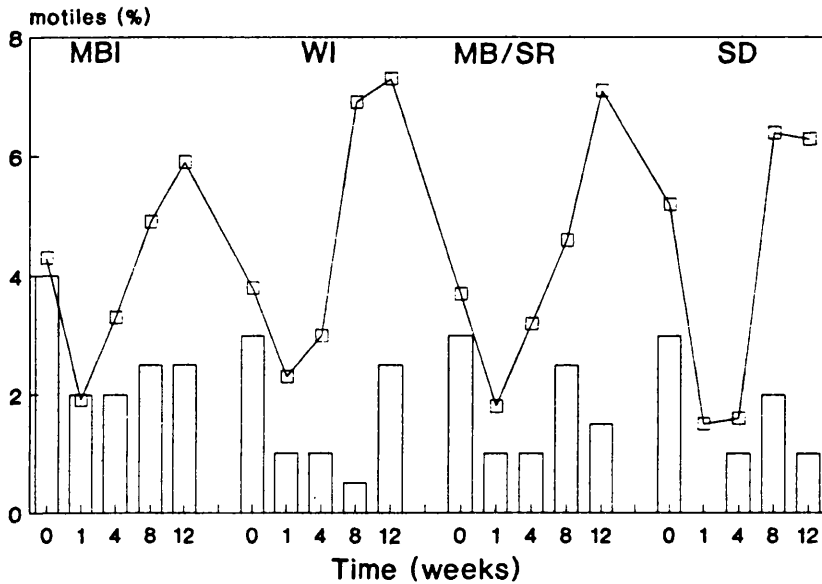


Fig. 6. Effect of treatment modality on the proportions of motile organisms in samples of subgingival plaque taken from the sites (MBI: sites irrigated with a 1% aqueous solution of methylene blue; WI: sites irrigated with sterile distilled water; MB/SR: sites receiving a methylene blue slow release device; SD: sites subjected to subgingival debridement. Vertical bars denote the median counts and open squares denote the mean counts.

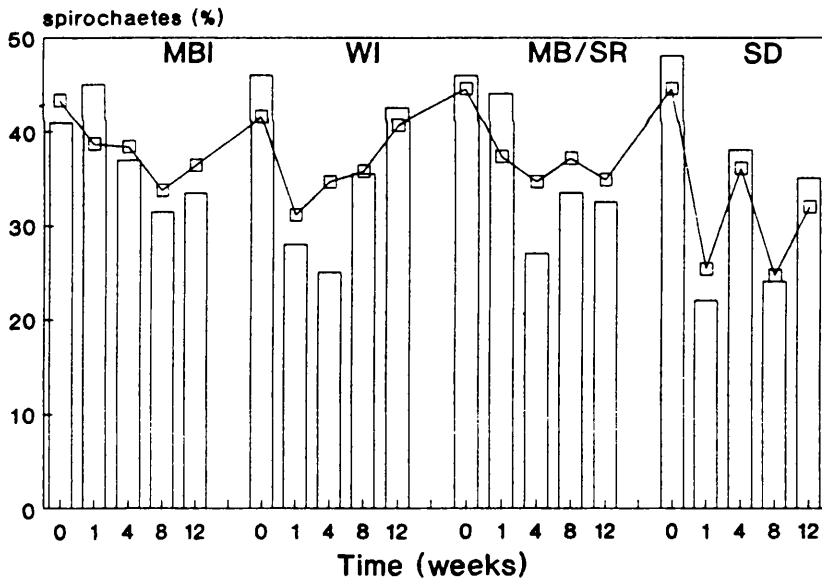


Fig. 7. Effect of treatment modality on the proportions of spirochaetes in samples of subgingival plaque taken from the sites (MBI: sites irrigated with a 1% aqueous solution of methylene blue; WI: sites irrigated with sterile distilled water; MB/SR: sites receiving a methylene blue slow release device; SD: sites subjected to subgingival debridement. Vertical bars denote the median counts and open squares denote the mean counts.

week. After 4 weeks, the ratio had increased to more than three times the baseline value, and this was statistically highly significantly different from the baseline value. The ratio then decreased progressively until it had returned almost to baseline after 12 weeks.

In sites receiving methylene blue irri-

gation, the ratio increased up to the cessation of treatment (week 4), at which point it was almost three-fold greater than at baseline. This was statistically highly significantly different from baseline. After cessation of treatment, the ratio then decreased, although it never returned to baseline.

The sites receiving the slow release device showed a similar pattern to those irrigated with methylene blue, except that the ratio increased to almost four times the baseline value after 4 weeks, and this increase was statistically highly significant.

% of black pigmented anaerobes. In all 4 groups, the proportion of black pigmented anaerobes (bpa) at baseline was between 7 and 22% (Fig. 9). In the water irrigation group, there was no statistically significant change in bpa during the course of the trial, although after 4 weeks, there was a decrease in the median value from 10 to 3%.

In the case of the debrided sites there was, likewise, no statistically significant change. In contrast, sites receiving methylene blue irrigation showed a significant decrease after 4 and 8 weeks. The proportion of bpa remained at a low level following the cessation of treatment so that, even after 12 weeks, the % was much lower than at baseline.

In sites receiving the slow-release device, the % of bpa decreased progressively until week 8, at which time the difference was significantly lower than that at baseline.

Adverse experiences

No serious adverse experiences were seen. 10/24 patients had no symptoms, and all symptoms had resolved by 8 weeks (Table 1). 3/24 patients experienced some pain during the week following subgingival debridement, a symptom not experienced with other treatments. 5/24 patients suffered a slight degree of discomfort. 3 related to irrigation of pockets and 2 to placement of the slow release device. 8/24 patients experienced hypersensitivity, all following subgingival debridement. 3/24 patients admitted difficulty in irrigating, and 6/24 confessed to imperfect compliance with irrigating regimes, usually missing 1 day of the 28, but in 1 case, 3 days were missed between days 21 and 24 due to the patient forgetting to take the equipment with him whilst attending a conference. 1 patient complained that a poorly finished composite had become slightly stained after irrigating with methylene blue for 28 days. (This was easily rectified with usual polishing techniques). Staining of oral tissues was never a voluntary complaint, although on questioning, 9 patients had noticed transient localised staining for less than 30 minutes after

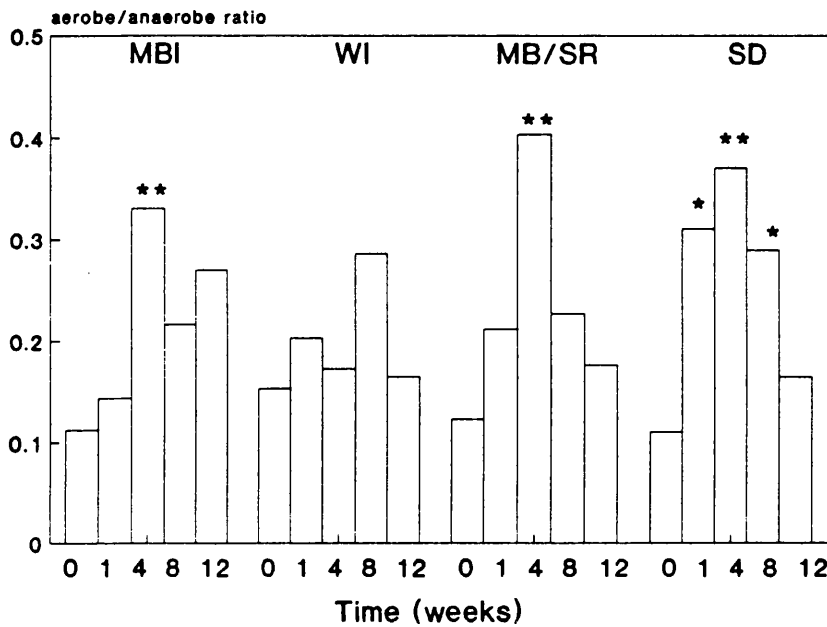


Fig. 8. Effect of treatment modality on the relative proportions of bacteria in subgingival plaque samples able to grow aerobically and anaerobically. WI: sites irrigated with sterile distilled water; MBI: sites irrigated with a 1% aqueous solution of methylene blue; MB/SR: sites receiving a methylene blue slow release device; SD: sites subjected to subgingival debridement.

*Significantly different from baseline ($p < 0.05$)
 **Significantly different from baseline ($p < 0.01$).

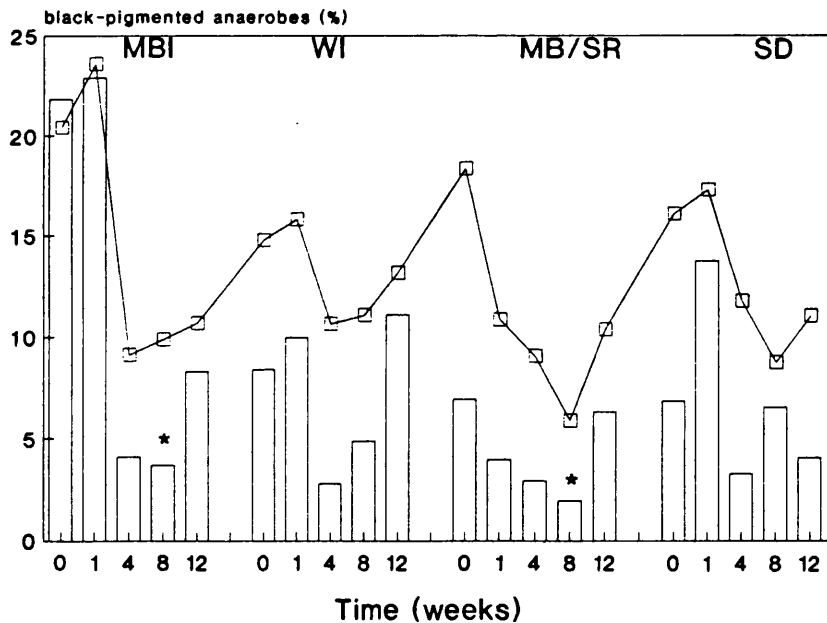


Fig. 9. Effect of treatment modality on the proportions of black pigmented anaerobes (expressed as a percentage of the total anaerobic count) cultured from subgingival plaque samples. WI: sites irrigated with sterile distilled water; MBI: sites irrigated with a 1% aqueous solution of methylene blue; MB/SR: sites receiving a methylene blue slow release device; SD: sites subjected to subgingival debridement. Vertical bars denote the median counts and open squares denote the mean counts.

*Significantly different from baseline ($p < 0.05$).

irrigating, when they felt they had overfilled the pockets. This was not a complaint with the slow-release methylene blue.

Discussion

The ability of bacteria to survive in the subgingival region is dependent on the presence of an adequate supply of nutrients, the absence of antagonistic substances and suitable environmental conditions. With regard to anaerobic bacteria, an important environmental requirement is the existence of a low (negative) redox potential (Socransky et al. 1964). Methylene blue has been shown to be able to raise the redox potential of an anaerobic environment to a more positive value, resulting in the death of obligate anaerobes, such as *Porphyromonas gingivalis* (Fletcher & Wilson 1993). Such agents, therefore, may be useful in controlling those anaerobic organisms implicated in the pathogenesis of chronic periodontitis. This particular redox agent was chosen because of its low toxicity for humans, and because promising results had been obtained in a small-scale, short-term, preliminary pilot study (Wilson et al. 1992).

There are several reasons why statistical differences may not have been observed for clinical parameters and, being a pilot study of 3 months duration, significance data need to be interpreted with considerable caution. This is because of the well known problems with current clinical periodontal variables, and the consequent need for adequate patient numbers to achieve statistical power, apart from marked site variability, and the delay between plaque and clinical changes. When the descriptive site data are considered, the proportion of sites showing improvement at the end of the study was actually highest for the slow-release methylene blue group as regards plaque index.

Debridement appeared superior as regards gingival index, although differences were again small. There was little difference concerning probing depth, with similar reductions of 0.6 to 1.2 mm (Fig. 3). In recent, similarly designed trials confirming the efficacy of metronidazole gel irrigation with subgingival debridement, Klinge et al. (1992) reported 1.0–1.3 mm reductions after 12 weeks with metronidazole or subgingival scaling. Pedrazzoli et al. (1992) and Ainamo et al. (1992) in 24-week studies

Table 1. Adverse experiences reported by subjects

Symptom	Treatment			
	MBI	WI	SRS	SGD
pain	0	0	0	3
discomfort/tenderness	2	1	2	0
hypersensitivity	0	0	0	8
initial difficulty irrigating	2	1	-	-
imperfect compliance	3	3	-	-
pain requiring extraction	-	-	-	1
staining of composite	1	-	-	-
transient tissue staining	9	0	0	0

10 patients no symptoms.

All symptoms resolved by 8 weeks.

reported probing depth reductions of 0.88–1.14 mm and 1.3–1.5 mm. Klinge et al. did not give oral hygiene instructions, Pedrazzoli et al. provided these after 3 weeks and Ainamo et al. after 2 weeks of the study period. It is possible that in the latter two studies, some of the reduction might have been due to improved oral hygiene introduced midway in the trial, as well as to the effect gained by the pocket treatment, since oral hygiene itself results in gingival tissue shrinkage due to reduction of inflammation (Cercek et al. 1983, Dahlen et al. 1992, Katsanoulas et al. 1992). The present study aimed to provide treatment as close as possible to that consistent with usual clinical practice, within the time constraints of the study. Therefore, since a gross superficial scale and polish and oral hygiene education were given 6 weeks prior to the study, it is likely that much gingival shrinkage would already have occurred prior to commencing the study. Although oral hygiene instruction was re-enforced only once 1–2 weeks later and a full month prior to baseline recordings, plaque scores were low at baseline as a consequence. Good clinical practice would normally entail interrupting the treatment programme to reinforce oral hygiene instruction where appropriate, although the protocol precluded this, which presumably explains the deterioration in supragingival plaque level towards the end of the study. Had oral hygiene instruction been given during the study, rather than before (Pedrazzoli et al. 1992; Ainamo et al. 1992), it is probable that the probing depth reduction would have been greater.

It is to be noted that the clinical effect of methylene blue applied by irrigation or slow-release was similar to that of subgingival debridement, although no mechanical therapy was per-

formed prior to MBI or MB/SR. Thus, plaque-retaining factors might have been present in these pockets during the follow-up period, possibly facilitating a faster rate of recolonisation in association with the increase in supragingival plaque observed. The split-mouth design of the study enabled direct comparison of the different treatments within individual mouths, and permits comparison also with similarly designed studies recently reported in which tetracycline (Christersson et al. 1993) and metronidazole (Klinge et al. 1992, Pedrazzoli et al. 1992, Ainamo et al. 1992) were used as treatments. A factor of concern in studies of this type is the putative risk of spill-over to other sites. This is unlikely to have been relevant in the present study for several reasons. Previous irrigation studies (Pitcher et al. 1980, Hardy et al. 1982) have shown that even vigorous application of dye, for example by mouthrinsing or direct irrigation interdentally at the gingival margin, did not effect significant pocket penetration of the dye. Further, professional irrigation in this study deliberately avoided overflow, and patients were meticulously instructed at the chairside so that they were able to irrigate in the same manner. Given the intense colour of methylene blue, and the fact that patients were questioned concerning adverse effects of treatments, including possible staining of oral tissues, it is unlikely that spill-over was a complicating factor. Finally, based on the cited penetration studies, even if there had in fact been spill-over, there would have been significant dilution by saliva and very limited, if any, entry to the pockets of sites in other quadrants.

A practical aspect not often considered in reports of subgingival therapy is the time taken to administer the

active agent. In this study, the time taken for operator irrigation, including isolation with cotton wool rolls, did not exceed 1 min. Patients offered no adverse comments concerning the time taken for irrigation in the questionnaire at the end of active treatment. This may be compared with the time for placement of the controlled release material, which varied depending on pocket depth and volume and tissue tone, and was 2–3.5 min. It may be observed, however, that both these are far less than the time taken for adequate subgingival debridement, which in our study varied between 4–8 min per tooth. This is in accordance with other studies, namely Badersten et al. (1981) (3.6–7.6 min) and Thornton & Garrick (1982) (3 min).

With regard to the culture data, methylene blue was clearly superior to subgingival debridement in reducing the proportion of black-pigmented anaerobes and was comparable to debridement in increasing the aerobic: anaerobe ratio. Darkfield microscopy showed that methylene blue treatment was also superior to subgingival debridement in decreasing the proportions of spirochaetes and motile bacteria. These results clearly demonstrate that, in altering the microflora to one more compatible with periodontal health, methylene blue treatment is comparable (or even better) than the currently standard treatment of subgingival debridement. This has to be considered promising, in view of the very low dose of the agent, and the fact that it was used without concomitant subgingival debridement, that is, with plaque-retaining factors within the pockets.

In view of the earlier pilot study (Wilson et al. 1992), it might have been expected that methylene blue irrigation might have performed better clinically. The microbiological data suggest a possible reason for this, in terms of abnormally high initial numbers of black pigmented anaerobes in the MBI group despite attempts to match pockets at the outset. The sustained-release methylene blue did not yield the clinical results one might have suspected from the bacteriological data. Possible reasons include the effect on plaque accumulation, gingival trauma and probing pocket depth of having a foreign body within the pocket. This may explain why the results tended to improve for this treatment some time after the last device had been placed (4 weeks).

The questionnaire revealed that a greater number of patients described adverse symptoms with subgingival debridement than with irrigation or use of the slow-release methylene blue. It is well-established that pain and hypersensitivity are significant hazards of scaling root surfaces, and are related to opening dentine tubule orifices (Branstrom 1962, 1966). Although compliance was generally excellent during this study, most of the adverse experiences related to methylene blue were due to omitting to irrigate or slight inadvertent trauma during irrigation, producing tenderness. It is for this reason that numerous attempts have been made to produce vehicles for local drug delivery which are more convenient, such as acrylic strips, dialysis tubing and monolithic fibres (Addy et al. 1982, Coventry & Newman 1982, Goodson et al. 1983). The slow-release device used in this study was well tolerated, with only 2 subjects describing mild transient discomfort on placement.

That this study showed improvements in all variables, even with sterile water irrigation, albeit generally less than with the test substance or subgingival debridement, is in accordance with some other studies in that irrigation itself, particularly if pulsating and subgingival, may have some benefit (Aziz-Ghandour et al. 1986, Sanders et al. 1986, Vignarajah et al. 1989, Schlagenhaut et al. 1987, 1990). In subgingival plaque, the existence of complex nutritional and environmental interrelationships among the constituent microflora is a prerequisite for the survival of many organisms, some of which may be important in the pathogenesis of chronic periodontitis. It has been shown that after a single course of scaling and root planing, it takes several weeks to months until a mature plaque is re-established (Slots et al. 1979). Therefore, the hydrodynamics of irrigation in combination with mechanical stirring action by inserting the tip to the bottom of the pocket may already help to disrupt the maturation process of the subgingival microflora.

In the present study, careful instructions were given prior to the study (on sites not included in the study), and at baseline for the test sites, which were reinforced at 1 week. It is likely, despite this, that irrigation techniques were imperfect throughout the trial, which may partly explain the variation in observed

changes. The metronidazole studies previously quoted (Klinge et al. 1992, Pedrozzi et al. 1992 and Ainamo et al. 1992) did not use a "negative control" in their studies for comparison.

The pockets in this study were generally deeper than in comparable studies (Klinge et al. 1992, Pedrozzi et al. 1992 and Ainamo et al. 1992) averaging 7.4 mm with 5.8 mm in all three of other studies. It may be concluded that the base of the pockets in this study were more anaerobic, and potentially more resistant to therapy. It is not known what dose of methylene blue is required to effect an adequate change in redox potential in the periodontal pocket, although in vitro studies show killing of *P. gingivalis* accompanied by a raised Eh at 1.0 mg/ml (Fletcher & Wilson 1993). Nonetheless, the results are encouraging in that they demonstrate that, in terms of altering the microflora to one more compatible with periodontal health, methylene blue treatment is at least as good as the currently standard treatment of subgingival debridement, and lacks the common side-effect of the latter of discomfort and hypersensitivity. The literature describes numerous studies of antibiotics (Slots & Rams 1990) and antiseptics (Addy 1990) in the treatment of chronic periodontitis. Whilst many report improvements, there are disadvantages such as emergence of resistant strains and hypersensitivity which argues against the long-term use of such agents. The results of this study have shown that methylene blue can produce equivalent clinical and bacteriological improvements over the short-term. One major advantage of such environmental-modifying agents is that resistance development in the target organisms would be extremely unlikely as this would have to involve fundamental changes in the biochemistry and physiology of anaerobic bacteria.

In summary, it appears that methylene blue alters the microflora of the periodontal pockets to one more conducive to health and this leads to clinical improvements nearly comparable with those achievable by the long-established therapy of sub-lingival debridement. It would seem likely that combining the ecologically-desirable effect of the administration of the redox agent, methylene blue with routine debridement would have an adjunctive outcome. This hypothesis begs further investigation.

Acknowledgements

We thank the British Technology Group for their support of this project and Johnson & Johnson Medical Biopharmers Ltd. for supplying the slow-release device. Local application of a redox agent for periodontal reasons has been granted a patent in the UK (number 2202442) and the USA (number 5087451) and is the subject of patent applications in Europe and Japan.

Zusammenfassung

Evaluation der Wirksamkeit eines Redoxmittels bei der Behandlung der chronischen Parodontitis

Ein Redoxfarbstoff, Methylenblau, wurde hinsichtlich der Behandlung der Erwachsenenparodontitis mit subgingivaler Kürettage und sterilem Wasser verglichen. Plaque- und Gingiva-Index, Sondierungsblutung sowie mikrobiologische Proben wurden bei der Eingangsuntersuchung als auch 1, 4, 8 und 12 Wochen nach der Behandlung bestimmt. Alle Versuchspersonen hatten vergleichbare Taschen von 5 mm und mehr in jedem der 4 Quadranten. Die eine Behandlung bestand aus einer professionellen Spülung mit einem 0,1%igen Methylenblaugel in Woche 0, 1 und 4 zusammen mit einer Spülung durch die Patienten an den übrigen Tagen für einen Zeitraum von 4 Wochen. Dies wurde an ausgewählten Zähnen innerhalb eines zufällig bestimmten Quadranten durchgeführt (Split-Mouth-Design). Eine zweite Behandlung bestand aus einer wie oben durchgeführten Spülung mit sterilem Wasser. Ein dritter Quadrant bekam eine subgingivale Kürettage. Die Taschen des vierten Quadranten erhielten Methylenblau, das in einen Medikamententräger, bestehend aus einem biologisch abbaubaren Kollagen-Alginat-Vicryl-Komposit, eingebracht war. Alle Taschen zeigten Verbesserungen der klinischen und mikrobiologischen Parameter. Jedoch wurden für die klinischen Messungen keine statistisch signifikanten Unterschiede zwischen den einzelnen Behandlungsarten gefunden. Obwohl sich der Plaque-Index nach der Woche 1 erhöhte, verminderte sich sowohl der Gingiva- als auch der Papillen-Blutungs-Index. Die Reduktion der Sondierungstiefe betrug für alle Behandlungen ungefähr 1,2 mm. Die mikrobiologischen Variablen zeigten einen Anstieg der Kokken und eine Reduktion der motilen Organismen bei allen Gruppen, wobei letztere für die subgingivale Kürettage statistische Signifikanz erreichten. Die Reduktion der Spirochäten war für subgingivale Kürettage und Methylenblau-Medikamententräger statistisch signifikant. Die Kultivierung zeigte für alle Gruppen eine Zunahme im Verhältnis aerob:anaerob, was initial (Woche 1 und 4) für die subgingivale Kürettage statistisch signifikant war. Auch Methylenblau war statistisch wirkungsvoll in der

Verbesserung dieses Verhältnisses, sowohl als Spülung als auch als Medikamententräger (Woche 4). Methylenblau reduzierte auch während des Versuchszeitraums signifikant die Anzahl an schwarz pigmentierten Anaerobiern durch Spülung und durch Medikamententräger. Hingegen wurde dies nicht durch steriles Wasser und subgingivale Kürettage erreicht. Es wurden keine ernstesten Nebenwirkungen beobachtet, jedoch lag die Morbidität bei subgingivaler Kürettage signifikant höher. Diese Ergebnisse zeigen deutlich, daß hinsichtlich Änderung der Flora in eine Flora, die besser mit parodontaler Gesundheit verträglich ist, Methylenblau-Behandlung vergleichbar, wenn nicht sogar besser als die aktuelle Standardbehandlung durch subgingivale Kürettage ist und besser toleriert wird.

Résumé

Evaluation de l'efficacité d'un agent redox dans le traitement de la parodontite chronique
Un colorant redox, le bleu de méthylène, a été comparé au débridement sous-gingival de la surface radiculaire et à l'eau stérile dans le traitement de la parodontite de l'adulte. On a enregistré l'indice de plaque, l'indice gingival et le saignement au sondage et prélevé des échantillons microbiologiques au début (baseline) et 1, 4, 8 et 12 semaines après le traitement. Tous les sujets avaient des poches apparues dans chacun des 4 quadrants, de 5 mm ou plus. L'un des traitements consistait en irrigations de gel de bleu de méthylène à 0.1%, faites par le personnel dentaire à 0, 1 et 4 semaines, et par les sujets eux-mêmes les autres jours, jusqu'à 4 semaines, dans des sites choisis au sein d'un des quadrants, sélectionné par tirage au sort (modèle en bouche divisée). Un 2^{ème} traitement consistait en irrigations d'eau stérile, faites comme ci-dessus. Un 3^{ème} quadrant subissait un débridement sous-gingival, et les sites du 4^{ème} quadrant recevaient le bleu de méthylène incorporé dans un dispositif à libération lente, en composé biodégradable de collagène alginat vicyrl. On a constaté dans tous les sites une amélioration des paramètres cliniques et microbiologiques. On ne constatait cependant pour les mesures cliniques pas de différences statistiquement significatives entre les types de traitement. Bien que l'indice de plaque ait eu tendance à augmenter après la semaine 1, l'indice gingival diminuait, ainsi que l'indice de saignement papillaire. Les réductions de la profondeur des poches étaient d'environ 1.2 mm pour tous les traitements. Pour les variables microbiologiques, on constatait pour tous les groupes une augmentation des cocci et une diminution des organismes motiles, cette dernière atteignant un niveau statistiquement significatif pour le débridement sous-gingival. Les réductions en spirochètes étaient significatives pour le débridement sous-gingival et pour le bleu de méthylène à libération lente. Les cultures mettaient en évidence une augmentation du rapport aérobie:anaérobies pour tous les groupes; elle

était statistiquement significative initialement (semaines 1 et 4) pour le débridement sous-gingival. Le bleu de méthylène était aussi statistiquement efficace pour améliorer ce rapport, tant en irrigation qu'en libération lente (semaine 4). Le bleu de méthylène réduisait aussi significativement les nombres d'anaérobies pigmentés en noir pendant la période de l'essai, tant en irrigation qu'en libération lente, ce que ni l'eau stérile ni le débridement sous-gingival ne pouvaient obtenir. Aucune réaction adverse sérieuse n'a été observée, cependant le débridement sous-gingival était associé à une morbidité significativement plus marquée. Ces résultats montrent clairement que le bleu de méthylène, en changeant la flore microbienne en une flore plus compatible avec la santé parodontale, est comparable, ou même supérieur, au traitement par débridement sous-gingival actuellement employé normalement, et est mieux toléré.

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THE EFFECT OF ENVIRONMENTAL CONDITIONS ON THE
PRODUCTION OF ACID AND ALKALINE PHOSPHATASES BY
ACTINOBACILLUS ACTINOMYCETEMCOMITANS

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Summary

Acid (AcP) and alkaline (AIP) phosphatases are produced by a number of oral bacteria. In addition to their nutritional role, these enzymes may also contribute to host tissue destruction. This study has shown that *Actinobacillus actinomycetemcomitans*, an organism associated with various forms of periodontitis, produces significant amounts of AcP and AIP, and that the production of these enzymes is affected by the nature of the growth medium and the incubation atmosphere.

Introduction

Actinobacillus actinomycetemcomitans is implicated in the etiology of several forms of periodontitis and has been shown to produce several factors which

contribute to its periodontopathic potential including acid phosphatases (AcP) and alkaline phosphatases (AIP) [9]. As bacteria are relatively impermeable to phosphate esters that are not actively transported, these enzymes are used to dephosphorylate substrates before they can be taken up by the cell. AcP and AIP have been found in dental plaque and in gingival crevicular fluid and some of this activity has been shown to be of bacterial origin [5,11]. Oral bacteria known to produce AcP and AIPs include *Streptococcus mutans*, *Treponema denticola*, *Capnocytophaga* spp., and *A. actinomycetemcomitans* [2,7,9,12]. In addition to a role in nutrition, AcP and AIPs may also be involved in the pathogenesis of periodontitis by dephosphorylating tissue phosphoproteins and by indirectly contributing to plaque mineralisation [5,7]. As the growth and virulence of bacteria are affected by environmental conditions and the availability of certain nutrients [6,10], the aim of this study was to determine the effect of incubation atmosphere and growth medium on the production of phosphatases by *A. actinomycetemcomitans*.

Materials and Methods

AcP and AIP activity has been demonstrated in many strains of *A. actinomycetemcomitans* [8]. For this study the type strain was used. *A. actinomycetemcomitans* NCTC 9710 was grown on Wilkins-Chalgren anaerobe (WC) agar containing 5% horse blood. Plates were incubated in a Compact anaerobic chamber (Don Whitley Scientific) at 37°C. After 72 h incubation bacteria were harvested from the plates, washed in sterile saline and used to inoculate a range of media: a semi-defined medium [6], Wilkins-Chalgren (WC) broth - as an example of a nutritionally-rich medium, and heat-inactivated (56°C for 30 minutes) foetal calf serum (FCS) - a medium similar in composition to that which would be encountered subgingivally. The phosphate concentrations of the different media were 1.7 mM, 3.1 mM and 3.0 mM for the semi-defined medium, WC broth and

FCS respectively. Cultures were incubated either anaerobically or in a CO₂-enriched atmosphere. A CO₂-enriched atmosphere was generated by placing a candle in with the cultures in a sealed jar. Samples were removed at the early stationary phase of growth and assayed for AcP and AIP using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate. The reaction mixture consisted of 0.45 ml 8 mM *p*-NPP and 0.45 ml of 0.2 M acetate, pH 4.8 or 0.2 M glycine NaOH, pH 9.4. All reagents were prepared in 0.85% saline containing 0.01% cysteine HCl [12]. To start the reaction, 0.3 ml of the culture was added and the samples were incubated at 37°C for 3h either anaerobically or in a CO₂-enriched atmosphere corresponding to the original growth conditions of the culture. Cultures which had been grown in the semi-defined medium incubated in a CO₂-enriched atmosphere were assayed for AcP under CO₂-enriched and anaerobic conditions to determine the effect of incubation atmosphere on the activity of the enzyme. Only AcP activity was determined since preliminary studies indicated that *A. actinomycetemcomitans* produced significantly greater amounts of AcP compared to AIP.

The effect of incubation atmosphere on the activity of a commercially available purified AcP enzyme from *Escherichia coli* was also determined. *E. coli* AcP was dissolved in 0.85% NaCl containing 0.01% cysteine HCl and 0.3 ml was added to the assay to give a final concentration of 2.5 µg of enzyme ml⁻¹ of reaction mixture. The reaction was stopped by the addition of 0.3 ml of 10 % NaOH. The samples were centrifuged at 10,000 g for 10 minutes to remove the cells and the absorbance of the supernatant at 420 nm was measured against an appropriate blank. The amount of *p*-nitrophenol (*p*-NP) liberated was determined by reference to a standard curve. Total protein content was determined by the method of Lowry *et al.* adapted for the assay of whole micro-organisms [4] using bovine serum albumin as the standard. WC agar and WC broth were obtained from Oxoid Ltd. and all other reagents were obtained from Sigma.

Results

Figure 1 shows the AcP and AIP activity of *A. actinomycetemcomitans* grown in the semi-defined medium. Anaerobically grown cells demonstrated significantly more AcP and AIP activity than cultures which had been incubated in a CO₂-enriched atmosphere. The AcP activity of cells grown and assayed under anaerobic conditions was 70.7 ± 8.7 units compared to 50.7 ± 5.1 units for cells grown and assayed in a CO₂-enriched atmosphere. The level of AIP activity was much lower than for AcP. A significantly greater AIP activity was obtained when the organism was grown anaerobically, 13.8 ± 1.0 units, compared to 7.1 ± 0.9 units for cells incubated in a CO₂-enriched atmosphere.

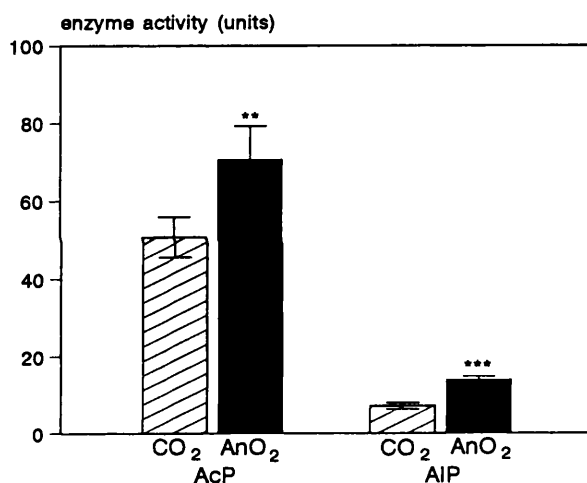


Fig. 1. AcP and AIP activities of *A. actinomycetemcomitans* grown and assayed in a CO₂-enriched atmosphere (▨) and anaerobically (■) when grown in a semi-defined medium. Values shown are the mean ± S.D. of 4 replicates. 1 unit is equivalent to the hydrolysis of 1 nmole *p*-nitrophenyl phosphate min⁻¹ mg⁻¹ bacterial protein. Student's *t* test; ** *p* < 0.01, *** *p* < 0.001.

The results in Figure 1 do not indicate whether the differences observed were due to differences in the amount of enzyme produced or differences in the activity of the enzyme under different incubation atmospheres, since reaction mixtures were

incubated either anaerobically or in a CO₂-enriched atmosphere corresponding to the original growth conditions of the culture. Figure 2 shows the activity obtained for *A. actinomycetemcomitans* grown in the semi-defined medium incubated in a CO₂-enriched atmosphere and assayed for AcP in the same incubation atmosphere (43.6 ± 1.5 units). When this culture was assayed for AcP anaerobically, the activity obtained was significantly higher, 75.9 ± 3.9 units. The level of activity was similar to that of cultures grown and assayed under anaerobic conditions. A similar pattern was observed for the *E. coli* AcP. Under anaerobic conditions the activity obtained was 514.7 ± 19.3 units which was significantly higher than the activity obtained when the enzyme was incubated in a CO₂-enriched atmosphere (352.0 ± 4.1 units).

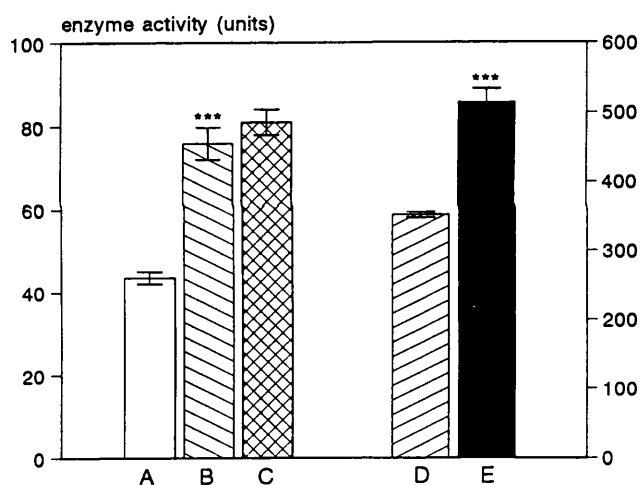


Fig. 2. Effect of incubation atmosphere on the AcP activity of *A. actinomycetemcomitans* cultures and purified AcP from *E. coli*. A, when grown in a CO₂-enriched atmosphere and assayed for AcP in the same environment; B, when grown in a CO₂-enriched atmosphere and assayed for AcP anaerobically; C, when grown anaerobically and assayed for AcP activity anaerobically; D, activity of 2.5 µg ml⁻¹ *E. coli* AcP assayed in a CO₂-enriched atmosphere; E, activity of 2.5 µg ml⁻¹ *E. coli* AcP assayed anaerobically. Values shown are the mean ± S.D. of 4 replicates. Units are as in Fig. 1. Student's t test; *** p < 0.001.

Figure 3 shows the AcP and ALP activities of *A. actinomycetemcomitans* grown

in three different media incubated anaerobically. Cells grown in FCS had the highest AcP activity, 100.6 ± 4.8 units, compared to 82.3 ± 2.6 units for cells grown in WC broth. Cells grown in the semi-defined medium demonstrated the least AcP activity, 70.7 ± 8.7 units. The highest level of AIP was obtained in WC broth, 27.8 ± 1.4 units. Cells grown in the semi-defined medium and in FCS had similar AIP activities, 13.8 ± 1.0 and 12.4 ± 1.6 units respectively.

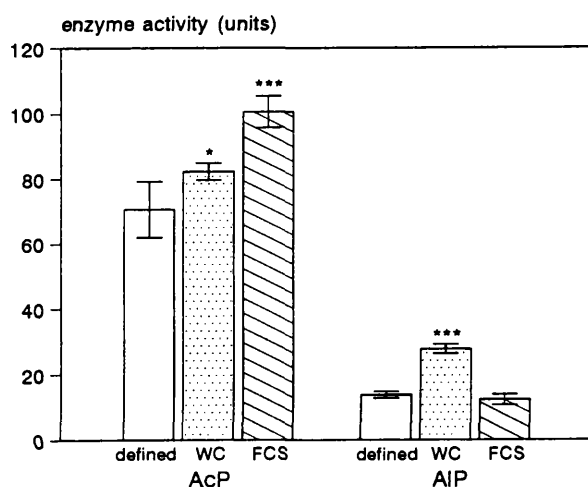


Fig. 3. Effect of growth medium on the AcP and AIP activities of *A. actinomycetemcomitans*. Semi-defined medium (\square), WC broth (\boxtimes), FCS (\boxminus). The values are the mean \pm S.D. of 4 replicates. Units are as in Fig. 1. Student's t test; * $p < 0.05$, *** $p < 0.001$.

Discussion

The AcP activity of *A. actinomycetemcomitans* grown and assayed in a CO_2 -enriched atmosphere was 57 % of that obtained when the same culture was assayed anaerobically. The activity of the culture grown in CO_2 -enriched atmosphere but assayed for AcP anaerobically was 94 % of that obtained for cultures which had been grown and assayed anaerobically. This suggests that cultures grown in both the CO_2 -enriched and the anaerobic atmosphere produced similar amounts of the

enzyme but that the enzyme was more active under anaerobic conditions. Purified AcP from *E. coli* was also more active under anaerobic conditions. One possible explanation for the greater activity of the phosphatase enzymes under anaerobic conditions is the presence of thiol groups. The protein tyrosine phosphatase of *Yersinia pestis* has been shown to possess an essential cysteine residue [3] and it is possible that thiol groups are involved in the phosphatase activity of *A. actinomycetemcomitans* and *E. coli*. Oxygen which would have been present in the CO₂-enriched atmosphere but not in the anaerobic atmosphere may have caused oxidation of thiol groups leading to a reduction in the activity of these enzymes. The substantial differences in phosphatase activity obtained when the organism was grown in different media did not appear to be related to the level of free phosphate in the medium. The amount of phosphatase produced by *A. actinomycetemcomitans* may be related to the proportion of phosphorylated substrates in the different media or the presence of a particular substrate. The AcP and AIP of *A. actinomycetemcomitans* may serve an important role in the nutrition of the organism in the periodontal pocket. Non-specific phosphatases could remove phosphate groups from non-transportable phosphate esters, thereby allowing the hydrolysed component to be transported and utilized by the cell. Such non-specific phosphatase activity could supply a variety of nutrients to the organism depending on the organic moiety of the ester, in addition to phosphate. It has been suggested that bacterial AcP and AIP may also play a role in the pathogenesis of periodontal disease. Poirier and Holt [7] demonstrated that AcP and AIP from *Capnocytophaga ochracea* were capable of hydrolysing phosvitin, a phosphoprotein similar in phosphoserine content to the phosphoproteins of dentin, enamel, cementum and bone. Dephosphorylation of such molecules could disrupt the integrity of mineralized and other connective tissues. Bacterial phosphatases may contribute to alveolar bone breakdown [1]. This study has shown that *A. actinomycetemcomitans* produces significant amounts

of AcP and ALP and that the production and activity of these enzymes is affected by the conditions under which it is grown. The production of such enzymes by *A. actinomycetemcomitans* may facilitate the survival and growth of the organism in the nutritionally competitive environment of the periodontal pocket and may also contribute to the pathogenesis of periodontal disease.

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Interactions between periodontopathogenic bacteria and cytokines

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Fletcher J, Reddi K, Poole S, Nair S, Henderson B, Tabona P, Wilson M: Interactions between periodontopathogenic bacteria and cytokines. J Periodont Res 1997; 32: 200–205. © Munksgaard, 1997.

Cytokines produced in response to plaque bacteria clearly play a key role in the periodontal diseases. However, we know very little about the interactions between cytokines and periodontopathogenic bacteria. The aims of this study were to determine whether the key pro-inflammatory cytokines interleukin-1 β (IL-1 β) and IL-6 could affect the growth of *Actinobacillus actinomycetemcomitans* or *Porphyromonas gingivalis* and to determine whether these organisms could hydrolyse IL-1 β , IL-6 or the anti-inflammatory IL-1 receptor antagonist (IL-1ra). Culture medium containing up to 100 ng/ml of IL-1 β or IL-6 was inoculated with *A. actinomycetemcomitans* (serotypes a, b and c) or *P. gingivalis* and growth was monitored by measuring changes in electrical conductivity every 3 min for up to 48 h. IL-1 β , IL-6 or IL-1ra were added to culture supernatants and incubated for up to 24 h. Samples were taken at various times, analysed by SDS-PAGE and the separated proteins transferred by Western blotting to PVDF membranes and probed with anti-cytokine antibodies. None of the cytokines tested had any effect on the rate of growth or yield of *A. actinomycetemcomitans* or *P. gingivalis*. Supernatants from *P. gingivalis* cultures, but not those from *A. actinomycetemcomitans*, hydrolysed IL-1 β , IL-6 and IL-1ra. The hydrolysate from the *P. gingivalis* supernatant-treated IL-1 β was unable to stimulate the release of IL-6 from human gingival fibroblasts showing that it had lost biological activity. These results suggest that *P. gingivalis* can perturb the cytokine network, not only by stimulating the release of cytokines from host cells, but also by removing them from its local environment.

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Key words: cytokines; proteases; *Porphyromonas gingivalis*; growth; conductivity

Accepted for publication May 16, 1996

Perturbation of the cytokine network existing in healthy gingival tissues by periodontopathogenic bacteria is thought to play a major role in initiating inflammatory periodontal diseases (1). Considerable effort, therefore, has been devoted to determining which cytokines are released by a variety of host cells when they are exposed to components or products of periodontopathogenic bacteria. Inevitably, lipopolysaccharide (LPS) has been the bacterial component receiving most attention (2) although in recent years an increasing number of other cell wall constituents and secretory products have also been shown to potently stimulate cytokine release from host cells (3, 4). Such studies have

revealed that a number of pro-inflammatory cytokines are synthesized in response to periodontopathogenic bacteria and their products, hence inducing and maintaining an inflammatory response in the periodontium. However, there is growing evidence to suggest that cytokines can promote bacterial growth (5, 6) or that bacterial proteases can activate or inactivate cytokines (7, 8). These represent additional ways in which bacteria can perturb the "normal" cytokine network responsible for maintaining periodontal health. The purpose of this study was to determine whether cytokines could either stimulate the growth of periodontopathogenic bacteria or could be degraded by them.

Materials and methods

Effect of IL-1 β and IL-6 on the growth of *A. actinomycetemcomitans* and *P. gingivalis*

The effect of human recombinant IL-1 β (rIL-1 β) and IL-6 (rIL-6) on the growth of *A. actinomycetemcomitans* and *P. gingivalis* was determined by monitoring conductivity changes in cultures using the Rapid Automated Bacterial Impedance Technique (RABIT, Don Whitley Scientific Ltd, Bradford, UK). Bacteria harvested from 24 h cultures of *A. actinomycetemcomitans* 670 (serotype a, a clinical isolate from the Eastman Dental Institute), 286 (serotype b, a clinical isolate from the Eastman Dental Institute) and NCTC 9710 (serotype c) and *P. gingivalis* W50 were resuspended in pre-reduced Whitley Impedance Broth (WIB) (Don Whitley Scientific Ltd). The *A. actinomycetemcomitans* suspension was used to inoculate tubes containing 2 ml of pre-reduced WIB or, in the case of *P. gingivalis*, Whitley Anaerobe Broth (WAB). Human rIL-1 β or human rIL-6 was added to give final concentrations of 0, 1, 10 and 100 ng/ml in a total volume of 2.5 ml. Tubes were inserted into the RABIT and changes in conductivity were recorded every 3 min for up to 48 h.

Hydrolysis of IL-1 β , IL-6 and IL-1ra by *P. gingivalis* or *A. actinomycetemcomitans*

P. gingivalis W50 and *A. actinomycetemcomitans* 670 were inoculated into a pre-reduced medium (BM broth) containing tryptone soya broth (10 g), proteose peptone (10 g), yeast extract (5 g), glucose (5 g), NaCl (5 g), cysteine-HCl (0.75 g), haemin (5 mg) and menadione (0.5 mg) per litre of distilled water. After 48 h anaerobic incubation at 37°C, cultures were centrifuged and the supernatants passed through a 0.22 μ m filter to provide a cell-free supernatant. Human rIL-1 β , rIL-6 or rIL-1ra was added to the culture supernatants to give a final concentration of 2.5 μ g/ml. In the case of IL-6 the culture supernatant was diluted 1 in 4 with BM broth prior to adding the cytokine. An aliquot was removed immediately and frozen at -70°C. This was designated as the t=0 sample. The culture supernatants containing either IL-1 β , IL-6 or IL-1ra were then incubated anaerobically at 37°C. Aliquots were removed at various time intervals and frozen immediately at -70°C to prevent further hydrolysis. Controls contained boiled (30 min) culture supernatant plus cytokine, and cytokine-free supernatant.

SDS-PAGE and Western blotting

Samples were separated by SDS-PAGE by the method of Laemmli (9) using gradient gels (4–20%

polyacrylamide, Bio-Rad Laboratories Ltd) and a running buffer consisting of 0.025 M Tris, 0.192 M glycine and 0.1% (w/v), SDS, pH 8.3. Before application to the gel, the samples were boiled for 5 min in a sample buffer consisting of 0.06 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.025% -bromophenol blue and 5% mercaptoethanol. Electrophoresis was carried out at a constant current of 30 mA at room temperature until the bromophenol blue dye reached the bottom of the gels. Proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) by electroblotting overnight in blotting buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol) at a constant voltage of 15 V using a TE Series Transphor Electrophoresis Unit (Hofer Scientific Instruments). Following electroblotting the membranes were washed in PBS containing 1% Triton-X-100 for 5 min and PBS containing 0.1% Triton-X-100 for 25 min. The membranes were then transferred to blocking buffer (2% FCS in PBS-0.1% Triton) for 45 min. The blocking buffer was removed and sheep monoclonal anti IL-1 β , IL-6 or IL-1ra diluted 1:1000 in blocking buffer was added and incubated at room temperature for 1.5 h. The membranes were then washed in PBS-0.1% Triton for 30 min and a rabbit anti-sheep HRP conjugate (DAKO) diluted 1:1000 in blocking buffer was added and incubated at room temperature for 1.5 h. The membranes were again washed and the peroxidase substrate 3,3'-diaminobenzidine tetrahydrochloride (1.7 mg/ml in dH₂O plus 0.8 μ l/ml 30% hydrogen peroxide) was added. The reaction was terminated by washing the membranes in dH₂O.

Assessment of bio-activity of IL-1 β

Induction of IL-6 release from human gingival fibroblasts (HGF) was used to assay the biological activity of IL-1 β following treatment with the bacterial culture supernatants. HGF were plated in 24-well plates at a density of 30,000 cells per well in DMEM (Sigma Ltd) containing 2% fetal calf serum. The plates were then incubated at 37°C in 5% CO₂ overnight to allow the cells to adhere. The following day the cells were washed and the supernatants (diluted 1:125,000 in DMEM) were added and incubated overnight. Controls consisted of IL-1 β at a concentration of 20 pg/ml as well as the *P. gingivalis* supernatant. The media were then assayed for IL-6 by ELISA as follows. Microtitre plates were coated with immuno-affinity purified polyclonal goat anti-IL-6 antibody at 1 μ g/ml, diluted in phosphate buffered saline (PBS), pH 7.2. Plates were incubated at 4°C overnight. The wells were decanted and washed with 0.01 M phosphate/0.05 M NaCl buffer (wash/dilution buffer,

pH 7.2) containing 0.1% Tween 20. IL-6 standards were added to wells over the dose range 0–3 ng/ml and the samples to be tested added to the remaining wells. Plates were incubated for 2 h at room temperature and washed 3 times with wash/dilution buffer. Biotinylated affinity purified polyclonal goat anti-IL-6 (100 μ l of a 0.014 μ g/ml solution) was added to each of the wells and incubated for 1 h at room temperature. The plates were washed 3 times and 100 μ l of a 1:5000 dilution of avidin-HRP was added to each well. Plates were then incubated for 15 mins at room temperature before washing 3 times with wash/dilution buffer. Wells were then developed with 100 μ l of 0.2 mg/ml orthophenylenediamine in 0.1 M citric acid-phosphate buffer pH 5.0 plus 0.4 μ l/ml 30% hydrogen peroxide. The reaction was terminated by the addition of 150 μ l of 1 M sulphuric acid, and the absorbance was measured at 492 nm on a Titertek Multiskan spectrophotometer.

Results

The effects of increasing concentrations of IL-1 β and IL-6 on the growth of *A. actinomycetemcomitans* 670 are shown in Fig. 1a,b. Concentrations of IL-1 β as high as 100 ng/ml had no effect on the rate of growth of *A. actinomycetemcomitans* 670 (slope = 2.57 ± 0.21 μ S/min compared to 2.40 ± 0.17 μ S/min for cytokine-free controls) or on the final conductivity of the medium (1269.33 ± 17.67 μ S and 1279.33 ± 40.70 μ S for medium containing 100 ng/ml IL-1 β and controls respectively) (Fig. 1a). IL-6 too, had no significant effect on the growth of the organism and the rate of growth in the presence of IL-6 at 100 ng/ml (2.27 ± 0.31 μ S/min) was similar to that of the IL-6-free controls (2.30 ± 0.10 μ S/min). The final conductivity was 1252.33 ± 12.70 μ S for medium containing 100 ng/ml IL-6 and 1282.33 ± 19.55 μ S for controls (Fig. 1b). Similar results (not shown) were obtained for the other 2 strains of *A. actinomycetemcomitans* and for *P. gingivalis*.

Supernatants from cultures of *A. actinomycetemcomitans* were unable to hydrolyse IL-1 β , IL-6 or IL-1ra (Fig. 2a,b,c). In contrast, supernatants from cultures of *P. gingivalis* were able to hydrolyse all 3 cytokines as shown by the disappearance from the Western blots of bands with molecular masses corresponding to those of the intact cytokines and the appearance of bands with lower molecular masses (Fig. 3a,b,c). No degradation was evident when the culture supernatants were boiled prior to addition of the cytokine. The appearance of lower molecular mass bands at $t=0$ with all 3 cytokines was indicative of some degree of cytokine degradation during sample preparation. It was not generally

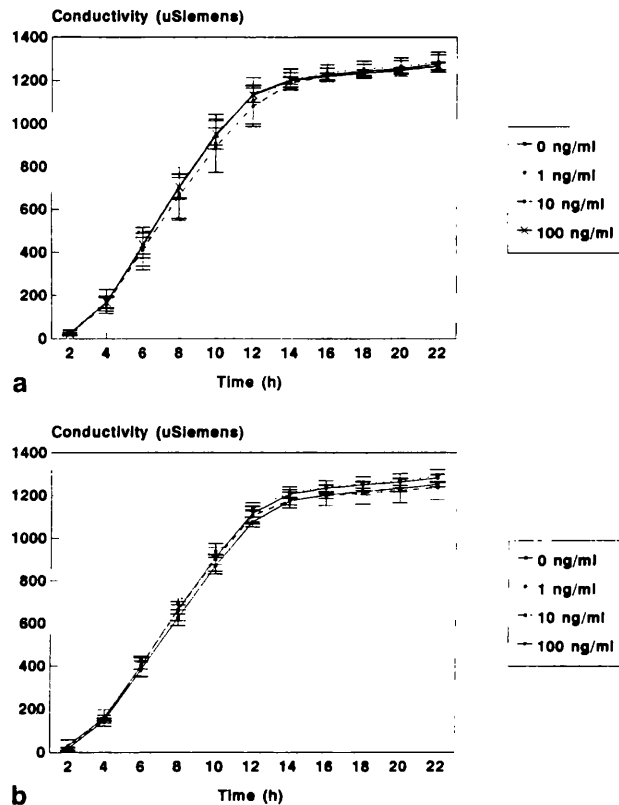


Fig. 1. Growth of *A. actinomycetemcomitans* in the presence of (a) IL-1 β and (b) IL-6 as determined by conductivity measurements. Each data point represents the mean value of 3 determinations. Vertical bars denote the standard deviations.

possible to reproducibly prevent cytokine degradation using a range of protease inhibitors (singly and together) including EDTA/phenanthroline, pepstatin A, trans-epoxy succinyl-L-leucylamido-(4-guanidino)-butane and phenyl-methyl-sulphonyl fluoride (data not shown). After 8 h incubation at 37°C, no IL-1 β was detectable by Western blotting (Fig. 3a). In contrast, although IL-1ra also underwent hydrolysis in the presence of the culture supernatant, some of the IL-1ra was still detectable after 8 h incubation (Fig. 3c). Lane 3 of Fig. 3c also shows that partial hydrolysis of IL-1ra had taken place during sample preparation, giving rise to molecules with slightly lower molecular masses. In contrast to the other antibodies used in these studies, the goat anti-IL-1ra serum appeared to cross-react with high molecular weight constituents of the *P. gingivalis* supernatant. IL-6 appeared to be particularly susceptible to hydrolysis as no cytokine was detectable within minutes of incubation with the undiluted culture supernatant (data not shown).

The effect of incubating IL-1 β with the *P. gingivalis* culture supernatant on the biological activity of the cytokine was determined by measuring its

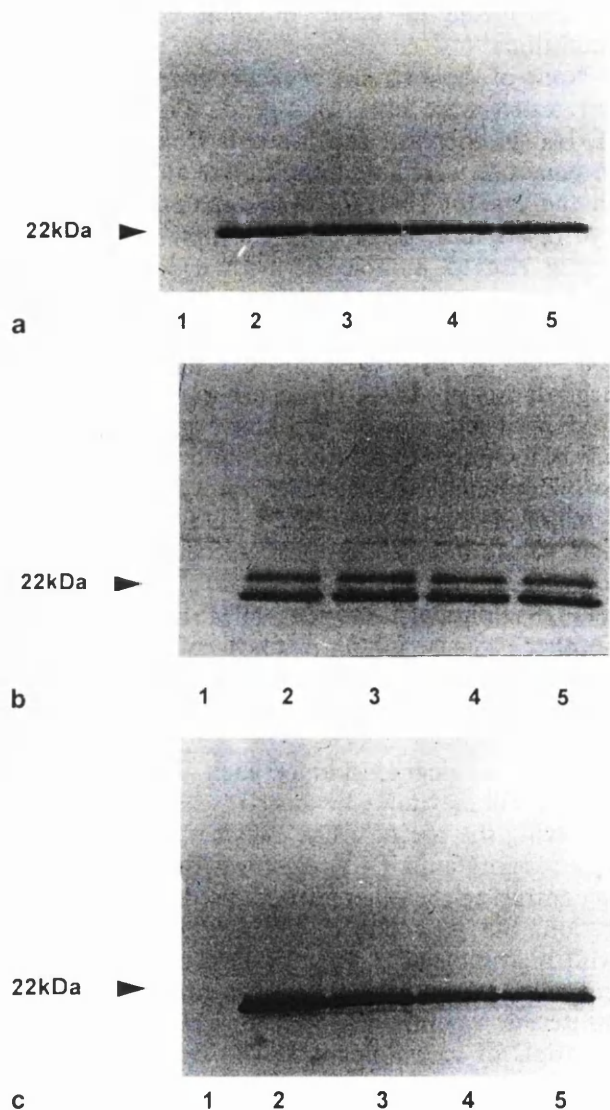


Fig. 2. Effect of culture supernatant from *A. actinomycetemcomitans* on (a) IL-1 β , (b) IL-6 and (c) IL-1ra as assessed by Western blotting. Lane 1, culture supernatant; lane 2, boiled supernatant + cytokine; lane 3, supernatant + cytokine at t = 0 h; lane 4, supernatant + cytokine at t = 6 h; lane 5, supernatant + cytokine at t = 24 h.

ability to stimulate the release of IL-6 from human gingival fibroblasts during a 16 h incubation period. The culture supernatants were diluted 1:125,000 which, if no cytokine degradation had taken place, would result in an IL-1 β concentration of 20 pg/ml. Using this dilution of the culture supernatants, the assay system used was sufficiently sensitive to detect the presence of biologically-active cytokine even if 99% degradation had taken place. The release of IL-6 from HGF following incubation of IL-1 β with the *P. gingivalis* culture supernatant for various periods of time is shown in Fig. 4. From this it can be seen that IL-1 β , after being incubated with the culture supernatant for 4 h, was unable to stimulate

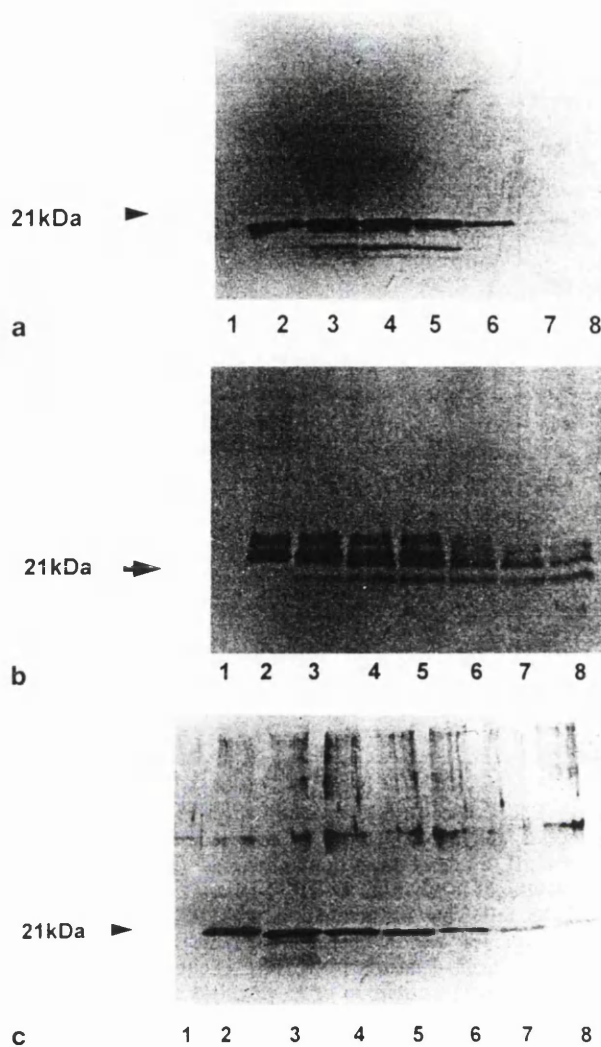


Fig. 3. Effect of culture supernatant from *P. gingivalis* on (a) IL-1 β , (b) IL-6 (culture supernatant diluted 1 in 4) and (c) IL-1ra as assessed by Western blotting after various periods of incubation. Lane 1, culture supernatant without cytokine; lane 2, boiled supernatant + cytokine; lane 3, supernatant + cytokine at t = 0 h; lane 4, supernatant + cytokine at t = 1 h; lane 5, supernatant + cytokine at t = 2 h; lane 6, supernatant + cytokine at t = 4 h; lane 7, supernatant + cytokine at t = 6 h; lane 8, supernatant + cytokine at t = 8 h.

the release of IL-6 from human gingival fibroblasts. The supernatant from *P. gingivalis* itself failed to stimulate HGF to release IL-6 while IL-1 β at a concentration of 20 pg/ml was able to stimulate the release of 820 pg/ml IL-6 following overnight incubation with the HGF.

Discussion

The ability of cytokines to stimulate the growth of bacteria (as reported for *Escherichia coli*) could have profound effects on the progression of an infectious process. Therefore, not only would this lead to a greater number of organisms, with all

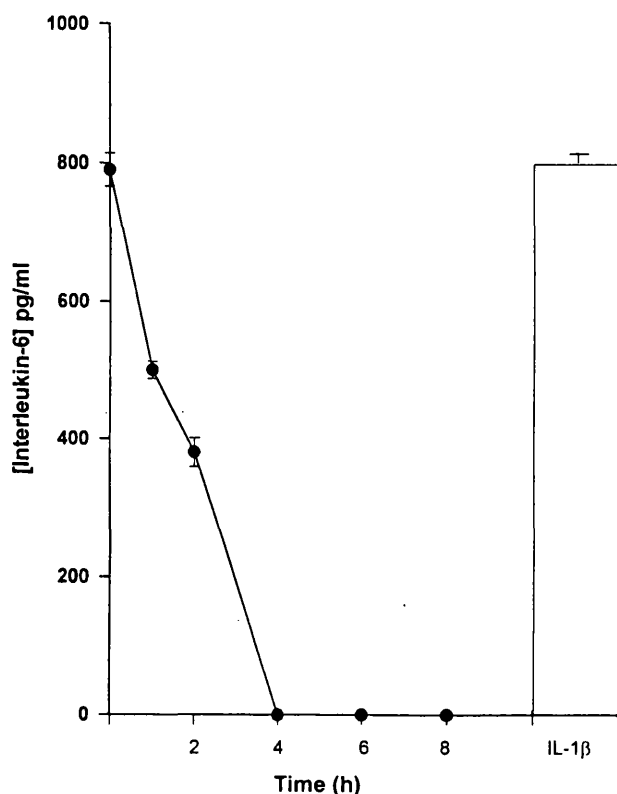


Fig. 4. Effect of culture supernatant from *P. gingivalis* on the biological activity of IL-1 β as assessed by its ability to stimulate IL-6 release from human gingival fibroblasts (HGF) after overnight incubation. ● = IL-6 release from HGF elicited by IL-1 β following its incubation with *P. gingivalis* culture supernatant for various periods of time. The vertical bar represents the IL-6 release from HGF elicited by 20 pg/ml of IL-1 β . Overnight incubation of *P. gingivalis* supernatant alone with the HGF did not induce the release of IL-6. Results are expressed as the mean and standard deviation of 6 replicate cultures.

that implies, but it could also result in impaired host defences as it may involve irreversible binding of the cytokine to the bacteria and hence the removal of the former from the local environment (6). We have previously studied the effect of cytokines on the growth of periodontopathogenic bacteria using viable counts to monitor growth. As the negative results obtained in such studies may have been attributable to the inability of this imprecise technique to distinguish between small changes in growth rate, we decided to repeat the experiments using conductivity measurements as a highly sensitive means of detecting changes in bacterial growth patterns. The results of this study have shown that neither IL-1 β nor IL-6, at concentrations as high as 100 ng/ml, were able to stimulate the growth of 3 strains of *A. actinomycetemcomitans* or of 1 strain of *P. gingivalis*. However, further studies are needed to determine whether similar results would be obtained using freshly isolated strains, different

culture media or under different environmental conditions.

None of the 3 strains of *A. actinomycetemcomitans* tested were able to degrade IL-1 β , IL-6 or IL-1ra. In contrast, supernatants from cultures of *P. gingivalis* were able to hydrolyse all 3 cytokines. In the case of IL-1ra it was apparent that rapid hydrolysis took place during sample preparation, giving rise to a molecule with a slightly lower molecular mass implying the presence of a uniquely sensitive site within the molecule. This was then followed by hydrolysis to low molecular mass degradation products. It was generally not possible to prevent reproducibly cytokine degradation using any of a number of protease inhibitors (either singly or in combination) which are known to inhibit the 4 major classes of proteases – EDTA/phenanthroline, pepstatin A, trans-epoxy succinyl-L-leucylamido-(4-guanidino)-butane (E-64) and phenylmethylsulphonyl fluoride (PMSF). However, another protease inhibitor, tosyllysyl-chloromethyl ketone (TLCK), was not tested. We are currently studying cytokine degradation by purified enzymes from *P. gingivalis* to identify the enzyme(s) responsible for the degradation of each cytokine. Such studies will inevitably be more revealing than those involving the use of crude enzyme-containing culture supernatants. Of interest was the finding that, in contrast to the other antisera used in this investigation, the goat anti-IL-1ra serum cross-reacted with high molecular weight constituents of the *P. gingivalis* supernatant. This was surprising as the antiserum was raised against a highly purified preparation of recombinant IL-1ra and showed no detectable cross-reactivity with IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-8, G-CSF, GM-CSF or TNF α . The hydrolysis of IL-1 β was accompanied by loss of bioactivity as determined by its inability to induce IL-6 release from human gingival fibroblasts. Such cytokine degradation, if it occurred *in vivo*, has implications regarding our understanding of the initiation and progression of those periodontal conditions with which *P. gingivalis* is associated. For example, if the integrity of the periodontium is maintained as a result of a balance of pro- and anti-inflammatory cytokines then the degradation of the anti-inflammatory cytokine IL-1ra may tip the balance in favour of a cytokine network capable of inducing disease. Such an event may constitute one of the “precipitating factors” postulated in the ecological plaque hypothesis (10). A decrease in the local concentration of the pro-inflammatory cytokines, IL-1 β and IL-6, could lead to a decrease in the host’s ability to mount an effective response to the organism. For example, IL-6 is a key cytokine in terms of the host’s acute phase response and is

also responsible for regulating the differentiation of B-cells into antibody-secreting plasma cells (11).

The results of the present study, however, are very preliminary findings as we have not yet determined which, if any, of the many other cytokines can be hydrolysed by *P. gingivalis*, nor have we established the relative susceptibilities of IL-1 β , IL-6 and IL-1ra to the proteases of this organism – apart from an indication that IL-6 may be the most easily degraded. Further work needs to be carried out to address these points and also to determine whether cytokine hydrolysis can take place under environmental conditions similar to those found *in vivo*. We are currently investigating the cytokine-degrading ability of purified enzymes of *P. gingivalis*.

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Cytokine Degradation by Biofilms of *Porphyromonas gingivalis*

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Received: 12 August 1997 / Accepted: 15 October 1997

Abstract. The aim of this study was to determine whether biofilms of *Porphyromonas gingivalis* could proteolytically degrade the cytokines interleukin (IL)-1 β , IL-6, or IL-1 receptor antagonist (IL-1ra). Biofilms were grown on membrane filters on the surface of Wilkins-Chalgren blood agar. The biofilms were removed from the plates, and solutions containing 2.5 μ g/ml of each cytokine were added. Following incubation for up to 4.0 h, supernatants from the biofilms were subjected to SDS-PAGE. The separated proteins were transferred by Western blotting to PVDF membranes and probed with peroxidase-conjugated antibodies recognizing both the intact cytokines and their degradation products. After 2 h, no intact IL-1 β , IL-6, or IL-1ra were detectable. Cytokine proteolysis also occurred in the presence of horse serum. These results demonstrate that biofilm-grown *P. gingivalis* can degrade both pro- and anti-inflammatory cytokines and so may be able to perturb cytokine networks in vivo by eliminating cytokines from the local environment.

Periodontitis is a chronic inflammatory disease that affects the tooth-supporting tissues and ultimately leads to tooth loss. The progression of this disease is characterized by the activation of inflammatory cells and tissue breakdown, and this may be related to excessive cytokine production by monocytes and other activated cells stimulated by bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetem-comitans*, which are among the main causative agents of periodontitis [10]. Cytokines are soluble proteins or glycoproteins that act like intercellular signals modulating immunoinflammatory processes. There is considerable evidence to suggest that the pro-inflammatory cytokines interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) are involved in the pathogenesis of periodontal diseases, and increased levels of IL-1 β have been demonstrated in gingival crevicular fluid and periodontal tissues of patients with these diseases [5]. Bacteria can stimulate the release of cytokines and interact with them in a number of ways. Hence, a wide range of bacterial components and products have been shown to stimulate the release of cytokines from monocytes and other cell types [4], while bacterial proteases can either activate cytokine precursors

or inactivate functional cytokines [8]. We have demonstrated degradation of several cytokines (IL-1 β , IL-6, and interleukin-1 receptor antagonist IL-1ra) by culture supernatants from *P. gingivalis* [2]. However, within the disease lesion in vivo, this organism constitutes part of a biofilm (i.e., subgingival plaque) formed between the teeth and the adjacent gingival tissues that is immersed in a serum exudate known as gingival crevicular fluid (GCF). Because biofilm-grown bacteria are known to differ from planktonic organisms in several respects, we decided to investigate whether biofilms of the periodontopathogen *P. gingivalis* could degrade the pro-inflammatory cytokines IL-1 β , IL-6, and the anti-inflammatory cytokine IL-1ra, and whether degradation could occur in the presence of serum, i.e., a fluid similar in composition to GCF. Such conditions more closely resemble those that would be encountered by the organism in vivo.

Materials and Methods

Biofilms of *P. gingivalis* were prepared as follows. Sterile nitrocellulose membranes (5-mm diameter) were placed on the surface of Wilkins-Chalgren agar plates containing 5% horse blood. The membranes were then inoculated with 10 μ l of a suspension of *P. gingivalis* and incubated anaerobically at 37°C for 48 h. The resulting biofilms were transferred to microtiter plates, and IL-1 β , IL-6, or IL-1ra was added. The cytokines were prepared at a final concentration of 2.5 μ g/ml in BM

broth (consisting of 10 g tryptone soya broth, 10 g proteose peptone, 5 g yeast extract, 5 g glucose, 5 g NaCl, 0.75 g cysteine-HCl, 5 mg hemin, and 0.5 mg menadione per liter of distilled water). Heat-inactivated biofilms (121°C, 15 min) plus cytokine, BM broth plus cytokine without any biofilm, and biofilm without cytokine were included as controls. Samples were removed at $t = 0, 0.25, 0.5, 1, 2$ and 4 h, centrifuged, and the supernatant stored at -70°C prior to analysis.

The experiments were repeated in a similar manner except that the cytokines were dissolved in horse serum (Unipath, Basingstoke, UK) instead of the BM broth. Controls consisted of horse serum plus cytokine without any biofilm, heat-inactivated biofilm plus cytokine, and the biofilm plus serum without cytokine.

Supernatants from the biofilms were separated by SDS-PAGE by the method of Laemmli [7] with Tris-HCl gradient gels (4–20%) (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Electrophoresis was carried out at a constant current of 30 mA at room temperature. Proteins were transferred onto Immobilon-P polyvinylidene difluoride membranes by Western blotting overnight at a constant voltage of 15V. Bands were detected with polyclonal antibodies to IL-1 β , IL-6, and IL-1ra followed by a peroxidase-conjugated rabbit anti-sheep antibody. 3,3'-Diaminobenzidine tetrahydrochloride was used as a substrate for peroxidase. All appropriate controls for immunoblotting were used.

Results

After 48 h incubation, substantial biofilms of *P. gingivalis* were present on the surface of the membrane filters, and these contained approximately 10^8 cfu/mm 2 .

The results of SDS-PAGE analysis of the supernatants from centrifuged biofilms of *P. gingivalis* exposed to various cytokines are shown in Fig 1. From this it can be seen that the biofilms were able to degrade IL-1 β , IL-6, and IL-1ra (Figs. 1a, 1b, and 1c respectively), as evidenced by the disappearance of the band corresponding to the cytokine with time and the appearance of bands with lower molecular masses representing breakdown products. After 2 h of incubation, neither IL-1 β nor IL-1ra was detectable. IL-6 appeared to be particularly sensitive to hydrolysis by *P. gingivalis* biofilms because after only 0.5 h virtually no IL-6 remained. There was no evidence of cytokine degradation by the heat-inactivated biofilms (lane 2).

The effects of exposing biofilms of the organism to the cytokines in the presence of horse serum are shown in Fig. 2. From this it can be seen that degradation of IL-1 β , IL-6, and IL-1ra also occurred in the presence of serum (Figs. 2a, 2b, and 2c). However, degradation occurred at a slower rate than in the absence of serum. Only a small amount of IL-1 β and IL-1ra remained after 4 h. Again, IL-6 was more rapidly degraded than the other cytokines and was undetectable after 2 h.

Discussion

The results of this study have demonstrated that biofilms of the periodontopathogen *P. gingivalis* could rapidly

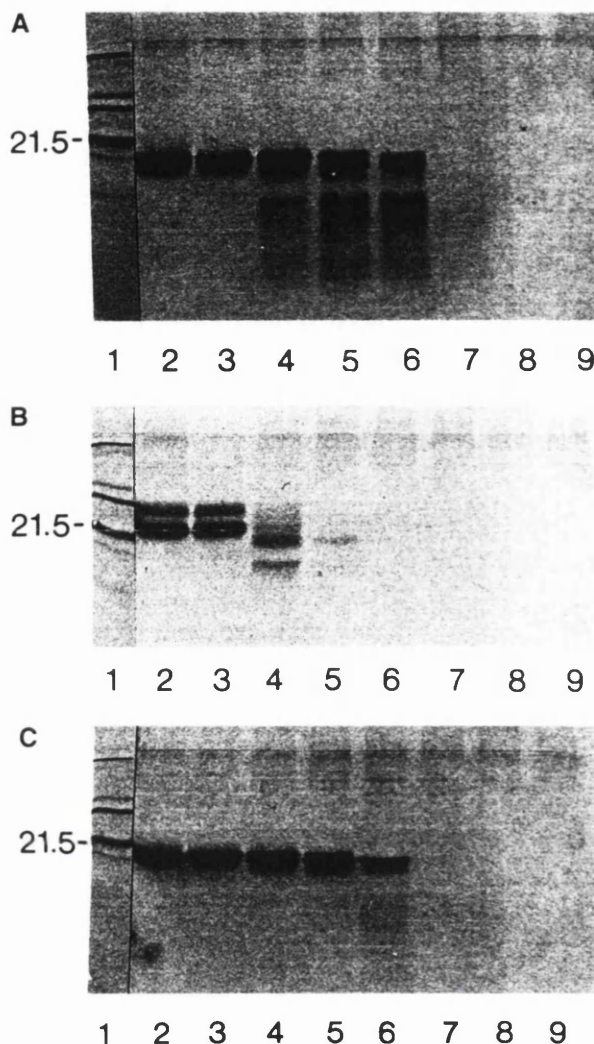


Fig. 1. Effect of *P. gingivalis* biofilms on (a) IL-1 β , (b) IL-6, and (c) IL-1ra as assessed by Western blotting. Lane 1, molecular weight markers; lane 2, heat-inactivated biofilm + cytokine; lane 3, biofilm + cytokine at $t = 0$ h; lane 4, biofilm + cytokine at $t = 0.25$ h; lane 5, biofilm + cytokine at $t = 0.5$ h; lane 6, biofilm + cytokine at $t = 1$ h; lane 7, biofilm + cytokine at $t = 2$ h; lane 8, biofilm + cytokine $t = 4$ h; lane 9, biofilm + BM broth.

degrade two key pro-inflammatory cytokines (IL-1 β and IL-6) as well as the anti-inflammatory cytokine IL-1ra. IL-6 appeared to be more susceptible to proteolysis than the other two cytokines. What was particularly interesting was that proteolysis also took place in the presence of serum despite its content of protease inhibitors such as α -1-antitrypsin and α $_2$ -macroglobulin. This may be a consequence of the ability of the proteases of *P. gingivalis* to degrade these, and other, host protease inhibitors [3].

IL-1 β and IL-6 play an important role in regulating the immune response to periodontal microorganisms. IL-1 β is responsible for activating myeloid and lymphoid

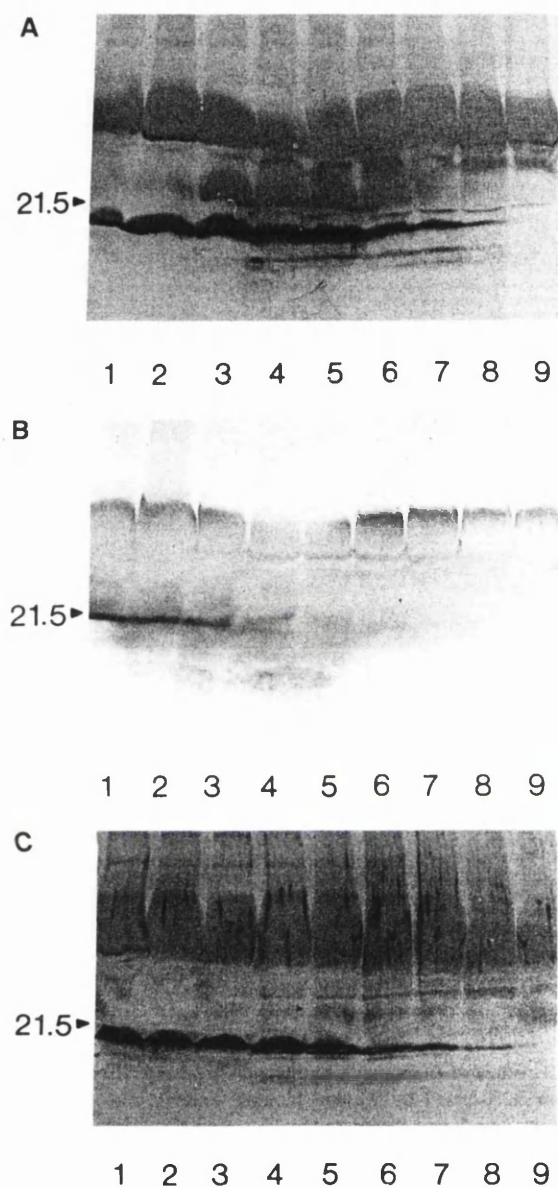


Fig. 2. Effect of *P. gingivalis* biofilms on (a) IL-1 β , (b) IL-6, and (c) IL-1ra in the presence of horse serum as assessed by Western blotting. Lane 1, serum + cytokine; lane 2, heat-inactivated biofilm + cytokine; lane 3, biofilm + cytokine at t = 0 h; lane 4, biofilm + cytokine at t = 0.25 h; lane 5, biofilm + cytokine at t = 0.5 h; lane 6, biofilm + cytokine at t = 1 h; lane 7, biofilm + cytokine at t = 2 h; lane 8, biofilm + cytokine t = 4 h; lane 9, biofilm + serum.

cells as well as mesenchymal cells, stimulating the release of other cytokines such as IL-6. IL-6 regulates the differentiation of B-cells into antibody-secreting cells and enhances T-cell proliferation. In addition to their role in the inflammatory response, cytokines may also contribute directly to the pathology of periodontal disease. IL-1 acts as a mediator of bone resorption (a characteristic feature of the disease) and also stimulates connective

tissue destruction via the release of metalloproteinases from target cells. IL-6 is responsible for the differentiation of osteoclasts, which are responsible for bone degradation. In vivo, the activities of cytokines are regulated by inhibitors and antagonists. IL-1ra binds to the same receptors on target cells as IL-1 but does not trigger intracellular transduction processes that normally occur when IL-1 binds. Thus, IL-1ra acts to suppress the effects of IL-1 on the cell. In vivo, cytokines, the cells producing them, and their target cells form an interacting network that serves to control both the production and biological activities of these potent immunomodulators. Proteolysis of cytokines by *P. gingivalis* may perturb the complex cytokine network responsible for maintaining periodontal health. Degradation of pro-inflammatory cytokines such as IL-1 β and IL-6 could lead to a decrease in the hosts' ability to mount an effective response to the organism, while degradation of the anti-inflammatory cytokine IL-1ra would decrease one of the mechanisms for controlling the activities of IL-1 β . It is, of course, difficult to predict the likely relevance of these findings to the situation in vivo, as we have little indication of the relative susceptibilities of these cytokines to proteolysis other than that IL-6 appears to be more susceptible than the other two cytokines, nor do we know much of the order of the secretion of the cytokines during the disease process.

In a previous study we demonstrated that planktonic cells of *P. gingivalis* displayed a similar ability to degrade IL-1 β , IL-6, and IL-1ra [2]. The interesting aspects of the results of this study are that this organism can also degrade these cytokines when it constitutes a biofilm and in the presence of horse serum. With the realization that bacteria in their natural environment are often in the form of a biofilm [1], there is currently great interest in investigating the effect of this mode of growth on bacterial activities. The vast majority of such studies have focused on the well-known resistance of biofilm-grown bacteria to antimicrobial agents [9]. In contrast, there have been remarkably few reports concerning the interactions of bacterial biofilms with host defense systems despite the fact that biofilms are responsible for a wide range of diseases with high morbidity and mortality, e.g., infective endocarditis, catheter-associated infections, caries and periodontal diseases. One of the few studies of this type is that of Jensen et al [6], who found that biofilms of *Ps. aeruginosa* induced a much lower oxidative burst from polymorphonuclear leukocytes than the planktonic form of the organism. The results of this study have shown that at least one other pathogenic organism, *P. gingivalis*, can interfere with host defense mechanisms when it is grown as a biofilm. The ability of biofilms of *P. gingivalis* to undermine host defenses, together with their

resistance to antimicrobial agents, may help to explain the recurrent nature of such infections and the difficulties encountered in their treatment.

Literature Cited

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