ERRATA

Page 9 PBMC are defined as peripheral blood mononuclear cells

Page 27 Kolliker, A. Die normale resorption des knochengewebes in ihre bedeutung fur die entstehung der typischen knochenformen. Leipzig, F.C.W. Vogel, 1873; p. 86.

Page 27 Mundy *et al* should read as Mundy 1989

Page 31 Maynard should read as Maynard

Page 32 Kornman *et al* should read as Kornman and Loesche

Page 32 Chen, Casey *et al* 1992 should read as Chen and Wilson

Page 32 Slots *et al* 1983 should read as Slots *et al* 1983a

Page 32 Zambon *et al* 1983 should read as Zambon *et al* 1983a

Page 32 The following should be entered in the reference list. Zambon JJ, Sunday GJ, Smutko JS. Molecular genetic analysis of *Actinobacillus actinomycetemcomitans*. *J Periodontol* (1990);**61**:75-80. and Zambon JJ. *Actinobacillus actinomycetemcomitans* in adult periodontitis. *J Periodontol* (1995); **65**:892-893.

Page 32 Listgarten *et al* 1994 should read as Listgarten 1994

Page 32 Mashimo *et al* 1985 should read as 1985b

Page 33 Chen *et al* should read as Chen and Wilson

Page 33 Savitt *et al* should read as Savitt and Socransky

Page 33 Mandell *et al* should read as Mandell

Page 34 Both references quoting Borinski *et al* should read as Borinski and Holt

Page 34 Armitage *et al* should read as Armitage and Holt

Page 35 Slots *et al* 1983b should read as Slots and Rosling 1983

Page 35 Slots *et al* 1984 should read as Slots and Genco 1984

Page 35 Zambon 1983a should read as Zambon *et al* 1983a

Page 35 Zambon 1988 should read as Zambon *et al* 1988

Page 35 Zambon JJ. *Actinobacillus actinomycetemcomitans* in adult periodontitis. *J Periodontol* (1995); **65**:892-893.

Page 37 Bramanti 1989 should read as Bramanti *et al* 1989

Page 37 Kennel *et al* 1990 should read as Kennel and Holt 1990

Page 37 Wilson *et al* 1995 should read as Wilson 1995

Page 37 Evans *et al* 1992 should read as 1992a, 1992b

Page 40 Slots *et al* 1978 should read as Slots and Gibbons

Page 43 Brock TD and Madigan MT (1991). Cell Biology. In; Biology of microorganisms. 6th edition. Prentice Hall International. Chapter 3. pp 39-91.

Page 43 Millar *et al* 1986 should read as 1986a

Page 46 Dinarello *et al* should read as Dinarello

Page 48 Bartold *et al* should read as Bartold and Haynes

Page 48 Mizutani in the reference list should read as Mitzutani

Page 50 Bickel et al should read as Bickel

Page 59 LAL is an abbreviation for *Limulus* amoebocyte lysate

Page 59 The word passages should be replaced by subcultures

Page 75 The exact migration distances from the top of the gel for the standards for Fig 3.4b and d are as follows:-66kDa-19mm, 45 kDa-37mm, 36kDa-45mm, 24 kDa-63mm, 20 kDa-68mm and 14kda-86mm. For Fig 3.4e the migration distances are as follows, 205kDa-30mm,116kDa-40mm, 97kDa-61mm, 66kDa-72mm and 45 kDa-82mm.

The migration distances for the standards for Fig 3.5 a 66kDa-32mm, 47kDa-47mm, 36kDa-57mm, 24kDa-67mm, 20kDa-73mm and 14kDa-83mm. For Fig 3.5b and c the following migration distances apply for the standards 66 kDa-19mm, 45kDa-37mm, 36kDa-45mm, 24kDa-63mm, 20kDa-68mm and 14kDa-86mm.

Page 98 The plot for *E. corrodens* on Fig 4.3 is now included

Page 103 Millar *et al* 1986 should read as 1986a

Page 104 White *et al* 1995 should be quoted in the reference section as White PA. Wilson M, Nair SP, Kirby AC, Reddi K and Henderson B. Characterization of an Antiproliferative Surface-Associated Protein from *Actinobacillus actinomycetemcomitans* Which Can Be Neutralized by Sera from a Proportion of Patients with Localized Juvenile Periodontitis. *Infec Immun* (1995);**63**:2612-2618.

Page 108 Kirby *et al* 1995 should be quoted in the reference section as Kirby AC, Meghji S, Nair S, White P, Reddi K, Nishihara T, Nakashima K, Willis A, Sim R, Wilson M, Henderson B. The potent bone resorbing mediator of *Actinobacillus actinomycetemcomitans* is homologous to the molecular chaperone GroEL. *J Clin Invest* (1995);**10**:726-734.

Page 126 Roodman *et al* 1992, 1995 should read as Roodman 1995

Page 156 The Legend for figure 8.1 should be amended as follows:- IL-6 release by HGF stimulated by 1- *E. coli* LPS alone and in the presence of an excess concentration of neutralising antibodies to 2-IL-1 (both α and β), 3-TNF-α, 4- a combination of anti IL-1 α, β and TNF-α and 5 IL-1ra.

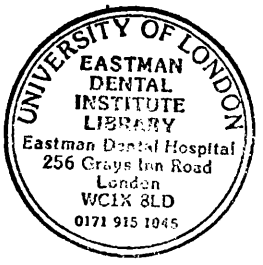
Page 157 The legend for figure 8.2 should be amended as follows IL-6 release by HGF stimulated by 1- SAM from *A. actinomycetemcomitans* alone and in the presence of an excess concentration of neutralising antibodies to 2- IL-1 (both α and β), 3-TNF-α, 4- a combination of anti IL-1 α, β and TNF-α and 5 IL-1ra.

Page 177 The symbol for interleukin concentration is ● which should be inserted in the legend

Page 186 Saito *et al* 1993 should read as 1993c

Page 189 Bramanti *et al* 1993 should have *J Periodontal Res* in the reference section

MODULATION OF CYTOKINE ~~RELEASE~~ SYNTHESIS BY BACTERIAL COMPONENTS



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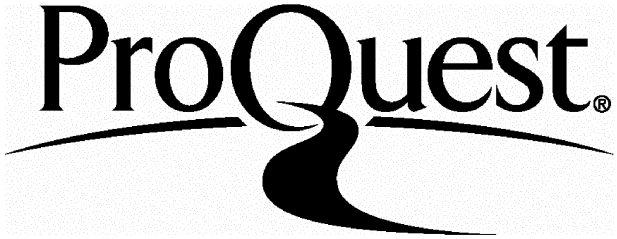

Dedicated to my dear mother
and
late father

ABSTRACT

The chronic inflammatory diseases (CIPDs) are mankind's most prevalent chronic inflammatory conditions. Inflammation in the gingivae is associated with the destruction of the alveolar bone and periodontal ligament which support the teeth. There is good evidence for the involvement of a range of Gram-negative anaerobic and capnophilic bacteria in tissue pathology including: *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, *Porphyromonas gingivalis*, *Campylobacter rectus* and *Prevotella intermedia*. Invasion of lesional tissues is not a conspicuous feature of this disease and the current paradigm is that soluble components or products of bacteria drive the tissue pathology. However, the nature of these virulence determinants has not been defined.

This study has examined the capacity of proteins, associated with the bacterial surface (termed surface-associated material- SAM) and the outer membrane (lipid A-associated proteins-LAP), from these bacteria to stimulate bone resorption and to induce the synthesis of cytokines by myelomonocytic and mesenchymal cell populations. These activities have been compared with those of the lipopolysaccharides from the respective organisms.

The bone resorbing activity of the SAMs from the bacteria studied varied widely in potency and efficacy. Some bacteria had SAMs which showed activity at concentrations as low as 1 ng/ml while others required microgram/ml concentrations to produce minimal bone resorption. In contrast, all the LAPs tested showed similar potencies and efficacies. The activity of the LPS from the various organisms was generally low.

The capacity of the SAMs to induce the release of the pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNF-α also showed a range of potencies and efficacies. In comparative studies of the cytokine-stimulating actions of the SAMs, LAP and LPS from *A. actinomycetemcomitans* the former generally showed the greatest potency with the LAP being somewhat less potent and the LPS showing very little activity. Comparison of the mechanism of stimulation of gingival fibroblast IL-6 release by SAMs or *E. coli* LPS revealed major differences. Stimulation by the SAMs was not affected by neutralizing IL-1 or TNF-α activity or by dexamethasone. In contrast LPS-induced IL-6 synthesis was totally abolished by these agents. This suggests that the active cytokine inducing component in the SAM from *A. actinomycetemcomitans* may interact with a novel transcriptional regulatory site in the IL-6 gene. This active IL-6 stimulating component has been purified by various chromatographic techniques and was found to be a 2 kDa peptide.

The results of this study have shown that bacterial components other than LPS are potent inducers of cytokine release and bone resorption *in vitro*, and so may play a role in initiating the tissue destruction characteristic of periodontitis.

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SYMBOLS AND ABBREVIATIONS

Symbol/abbreviation	Description
α	Alpha
β	Beta
BPA	Black pigmented anaerobes
EAP	Endotoxin-associated-proteins
EP	Endotoxin proteins
FGF	Fibroblast growth factor
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-11	Interleukin-11
KDO	2-keto-3-deoxyoctonic acid
LAP	Lipid-A-associated protein
LPS	Lipopolysaccharide
LJP	Localised juvenile periodontitis
MMPs	Matrix metalloproteinases
PMN	Peripheral blood monocytes
SAM	Surface-associated-material
TGFα	Transforming growth factor α
TGF β	Transforming growth factor β
TIMP	Tissue inhibitors of metalloproteinases
TNF	Tumour necrosis factor

ACKNOWLEDGEMENTS

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DECLARATION

This thesis is the result of my own original investigations, except where otherwise stated. The mouse calvarial bone resorption assays were kindly performed by Dr. Sajeda Meghji of the Oral and Maxillofacial Surgery Department at the Eastman Dental Institute. The electron microscopy work mentioned in chapter 3 was performed by Ms. Pauline Barber and Ms. Nicky Morden of the EM Unit at the Eastman Dental Institute.

PUBLICATIONS AS A RESULT OF THIS THESIS

Full papers

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Chapter 1

Introduction

1.1 Anatomy of the periodontium

The periodontium is an intricate mosaic of various tissue types which are primarily responsible for the attachment of teeth in the oral cavity. The principle structures of the periodontium are the gingiva, root cementum, alveolar bone and the periodontal ligament (PDL, figure. 1.1, Hasell, 1993).

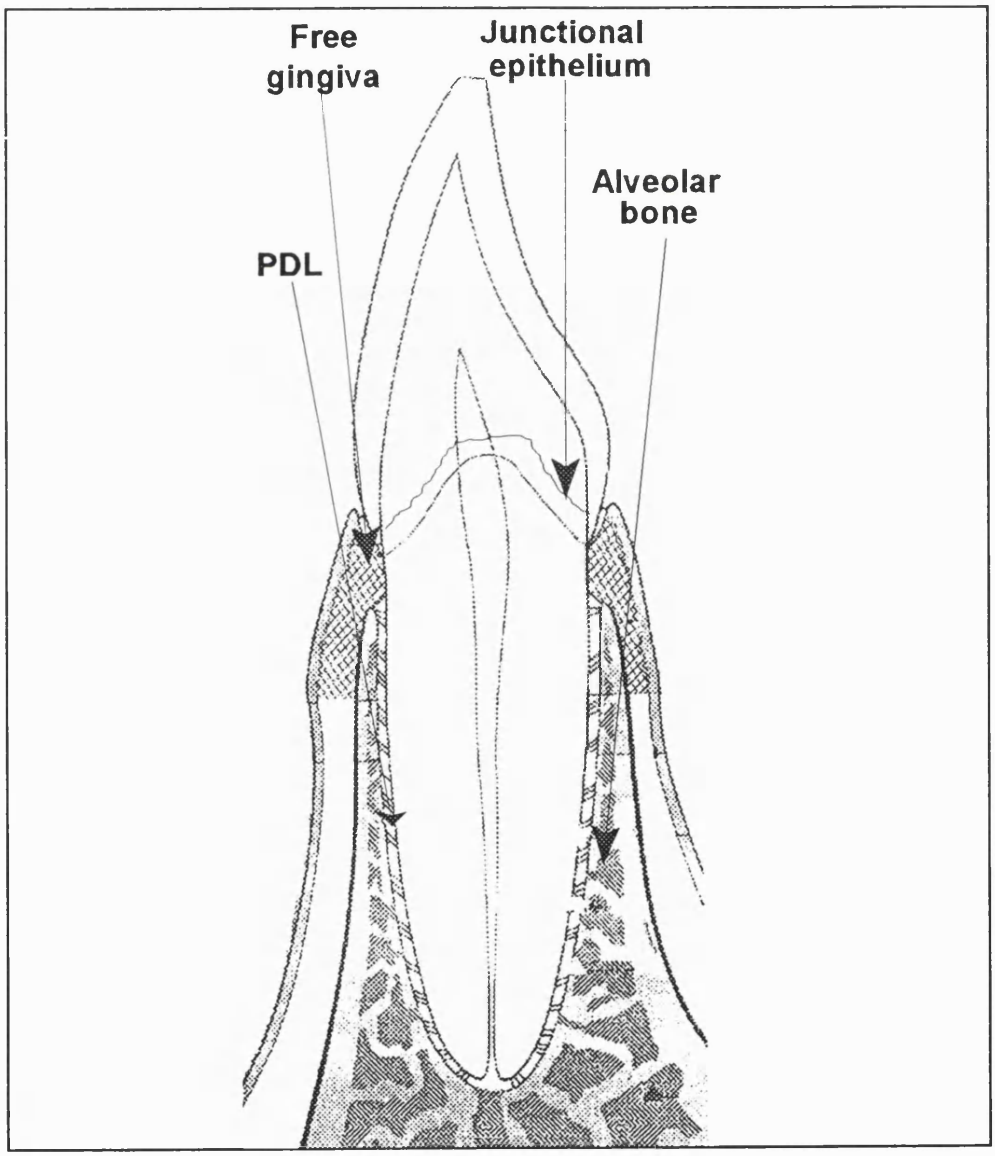


Figure 1.1 **Diagrammatic representation of the principal structures of the periodontium (gingiva, root cementum, alveolar bone, PDL) and the tooth.**

1.1.1 Gingiva

The most peripheral layer of the periodontium is the gingiva. This layer begins at the mucogingival line and terminates at the neck of the tooth, which is covered with epithelium and connective tissue. Here the gingiva is detached from the tooth surface for approximately 1.5 mm and is known as the free or marginal gingiva. Between the gingiva and the tooth is an invagination of the epithelium measuring approximately 1 mm. This invagination is closely fitted around the tooth forming the gingival crevice. The gingiva in between the teeth is known as the interdental papilla. In healthy gingiva the interdental papilla completely fills the spaces between the teeth.

The next zone of the gingiva, consists of the attached gingiva, which is firmly bound to the cementum and the bone by collagen fibres. This zone may be quite variable in depth and terminates in a well-marked line. At this line a sharp colour change occurs from the pink of the attached gingiva to the deeper red of the alveolar mucosa. This line of demarcation is known as the mucogingival junction. Healthy gingiva appears as salmon or coral pink and is firm in consistency (Mariotti, 1993).

The surface of the gingiva is covered by a keratinising stratified squamous epithelium, of which there are three major types: the oral gingival epithelium, the oral sulcular epithelium and the junctional epithelium. The oral sulcular epithelium is an extension of the oral gingival epithelium. The function of the oral gingival epithelium is to provide protection for the periodontium, as keratinocytes, melanocytes and Langerhans cells are found in this region. The Langerhans cells have surface antigens which are similar to those of lymphocytes and macrophages, as well as possessing receptors for immunoglobulin and complement. Thus the Langerhans cells have been reported to play an important role in the early host response to plaque bacteria (Newcomb, 1982). The junctional epithelium is a specialised collar of gingival epithelium which serves to anchor the gingival soft tissue and the calcified structure of the tooth.

The free marginal gingiva, the interdental gingiva and the attached gingiva are largely comprised of connective tissue, of which approximately 65 % is collagen.

The individual fibrils of the collagen are organised into fibre bundles which course in various directions. This arrangement of collagen fibrils provides the gingiva with resistance to external forces and stabilises the positions of the teeth. The other components of the connective tissue compartment include fibroblasts, leukocytes, mast cells, tissue macrophages, blood, lymph and nerve cells (Schroeder *et al.*, 1973).

1.1.1.1 Fibroblasts

65 % of the cells contained in the gingival connective tissue are fibroblasts (Schroeder *et al.*, 1973). These cells produce the structural connective tissue proteins, collagen and elastin, as well as the glycoproteins, glycosaminoglycans and proteoglycans which comprise the gingival and the periodontal ligament extracellular matrices.

Both gingival fibroblasts, and periodontal fibroblasts secrete an active collagenase which belongs to the family of enzymes known as matrix metalloproteinases (MMPs) or matrixins (Birkedal-Hansen, 1993a). The function of the MMPs is to degrade the extracellular matrix (Page, 1991; Henderson and Blake, 1992). These MMPs are secreted by fibroblasts in an inactive precursor form which are activated by plasmin, also secreted by fibroblasts. In addition, a variety of tissue inhibitors of metalloproteinases (TIMPs) which serve to inhibit the actions of the MMPs are also secreted by these cells (Goldberg *et al.*, 1989). As well as MMPs, fibroblasts also secrete other inflammatory mediators such as cytokines (Birkedal-Hansen, 1993b).

In healthy gingiva, fibroblasts produce the extracellular matrix and also maintain homeostasis (Page, 1991). Maintaining homeostasis is very important, particularly in the gingival sulcular region, as it is at this site that dental plaque accumulates eliciting a response from the body's immune system.

1.1.2 Periodontal Ligament

The periodontal ligament is embedded in the cementum of the tooth root and in the bone of the socket. During health, the ligament is mainly composed of coarse

collagenous fibre bundles known as Sharpey's fibres which are closely interlaced. Thus, the PDL suspends the tooth in its socket by the Sharpey's fibres, attaching the root cementum to the alveolar bone. The PDL contains cells such as fibroblasts, osteoblasts and cementoblasts which maintain homeostasis in the alveolar segment of the periodontium. The other functions attributable to the PDL include development of fibrous and calcified tissue, as well as the transport of nutrients and metabolites. These functions are achieved by the osteogenic layer which borders on one side of the periodontal ligament and the cementogenic layer on the other side. The osteogenic layer is involved in bone formation, whilst the function of the cementogenic layer is in the formation of new cementum. The vascular and lymphatic networks within the PDL supply nutrients to, and remove by-products from, the soft tissues of the periodontal ligament and, in part, the gingiva.

1.1.3 Cementum

The cementum is the calcified tissue which covers the dentine of the roots of each tooth. Anatomically, it is part of the tooth, and functionally it belongs to the tooth-supporting structures because, as mentioned above, the gingival and periodontal fibres are anchored in cementum as Sharpeys fibres. Cementum resembles bone in its physical properties. It is always covered by a very thin layer of cementoid substance that is synthesised and secreted by resident cementoblasts. These cementoblasts are believed to be derived from fibroblast-like cells in the periodontal ligament. The principal difference between the composition of bone and cementum is that cementum is made up of only 46 % inorganic salts whereas bone is made up of 70 % inorganic salts. The cementum contains type I and type III collagen and it also contains a proteoglycan interfibrillar substance, which is unique to cementoblasts.

1.1.4 Alveolar bone

Alveolar bone is that bone in the jaw which supports the teeth. Its loss is the most serious consequence of advanced periodontal disease. Like the rest of the skeleton, alveolar bone continues to be remodelled throughout life. Therefore at any given time, in the absence of disease, the processes of bone synthesis and

bone breakdown are taking place simultaneously (ie. remodelling). Bone remodelling consists of bone resorption (by osteoclasts) followed by bone formation (by osteoblasts). As described by Vaananen (1993) and Meghji (1992a), initiation of the remodelling sequence involves the recruitment of osteoclast progenitor cells to the remodelling site; these cells fuse and differentiate into the mature osteoclastic phenotype. Very little is known about the activation of the bone remodelling process. However, there is growing evidence that interactions occur between osteogenic and osteoclastic cells which may provide the physiological and anatomic basis for the coupling of bone formation and resorption (Price *et al.*, 1994). Net loss of bone can occur when an increased rate of bone resorption is not accompanied by an increased rate of bone deposition, eg. during disease or when inhibition of bone formation occurs.

1.1.4.1 Osteoblasts

Osteoblasts are cuboidal specialised bone forming cells of mesenchymal origin, related to fibroblasts (Arnett and Stevenson, 1993). In adults, osteoblasts are situated on surfaces involved in remodelling or repair as continuous sheets of cells. The function of the active osteoblast is to secrete type I collagen which is the major organic component of the bone matrix, as well as other matrix proteins such as osteocalcin, phosphorylated glycoproteins (eg. osteonectin), bone sialoproteins (eg. osteopontin) and proteoglycans.

1.1.4.2 Osteoclasts

The primary bone resorbing cell is the differentiated large multinucleated osteoclast which belongs to the haemopoietic cell lineage and was originally observed on bone surfaces by Kolliker in 1873. The other cell types which have been reported to resorb bone *in vitro* include osteocytes (Belanger, 1972) and macrophages (Kahn *et al.*, 1978; Mundy *et al.*, 1989). Osteoclasts are found in small numbers in normal bone and are nearly always found in the vicinity of mineralised matrix. However, the osteoclasts are motile and are thought to move on the bone surface to extend erosion.

1.2 Dental Plaque Formation

From infancy until death, people are in continuous contact with various micro-organisms (Moore and Moore, 1994). However, in spite of this, each person tends to maintain his or her own unique periodontal, skin and intestinal floras (Moore *et al.*, 1984).

The earliest microbiota to colonise the mouth of the new-born infant is derived from the mother's genital tract, oral cavity and skin. As there are no teeth in the new-born baby the first microbial colonisers are those bacteria which are able to adhere to the available surfaces. *Streptococcus salivarius* is such an organism (Long and Swenson, 1976), which becomes established in the mouth within one day of birth (Carlsson *et al.*, 1975).

As the teeth erupt, the dominant species which colonise the tooth surfaces are *Lactobacillus casei, Streptococcus sanguis* and *Streptococcus mutans* (Carlsson *et al.*, 1975; Kononen *et al.*, 1992a; 1992b). Although most infants harbour a predominantly Gram-positive, facultative microbiota, anaerobes can also be recovered, particularly after tooth emergence. As many as 61 % of children aged 5-7 years harbour black-pigmented Gram-negative anaerobes (Mackler and Crawford, 1973; Friskin *et al.*, 1990) as well as spirochaetes (Mackler and Crawford, 1973). The proportion of these organisms and other strict anaerobes increases in adolescence and adulthood (Wojcicki *et al.*, 1987) but may show a great deal of site-to-site variability (Bowden *et al.*, 1975), with local factors such as pH, Eh and assorted bacterial interactions playing an important role (Midtvedt, 1990). Some variability is also due to such factors as the state of health of the dentition, the periodontal status (Asikainen *et al.*, 1991a; 1991b) the medical status of the patients and their racial origin. Although the suspected periodontal pathogens such as the Gram-negative anaerobic rods of the *Porphyromonas*, *Fusobacterium*, *Prevotella* and *Wolinella* genera and the Gram-negative rods of the genera *Capnocytophaga*, *Eikenella* and *Actinobacillus* are members of the normal oral flora, there remains some confusion as to how these bacteria cause periodontal disease. This gives rise to the specific, non-specific and the ecological plaque hypotheses.

1.2.1 Plaque and oral diseases

The specific plaque hypothesis states that, out of the diverse collection of organisms comprising the resident plaque microflora, only a limited number of species are involved in disease. In contrast, the non-specific plaque hypothesis considers that any of a heterogeneous mixture of micro-organisms can play a role in disease aetiology (Marsh and Martin, 1992). The ecological plaque hypothesis concentrates on the bacterial environment, where for example lactic acid production by oral *Streptococcus mitis* inhibits the growth of oral *Capnocytophaga* (Mashimo, 1985a). In contrast the rise in lactic acid production however, would be favourable for the increased growth of *Veillonella* spp.

1.2.2 Formation of supragingival dental plaque

After a tooth surface is freshly cleaned, an acquired pellicle is formed, composed primarily of salivary proteins (Listgarten, 1976). The earliest colonisers are streptococci, which are found during the first 4 h after professional tooth cleaning (Nyvad and Kilian, 1987). However, within 12 h the population diversifies to include actinomyces, capnocytophaga, haemophili, prevotellae, propionibacteria, and veillonellae (Kolenbrander *et al.*, 1993).

1.2.3 Formation of subgingival dental plaque

Within a few days of undisturbed plaque formation, the gingival margin begins to show typical inflammatory changes, including redness and swelling. This then results in the creation of a deepened gingival sulcus (pseudopocket), which provides a relatively anaerobic environment for the development of an anaerobic microbiota. Anaerobic bacteria that colonise this subgingival region include motile rods and spirochaetes. They are able to increase their mass hence contributing to the deepening of the sulcus, thereby increasing the volume of their habitat. Because many of the subgingival micro-organisms are motile, the structural organisation of this microbial population is quite different from that seen supragingivally. Rods and filaments tend to be arranged in a palisading pattern, with the long axes of the cells perpendicular to the tooth surface. The bulk of the subgingival microbiota consists of a complex mixture of predominantly anaerobic bacteria that surround and cover the bacterial aggregates.

The bottom of the sulcus or pocket is formed by the coronal, desquamative surface of the junctional epithelium, which is attached to the tooth surface on one side and to the gingival connective tissue on the other. This portion of the junctional epithelium is subject to bacterial as well as mechanical injuries, which may result in enlarged intercellular spaces and vertical tears in the epithelium. These alterations in the integrity of the junctional epithelium allow a gradual apical colonisation of the tooth surface by coccoid cells and rods (Vrahopoulos *et al.*, 1992a; 1992b; 1995). Irregularities in the root surface, such as those caused by localised root resorption may shelter plaque micro-organisms and contribute to their retention at such sites. The tendency for bacteria to colonise tooth surfaces freshly exposed, because of disruptions in the junctional epithelium, leads to a gradual deepening of the sulcus or pocket. Thus, a distinctive subgingival microbiota, predominantly composed of Gram-negative anaerobic bacteria, including a number of motile species, becomes established in the gingival sulcus between 3-12 weeks after the beginning of supragingival plaque formation. Each of these microbial populations appears to facilitate the colonisation of this region by the next wave of bacterial settlers, with the ultimate establishment in the subgingival region of a predominantly anaerobic, Gram-negative microbiota. In this protected environment they are in an excellent position to participate in the destruction of the periodontal tissues, with the resulting maintenance and expansion of their subgingival habitat.

1.3 Periodontal Diseases

Periodontal diseases are those inflammatory processes primarily associated with dental plaque which affect the tissues of the periodontium. The vast majority of the adult population suffer some degree of periodontal disease, with between 7 % and 15 % of dentate adults experiencing advanced forms of periodontitis (Johnson *et al.*, 1988).

Periodontal diseases are generally classified as gingivitis and periodontitis. Gingivitis is the inflammation of the tissues of the marginal gingiva. It is characterised by oedema, bleeding and redness of the tissues but does not affect the attachment of the tooth to the alveolar bone. At the onset of gingivitis a lesion appears after approximately four days of plaque accumulation (Page and Schroeder, 1976). Due to an increase in the flow of gingival fluid and

transmigration of blood cells (mainly neutrophils) through the tissues to the gingival sulcus, an acute inflammation of the tissues occurs. Within seven days, an influx of lymphocytes and macrophages occur. Destruction of the tissues of the periodontium occurs and the condition becomes recognisable as clinical gingivitis. The development of the established lesion is accompanied by an increase in the proportion of B cells present and a decrease in T cells and a pocket forms (Page, 1986). These lesions may remain stable for many years or may develop into the more destructive lesions observed in periodontitis. The reasons why only some sites in some individuals progress to periodontitis are not yet known. However, it is thought that periodontitis may occur due to a shift in the microbial population.

1.3.1 Periodontitis

Periodontitis is characterised by the destruction of periodontal tissues resulting in the loss of gingival attachment (Williams *et al.*, 1992). There are several distinct forms of periodontitis which are associated with dental plaque. These include chronic (adult) periodontitis, which is the main cause of tooth loss in the adult population (Page and Schroeder, 1976), and various types of early onset periodontitis, namely chronic periodontitis, pre-pubertal periodontitis, localised juvenile periodontitis and rapidly progressive periodontitis (Listgarten, 1986; 1994).

Chronic periodontitis appears not to progress at a continuous slow rate but is thought to occur with distinct periods of disease activity over relatively short periods of time, followed by phases of quiescence or even repair. As already mentioned, during periodontal disease a pocket is formed and fibrosis of the gingiva occurs as well as loss of collagen. This results in the breakdown of the periodontal ligament. Resorption of the alveolar bone then takes place leading to tooth mobility and eventual tooth loss.

1.4 Microflora associated with chronic periodontitis

Black-pigmented anaerobes (BPA) are one of the predominant Gram-negative isolates encountered in human periodontitis. As early as 1928, BPA were regarded as being important in the pathogenesis of periodontal disease (Burdon, 1928). Of the BPA *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*) has

been implicated in many cases of severe adult periodontitis (Spiegel *et al.*, 1979; White and Maynard, 1981; Slots, 1982; Loesche *et al.*, 1985), and *Prevotella intermedia* (formerly *Bacteroides intermedius*) in acute necrotising ulcerative gingivitis (Loesche *et al.*, 1982, Chung *et al.*, 1983). *Prev. intermedia* has also been associated with pregnancy gingivitis (Kornman *et al.*, 1980). Other species such as *Eikenella corrodens* (Chen Casey *et al.*, 1992), *Campylobacter rectus* (formerly *Wolinella recta*; Lai *et al.*, 1992), *Fusobacterium* spp (Slots *et al.*, 1983) and *Eubacterium* spp. have been reported to participate in destructive periodontal disease (Slots and Genco, 1984). *Actinobacillus actinomycetemcomitans* has been implicated in localised juvenile periodontitis (Zambon *et al.*, 1983; 1985; 1988; 1990; 1995) and in more recent years with other forms of periodontal disease (Listgarten *et al.*, 1994).

1.4.1 Black pigmented anaerobes

The BPA are Gram-negative obligately anaerobic, nonmotile, nonspore-forming rods which were first isolated from the oral cavity in 1921 by Oliver and Wherry. The organisms isolated by them produced a black pigment which they referred to as melanin, therefore the organisms were named *Bacterium melaninogenicum* ("melanin producing"). In later studies it was found that the black pigmentation was not melanin, which led to the subdivision of this species and the evolution of the taxonomy of oral *Bacteroides* species. Oral species which have been classified as *Bacteroides* species have recently been recategorised into the genera *Porphyromonas* and *Prevotella*.

1.4.1.1 Porphyromonas gingivalis

Por. gingivalis is an anaerobic, non-fermenting Gram-negative short rod. This organism produces a number of virulence factors with which it may initiate the pathology observed in chronic periodontitis. These virulence factors include a capsule (McKee *et al.*, 1986), fimbriae (Hanazawa *et al.*, 1988; 1995; Murakami *et al.*, 1994), lipopolysaccharide (Mashimo *et al.*, 1985; Millar *et al.*, 1986) and vesicles (Haapasalo *et al.*, 1989; Smalley and Birss, 1991) . In addition this organism also possesses trypsin-like activity (known as gingivain; Smalley *et al.*,

1987; Shah *et al.*, 1992) as well as argingipain (Okamoto *et al.*, 1995) and collagenolytic activity (Grenier and Mayrand, 1987; Smalley *et al.*, 1989).

1.4.1.2 Prevotella intermedia

The genus *Prevotella* was named after the French microbiologist, Prevot. The organisms in this group include those which were formerly classified as *Bacteroides intermedius.* These organisms are anaerobic, Gram-negative rods and include both pigmented and non-pigmented species. *Prev. intermedia* also possesses virulence factors which may initiate the pathology observed in chronic periodontitis. These factors include a lipopolysaccharide (Sveen and Skaug, 1980; Mashimo, 1985b; Wilson, 1995) and an epitheliotoxin (Birkedal-Hansen *et al.*, 1982). These organisms also produce a number of enzymes such as gelatinase (Sorsa *et al.*, 1992) a trypsin-like enzyme (Moncla *et al.*, 1991), fibrolysin, IgA and IgG proteases and superoxidase dismutase (Zambon and Nisengard, 1994).

1.4.2 Eikenella corrodens

Eikenella corrodens is a Gram-negative, capnophilic, asaccharolytic, small rod. The organism was first characterised by Eiken in detail in 1958. It was originally named '*Bacteroides corrodens*', however, in 1978, due to the fact that this organism is a facultative anaerobe, a new name, *Eik. corrodens* was assigned to it. This organism exhibits corroding colony morphology characterised by a circular and irregular margin hence its name.

As well as periodontitis, *Eik. corrodens* has been recognised as a pathogen in many other diseases, particularly osteomyelitis (Johnson *et al.*, 1976), infections of the central nervous system (Brill *et al.*, 1982) and root canal infections (Goodman., 1976; 1977). This species has been found more frequently in sites of periodontal destruction than in healthy sites (Savitt *et al* ., 1984) and is a common inhabitant of the intestinal and genital tracts (Chen *et al.,* 1992). It has also been found in close association with *A. actinomycetemcomitans* in some lesions of localised juvenile periodontitis (Mandell *et al.*, 1984).

1.4.3 Campylobacter rectus

Campylobacter rectus is a small, Gram-negative, asaccharolytic rod which is capable of motility via a single polar flagellum. In a study by Rams and colleagues (1993), *C. rectus* was detected in 80% of periodontitis patients of various age groups. However, they found that it tended to occur in higher proportions in younger than in older infected individuals. Similar to the other organisms already mentioned, *C. rectus* also possesses a number of virulence factors. In particular, it forms an extracellular proteinaceous cytotoxin which acts on human polymorphonuclear neutrophils (Gillespie *et al.*, 1992). Fresh isolates of *C. rectus* have been shown to exhibit a proteinaceous surface structure (S-layer) external to the outer membrane (Kerosuo *et al.*, 1989; Borinski *et al.*, 1990) that enhances the organisms pathogenicity in necrotic skin lesions in animal models (Borinski *et al.*, 1990). It also produces an LPS which has been shown to stimulate plasmin activity in gingival fibroblasts (Ogura *et al.*, 1992; 1995) . In a number of studies *C. rectus* was found to possess the ability to resist *in vitro* phagocytosis by granulocyte-like HL-60 cells (Armitage *et al.*, 1990, Gillespie *et al.*, 1992). This organism has also been reported to suppress human lymphocyte proliferation *in vitro* (Saito *et al.*, 1993a; 1993b), produce the potentially tissue-damaging enzyme arylsulfatase (Wyss, 1989) and to release the cytotoxic volatile sulphur compound hydrogen sulphide (Persson *et al.*, 1990; Gillepsie *et al.*, 1993). These studies suggest that *C. rectus* could be a potential pathogen in human periodontitis.

1.5 Microorganisms involved in localised juvenile periodontitis (LJP)

Localised juvenile periodontitis begins in puberty but is usually not diagnosed until several years later. The disease affects approximately 0.1 % of the United States population, but it has also been found in up to 10 % of the population in certain parts of the world.

LJP attacks the permanent dentition with the initial sites of tissue destruction being the mesial or distal surfaces of one or more of the first permanent molars. As the disease progresses, the affected teeth may become increasingly mobile, with labial movement and spacing of incisors. Later, other teeth may also be involved in the disease process. An interesting difference between LJP and other periodontal

diseases is that in its earliest stages, LJP seldom manifests as gingival inflammation (Hall *et al.*, 1991). Between 75 and 80 % of patients with LJP have depressed neutrophil chemotaxis and this is believed to be an intrinsic defect of the cell. Such neutrophils have been shown to have a reduced number of cell surface receptors for chemotactic factors. Despite the high prevalence of neutrophil dysfunction in LJP patients, approximately 20 % of affected individuals have normal neutrophils and some may exhibit a cell-directed inhibitor of chemotaxis in their serum. Thus there appear to be at least two types of familial LJP: those with neutrophil chemotactic defects and those without.

Most affected individuals have clean mouths and minimal tooth deposits and the involved sites harbour a sparse subgingival flora. Considerable progress has been made in identifying the micro-organisms associated with LJP and their pathogenic potential. Evidence suggests that *A. actinomycetemcomitans*, a Gram-negative capnophilic rod, plays a dominant role in the disease process (Slots *et al.*, 1976; 1983b; 1984; Slots and Listgarten, 1988; Zambon, 1983a; 1985; 1988; 1995; Wolff *et al.*, 1985; Slots and Schonfeld, 1991; Aass *et al.*, 1992; Hall *et al.*, 1994; Muller *et al.*, 1995).

1.5.1 Actinobacillus actinomycetemcomitans

A. actinomycetemcomitans is present in large numbers in the gingival pockets of patients with LJP, and resolution of the disease is positively correlated with its elimination from subgingival plaque. Moreover, the re-establishment of the organism is associated with the recurrence of the disease. LJP patients have elevated serum immunoglobulin levels to this organism and high levels of antibody to *A. actinomycetemcomitans* in crevicular fluid and gingival tissue. Following successful treatment, the levels of antibody to this species decrease significantly in serum and crevicular fluid. *A. actinomycetemcomitans* was named in 1912 by Klinger because of its association with *Actinomyces israelli* which caused *actinomycosis*. Originally it was named as *Bacterium actinomycetum comitans* (Lieske, 1921) and finally *Actinobacillus actinomycetemcomitans* (Topley and Wilson, 1929).

As already mentioned, numerous reports in the literature have demonstrated the presence of *A. actinomycetemcomitans* in the gingival crevices of LJP patients, and many of these reports are often cited as evidence for an aetiologic role of the organism in LJP. The pathogenic potential of *A. actinomycetemcomitans* has been recognised for many years in diseases other than LJP as it has also been isolated from many non-oral sites. There has been evidence since 1964 that it is the sole aetiological agent in certain cases of subacute bacterial endocarditis. Other infectious diseases in which *A. actinomycetemcomitans* has been implicated include septicaemia, facial abscesses and pleural abscesses (Page and King, 1966). Although there was a wealth of information demonstrating the pathogenic capacities of this organism, almost nothing was known of the pathogenic mechanisms involved until dental researchers discovered its role in periodontal disease.

A. actinomycetemcomitans possesses considerable potential to destroy the periodontal tissues. These factors include a bacterial collagenase (Robertson *et al.*, 1982), a capsule (Wilson *et al.*, 1985) an epitheliotoxin (Birkedal-Hansen *et al.*, 1982), a fibroblast-inhibiting factor (Stevens *et al.*, 1983) a leukotoxin (Tervahartiala *et al.*, 1989), lipopolysaccharide (Kiley and Holt, 1980) and a bone-resorption inducing toxin (Iino and Hopps., 1984; Nowotny *et al.*, 1982).

1.6 Bacterial components which may initiate tissue pathology associated with periodontitis

Electron-microscopic studies by Listgarten and colleagues (1967;1975) of acute necrotising ulcerative gingivitis lesions revealed the presence of spirochaetes invading the underlying tissues. However, this appears to be one of the few forms of periodontal disease in which invasion of the underlying connective tissues actually takes place. The only other reports in humans of invasion by microorganisms into underlying tissues have been in terminal stages of advanced periodontitis, in which microorganisms were observed adjacent to the alveolar bone (Frank and Voegel, 1978). Later in 1982, Gillett and Johnson, observed microorganisms in the periodontium in a case of juvenile periodontitis, as well as in 1986, where organisms were found in the

oral epithelium (Saglie *et al.*, 1986). However, in spite of these findings, these cases of bacterial invasion are still relatively rare. In the late 70's and during the 80's it was recognised that the tissue destruction observed in periodontitis may be due to bacterial components rather than due to bacterial invasion. However, much of the work on the pathogenesis of periodontal disease has concentrated in particular on the part played by LPS/endotoxin (Daly *et al* ., 1980; Nair *et al.*, 1983; Sismey-Durant *et al.*, 1987; Bramanti, 1989). One of the reasons for this may have been due to the discovery by Elin and colleagues (1976) and Morrison and co-workers (1978; 1979) that LPS has the ability to activate the alternate pathway of complement, with the generation of biologically active fragments such as anaphylatoxins, chemotaxins, and opsonins. However, many researchers have recently realised that other bacterial components may also contribute to the periodontal destruction observed. In this context the outer membrane proteins have been studied (Bolstad *et al* ., 1990; Kennel *et al.*, 1990; 1991; 1992; Bramanti *et al.*, 1993) as well as the surface-associated material (SAM) which includes structures such as the capsule, fimbriae, pili and fibrils (Wilson *et al.*, 1985; 1988; 1993b; 1995; Kamin *et al* ., 1986; Okuda *et al* ., 1987; Hanazawa *et al.*, 1988; 1991; 1995; Borinski *et al* ., 1990; Meghji *et al.*, 1992b; 1992c; 1993; Evans *et al.*, 1992).

The mediators of tissue destruction observed in periodontitis has also become a very active area of research mainly from the late 80's to the present date. In this respect many researchers have studied the role of cytokines and inflammatory mediators in tissue and bone destruction (Raisz *et al.*, 1981; Nishihara *et al.*, 1989; Bom-Van Noorloos *et al.*, 1990; Ishimi *et al.*, 1990; Masada *et al.*, 1990; McFarlane *et al.*, 1990; Ishihara *et al.*, 1991; Takada *et al.*, 1991; Meghji *et al.*, 1992d; Takashiba *et al.*, 1992; Birkedal-Hansen., 1993b; Gemmell *et al.*, 1993; Hanazawa et al., 1993; Ishida., 1993; Payne *et al.*, 1993; Reinhardt *et al*, 1993; Takahashi *et al.*, 1991; 1994; Smalley 1994; Nishihara *et al.*, 1994; Manolagas and Jilka; 1995). Although there have been numerous reports which have studied the tissue pathology initiated by bacterial components in periodontitis, very few investigations have isolated and purified the component(s) responsible for the observed pathology (Progulske and Holt,

1984; Hammond *et al.*, 1987; Takahashi *et al.*, 1991; Takada *et al.*, 1991; Matsushita *et al.*, 1994).

With the evidence presented above, it becomes increasingly apparent that various bacterial components, particularly those associated with the surface of the organism may have a fundamental role in the pathogenesis of periodontal disease. Therefore, in this section the bacterial components are described. Figure 1.2 shows the basic structures of the cell wall of a Gram-negative bacteria.

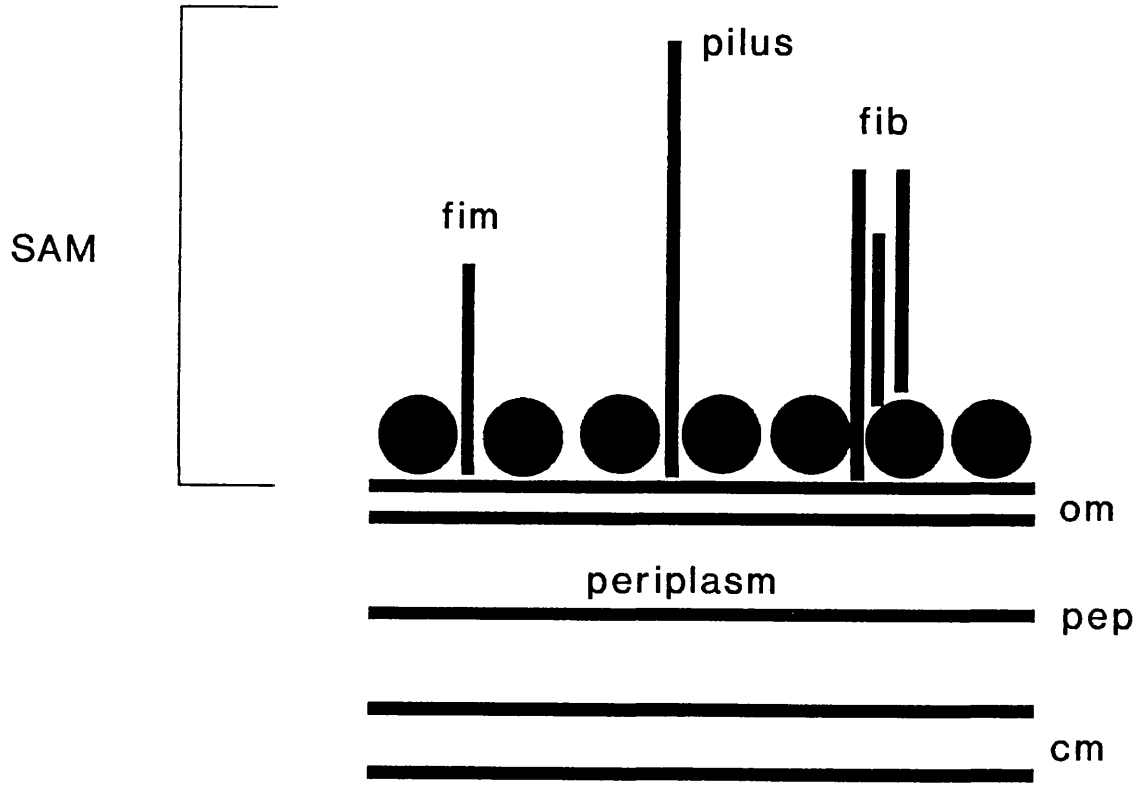


Figure 1.2 **Diagram showing the structure of the Gram-negative cell wall. (fim)-fimbriae, (fib)-fibrils; (cap)-capsules; (om)-outer membrane; (pep)-peptidoglycan; (cm)-cytoplasmic membrane.**

1.6.1 Surface associated material

In this thesis the term SAM will be used to describe those components of an organism removed by saline extraction at 4^0 C. SAM may contain, depending on the particular organism, capsule, fimbria, pili, fibrils, surface-associated proteins and components of the outer membrane and periplasm.

1.6.1.1 Capsules

Capsules and capsular antigens are considered to be outer envelope polymers which surround the bacterial cell in the form of a hydrophilic gel. The size of the capsules may vary with the physiological state of the cell. Bacterial strains which possess capsules are almost always more resistant to phagocytosis than unencapsulated strains of the same species..

1.6.1.2 Fimbriae and Pili

There are five types of fimbriae of which type 1 is the most common. Fimbriae are responsible for the adhesive properties of the organism, in particular the ability to agglutinate red blood cells. A single bacterium may contain between 100 and 1,000 fimbriae of varying length and width, distributed evenly on the cell surface. Preus and colleagues (1988) revealed the presence of surface appendages closely resembling fimbriae in clinical strains of *A. actinomycetemcomitans*. It was reported that in this organism the fimbria-like structures appeared in bundles and were anchored in the bacterial membrane and that initial adherence by this bacterium to the periodontal pocket epithelium may be mediated by fimbriae. Fimbriae have also been shown to possess other functions. Kawata and colleagues (1994) have shown that a 43 kDa fimbrial protein from *Por. gingivalis* stimulated embryonic mouse calvarial bone resorption. They observed by northern (RNA) blotting that the fimbriae-treated calvarial bone cells expressed genes for both interleukin-1 (IL-1) and granulocyte macrophage colony-stimulating factor (GM-CSF). Both of these cytokines are known to induce differentiation of osteoclast lineage cells. Therefore this group concluded that *Por. gingivalis* fimbriae-stimulated bone resorption may be regulated by a network of inflammatory cytokines produced by calvarial bone cells. In addition, Ogawa and colleagues (1991) have shown that fimbriae from *Por. gingivalis* induces interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) synthesis by human peripheral mononuclear cells. Murakami and co-workers (1994) have demonstrated that *Por. gingivalis* fimbriae specifically induce a 68 kDa phosphorylated protein in mouse peritoneal macrophages.

Pili are structurally similar to fimbriae but are generally longer and only present in small numbers on the surface. They exhibit a distinct axial hole and often bear a terminal knob. The proteins that make up the pili and fimbriae are biochemically and antigenically distinct. Pili play an essential role during bacterial conjugation. This process involves the transfer of genetic information from one bacterial cell to another. The sex pili make specific contact between the donor and recipient cells and then retracts. This in turn pulls the two cells together so that a conjugation bridge forms through which DNA passes from one cell to another. Of the periodontopathogens investigated in this thesis the BPA have been found to possess pili and/or fimbriae (Slots *et al.*, 1978; Okuda *et al.*, 1981; Yamamoto *et al.*, 1982). In a recent paper by Hood and Hirschberg (1995), the type IV pilin of *Eik. corrodens* has been purified and characterised.

Fibrils represent a new class of adesins whose function has not yet been elucidated (Handley and Tipler, 1986). Of the BPA investigated in this thesis, *Prev. intermedia* is the only microorganism which possesses fibrils.

1.6.2 Endotoxin

Endotoxin was described as early as the end of the 19th century by Pfeiffer, who reported that the toxicity encountered in response to Gram-negative bacteria was present within the bacterial cell and, hence it was termed endotoxin. Later studies, showed that endotoxins are localised on the surface of bacterial cells and together with phospholipids and proteins form part of the outer membrane of Gram-negative bacteria. Endotoxin is defined as "products of extraction procedures which result in macromolecular complexes of LPS, protein, and phospholipid" (Hitchcock *et al.*, 1986; Wilson, 1993a).

1.6.2.1 Lipopolysaccharide (LPS)

The term LPS denotes any molecular species that consists mainly of polysaccharide, but also possesses a significant proportion of lipid (Lipid-A). In contrast to endotoxin LPS is free of detectable contaminants such as proteins. LPS is composed of four covalently linked regions (figure 1.3), each with a distinctive composition and biological function. These four regions are the O-

specific chain, the core oligosaccharide (consisting of the inner and outer core) and the lipid A component.

1.6.2.2 O-specific side chain

The O-specific side chain is made up of repeating oligosaccharide units containing up to five sugar residues which are characteristic and unique for a given lipopolysaccharide. The O-specific polysaccharide mediates several biological properties of LPS of which a few are described. It carries the epitopes of LPS and the parental bacterial strain and functions as a receptor for bacteriophages (Lindberg *et al.*, 1983), and modulates activation of the alternative complement pathway (Grossman and Leive, 1984).

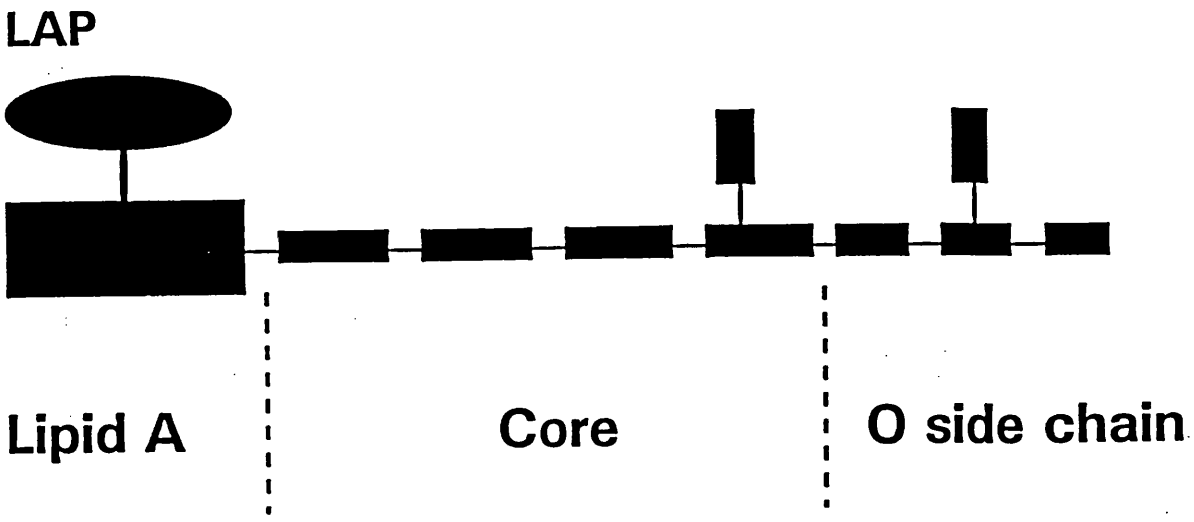


Figure 1.3 **Diagrammatic representation of endotoxin showing the LAP, Lipid A, the core and the O-specific side chain**

1.6.2.3 Outer core

The outer core, in general, contains the common hexoses, hence this region is often referred to as the hexose region. Very little is known about the activity or function of this region.

1.6.2.4 Inner core

Structurally, the inner core region of LPS is a conserved region. All LPSs, independent of their bacterial origin, possess at least one 2-keto-3-deoxyoctonoic acid (KDO) residue in this region. This KDO group occupies the lipid-A proximal position of the reducing terminus of the inner core via a free carboxyl group. The KDO-containing region is very important for the biological activities and functions of the LPS molecule. The structures within the inner core are thought to modulate the biological activity of lipid A (Haeffner-Cavaillon *et al.*, 1989).

1.6.2.5 Lipid A

Lipid A represents the covalently bound lipid component of LPS and is essential for its biological effects *in vitro*. In addition to causing the classical endotoxic effects such as fever, local Schwartzman reactivity and lethal toxicity, lipid A also carries common LPS epitopes (Galanos *et al.*, 1984a; 1984b), activates the classical complement cascade (Morrison and Kline, 1977) and binds to serum proteins such as lipoproteins (Flegel *et al.*, 1989). The degree of lipid A bioactivity may be modulated by the polysaccharide portion, particularly by the KDO-containing inner core. This is illustrated by the fact that IL-1 from adherent human mononuclear cells cannot be induced by polysaccharide-free lipid A.

Despite its remarkable spectrum of biological activities mediated through various cascade systems, the concept of bacterial LPS as an essentially protective impermeable layer is helpful in coming to terms with the possibility that it is not a primary virulence factor.

1.6.2.6 Lipid-A-associated proteins

Lipid A-associated proteins (LAPs), also known as endotoxin proteins (EP) or endotoxin associated proteins (EAP) are a complex of proteins intimately associated with the LPS of Gram-negative bacteria (Sultzer and Goodman, 1976; Porat *et al.*, 1992). The LAPs do not contain KDO groups which indicates that there is no core LPS present. The LAPs were first thought to be a superfluous carrier of LPS, however, they are now recognised to elicit a number of biological responses. The biological activities attributable to the LAPs include being potent B

cell mitogens and polyclonal activators of immunoglobulin synthesis (Goodman and Sultzer, 1979) and activators of murine macrophages to produce prostaglandins and the release of haematopoietic growth factors. Additionally, in a study by Mangan and co-workers (1992), it was found that LAP was consistently three to four times more active than LPS in promoting the synthesis and release of bioactive IL-1. This group has postulated that, as mononuclear phagocytes are a crucial cellular component of the host inflammatory and immune responses, the activation of monocytes by LAP may play a significant role in the pathogenesis associated with Gram-negative infections.

1.6.3 Peptidoglycan

The peptidoglycan present in the cell walls of eubacteria provides mechanical strength to the wall. The peptidoglycan is composed of two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid as well as a small group of amino acids. The basic structure of the peptidoglycan is a thin sheet in which the glycan chains formed by the sugars are connected by peptide cross-links formed by the amino acids. The shape of a cell is determined by the lengths of the peptidoglycan chains and by the manner and extent of cross-linking of the chains (Brock and Madigan, 1991).

1.6.4 Lipoprotein

A lipoprotein complex is found on the inner side of the outer membrane of a number of Gram-negative bacteria. This lipoprotein is a small (7200 daltons) molecule which serves as an anchor between the outer membrane and peptidoglycan. It possesses a hydrophobic-rich end which is thought to be associated with the outer membrane phospholipids. In addition to the ability to bind eukaryotic cells, the lipoprotein complex also possesses very potent mitogenic activity. The other biological activity attributable to lipoprotein was demonstrated by Millar and colleagues (1986) who showed that this component from *Escherichia coli* possessed the ability to stimulate bone resorption.

1.7 Pathology of periodontitis

In the early stages of the inflammatory process, most of the cells migrating to the injured area are neutrophils. Generally, the early peak in the migration of neutrophils to an inflammatory focus is followed some hours later by another wave of cell migration, this time by mononuclear phagocytes (macrophages). An intermediate form of macrophage known as the monocyte, circulates in the blood and when it migrates into tissues it either matures or differentiates into the tissue macrophage.

Bacterial plaque appears to initiate inflammatory events which lead to the accumulation of polymorphonuclear leukocytes in the gingival sulcus. The persistence of inflammation which ultimately leads to the loss of bone in periodontal disease is probably, in part, the result of activation of both the humoral and cellular immune systems in response to metabolic products, SAM, endotoxins and other bacterial products (Lavelle, 1992). The infiltration of the diseased periodontal tissue with the immunologically competent macrophages and lymphocytes also provides a potential source of inflammatory mediators such as cytokines and other effector molecules (eg. prostaglandins) which influence the loss of bone. The activated macrophage releases arachidonic acid from the cells suggesting that a membrane phospholipase has been activated. The arachidonic acid is further metabolised by the cycloxygenase and lipoxygenase pathways into prostaglandins and leukotrienes. The protein kinases are then activated and stimulation of bone resorption occurs (figure 1.4).

The osteoblast is the cell which contains the receptors for the major bone resorbing agents, such as PTH, the eicosanoids, 1,25- dihydroxy vitamin D_3 and cytokines (IL-1 and TNF). The osteoblasts, having recognised the resorptive signal, somehow transmit it to the osteoclast. Osteoclastic bone resorption does not appear to directly involve matrix metalloproteinases (MMP), but evidence suggests that bone resorption is initiated by removal of the osteoid layer by osteoblasts by means of a collagenase-dependent process. However, in a recent study by Okada and co-workers (1995), it was suggested that MMP-9 is produced by osteoclasts in the human bone tissues and that it can degrade bone collagen in concert with MMP-1.

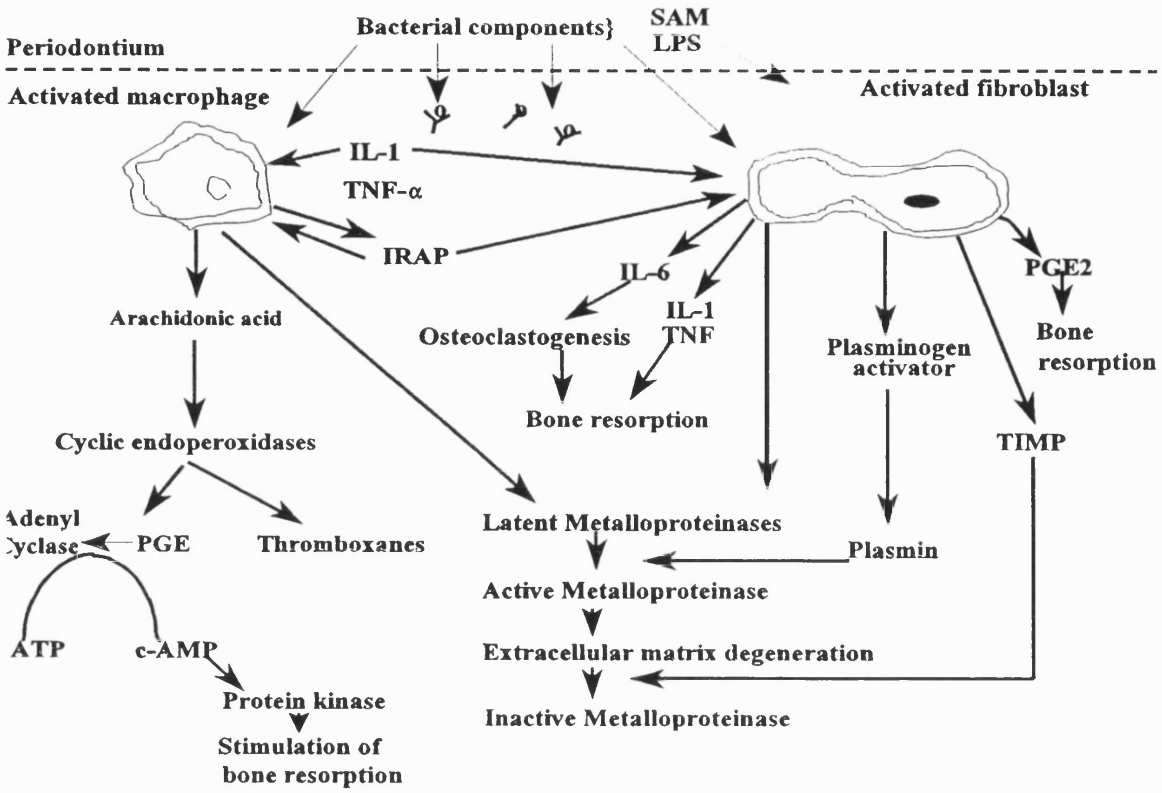


Figure 1.4 **Diagram showing the infiltration of bacterial components through the periodontium subsequently activating macrophages and fibroblasts to release inflammatory mediators ultimately leading to the stimulation of bone resorption.**

1.7.1 Cytokines

Cytokines function as intercellular signals that regulate local and, at times, systemic inflammatory responses. Cytokines differ from endocrine hormones in that they are produced by a range of cells rather than by specialised gland cells. In general, cytokines act in a paracrine (ie. near to the producing cells) or autocrine (ie. directly on the producing cells) manner rather than in an endocrine manner on distant target cells. Generally, cytokines are peptides or glycoproteins with molecular weights ranging from 6 kDa to 60 kDa. More than 100 cytokines have been identified (Henderson and Poole, 1994), however, for the purpose of this thesis the focus will be mainly on the pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNF-α.

1.7.2 Interleukin-1

During an immune response macrophages or monocytes are probably the major source of IL-1 (Durum *et al.*, 1990). Generally macrophages do not synthesise IL-1 constitutively, but require a stimulus for IL-1 gene transcription. Many factors can stimulate IL-1 including complement products generated in the course of humoral immunity. Historically, three biological activities were initially related to the inflammation-associated breakdown of connective tissues: mononuclear cell factor (Dayer *et al.*, 1977), catabolin (Saklatvala, 1981) and osteoclast-activating factor (Horton *et al.*, 1972). Subsequently these three activities were shown to be mediated by IL-1. At least two distinct IL-1 proteins (α and β) exist (Lomedico *et al.*, 1986). Both forms are produced by the cell as 31 kDa precursors that undergo enzymatic processing by pro-IL-1 converting enzyme (ICE) to the mature 17.5 kDa forms (Mitzutani *et al.*, 1991; 1994).

The biological effects of IL-1 include, among others, lymphocyte (T- and B-cell) activation, macrophage activation, natural killer cell stimulation, prostaglandin formation, fever induction, anorexia, acute phase protein release (hepatocyte activation), adrenocorticotropin release, corticosteroid release, cytokine gene expression (IL-1, TNF, IL-6), plasminogen activator and activator inhibitor gene expression, endothelial cell activation, tumour cell growth inhibition, and suppression of lipoprotein lipase gene expression (Durum *et al.*, 1990; Cavaillon *et al.*, 1990; Dinarello *et al* ., 1991;1994).

In 1983, Gowen and co-workers were the first to describe the bone resorbing activity of highly purified IL-1. Besides stimulating resorption through increased osteoclastic activity, IL-1 was shown to inhibit the synthesis of collagen and osteonectin in calvaria (Canalis *et al.*, 1986). Bone resorbing agents act in a complex way. They cause precursor cells to fuse to form osteoclasts, which they then may also activate. IL-1, like other bone resorbing agents, induces the formation of multinucleate osteoclast-like cells in cultures of bone marrow (Takahashi *et al.*, 1986; Pfeilshifter *et al.*, 1989; Kurihara *et al.*, 1990). The IL-1-stimulated osteoblasts are thought to produce a soluble factor which activates the osteoclasts. The osteoclast then degrades the bone matrix under its ruffled border by generating a low pH and releasing lysosomal proteinases. As

mentioned previously, loss of bone may be the result of stimulation of resorption or inhibition of formation. It is the balance between these two processes that maintains the tissue during normal bone turnover (Heersche *et al.*, 1989). In addition, IL-1 has been shown to inhibit bone nodule formation (Stashenko *et al.*, 1987; 1989) as well as mediating the IL-6-induced formation of osteoclasts (Kurihara *et al.*, 1990).

1.7.3 The role of Interleukin-1 in periodontal tissue destruction

Charon and co-workers (1982) were the first to demonstrate increased levels of IL-1 in gingival crevicular fluid (GCF) from the inflamed sites of patients with gingivitis. Kabashima and colleagues (1990), partially characterised the IL-1-like activity from periodontitis patients and found it to be predominantly 'IL-1α like'. However, in 1990, Masada and co-workers (1990) reported the presence of elevated levels of IL-1α and IL-1β in the GCF from both early onset and adult periodontitis patients. Previously, Honig and co-workers (1989) and more recently Hou and colleagues (1995) have detected IL-1β in all tissue extracts from adult periodontitis patients, whereas they did not detect any IL-1 in healthy gingival biopsies. Stashenko and colleagues (1991a; 1991b) reported that IL-1β is the predominant form present in periodontal tissues and that disease-active sites have higher IL-1β levels than inactive and healthy sites. This group further reported that IL-1β levels were much higher than TNF levels in diseased tissues.

The evidence implicating IL-1 in periodontal disease had prompted investigators to examine whether peripheral blood mononuclear cells from periodontitis patients produced more IL-1 than cells from healthy patients. The available results are conflicting (Garrison *et al.*, 1989; McFarlane *et al.*, 1990). This has been explained by the differences in methodology used and also in the difficulties associated with the assessment of *in vitro* IL-1 production by peripheral blood cells (Dinarello *et al.*, 1991). Based on the evidence for the cellular source of IL-1 in diseased gingiva, the capacity of peripheral monocytes to produce IL-1 may be an important factor.

Recently there have been a number of observations that IL-1 has profound *in vitro* effects on cells from the gingiva (Mochan *et al.*, 1988; Richards and Rutherford.,

1990; Tipton *et al.*, 1990) and the PDL (Richards *et al.*, 1990; Saito *et al.*, 1990a; 1990b; 1990c). Interleukin-1 stimulates antigen expression on gingival Langerhans cells, production of plasminogen activator (Mochan *et al.*, 1988), metalloproteinases (Heath *et al.*, 1982) and IL-6 production (Bartold *et al.*, 1991) by gingival fibroblasts.

A number of studies have specifically addressed the involvement of IL-1 as a mediator of periodontopathogenic bacteria-stimulated bone resorption (Iino and Hopps., 1984; Nishihara *et al.*, 1989; Bom van Noorloos *et al.*, 1990; Ishihara *et al.*, 1991; Nishihara *et al.*, 1994). However, the majority of these studies have used LPS as the bacterial component responsible for IL-1 synthesis. In this thesis the bacterial components SAM, LAP and LPS were studied for their ability to stimulate cytokine release by human gingival fibroblasts and peripheral blood mononuclear cells.

1.7.4 Interleukin-6

IL-6 is a major mediator of the host response to tissue injury and infection (Kishimoto, 1989). This cytokine plays a major role in B-cell differentiation in the immune system. It is accepted that this cytokine has multiple biological activities, such as the enhancement of T cell proliferation (Lotz *et al.*, 1988) the acceleration of bone resorption (Ishimi *et al.*, 1990) and the inhibition of *in vitro* bone formation (Hughes and Howells, 1993a). It is, therefore, generally recognised that IL-6 is involved in the pathogenesis of various inflammatory diseases. However, the exact contribution of cytokines, including IL-6 to the progression of periodontal disease is unknown. The pathogenesis of periodontitis, therefore, should be considered in relation to the cytokine network, including IL-6 expression.

IL-6 is a glycoprotein with a molecular mass in the range of 20 to 30 kDa, depending on the cellular source. IL-6 is produced by a wide variety of cells including T-cells, B-cells, monocytes and fibroblasts. cDNA's encoding human IL-6 have been cloned and it has been found that, with the exception of the short segment (amino acids 1-28) at the N terminus, the entire primary sequence of human IL-6 is required for biological activity (Brakenhoff *et al.*, 1989).

IL-6 is known to be an important regulator of bone remodelling, produced locally in bone by osteoblasts under the direction of parathyroid hormone and cytokines such as IL-1 and TNF (Lowik *et al.*, 1989; Ishimi *et al.*, 1990). Osteoclasts are also thought to produce IL-6. As previously mentioned, IL-6 stimulates early osteoclast precursor formation (Kurihara *et al.*, 1990) and stimulates the recruitment as well as the formation of osteoclasts. Black and colleagues in 1991 demonstrated that IL-6 stimulates bone resorption *in vitro*. Evidence for the direct role of IL-6 in osteoclastogenesis *in vivo* also has been demonstrated (Girasole *et al.*, 1992) and a number of investigators have recently started to acknowledge the importance of IL-6 in periodontal disease (Ishimi *et al.*, 1990; Gemmel and Seymour, 1993; and Reinhardt *et al.*, 1993).

1.7.5 Tumour necrosis factor-α

TNF is a 17 kDa protein (determined by SDS-PAGE) which exists in two forms, α and β. However, for the purpose of this thesis only TNF-α has been investigated. This cytokine is produced in response to stimuli by a wide variety of cells including macrophages, fibroblasts, natural killer cells, astrocytes and mast cells. TNF-α has been shown to have a number of *in vivo* biological effects. It has a pro-inflammatory role and, as its name suggests it has direct *in vitro* growth inhibitory effects on tumour cells. Apart from these effects, because of its ability to stimulate new blood vessel formation and its proliferative effects on fibroblasts, TNF-α is considered to play a part in wound repair similar to that of other macrophage-derived growth factors, such as fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β). To date there are very few studies which have investigated the involvement of TNF-α in periodontal diseases.

1.7.6 Interleukin-8

Interleukin-8 was first discovered by Baggiolini and colleagues (1989), who described it as a 72 amino acid (8 kDa) polypeptide produced by *E. coli* LPS-stimulated human peripheral blood monocytes (PMN). IL-8 is also produced by a number of other cells including fibroblasts, endothelial cells, epithelial cells, chondrocytes, synovial cells and various tumour cells. This cytokine is different from others in that it has a distinct target, the neutrophil, which it attracts and

activates in inflammatory sites (Bickel *et al.*,1993). As previously described, the recruitment of neutrophils to a damaged site is an important step in the inflammatory process since this leads to the release of antimicrobial and inflammatory products by exocytosis of storage granule proteins and the respiratory burst.

Although a number of studies as early as 1970 investigated the importance of neutrophil infiltration during periodontal disease (Attstrom 1970; Attstrom and Egelberg 1970; Ashkenazi *et al.*, 1992), it was only recently shown that IL-8 is an important cytokine in periodontitis (Takashiba *et al.*, 1992; Tamura *et al.*, 1992; Hanazawa *et al.*, 1993; Payne *et al.*, 1993; Liebler *et al.*, 1994). The majority of these studies have investigated the release of IL-8 in response to LPS.

1.8 Aims of this thesis

A major difference between the majority of human bacterial infections and periodontitis is that in the former the acute infectious agent is normally eliminated from the host at the time of convalescence. In contrast, the pathogens associated with periodontitis may be acquired early in life, and there is scant evidence that treatment can permanently eliminate these bacteria from the oral cavity.

For periodontal treatment to be effective, specific periodontal pathogens and their effects on the host need to be either controlled or eliminated. However, to achieve this goal it is important to know more about the nature of periodontal infections, the characteristics of the pathogenic microorganisms and their components, as well as the host's response to these organisms. Only with this information can therapy for the specific periodontal infections be devised. The aims of this thesis were therefore to investigate the pathological effects of bacterial components from periodontopathogenic bacteria. In the past related research has been concerned primarily with the effects of LPS on the periodontium. However, in this investigation, attention has been focused on other surface components of five periodontal pathogens. The periodontopathogens chosen for this investigation were *A. actinomycetemcomitans*, *Eik. corrodens*, *Por. gingivalis*, *Prev. intermedia* and *C. rectus*. As the resorption of alveolar bone is one of the characteristics of

periodontal disease, the osteolytic potential of the SAM, LAP and LPS from these periodontopathogens was determined. Following this, the ability of these components to stimulate cytokine release by a number of human cells was also determined. Additionally, isolation of the osteolytic protein and the IL-6 stimulating protein from *A. actinomycetmcomitans* was attempted.

Chapter 2

General Methods

2.1 Bacterial cultivation

Eik. corrodens NCTC 10596, *Por. gingivalis* W50, *Prev. intermedia* NCTC 9336 and *C. rectus* NCTC 11489 were all cultured on Wilkins Chalgren agar (Oxoid, Hampshire, UK), supplemented with 5 % (v/v) horse blood (Oxoid, Hampshire, UK) and incubated at 37^{o} C for 72 h under anaerobic conditions. *A. actinomycetemcomitans* NCTC 9710 was cultured on brain heart infusion agar (Oxoid, Hampshire, UK) supplemented with 5 % (v/v) horse blood and incubated in a carbon-dioxide rich atmosphere for 48 h. Cultures were checked visually and by Gram-staining, for contamination with other bacteria. Pure cultures were harvested by gentle scraping with a glass rod and the cells washed with 0.85 % (v/v) saline prior to freeze drying and extraction of the SAM.

2.2 Extraction of SAM

The procedure used was that described by Wilson and colleagues (1985). The lyophilised bacteria were suspended in 0.85% (w/v) saline and the SAM was removed by gentle stirring at 4^{o} C for 1 h. The supernatant was collected after centrifugation of the suspension at 3000 g for 1 h and an equal volume of acetone at -20^{0} C was added. The extraction process was repeated twice more and the SAM was pooled, exhaustively dialysed (using a 2 kDa cut-off dialysis membrane (Sigma, Dorset, Poole, UK) against distilled water for 48 h at 4^{o} C and then lyophilised.

The SAM from *A. actinomycetemcomitans* was also extracted by omitting the addition of acetone at -20^{o} C. The supernatant was collected after the first centrifugation step and the extraction process repeated twice more. The SAM was pooled and exhaustively dialysed against distilled water for 48 h at 4^{o} C using a 2 kDa cut-off dialysis membrane and then lyophilised and weighed.

2.3 Electron microscopy

Electron microscopy was performed by Ms. Pauline Barber and Ms. Nicky Morden of the Electron Microscopy Unit at the Eastman Dental Institute.

Cells were stained and examined by transmission electron microscopy (tem) prior to and after saline extraction. A portion of each specimen, before and after saline extraction, was fixed in 3 % (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at room temperature; these served as controls. The remaining portion of each specimen was fixed in the same manner except that the buffer also contained 0.15 % (w/v) ruthenium red. All specimens were then centrifuged at low speed, the fixative decanted, and the pellets washed in 0.1 M cacodylate buffer. The control cells were then fixed in 1 % (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at room temperature in the dark. The test cells were treated likewise except that the buffer also contained 0.15 % (w/v) ruthenium red. The fixative was decanted after centrifugation and the pellets washed in 0.1 M cacodylate buffer. All specimens were then dehydrated in a graded series of concentrations of ethanol and embedded in Araldite. Sections were cut on an LKB ultramicrotone using a diamond knife. They were then examined (unstained) in a JEOL 100CXII electron microscope.

2.4 Extraction of LAP and LPS

Endotoxin was obtained from the SAM-extracted bacteria by the butanol extraction method of Morrison and Leive (1975). Cells were suspended in 0.15 M NaCl at 4^{o} C, an equal volume of n-butanol (BDH) added and the suspension mixed thoroughly for 10 min and centrifuged for 20 min at 35,000 g on a Sorvall centrifuge. The aqueous phase was removed and the butanol, together with the insoluble residue, was re-extracted twice with half the initial volume of saline. The combined aqueous phases were centrifuged at 35,000 g for 20 min to remove particulate matter and dialysed against distilled water for 48 h. This crude preparation, which contains endotoxin (ie. LAP and LPS), was ultracentrifuged at 100,000 g for 1 h at 4^{o} C and lyophilised. This material was then subjected to hot phenol/ water extraction (Westphal and Jann, 1965). The aqueous phase was digested by addition of RNase and DNase (Sigma:20 mg/ml in 0.05M Tris buffer

pH 7.3) overnight at 37° C and then with 1 mg/ml pronase (Sigma) for 6 h. The suspension was then ultracentrifuged at 100,000 g for 1 h and the LPS, which appeared as a clear gel at the base of the centrifuge tube, was removed and lyophilised. The phenol layer which contained the LAP was removed, dialysed against distilled water and lyophilised.

2.5 Determination of the composition of SAM and LAP

The composition of the SAMs and LAPs were determined by assaying the protein, carbohydrate, lipid and endotoxin content of each of these preparations. The heterogenity of the protein composition was determined by SDS-PAGE and High Performance Liquid Chromatography (HPLC).

2.5.1 Determination of the protein content of SAM and LAP

The protein content of each of the SAM and LAP preparations was determined by three techniques: (i) the enhanced alkaline copper (Lowry) protein assay; (ii) the Bio-Rad Detergent Compatible (DC) protein assay; and (iii) the Bradford protein assay.

2.5.1.1 Enhanced alkaline copper (Lowry) protein assay

This assay primarily detects the tyrosine and tryptophan content of proteins. The assay is based on the fact that peptide bonds form a complex with Cu^{2+} under alkaline conditions and become reduced to Cu^{+}. Upon addition of the Folin reagent, Cu^{+} and the R groups of the tyrosine and tryptophan residues react to produce an unstable product, which is slowly reduced to become molybdenum/tungsten blue. This colour change is then monitored by measuring the absorbance at 280 nm. Agents which acidify the solution, chelate the copper, or cause the reduction of copper are known to interfere with this assay.

The assay protocol was as described by Lowry and colleagues (1951). The standard used was bovine serum albumin (BSA) diluted at concentrations from 2 to 100 μg/ml. To a 400 μl sample of standard or test material, 400 μl of Lowry concentrate (2x) was added and incubated at room temperature for a minimum of 10 min. To this, 200 μl of the 0.2 N Folin reagent was added and vortexed

immediately. This rapid mixing was an important step since the reagent decomposes rapidly. After an additional 30 min incubation at room temperature the absorbance was measured at 750 nm.

2.5.1.2 Bio-Rad DC protein assay

The principle of this assay is similar to the Lowry assay but has been slightly modified to save time. This procedure only requires 15 minutes incubation time and the reaction colour complex is stable for 2 h. The advantage of using this assay is that it is compatible with many detergents which may be present in the sample. The standard Lowry assay, which is performed in cuvettes requires 100 µl of sample whereas the microassay system requires only 20 µl of sample, another advantage of using this assay .

BSA standards were prepared at concentrations ranging from 2 to 1400 µg/ml. This assay was obtained from Bio-Rad in the form of a kit. In microtitre plates, 20 µl of standard or sample were added, and 10 µl of reagent A and 80 µl of reagent B added. The plates were incubated at room temperature for 15 min and the absorbance at 650 nm measured on a Titertek plate reader.

2.5.1.3 Bradford protein assay

The absorbance maximum of the dye (Coomassie blue) in an acidic solution shifts from 465 to 595 nm after adding protein. This is due to stabilisation of the anionic form of the dye by both hydrophobic and ionic interactions. The dye principally reacts with arginine residues and to a lesser extent with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues.

BSA was used as standard at a concentration range of 2 to 1400 µg/ml. The assay protocol was as described by Bradford (1976). 20 µl of sample or standard was dispensed into polystyrene cuvettes. To this, 50 µl of 1 M NaOH was added and 1 ml of the dye reagent added. After incubating for 5 min the absorbance at 590 nm was then measured.

2.5.2 Sodium-Dodecyl-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This is a discontinuous system consisting of two gels, one resolving and one stacking. The two gels are cast with different porosities, pH and ionic strength. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate the large volume of sample in the stacking gel, resulting in improved resolution. Proteins, once concentrated in the stacking gel, are separated in the resolving gel. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. Molecular sieving causes the SDS complexes to separate on the basis of their molecular weights. The molecular weights of the proteins were determined by performing Ferguson plots.

The number of proteins present in the SAM and LAP preparations were determined by SDS-PAGE by the method of Laemmli (1970). 12 % (v/v) gels were used and proteins separated in the resulting gel were stained with Coomassie Colloidal blue (Sigma) or silver stain (Sigma). To obtain adequate separation of the proteins in the LAP extracts it was necessary to increase the SDS concentration in the sample buffer to 16 % (w/v).

2.5.3 Determination of the protein profiles of the SAM by Ion Exchange High Performance Liquid Chromatography (HPLC).

Ion exchange HPLC is designed specifically for the separation of ionic or ionizable compounds. Ion exchange HPLC has both stationary (column packing) and mobile phases. It differs from other types of liquid chromatography in that the stationary phase carries ionizable functional groups, fixed by chemical bonding to the stationary phase. In anion exchange HPLC the fixed charges are positive and in cation exchange chromatography the fixed charges are negative. Elution in ion exchange HPLC is generally accomplished at a constant pH with increasing mobile-phase ionic strength to displace the protein from the stationary phase. NaCl is the most widely used displacing salt.

2.5.3.1 Anion exchange HPLC

An anion exchange MA7Q (Bio-Rad) HPLC column (5 cm x 0.78 cm) was equilibrated with 20 mM Tris buffer pH 8.5 (buffer A). Aliquots (500 µl) of 1 mg/ml SAM in buffer A were injected onto the column and eluted with 8 ml of buffer A, followed by a linear gradient from 0-50 % buffer B (buffer A + 2 M NaCl) in 30 ml, 50-100 % buffer B in 15 ml volume and finally 6 ml of buffer B. The flow rate was 1 ml/min and 1 ml fractions were collected in Eppendorf tubes with the protein absorbance being monitored at 280 nm. Fractions were dialysed against distilled water for 48 h, lyophilised and the biological activity of each fraction determined.

2.5.3.2 Cation exchange HPLC

Cation exchange chromatography used an identical protocol to anion exchange chromatography. The cation exchange column used was a Bio-Rad MA7S column (5 cm x 0.78 cm) and the buffers used were 20 mM Bis-Tris Buffer (buffer A) and Buffer A plus 2M NaCl (buffer B).

2.5.4 Size exclusion (SEC) HPLC

SEC is based on the separation of protein molecules according to their relative size. SEC is performed using porous beads as the chromatographic support. The column has two liquid volumes, the external consisting of the liquid between the beads, and the internal volume, consisting of the liquid within the pores of the beads. Large molecules will equilibrate only with the external volume while small molecules will equilibrate with both the external and internal volumes. A mixture of proteins is applied at the top of a gel filtration column and allowed to go through. The large protein molecules are excluded from the internal volume and therefore emerge first from the column while the smaller protein molecules, which can access the internal volume, emerge later.

A TSK-250 (Bio-Rad, 30 cm x 0.75 cm) column was equilibrated with 50 mM sodium phosphate buffer, pH 6.7. SAM (1 mg/ml) was dissolved in this buffer and injected onto the column. 1 ml fractions were collected in Eppendorfs with the absorbance being monitored at 280 nm. To determine the molecular weights of the

eluted proteins it was necessary to run a set of standards ranging from 1.35 kDa to 669 kDa. A protein pak 125 SEC column was also used in further studies.

2.5.5 Determination of the carbohydrate content

The carbohydrate content was determined by the method of DuBois and colleagues (1956) using glucose as a standard. The principle of this assay is that simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl ethers with free or potentially free reducing groups, give an orange-yellow colour when treated with sulphuric acid. This colour is monitored spectrophotometrically at a wavelength of 490 nm for hexoses and 480 nm for pentoses and uronic acids.

In glass tubes (Pyrex) 1 ml of standard (ranging from 20 to 100 μg/ml) or sample and 1 ml of 5 % (w/v) phenol was added. To each tube, 5 ml of concentrated sulphuric acid was then added and the tubes left to stand for 10 min. Blank tubes were prepared by substituting distilled water in place of the standard. These steps were carried out in the fume cupboard as both phenol and sulphuric acid are corrosive. After the incubation period the tubes were shaken and placed for 10 to 20 min in a water bath at 25° to 30° C. A characteristic yellow/orange colour developed if there were any sugar derivatives present. 1 ml aliquots from each tube were dispensed into glass microcuvettes and the absorbance measured at a wavelength of 490 nm for hexoses and 480 nm for pentoses and uronic acids using a Cecil spectrophotometer.

2.5.6 Extraction of the Lipid from the SAM and LAP

SAM or LAP (10 mg) was dissolved in 5 ml of HPLC grade water and 5 ml of chloroform:methanol (2:1) added to this solution. After vortex mixing for 10 min the mixture was centrifuged at 3000 g for 10 min to separate the two layers. The chloroform layer (lower) was then carefully removed into a pre-weighed glass bijoux. The sample was placed in the fume cupboard with the lid open to remove the chloroform:methanol and then weighed to measure the amount of lipid present. The sample was then re-dissolved in ethanol:acetonitrile (1:1) and stored at -70° C.

2.5.7 Determination of the endotoxin content

The endotoxin content of each SAM, LAP and LPS preparation was determined by the LAL chromogenic assay using *E. coli* LPS (international standard, preparation 84/650, NIBSC) as standard. The endotoxin assay used was a kinetic assay based on the release of the chromophore, para-nitro aniline (PNA) from the substrate. The substrate consists of a short chain peptide linked to PNA offering a cleavage site which mimics that of the natural protein, the coagulogen. The colour produced was measured spectrophotometrically at an absorbance of 405 nm. The colour development is repeatedly measured until the assay is terminated by the lowest standard used in the assay reaching a fixed increase in optical density.

2.5.8 Nucleic acid determination

The percentage nucleic acid present in the SAM and LAP preparations was determined by measuring the absorbances at 260 nm and 280 nm and the ratios calculated.

2.6 Cell culture

The cells used throughout this thesis were human gingival fibroblasts (HGF), human peripheral blood mononuclear cells (PBMCs), a human myelomonocytic cell line (Mono-Mac-6 cells) and human neutrophils.

2.6.1 Human gingival fibroblasts

HGF were cultured from explants obtained during minor oral surgical procedures. Cells were maintained in Dulbecco's modified Eagles medium (DMEM) plus 3.70 g/l sodium bicarbonate, (Sigma) supplemented with 10 % (v/v) foetal calf serum (FCS; ICN Flow, Oxfordshire, UK), 100 U/ml penicillin/streptomycin (Gibco Ltd), and 2 mM glutamine (Sigma). The cells were subcultured at twice-weekly intervals with 0.25 % (w/v) trypsin (Sigma), stained with trypan blue for cell viability and used for experiments between passages 6 and 12. To determine the cytokine-stimulatory effect of bacterial constituents, cells were cultured into 24-well plates (ICN, Flow) at a concentration of 30,000 cells/500 µl/well (supplemented with 2 % (v/v) FCS) and incubated overnight at 37° C in 5 % CO_2 95 % air. Plates were washed with Hanks balanced salt solution (HBSS, Sigma) and further incubated

with serially diluted stimulants (500 μl) of either the bacterial component or *E. coli* LPS in groups of three wells per concentration, at 37° C for 24 h in 5 % CO_2 95 % air. Control cultures received only fresh medium. At the end of the culture period, cytokines released into the medium were assayed by enzyme linked immunosorbent assay (ELISA).

2.6.2 Peripheral Blood Mononuclear Cells (PBMC)

These were prepared by Ficoll density gradient centrifugation as described by Bristow and colleagues (1991). 20 ml of blood was obtained from periodontally-healthy volunteers, transferred into heparinised sterile Universals (Sterilin) to which an equal volume of RPMI 1640 medium (Sigma) at 37° C was immediately added. To 15 ml of Ficoll-Histopaque 1077 (Sigma), 35 ml of the blood/RPMI suspension was carefully layered on and centrifuged at 400 g for 30 min at room temperature. The mononuclear cell layer was collected into Falcon tubes, washed with RPMI 1640 medium and centrifuged at 500 g for 15 min. The wash step was repeated and the pellet resuspended in RPMI 1640 medium containing 2 % (v/v) FCS. Cells were stained for viability using 0.4 % (w/v) trypan blue (Sigma). The PMNs were counted and cultured at $2x10^6$ cells/500 μl/well in 24 well plates. Plates were incubated for 2 h, washed with HBSS (Sigma). To calculate the percentage PMNs, cells were stained with a three step haematology stain (Midatlantic Biomedical incorporation) and then incubated with serially-diluted stimulants of either SAM or *E. coli* LPS (international standard, preparation 84/650) at 37° C in 5 % CO_2, 95 % air. Control wells received only medium alone. At the end of the culture period cytokines released into the medium were measured by an ELISA.

2.6.3 Mono-Mac-6 cells

The myelomonocytic cell line Mono-Mac-6 was a kind gift from Dr Ziegler-Heitbrock, (Munich, Germany). Cells were maintained in RPMI 1640 medium containing 2 mM L-glutamine, 5 % (v/v) heat inactivated FCS, insulin (9 μg/ml), oxaloacetic acid (1 mM), sodium pyruvate (1 mM) and non-essential amino acids (0.1 mM, Sigma). Cells were seeded at $2x10^5$ cells/ml and fed twice weekly. To determine the response of these cells to bacterial constituents, cells were centrifuged (400 g for 10 min) in 50 ml sterile Falcon tubes and resuspended in

media with 2 % (v/v) FCS. Viable cells were dispensed into 24 well tissue culture plates (Falcon, 3047) at $2x10^6$ cells/500 µl/well and stimulated with graded concentrations of either SAM or *E. coli* LPS (84/650) for 16 h at 37° C in 5 % CO_2 95 % air. At the end of the culture period cytokines released into the medium were assayed by ELISA.

2.6.4 Human neutrophils

Human neutrophils were extracted from the blood of periodontally-healthy volunteers by discontinuous Percoll gradient centrifugation. 50 ml of blood was collected and placed into heparinised containers to prevent clotting. To 45 ml of blood, 5 ml of 100 % (v/v) acid citrate dextrose (ACD) was added. The blood/ACD mixture was then divided equally into two 50 ml Falcon tubes. To these, 5 ml of 1:10 ACD and 20 ml of Hespan were added. The tubes were mixed carefully avoiding the formation of air bubbles as these could stimulate the neutrophils to release IL-8. Any bubbles formed were gently removed with a sterile Pasteur pipette. The tubes were left to stand at room temperature for 45-60 min. After this period the top layer of blood was collected into 3 x 20 ml Sterilin tubes and any air bubbles removed. The tubes were centrifuged for 7 min at 312 g and the supernatant discarded. The pellets were then resuspended in 55 % (v/v) Percoll to give a final volume of 5-7 ml in one tube and then layered onto Percoll gradients ranging from 55 % to 100 % Percoll (v/v). Using 10 ml Sterilin gradient tubes, 3 ml of 70 % (v/v) Percoll was layered on top of 5 ml of 81 % (v/v) Percoll, two gradient tubes were used per 50 ml of blood. Three layers formed of which the top layer contained the monocytes, the second layer, neutrophils and the last layer red blood cells. The top layer was discarded and the neutrophils collected (approximately 4 ml). The neutrophils were stained with Kimera stain for viability, counted and washed three times (7 min at 367 g) with culture medium. The cells were plated at 5 x 10^4 neutrophils/ well and allowed to adhere for 24 h at 37^0 C, 5 % CO_2/ 95 % air. After this period the stimulants were added and the cells incubated overnight. The following day the media from the stimulated cells were collected and stored in Eppendorf tubes at -70^0 C until the radioimmunoassay (RIA) was performed. To detect intracellular IL-8, the cells were lysed by addition of 500 µl of 1.1 % (v/v) Triton in saline and then centrifuged for 10 min at 367 g.

These lysed cells were also stored in Eppendorf tubes at -70^0 C until the RIA was performed.

2.7 Cytokine Assays

The media from the stimulated cells were assayed for the presence of IL-6, IL-1β, TNF-α and IL-8. Cytokines were assayed using an ELISA apart from IL-8 release from neutrophils for which a RIA was employed.

2.7.1 ELISA for IL-6

Microtitre plates (Immulon 4, Dynatech) were coated with immuno-affinity purified goat polyclonal anti-IL-6 antibodies (Taktak *et al.*, 1991) at 1 μg/ml, diluted in phosphate buffered saline (pH 7.2). Plates were incubated at 4° C overnight. The wells were then decanted and washed with 0.01 M phosphate/0.05 M NaCl buffer (wash/dilution buffer, pH 7.2) containing 0.1 % (v/v) Tween 20. Standards of IL-6 (100 μl human recombinant IL-6 standard, preparation 88/514: NIBSC) were added to wells over the concentration range of 0-3ng/ml and the supernatants to be tested were added to the remaining wells. Plates were incubated for 2 h at room temperature and washed three times with wash/dilution buffer. Biotinylated affinity-purified polyclonal goat anti-IL-6 antibody (100 μl of a 0.014 mg/ml solution) was added to each of the wells and incubated for a further 1 h at room temperature. The plates were washed 3 times and 100 μl of a 1:5000 dilution of avidin-HRP (Dako Ltd) were added into each well. Plates were then incubated for 15 min at room temperature before washing 3 times with wash/dilution buffer. Wells were then developed with 100 μl of 0.2 mg/ml orthophenylenediamine (OPD, Sigma) in 0.1 M citric acid-phosphate buffer pH 5.0 plus 0.4 μl/ml 30 % H_2O_2 (Sigma). The reaction was terminated by the addition of 150 μl of 1 M H_2SO_4, and the absorbance at 492 nm was measured on a Titertek Multiscan spectrophotometer. A standard curve was plotted of the absorbance versus the concentration of IL-6.

2.7.2 ELISA for IL-1β

The assay of IL-1β used a similar protocol to the IL-6 assay described above with the following modifications. Microtitre plates were coated with an immuno-affinity purified goat polyclonal anti-human IL-1β antibody (Rafferty *et al.*, 1991) diluted at

5 μg/ml. The plates were incubated overnight at 4° C. The IL-1β standard used was the international standard for IL-1β (NIBSC: 86/680) at a concentration range of 0-8 ng/ml. The detecting antibody was biotinylated immuno-affinity purified polyclonal anti-IL-1β antibody (Rafferty *et al.*, 1991) used at a dilution of 1:4000. A curve was plotted of the absorbance versus the concentration of IL-1β.

2.7.3 ELISA for TNF-α

Microtitre plates were coated with a mouse monoclonal antibody to human TNF-α at a concentration of 2 μg/ml in bicarbonate buffer at pH 9.6 (Meager, 1987). These plates were allowed to incubate overnight at 4° C. The following day the plates were washed with wash buffer and then blocked overnight using 1 % human serum albumin (HSA) in PBS at pH 7.2-7.4. The TNF-α standard (international standard for TNF-α:NIBSC 86/659) was diluted in PBS plus 1 % human serum albumin (HSA) at a concentration range of 0-200 ng/ml. The standards and the samples were aliquoted into the washed wells and allowed to incubate for 1 h at 37° C. The washed plates then received 100 μl of biotinylated goat polyclonal anti-TNF-α antibody used at a dilution of 1:200 and were allowed to incubate for 1 h at 37° C. After this period the plates were washed, streptavidin-HRP at a dilution of 1:5000 added to each well and the plates allowed to incubate at 37° C for 30 min. The plates were then washed, the substrate OPD added and the colour allowed to develop in the dark for 15 min. The reaction was terminated by addition of 1M sulphuric acid and the absorbance for each well monitored at 492 nm. A standard curve was plotted of the absorbance versus the concentration of TNF-α.

2.7.4 ELISA for IL-8

The assay protocol was followed according to the manufacturers (R and D systems) instructions. A standard curve was plotted of the absorbance versus the concentration of IL-8.

2.7.5 Radioimmunoassay for IL-8

The samples to be tested were thawed out and an equal volume of 22 % (w/v) PEG/ 1 % Protamine sulphate (PEG/PS) was added to each tube and mixed well by vortexing. The tubes were then left for 1 h at 4° C. After this period, the tubes

were centrifuged for 5 min at 5000 rpm at 4° C and the supernatants transferred into labelled luckham tubes. The human IL-8 standard was diluted in doubling dilutions over the concentration range 40000 pM to 20 pM in spun sample buffer (SSB). 1.5 ml Eppendorf tubes without lids were labelled and placed in Eppendorf transfer tubes. To each sample tube the following were added: 100 µl of sample, 100 µl of anti-human IL-8 antibody at 1:50 dilution and 50 µl of ^{125}I-human IL-8. To the standard tubes, all the above were added except that various standards were added instead of the sample. Total tubes or label only tubes contained 50 µl of ^{125}I-human IL-8. The "O" tubes or reference binding tubes contained the following: 100 µl of SSB, 100 µl of IL-8 antibody (1:50 dilution) and 50 µl of ^{125}I-human IL-8. To the non-specific-binding (NSB) tubes the following were added: 100 µl of SSB, 100 µl PBS.GP (PBS containing gelatin and protamine sulphate) and 50 µl of ^{125}I-human IL-8. After an overnight incubation at 4° C, 50 µl of a 1:30 dilution of donkey anti-goat IgG was added to all the tubes. The tubes were then incubated for 8 h or overnight at 4° C. After this incubation period 1 ml of PBS/azide was added to each tube and then spun at 5420 g for 10 min. The supernatants were discarded and the pellet counted on a Canberra Packard Cobra multigamma counter (model 5005).

2.8 Calvarial Bone Resorption Assay

Bone resorption was assayed by measuring the release of calcium from 5-day old mouse calvaria *in vitro* (Zanelli *et al.*, 1969). Halved calvaria were cultured singly on stainless steel grids in 30 mm dishes (5 per group) with 1.5 ml BGJ medium (ICN Flow) supplemented with 5 % complement-inactivated rabbit serum (Gibco Ltd) and 50 µg/ml ascorbic acid (Sigma). After 24 h, the media were replaced by fresh medium containing various concentrations of bacterial extracts. Prostaglandin (PG)E_2 was added at 1 µM to five wells in each experiment to act as a positive control for the responsivity of the bone. The calvaria were cultured for a further 48 h and then the calcium content of the media was measured by automated colorimetric analysis (Gitelman, 1967).

2.9 Trypsin and heat treatment of SAM and LAP

To determine if the active moieties in the SAM or LAP preparations were proteinaceous, 1 mg/ml solutions of the preparations from each organism were treated either by heating at 100^0 C for 1 h or by exposure to 0.25 % trypsin for 18 h. After trypsin treatment, soya bean trypsin inhibitor (Sigma) was added to the samples, which were then tested for their ability to stimulate cytokine release or bone resorption. Controls were treated with equivalent amounts of trypsin inhibitor.

Chapter 3

Composition of the Bacterial Components

3.1 Introduction

The hypothesis being tested in this thesis is that easily solubilised molecules present on the surface of oral bacteria have biological activities consonant with their being involved in the pathogenesis of the periodontal diseases. As these components are not well-characterised, in this chapter their content of protein, carbohydrate, lipid or nucleic acid is described.

3.2 Aims

The aims of the work described in this chapter were to determine the composition of each of the SAM and LAP preparations as well as to determine the relationship of the SAM to the bacterial cell wall by Electron Microscopy.

3.3 Materials and Methods

The methods were as described in chapter 2. Electron Microscopy of the bacteria was performed before and after SAM extraction to show that the extraction was successful and did not damage the bacteria. To determine the protein concentrations of the SAM and LAP preparations three different methods were used: the classic Lowry assay, the detergent compatible Lowry assay and the Bradford assay. The carbohydrate content of the SAM and LAP preparations was determined by the method of DuBois *et al.*, (1956), the lipid content by extraction with chloroform/methanol and the endotoxin content was measured by the LAL chromogenic assay. The nucleic acid content was determined by measuring the absorbances at 260 nm and 280 nm and the ratio between them, calculated.

Statistical analysis

The results are expressed as the mean $\pm$ 2SD.

3.4 Results

3.4.1 Electron microscopy of the cells prior to and after saline extraction

Cells were fixed and viewed by transmission electron microscopy (tem). It was found that the cells were intact and that after saline extraction only the surface coating had been removed (figures 3.1-3.3).

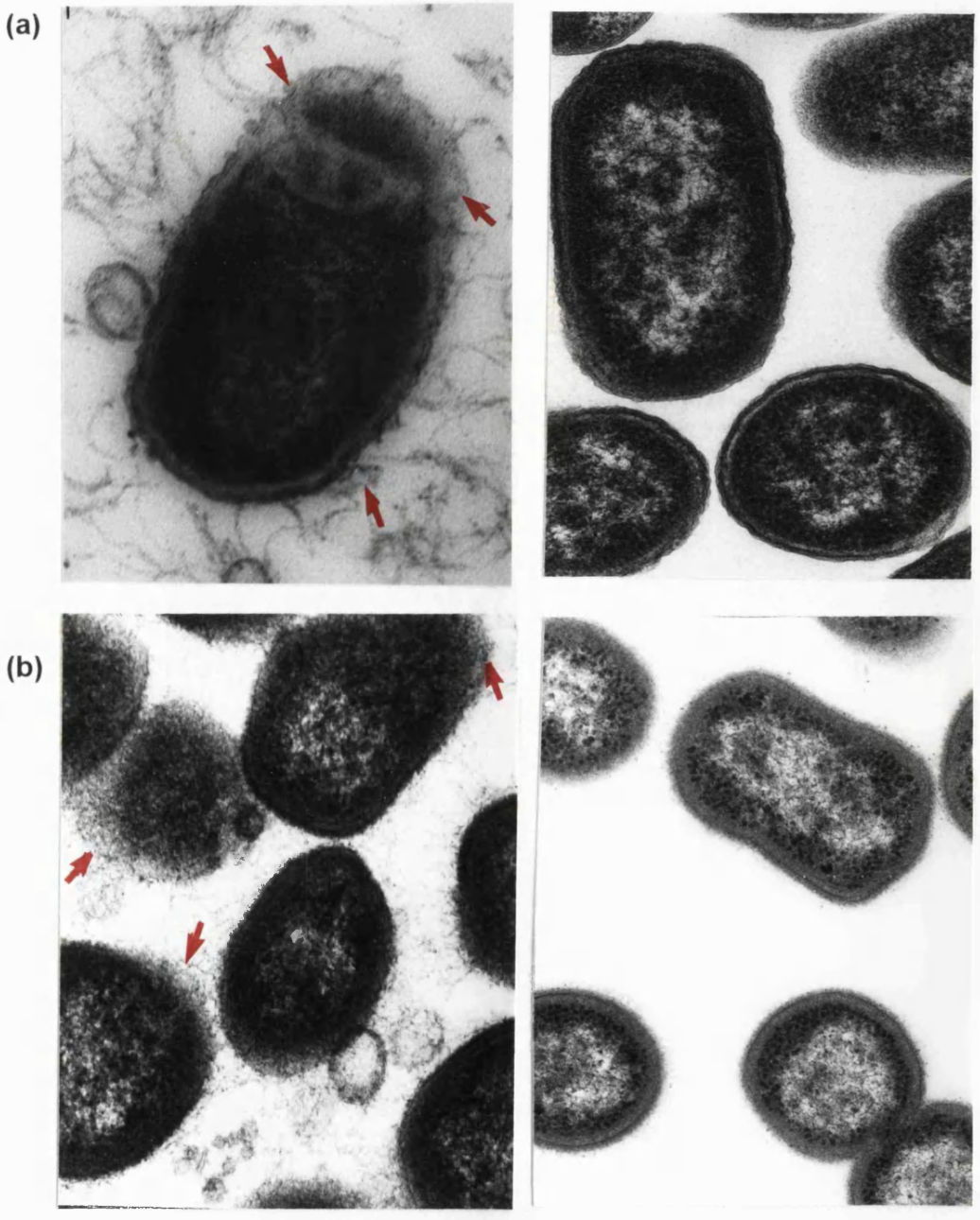
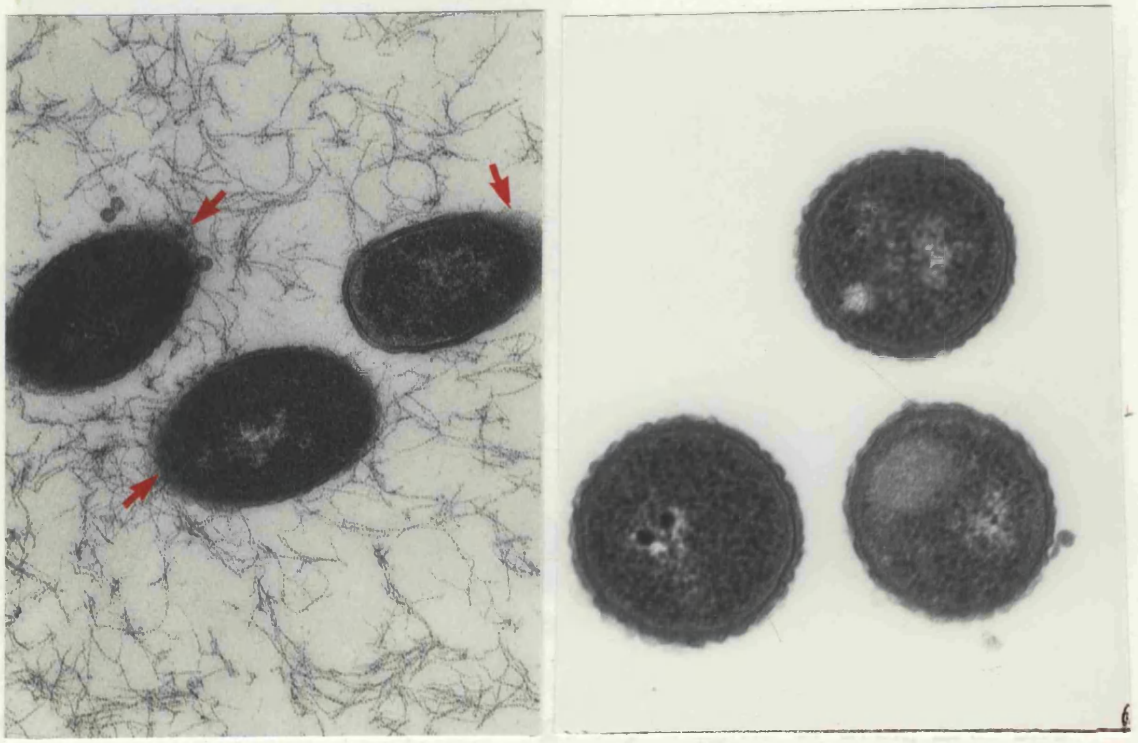


Figure 3.1 **Electron microscopy of (a): *A. actinomycetemcomitans* and (b): *Por. gingivalis* prior to (red arrows show the SAM) and after) saline extraction.**

(c)



(d)

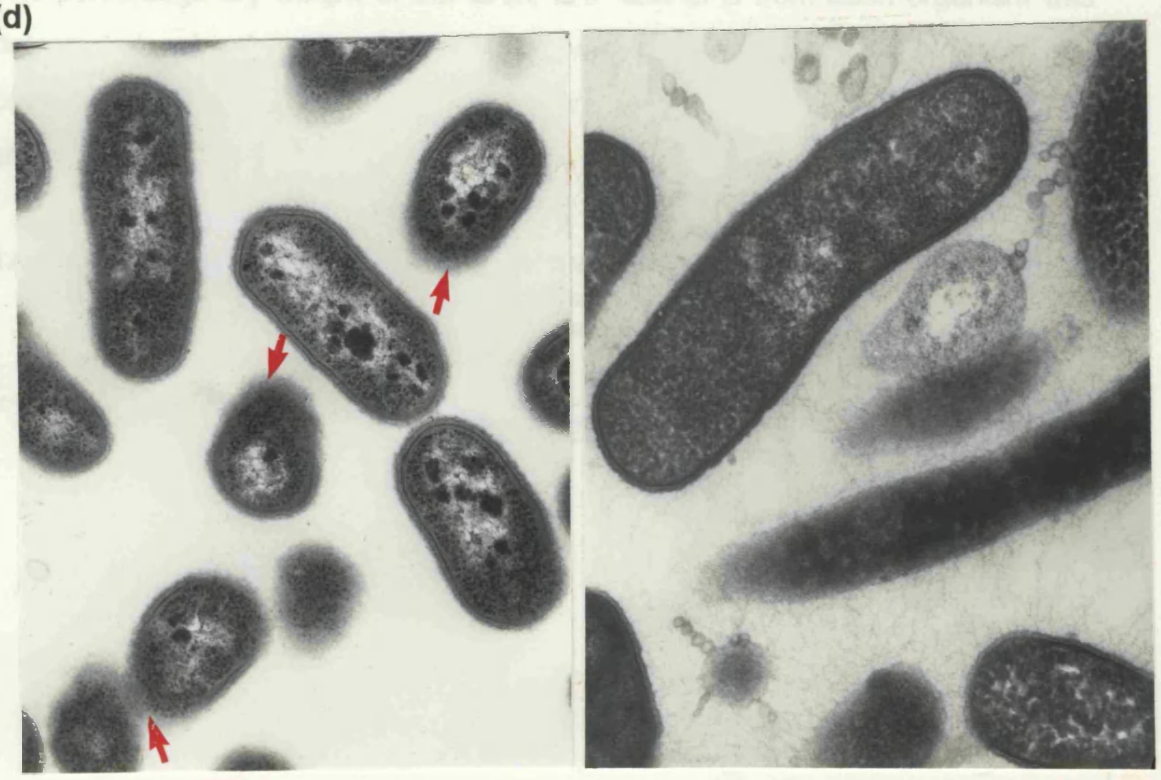


Figure 3.2 Electron microscopy of (c): *Eik. corrodens* and (d): *Prev. intermedia* prior to (red arrows show the SAM) and after saline extraction.

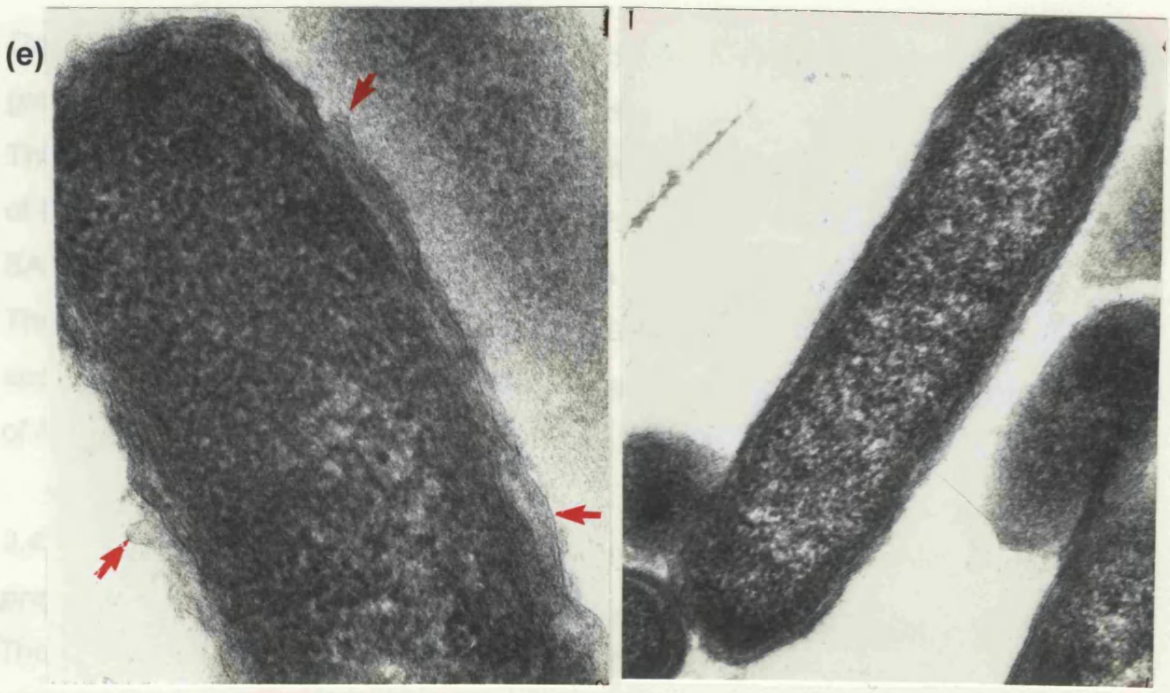


Figure 3.3 **Electron microscopy of (e): *C. rectus* prior to (red arrows show the SAM) and after saline extraction.**

3.4.2 Determination of the percentage dry weights of SAM, LAP and LPS

The percentage dry weight of the SAM, LAP and LPS from each organism was determined, by lyophilising and then weighing the extracted material and comparing with the dry weight of the starting material. The results obtained are shown in table 3.1.

Table 3.1 **The percentage dry weight of SAM, LAP and LPS obtained from each of the five periodontopathogens. The results are expressed as the % dry weight and % ± 2SD of three preparations.**

Periodontopathogen	Dry weight of SAM % (w/w)	Dry weight of LAP % (w/w)	Dry weight of LPS % (w/w)
A.actinomycetemcomitans	15 ± 1.2	6.7 ± 1.3	1.9 ± 0.3
Eik. corrodens	10 ± 1.1	n/m	n/m
Por. gingivalis	11 ± 0.7	1.1 ± 0.4	0.8 ± 0.4
Prev. intermedia	4 ± 0.4	0.3 ± 0.1	2.0 ± 0.5
C. rectus	11 ±1.5	n/m	n/m

n/m - not measured

On a dry weight basis, the SAM was found to be the constituent present in the greatest proportions in all of the organisms, followed by the LAP and then the LPS. This was with the exception of *Prev. intermedia*, where the percentage dry weight of the LPS, was greater than that, of the LAP. The percentage dry weights of the SAMs ranged from 4 % for *Prev. intermedia* to 15 % for *A. actinomycetemcomitans*. The percentage dry weights of the LPS preparations was, as expected approximately 2 % of the total dry weight of the starting material with the exception of *Por. gingivalis* where the LPS accounted for only 0.8 % of the dry cell weight.

3.4.3 Determination of the endotoxin levels of the SAM and LAP preparations

The endotoxin levels of each of the SAM and LAP preparations were found to be low, ranging from 3×10^{-6} to 9×10^{-3} IU/ng (table 3.2). These values were obtained by using *E. coli* LPS as standard. This preparation is known to have an endotoxin level of 7000 IU/μg. The endotoxin levels of the LPS preparations ranged from 2.9 IU/ng for *A. actinomycetemcomitans* to 4.2IU/ng for *Por. gingivalis*.

Table 3.2 **The endotoxin levels (IU/ng) present in each SAM and LAP preparation measured by the LAL chromogenic assay. The results are expressed as the mean of four replicates.**

Periodontopathogen	Endotoxin content of SAM [IU/ng]	Endotoxin content of LAP [IU/ng]	Endotoxin content of LPS [IU/ng]
A. actinomycetemcomitans	9×10^{-3}	9×10^{-3}	2.9
Por. gingivalis	3×10^{-6}	3×10^{-4}	4.2
Prev. intermedia	6×10^{-4}	3×10^{-4}	3.7
Eik. corrodens	2×10^{-5}	n/m	n/m
C. rectus	3×10^{-4}	n/m	n/m

n/m-not measured

3.4.4 Determination of the protein composition of each preparation

The assays used to determine the protein content were (i) Enhanced alkaline copper (Lowry) protein assay (ii) Bio-Rad detergent compatible (DC) protein assay and (iii) Coomassie blue (Bradford) protein assay.

3.4.4.1 Enhanced alkaline copper (Lowry) protein assay

On the basis of this assay, the SAM from *A. actinomycetemcomitans* contained the greatest protein content (70 %), followed by the SAM from *Por. gingivalis* (42 %). In comparison the protein contents for the SAMs from *Prev. intermedia* (14 %), *Eik. corrodens* (12 %) and *C. rectus* (3 %) were found to be low. The protein contents obtained for the LAPs of both *Por. gingivalis* (4.1 %) and *Prev. intermedia* (4.0 %) were low, however the protein content of the LAP from *A. actinomycetemcomitans* was found to be 82 %.

Table 3.3 **The percentage protein content of each SAM and LAP preparation obtained by the Lowry protein assay. The results are expressed as the % protein content and the % ± 2SD of four replicates.**

Periodontopathogen	Protein content of SAM	Protein content of LAP
	% (w/w)	% (w/w)
A. actinomycetemcomitans	70 ± 1.8	82 ± 2.6
Por. gingivalis	42 ± 2.4	4.1 ± 1.6
Prev. intermedia	14 ± 1.4	4.0 ± 1.0
Eik. corrodens	12 ± 1.0	n/m
C. rectus	3.0 ± 0.4	n/m

n/m - not measured

3.4.4.2 Bio-Rad Detergent Compatible (DC) protein assay

On the basis of this assay, the SAM from *Eik. corrodens* (80 %) and *A. actinomycetemocomitans* (60 %) contained the greatest protein content (table 3.4). In contrast to the classic Lowry assay described above, the SAM from *Eik. corrodens*, gave a greater protein content than the SAM from *A. actinomycetemcomitans*. Although, on the basis of this assay a higher protein content was obtained for the SAMs from *Prev. intermedia* (21 %) and *C. rectus* (11

%) compared to the classic Lowry assay, the values were still relatively low. The protein content of the SAM from *Por. gingivalis* (18 %) was approximately half on the basis of this assay compared with the value obtained by the classic Lowry assay. The protein concentrations of the LAP preparations from *Por. gingivalis* and *Prev. intermedia* were found to be 7.5 % and 21 % respectively (compared with 4.1 and 4.0 %). However, the LAP from *A. actinomycetemcomitans* contained a similar protein content as derived by the Lowry assay.

Table 3.4 **The percentage protein content of each SAM and LAP preparation obtained by the Bio-Rad DC protein assay. The results are expressed as the % protein content and the % ± 2SD of four replicates.**

Periodontopathogen	Protein content of SAM	Protein content of LAP
	% (w/w)	% (w/w)
A. actinomycetemcomitans	60 ± 3.4	86 ± 2.2
Por. gingivalis	18 ± 1.0	7.5 ± 1.6
Prev. intermedia	21 ± 0.2	21 ± 0.6
Eik. corrodens	80 ± 1.4	n/m
C. rectus	11 ± 2.4	n/m

n/m - not measured

3.4.4.3 Bradford protein assay

On the basis of the Bradford assay the protein contents of the SAMs from *A. actinomycetemcomitans* and *Eik. corrodens* were 35 % and 44 % respectively (table 3.5). The protein content of the SAMs from *Por. gingivalis* (7 %), *Prev. intermedia* (5 %) and *C. rectus* (9 %) still gave low values. The protein content of the LAP from *Por. gingivalis* obtained by the Bradford assay was found to be only 1 %, whereas the LAP from *Prev. intermedia* was found to be 10 %, the greatest value obtained for this preparation compared with any of the other assays used. The protein content obtained for the LAP from *A. actinomycetemcomitans* (52 %) by this assay was low in comparison with the other two assays.

Table 3.5 **The percentage protein content of both the SAM and LAP preparations from the five periodontopathogens obtained using the Bradford protein assay. The results are expressed as the % protein content and the % ± 2SD of four replicates.**

Periodontopathogen	Protein content of SAM	Protein content of LAP
	% (w/w)	% (w/w)
A. actinomycetemcomitans	35 ± 2.2	52 ± 3.0
Por. gingivalis	7 ± 0.8	1 ± 0.26
Prev. intermedia	5 ± 0.66	10 ± 1.8
Eik. corrodens	44 ± 0.2	n/m
C.rectus	9 ± 3.8	n/m

n/m - not measured

As there were some discrepancies between the protein values using the various assays, the amino acid composition of three of the SAM preparations was investigated (table 3.6). Amino acid analysis revealed, that in general, there were few tyrosine residues present in any of the three SAM preparations. In contrast, however, the aspartate content was high for all three SAMs. *Prev. intermedia* had no arginine or tyrosine residues present in its SAM which may explain the low protein concentrations obtained for this preparation in the various assays.

Table 3.6 The amino acid composition of the SAM from three periodontopathogens.

Amino acid	*A.actinomycetemcomitans*	*Por. gingivalis*	*Prev. intermedia*
Aspartate	56	42	23
Threonine	14	9	10
Serine	14	11	20
Glutamine	25	38	36
Proline	10	8	15
Glycine	26	16	38
Alanine	20	19	24
Valine	12	14	12
Met	4	2	2
Isoleucine	10	8	8
Leucine	14	18	15
Tyrosine	8	4	0
Phenylalanine	8	6	5
Histamine	4	4	4
Lysine	12	23	17
Arginine	6	8	0

3.4.5 SDS-PAGE analysis of the SAM and LAP preparations

SDS-PAGE analysis of the SAMs revealed the presence of protein bands ranging from <14 kDa to >66 kDa (figure 3.3). The SDS-PAGE of the LAP from *A. actinomycetemcomitans* also showed many protein bands. However, in contrast, the LAP from *Por. gingivalis* only revealed the presence of one major band at 17 kDa whereas the LAP from *Prev. intermedia* showed two major bands at 20 and 45 kDa and two minor bands (figure 3.4).

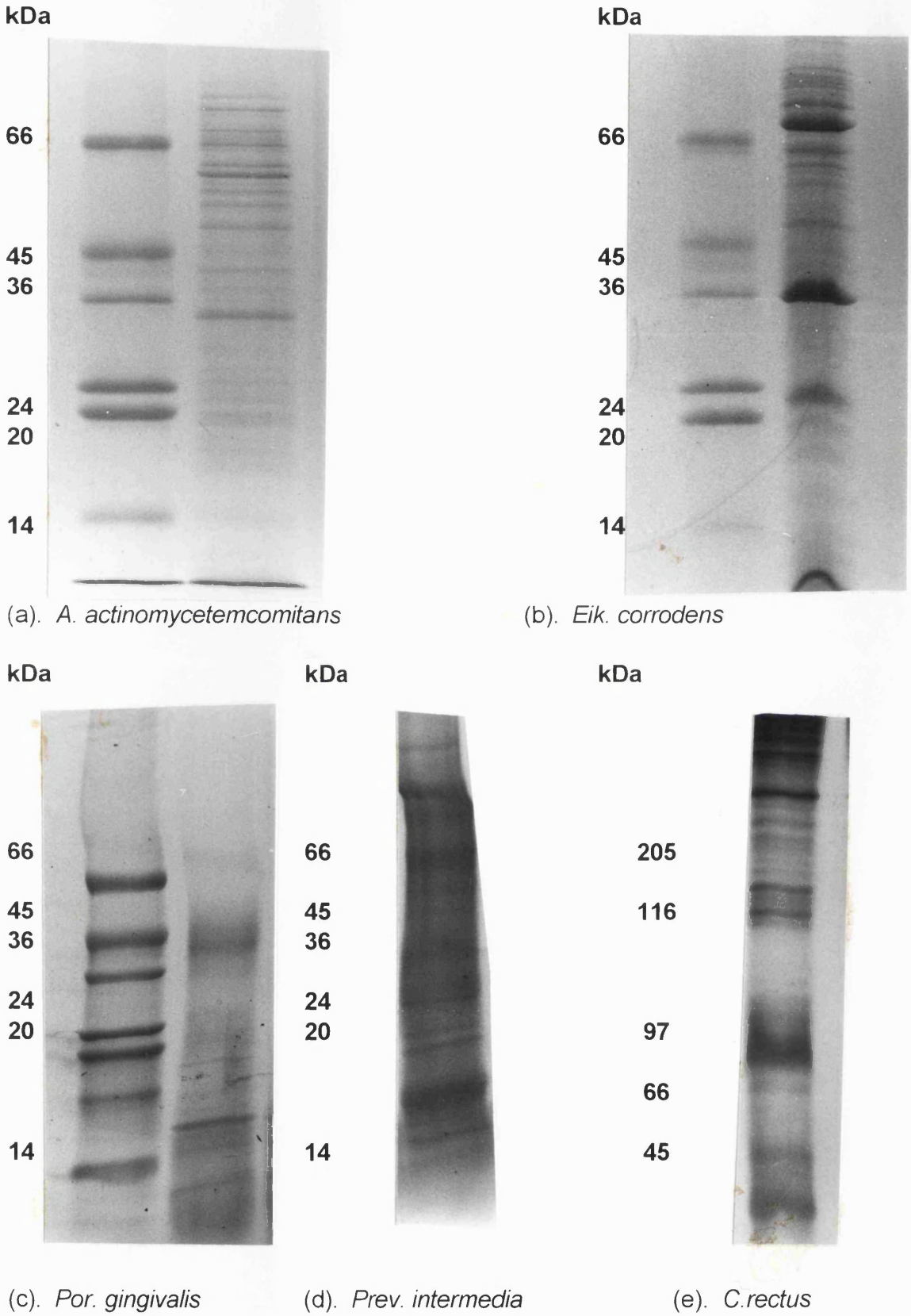
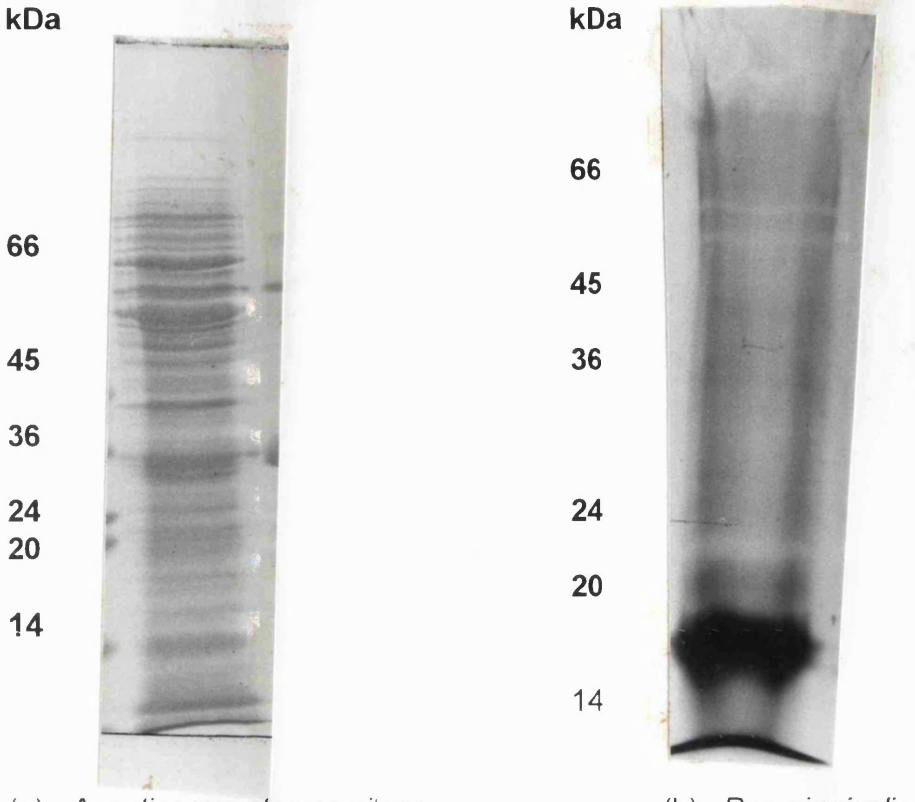
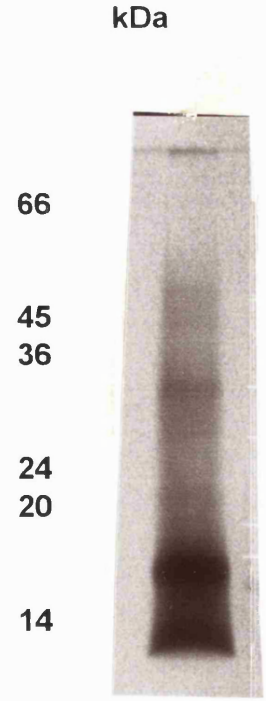


Figure 3.4 **SDS-PAGE of the SAMs from (a) *A. actinomycetemcomitans*; (b) *Eik. corrodens*; (c) *Por. gingivalis*; (d) *Prev. intermedia* and (e) *C. rectus* stained with Coomassie Colloidal blue.**



(a). *A. actinomycetemcomitans* (b). *Por. gingivalis*



(c). *Prev. intermedia*

Figure 3.5 **SDS-PAGE of the LAPs from (a) *A. actinomycetemcomitans*; (b) *Por. gingivalis* and (c) *Prev. intermedia* after staining with silver.**

3.4.6 Determination of the protein profiles of the SAM from A. actinomycetemcomitans, Eik. corrodens and Por. gingivalis by IEC.

The protein profiles of the three SAM preparations which contained the largest proportions of proteins were investigated by using IEC.

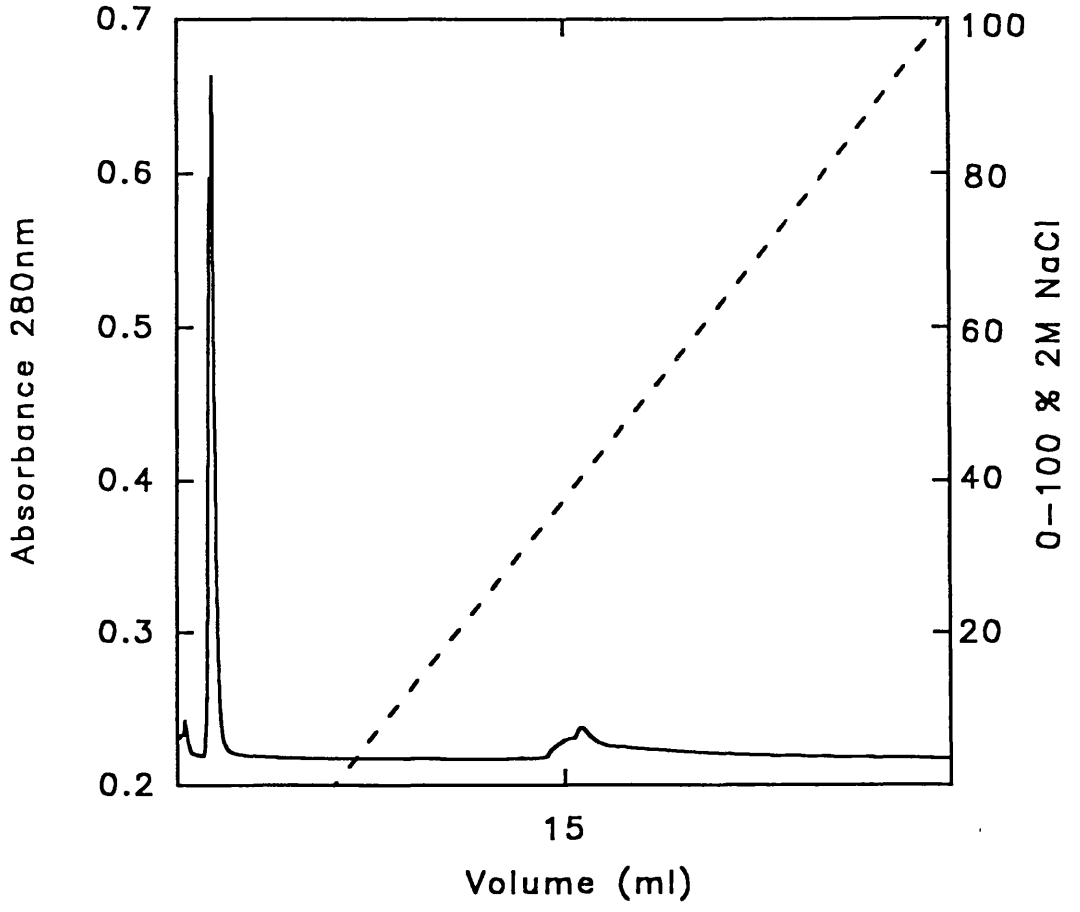


Figure 3.6 **Cation exchange HPLC of the SAM from *A. actinomycetemcomitans* using a MA7S Bio-Rad column. (——) 0-100 % 2M NaCl gradient. A large proportion of the SAM failed to bind to the column.**

Cation exchange chromatography of the SAM from *A. actinomycetemcomitans* revealed that a large proportion of the SAM did not bind to the column, implying that the proteins present in this preparation were anionic in nature under the conditions employed. Therefore, anion exchange chromatography was performed (figure 3.7).

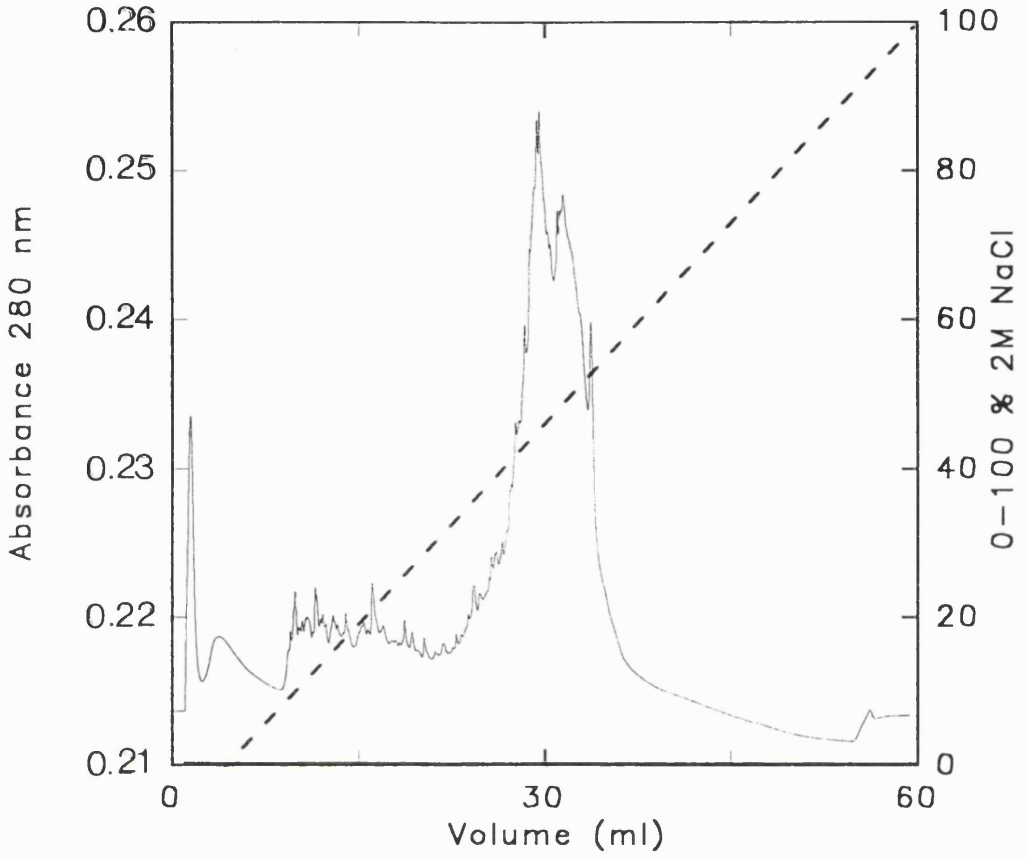


Figure 3.7 **Anion exchange HPLC of the SAM from *A. actinomycetemcomitans*. (——) 0-100 % 2M NaCl gradient. A small number of proteins eluted between 0.14M and 0.64M NaCl. However, the majority of proteins eluted between a NaCl concentration of 0.64M and 0.75M.**

Anion exchange HPLC of the SAM from *A. actinomycetemcomitans* revealed a number of protein peaks. Although some of the SAM did not bind to this column, a small number of proteins eluted between a NaCl concentration of 0.14M and 0.64M. However, the majority of peaks eluted between a NaCl concentration of 0.64M and 0.75M indicating that these proteins were strongly anionic.

Cation exchange chromatography of the SAM from *Eik. corrodens* revealed that the proteins present in this preparation were also anionic in nature under the conditions used in this experiment. This is shown in figure 3.8 where the majority of the proteins in the sample applied to the column passed through the column without binding to it.

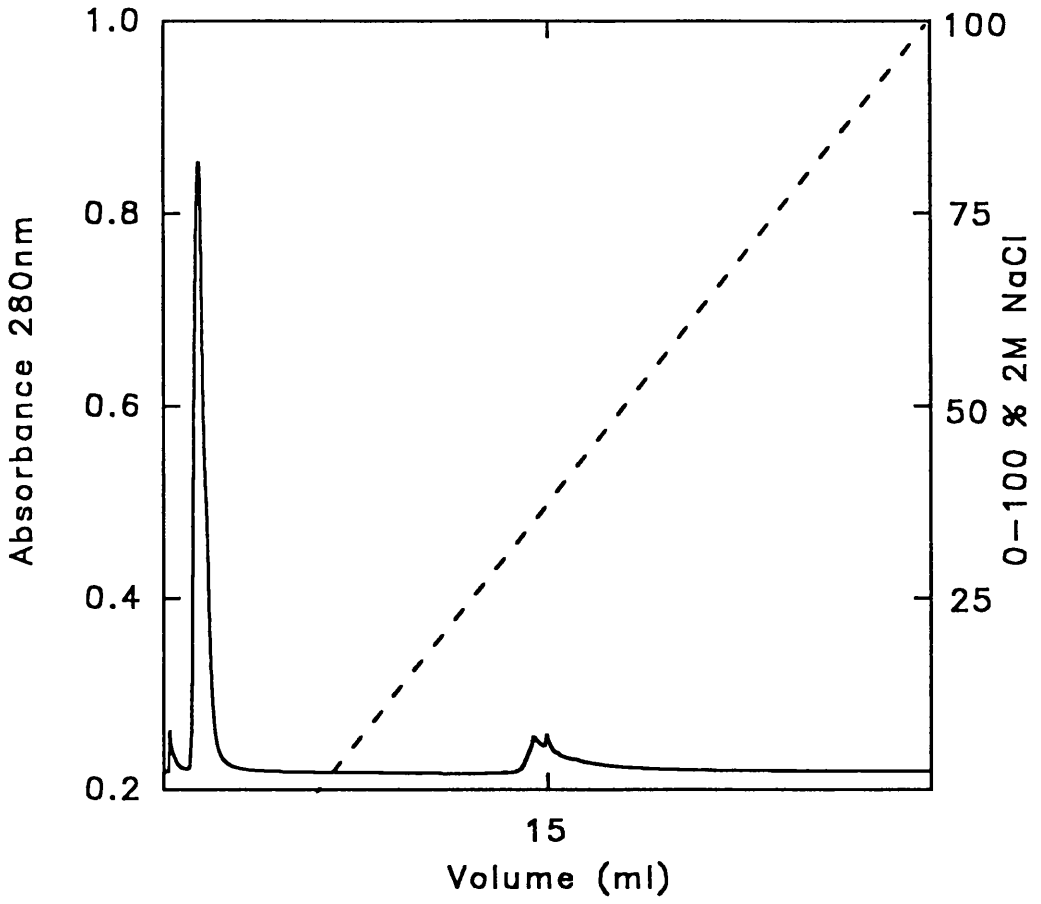


Figure 3.8 **Cation exchange HPLC of the SAM from *Eik. corrodens* using a MA7S Bio-Rad column. (——) 0-100 % 2M NaCl gradient. The SAM from *Eik. corrodens* was found to be anionic in nature under the conditions used in this experiment.**

Anion exchange HPLC of the SAM from *Eik. corrodens* showed that the proteins present in this preparation were strongly anionic in nature (figure 3.9). From the elution profile it was seen that a small number of proteins eluted between 0.4 M and 0.86 M NaCl. However, the majority of proteins eluted between a NaCl concentration of 0.86M and 1.22M.

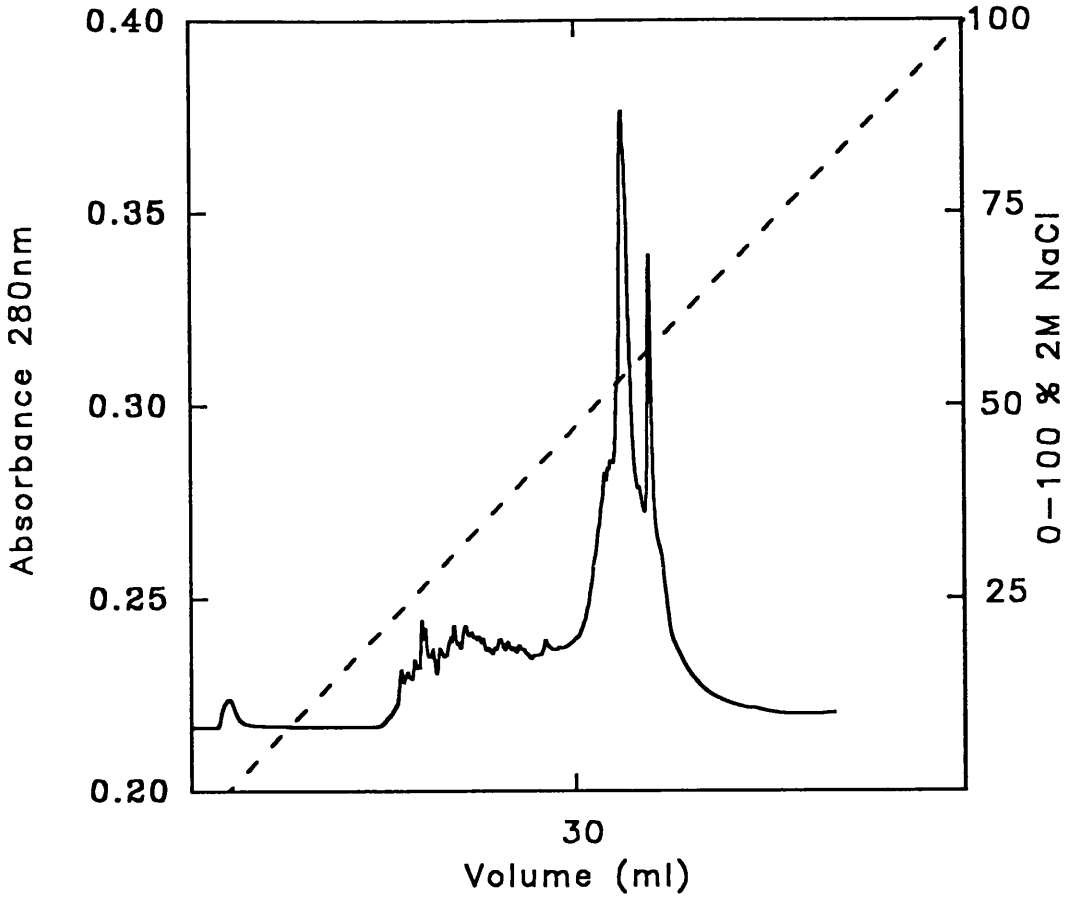


Figure 3.9 **Anion exchange HPLC of the SAM from *Eik. corrodens* using a MA7Q column. (——) 0-100 % 2M NaCl gradient. The SAM from *Eik. corrodens* was found to be strongly anionic in nature. The first set of proteins eluted between 0.4M and 0.86M NaCl. However, the majority of proteins eluted between 0.86M and 1.22M NaCl.**

Cation exchange HPLC of the SAM from *Por. gingivalis* revealed a similar pattern of elution to the SAMs from the other two bacteria (figure 3.10). The majority of the proteins failed to bind to this column, indicating that the proteins were anionic in nature under the conditions employed for this experiment.

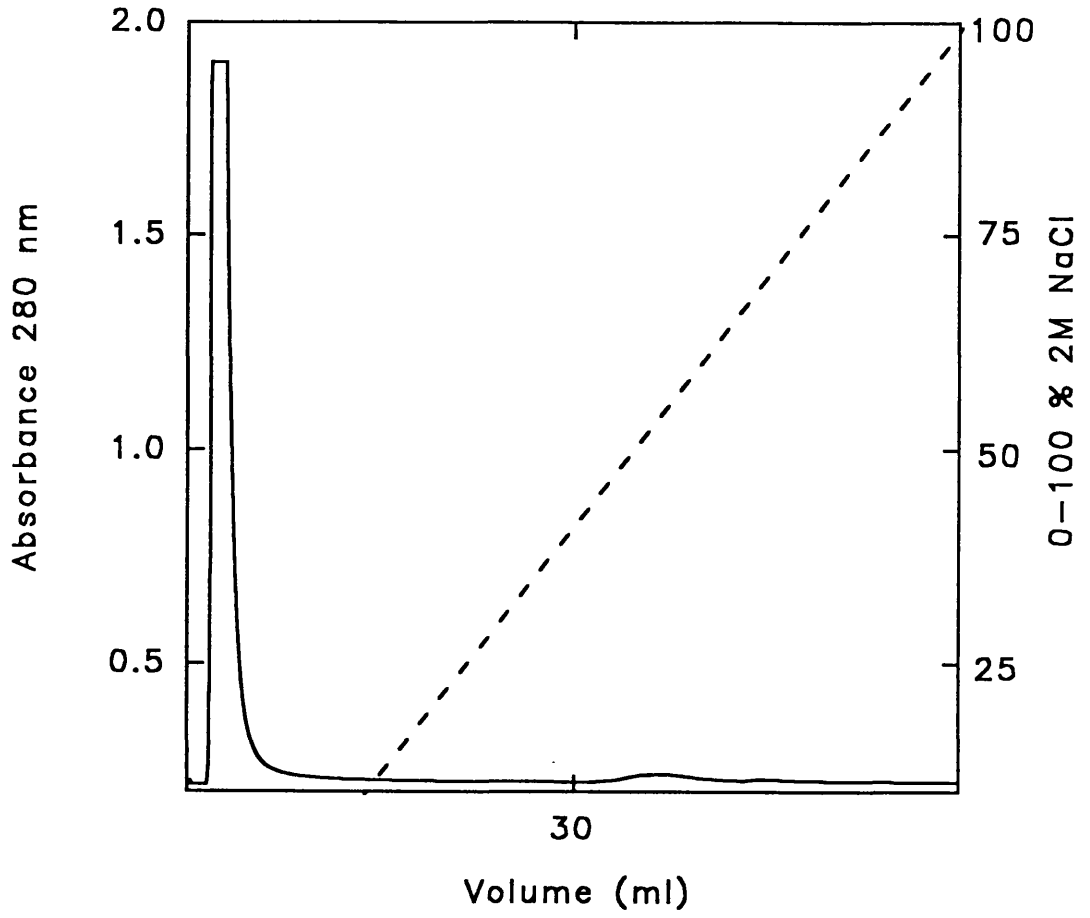


Figure 3.10 **Cation exchange HPLC of the SAM from *Por. gingivalis* using a MA7S Bio-Rad column. (——) 0-100 % 2M NaCl gradient. The majority of the proteins f rom this SAM preparation failed to bind to cation exchange column under the conditions used.**

After performing anion exchange HPLC of the SAM from *Por. gingivalis* it was found that the proteins present were strongly anionic in nature (figure 3.11). The elution profile revealed that the majority of proteins eluted at concentrations of NaCl between 0.66M and 1.3M. In contrast to the SAMs from *A. actinomycetemcomitans* and *Eik. corrodens* the vast majority of the proteins from the SAM from *Por. gingivalis* only eluted at high concentrations of NaCl.

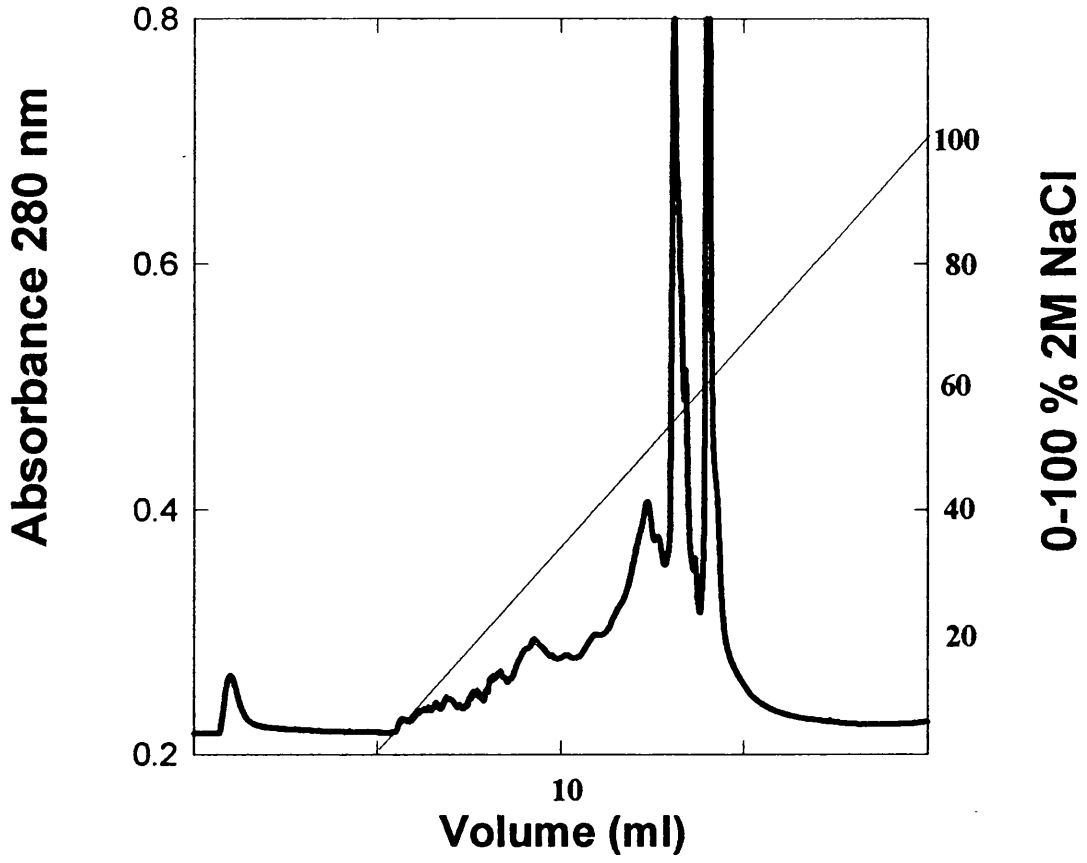


Figure 3.11 **Anion exchange HPLC of the SAM from *Por. gingivalis* using a MA7Q Bio-Rad column. (——) 0-100 % 2M NaCl gradient. The majority of peaks eluted between 0.66M and 1.22M NaCl.**

3.4.7 Determination of the carbohydrate composition of the SAM and LAP preparations

On the basis of the DuBois assay, none of the three LAP preparations contained measurable carbohydrate (table 3.7). The SAM preparations also had low carbohydrate contents ranging from 1-18 % (w/w).

Table 3.7 **The percentage carbohydrate content of both the SAM and LAP preparations. The results are expressed as the % carbohydrate content and the % ± 2SD of four replicates.**

Periodontopathogen	Carbohydrate content of SAM % (w/w)	Carbohydrate content of LAP % (w/w)
A. actinomycetemcomitans	13 ± 3.4	0
Por. gingivalis	18 ± 2.8	0
Prev. intermedia	17 ± 2.4	0
Eik. corrodens	6 ± 0.6	n/m
C. rectus	1 ± 0.2	n/m

n/m- not measured

Spectral scans of the SAMs following phenol-sulphuric acid treatment were also performed. From these spectral scans, the SAMs from *A. actinomycetemcomitans*, *Por. gingivalis* and *Eik. corrodens* were found to absorb maximally at 480 nm suggesting that one or many of the following carbohydrates may be present in these SAMs:-D-galacturonic acid, D-glucarone, L-arabisone, D-xylose, L-rhamnose and L-fructose (figure 3.12). The spectral scans obtained for the SAMs from *Prev. intermedia* and *C. rectus* showed an absorbance maximum of 485 nm suggesting that either D-glucose, and/or D-mannurone may be present in these SAMs.

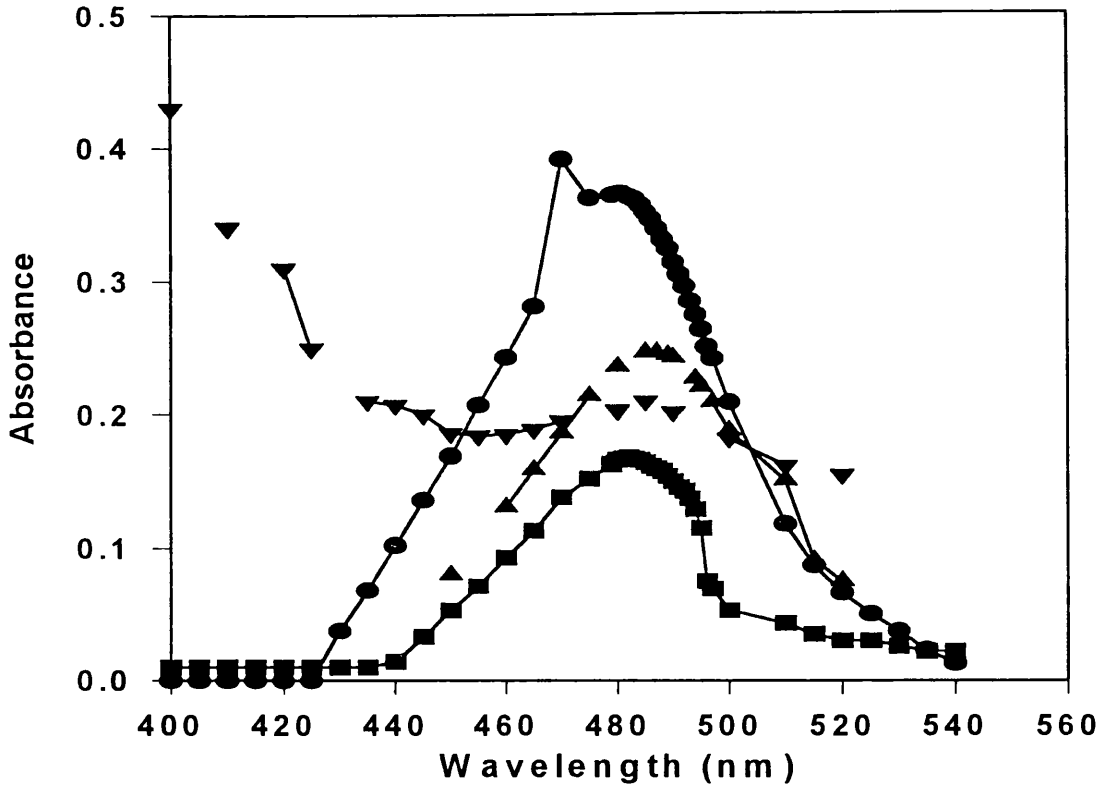


Figure 3.12 **Spectral scan of the SAMs from (●) *A. actinomycetemcomitans*; (■) *Por. gingivalis;* (▲) *Prev. intermedia* and (▼) *C. rectus* after treatment with phenol-sulphuric acid reagent.**

The spectral scan obtained for the SAM from *Eik. corrodens* treated with phenol-sulphuric acid reagent showed the presence of two peaks at 485 nm and 500 nm (figure 3.13). The peak at 485 nm indicates the presence of either D-glucose, and/or D-mannurone, however, it is not known which carbohydrates peak at 500 nm following treatment with phenol-sulphuric acid reagent and absorption may be due to some inherent chromophore.

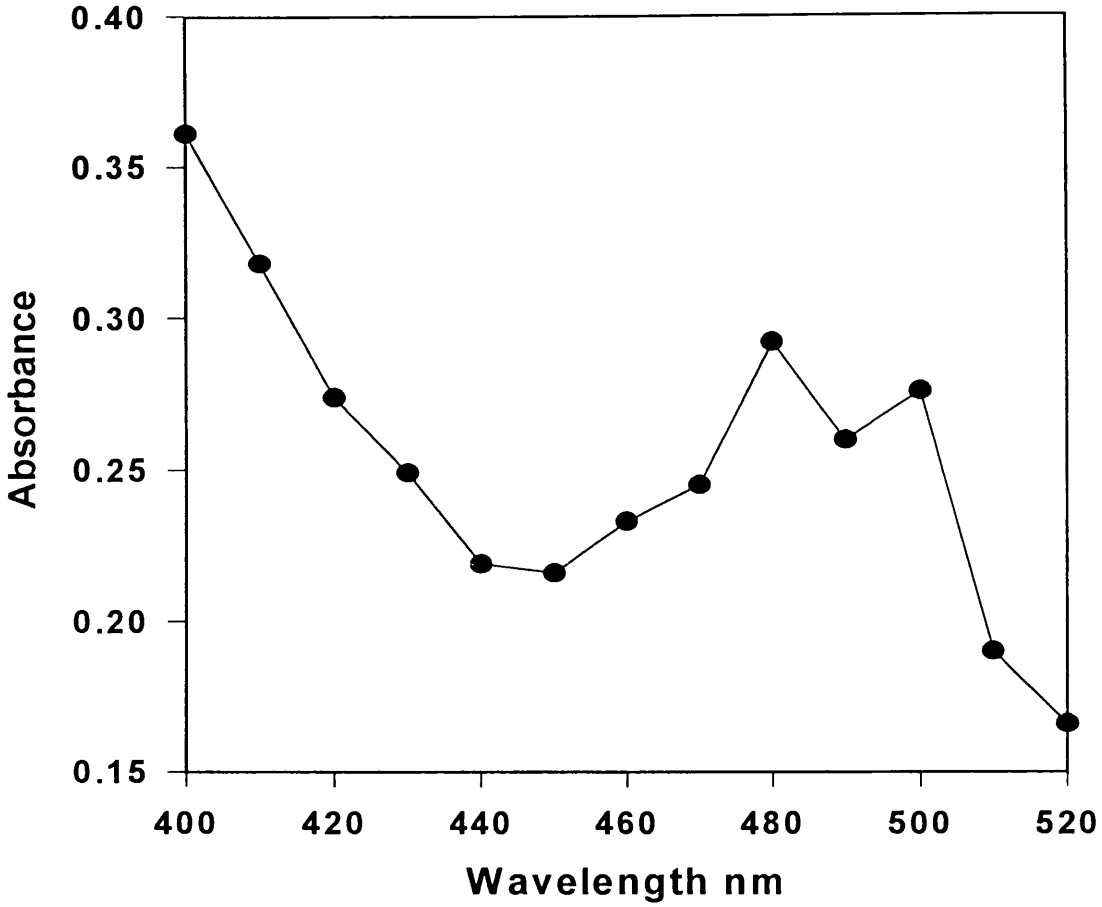


Figure 3.13 **Spectral scan of the SAM from *Eik. corrodens* treated with phenol-sulphuric acid reagent. The presence of two peaks, one at 485 nm and one at 500 nm were identified.**

3.4.8 Determination of the lipid content of the SAM and LAP preparations

In general, the lipid content of the SAMs were low (table 3.8). In contrast, the LAP preparations from *Por. gingivalis* (85 %) and *Prev. intermedia* (70 %) contained a high percentage of lipid. However, the lipid content of the LAP from *A. actinomycetemcomitans* was found to be only 2 %.

Table 3.8 **The percentage lipid content of both the SAM and LAP preparations. The results are expressed as the % lipid content and % ± 2SD of three preparations.**

Periodontopathogen	Lipid content of SAM	Lipid content of LAP
	% (w/w)	% (w/w)
A. actinomycetemcomitans	4 ± 0.4	2 ± 0.2
Por. gingivalis	8 ± 0.6	85 ± 5.6
Prev. intermedia	22 ± 2.9	70 ± 4.9
Eik. corrodens	14 ± 1.7	n/m
C. rectus	5 ± 0.7	n/m

n/m - not measured

3.4.9 Nucleic acid content of SAM and LAP preparations

The nucleic acid content of the SAM and LAP preparations were low with the exception of the SAM from *Eik. corrodens* which contained 14 % nucleic acid. Unfortunately most of the LAP preparations could not be measured as these had already been used up in other studies.

Table 3.9 **The percentage nucleic acid content of both the SAM and LAP preparations. The results are expressed as the percentage nucleic acid and % ± 2SD of three preparations.**

Periodontopathogen	Nucleic acid content of SAM	Nucleic acid content of LAP
	% (w/w)	% (w/w)
A. actinomycetemcomitans	8.2 ± 1.0	n/m
Por. gingivalis	3.2 ± 0.6	0.5 + 0.2
Prev. intermedia	1.7 ± 0.2	n/m
Eik. corrodens	14.3 ± 2.8	n/m
C. rectus	1.3 ± 0.6	n/m

n/m - not measured

3.5 Summary

The following three figures summarise: (i). the results obtained for the % protein content for the SAM and LAP preparations using the three different protein assays and (ii). the % total composition of each SAM preparation. The protein values obtained by using the Bio-Rad protein assay were used to calculate the % total composition of each preparation.

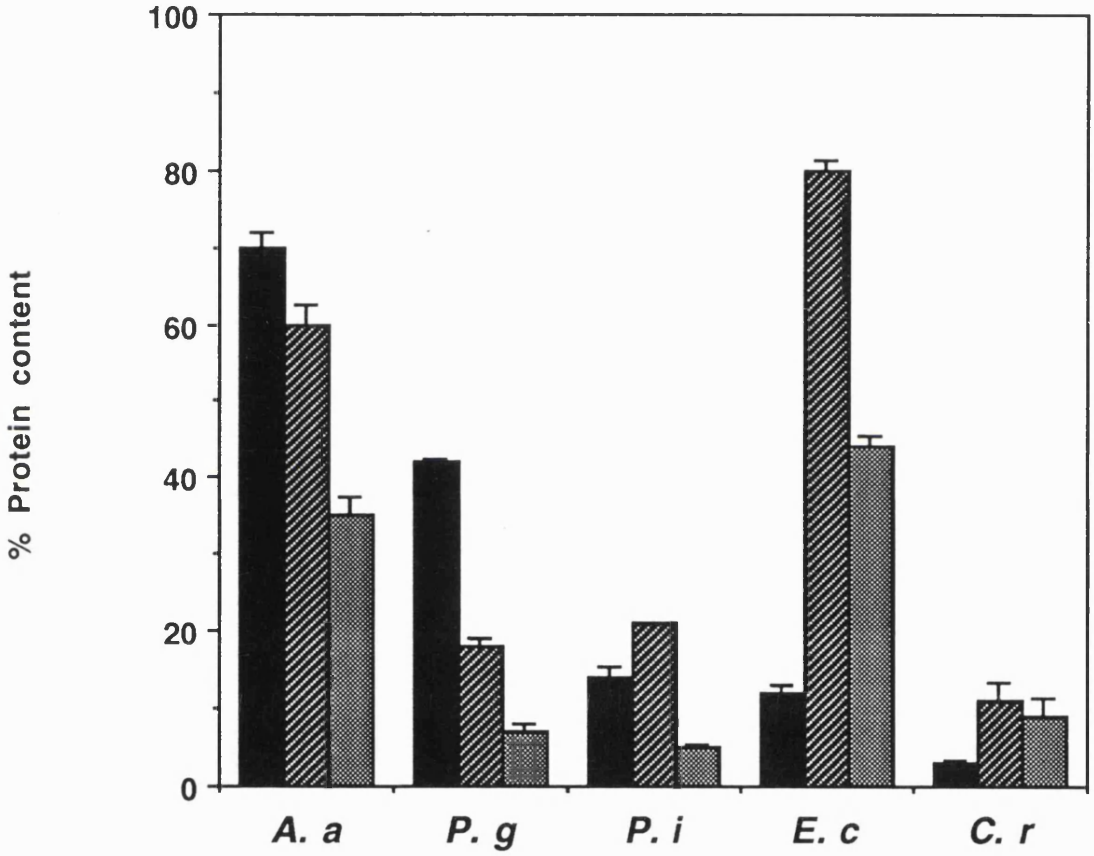


Figure 3.14 **Comparison of the % protein content (w/w) of the SAM preparations obtained by the three different protein assays. (filled columns)- Lowry assay; (hatched column)- Bio-Rad protein assay; (dotted column)- Bradford protein assay. The results are expressed as the % protein content and % ± 2SD of four replicates.**

The following figure shows the comparison of the % protein content of the LAP preparations obtained by the three different protein assays (figure 3.15).

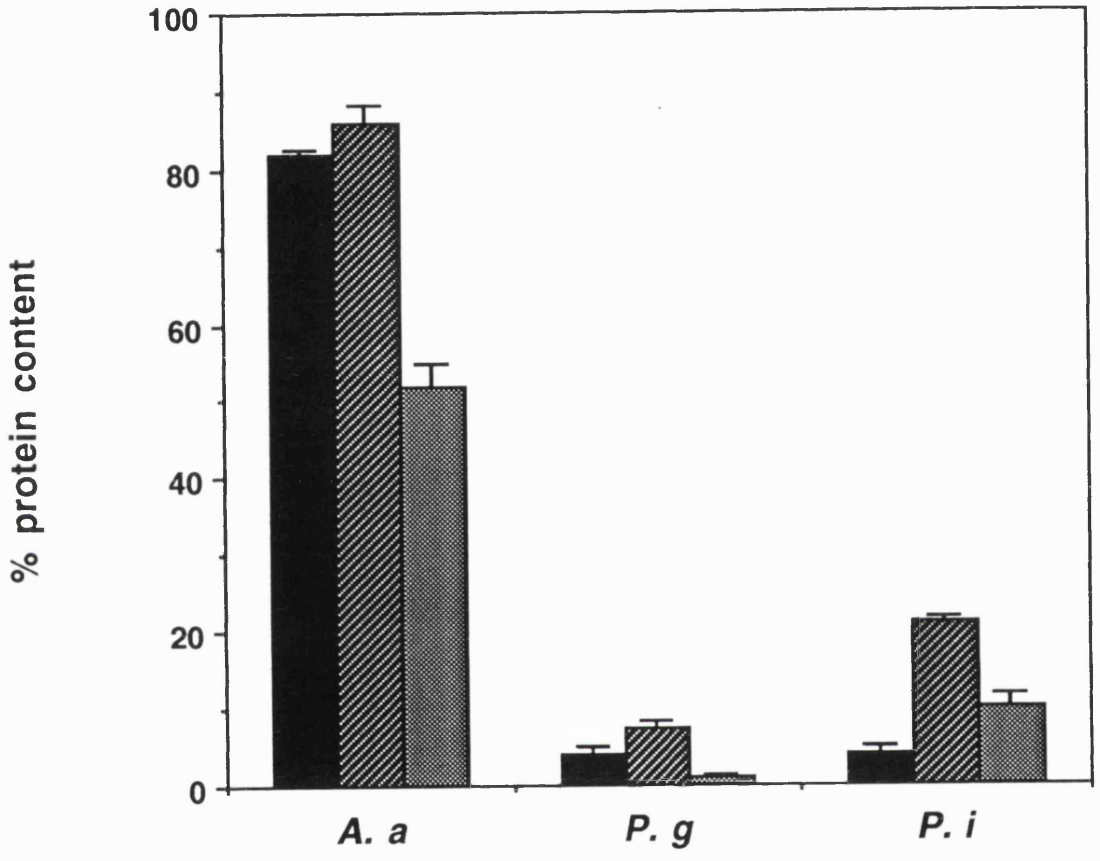


Figure 3.15 **Comparison of the % protein content (w/w) of the LAP preparations obtained by the three different protein assays. (filled columns)- Lowry assay; (hatched column)- Bio-Rad protein assay; (dotted column)- Bradford protein assay. The results are expressed as the % protein content and % + 2SD of four repl**

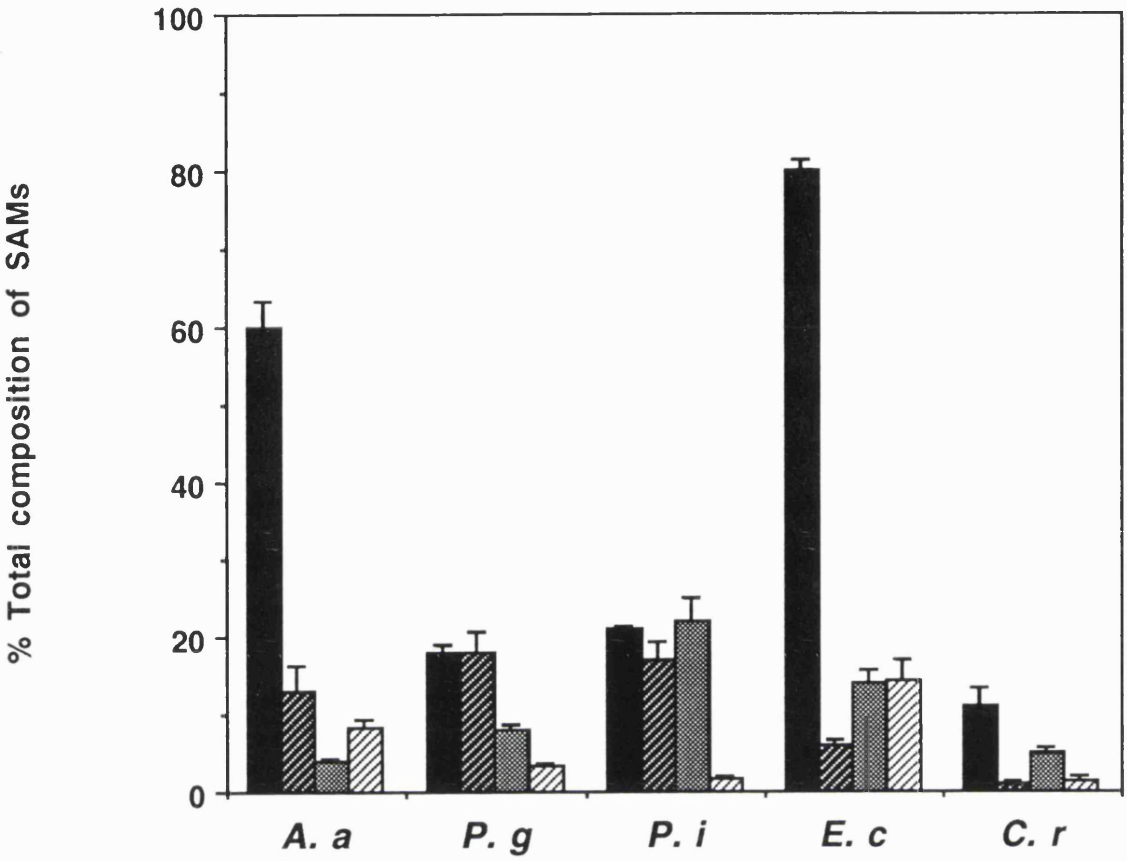


Figure 3.16 **The % total composition of the SAM preparations. (filled columns)-% protein content determined by the Bio-Rad assay; (black hatched columns)-% carbohydrate content; (dotted columns)- % lipid content; (white hatched columns)- % nucleic acid content.**

3.6 Discussion

Prior to extraction with saline, ruthenium red staining revealed the presence of a thick electron-opaque layer external to the cell wall. Saline extraction resulted in the complete removal of this layer. Detailed inspection of cells after extraction showed no signs of membrane damage. Thus simply stirring cells in saline completely removed SAM without causing cellular damage.

Of the three components studied (SAM, LAP, LPS), the former was found to be present in the greatest amount in each of the bacteria. The LAP was the second

most abundant, followed by the LPS. The only exception was *Prev. intermedia* where the percent LPS obtained was far greater than that of the LAP from this organism. The percent dry weight of the SAMs ranged from 4 % (w/w) for *Prev. intermedia* to 15 % (w/w) for *A. actinomycetemcomitans*, whereas the percentage dry weight of the LAP from *A. actinomycetemcomitans* was found to be 6.7 % (w/w) which was greater than the LAP obtained from *Prev. intermedia* or *Por. gingivalis* (1.1% (w/w) and 0.3 % (w/w) respectively). The percentage dry weight obtained for the SAM from *A. actinomycetemcomitans* appeared to be relatively high, however, the percentage dry weight of the LAP (6.7%) from this organism was also found to be greater than the dry weights of the LAP from *Por. gingivalis* or *Prev. intermedia*. The reason for the high yields is not clear. However, after examining the electron micrograph of the SAM- depleted *A. actinomycetemcomitans* it was clear that saline extraction did not damage cells indicating that SAM was indeed surface bound. Although the successful removal of the SAM can be independently verified it is not so easy to determine the percent yield of the LPS and the LAP and thus the extraction efficiency is not known.

The endotoxin levels of the LPS preparations ranged from 2.9IU/ng to 4.2IU/ng for *A. actinomycetemcomitans* and *Por. gingivalis* respectively. The endotoxin levels of each of the SAM and LAP preparations were low ranging from 3×10^{-6} to 9×10^{-3} IU/ng further supporting the claim that saline extraction is not causing damage to the cell wall. *E. coli* LPS is known to have an endotoxin level of 7000 IU/μg.

To determine the protein content of the SAMs and LAPs, three different assays were used. These were the Lowry assay, the Bio-Rad DC Lowry assay and the Bradford assay. In general, the most widely used protein assay is the Lowry assay. However, as this gave very low protein concentrations for some of the SAM preparations other methods were employed, and the results compared. The SAM and LAP preparations from *A. actinomycetemcomitans* had the greatest protein contents regardless of which assay was used. However, there were some discrepancies within the different protein assays used for these preparations. Both the Lowry and the Bio-Rad protein assays gave similar results, 60 - 70 % for the SAM and 86 % for the LAP. However, the Bradford assay appeared to underestimate the protein content giving only 35 % and 52 % respectively for SAM

or LAP. In general, the Bradford assay gave the lowest values for the protein content of all the SAM and LAP preparations. Using this assay the percent protein content ranged from 5 % (w/w) for *Prev. intermedia* to only 35 % for *A. actinomycetemcomitans*, and the LAPs ranged from 1.1 % for *Por. gingivalis* to 52 % for *A. actinomycetemcomitans*. The reason for the low protein values obtained with the Bradford assay may be because this assay primarily reacts with arginine residues. Amino acid analysis of the SAM from *A. actinomycetemcomitans*, *Por. gingivalis* and *Prev. intermedia* revealed that there were very few arginine residues (8, 6 and 0 respectively) in these preparations. The Lowry assay primarily detects tyrosine and tryptophan residues which may account for the higher protein values obtained when either the Lowry or the Bio-Rad DC kit was used. However, the tyrosine content was found to be very low for all the three SAM preparations analysed. The Bio-Rad DC kit gave the greatest protein values for the all of the SAM preparations, therefore this assay was used in all subsequent studies in this thesis.

SDS-PAGE analysis of the SAM preparations after staining gels with colloidal Coomassie blue revealed a large number of protein bands ranging from <14 kDa to > 66 kDa. The SAM from *Por. gingivalis* appeared to only have a few bands which were visible on the gels stained with colloidal Coomassie blue stain. Although the SAM from *C. rectus* only had a protein content of 11 % (w/w), a large number of protein bands were seen on the SDS-PAGE gel. This perhaps suggests that none of the assays employed for the estimation of the protein content of this preparation was suitable, therefore alternative assays need to be found for this particular preparation. The LAP from *Por. gingivalis* only revealed the presence of one major band at 17 kDa, however, the LAP from *Prev. intermedia* showed two major bands at 20 and 45 kDa as well as two minor bands.

Cation exchange HPLC of the SAM from *A. actinomycetemcomitans*, *Por. gingivalis* and *Eik. corrodens* showed that the majority of the proteins failed to bind to the column indicating that these proteins were anionic in nature under the conditions employed. Hence, anion exchange HPLC was performed on each of these preparations. The anion exchange elution profiles of the SAMs from *A. actinomycetemcomitans*, *Eik. corrodens* and *Por. gingivalis* revealed

that the proteins present in these preparations were strongly anionic. This was particularly so for the proteins present in the SAM from *Por. gingivalis*. It was found that some degree of separation of these proteins was attainable under the conditions used. This has been utilised in subsequent chapters for the isolation of individual proteins.

The accepted paradigm is that the bacterial capsule is rich in carbohydrate. However, analysis of the carbohydrate content of the SAM revealed only between 1 and 18 % using the commonly used assay developed by Dubois and co-workers (1956). After performing spectral scans of the SAMs following phenol-sulphuric acid treatment it was revealed that the SAMs from *A. actinomycetemcomitans* and *Por. gingivalis* gave a maximum absorbance of 480 nm, whereas the SAM from *Prev. intermedia* and *C. rectus* absorbed maximally at 485 nm. The carbohydrates which absorb maximally at 480 nm after phenol-sulphuric acid treatment are D-galacturonic acid, D-glucarone, L-arabisone, D-xylose, L-rhamnose and L-fucose indicating that the SAMs from *A. actinomycetemcomitans* and *Por. gingivalis* may have contained one or more of these sugars. D-glucose, and D-mannurone peak at 485 nm following phenol-sulphuric acid treatment, suggesting that the carbohydrates present in *Prev. intermedia* and *C. rectus* SAM may contain either one or both of these sugars.

The LAP from *A. actinomycetemcomitans* contained only 2 % lipid, whereas the lipid contents of the LAP from *Por. gingivalis* and *Prev. intermedia* were 85 % and 70 % respectively. This high lipid content in the LAPs from both *Por. gingivalis* and *Prev. intermedia* caused the protein bands to smear whilst running SDS-PAGE gels. Therefore the SDS concentration was increased to prevent this. In contrast to the LAPs, the percent lipid content of the SAMs ranged from only 4 % for *A. actinomycetemcomitans* to 22 % for the SAM from *Prev. intermedia*.

The nucleic acid content based solely on OD_{260}/OD_{280} nm ratios of each of the SAM and LAP preparations ranged from approximately 0.5 % for the LAP from *Por. gingivalis* to 14.3 % for the SAM from *Eik. corrodens*.

3.7 Conclusion

The percentage total composition of each SAM preparation was calculated and the following values were obtained:- 93% for *A. actinomycetemcomitans*; 98 % for *Eik. corrodens*; 78 % for *Por. gingivalis*; 62 % for *Prev. intermedia*, and 18 % for *C. rectus*. For the majority of the SAM preparations the percentage total composition was found to be above 60 %. This was with the exception *of C. rectus* where the percentage total composition was found to be only 18 %. It is difficult to account for the low percentage total composition of this SAM. One reason may be that this preparation may have very low amounts of tyrosine, tryptophan and arginine residues, therefore giving low readings in all of the protein assays. However, this needs to be further investigated. The percentage total composition was calculated using the protein concentrations determined by the Bio-Rad DC kit. In general this assay gave the greatest protein values therefore it was used in subsequent investigations throughout this study.

Chapter 4

Part 1:The osteolytic activity of the bacterial surface components

4.1 Introduction

The role of bacteria in the initiation of periodontitis is well-documented, and the ensuing destruction of the tissues of the periodontium is readily observed (Page, 1991). However, the events occurring between bacterial colonisation and tissue destruction remain obscure. As there is very little evidence that bacteria invade the tissues of the periodontium and reach the alveolar bone, it has been postulated that diffusable bacterial components or products are the cause of the bone destruction observed in the periodontal diseases.

In previous studies by Wilson and colleagues (1985; 1988; 1993b) and Meghji and colleagues (1994) only the SAM from *A. actinomycetemcomitans*, *Eik. corrodens* and *Por. gingivalis* were assessed for their ability to stimulate bone resorption in the murine calvarial bone resorption assay. These investigations showed that the SAMs from *A. actinomycetemcomitans* and *Eik. corrodens* were extremely potent stimulators of calvarial bone resorption with significant activity being observed at concentrations as low as 1-10 ng/ml (w/v). In contrast, the SAM from *Por. gingivalis* was not as active, only stimulating mouse calvarial bone resorption at concentrations in excess of 1 μg/ml. This investigation has been expanded by determining the comparative osteolytic activity of the SAM from five Gram-negative bacteria implicated in the pathology of periodontal disease: *A. actinomycetemcomitans*, *Por. gingivalis*, *Eik. corrodens*, *Prev. intermedia* and *C. rectus*. In addition the osteolytic activity of the LPS from *A. actinomycetemcomitans*, *Eik. corrodens*, *Por. gingivalis* and *Prev. intermedia* has been investigated.

The LAP family of cell surface-associated molecules has not previously been assessed for bone resorbing activity. Therefore, for the first time, the osteolytic activity of LAP from *A. actinomycetemcomitans*, *Por. gingivalis* and *Prev. intermedia* has been studied.

4.2 Aims

The aims of this part of the study were to determine whether the SAM, LAP and LPS from *A. actinomycetemcomitans*, *Por. gingivalis* and *Prev. intermedia* possessed the ability to stimulate bone resorption *in vitro*. Additionally, the osteolytic potential of the SAM and LPS from *Eik. corrodens* as well as the SAM from *C. rectus* was investigated.

4.3 Materials and Methods

The mouse calvarial bone resorption assay was performed as described in chapter 2. The calvaria was incubated with SAM, LAP and LPS from the periodontopathogens and the calcium released after 48 h measured. The control was to culture calvaria with media alone.

Statistical analysis

The data obtained are expressed as the mean $\pm$ 2SD, n=5. The calcium released by calvaria stimulated with SAM, LAP and LPS from the periodontopathogens was compared with calcium release from the control calvaria (media alone) using Students t-test.

4.4 Results

4.4.1 Osteolytic activity of the SAM from five periodontopathogens

The SAM preparations from the 5 bacteria demonstrated a wide range of osteolytic activity in terms of potency and efficacy (figure 4.1). The SAM from *Eik. corrodens* was the most potent with a capacity to stimulate 50 % of the maximum activity from calvaria at a concentration of 1 ng/ml. A bell-shaped dose response was observed with a loss of response at concentrations greater than 1 μg/ml. The SAM from *A. actinomycetemcomitans* produced a linear dose response over the concentration range 10 ng/ml to 10 μg/ml. In contrast, the SAM from the other bacteria were both less potent and less efficacious.

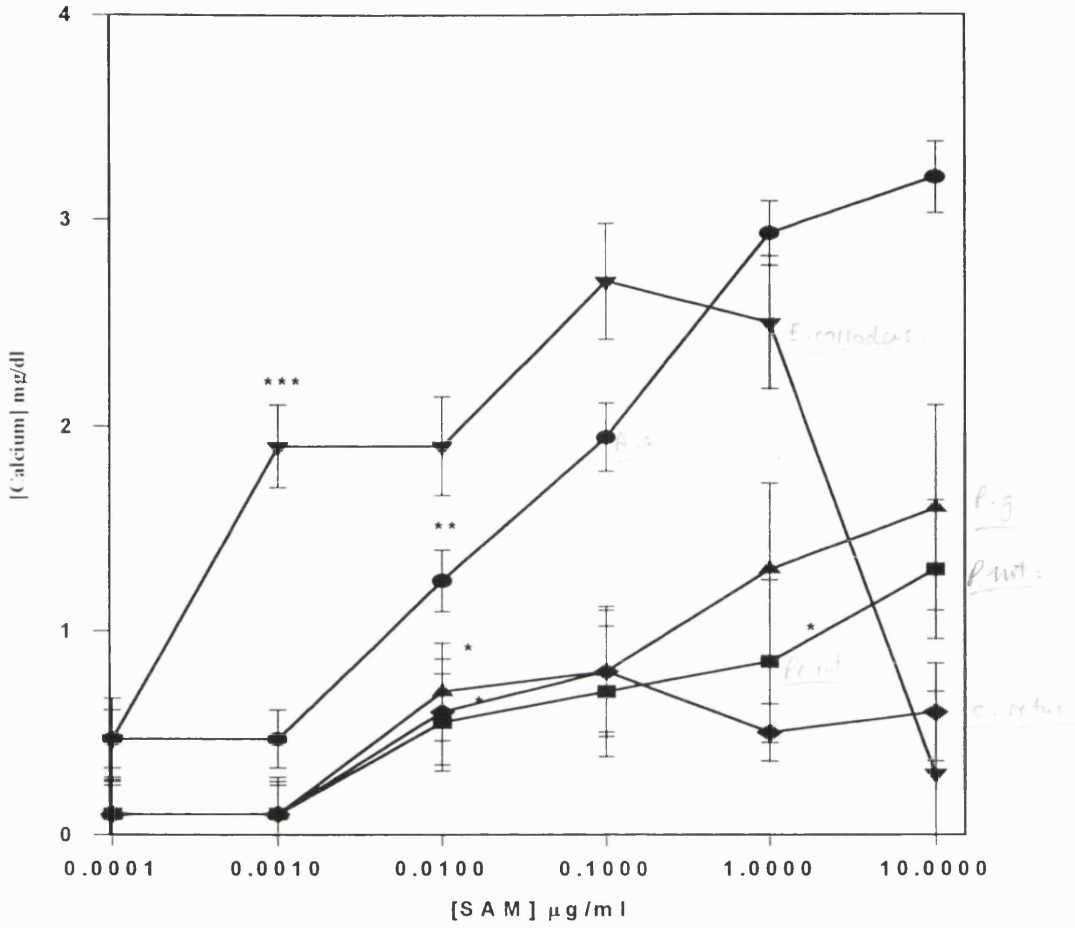


Figure 4.1 **The osteolytic activity of the SAM from five oral bacteria over the concentration range 100 pg/ml to 10 µg/ml. (▼) *Eik. corrodens*; (●) *A. actinomycetemcomitans*; (■) *Prev. intermedia*; (▲) *Por. gingivalis* and (◆) *C. rectus*. The SAMs from *Eikenella corrodens* and *A. actinomycetemcomitans* possessed potent osteolytic activity. The results are expressed as the mean ± 2SD of five replicate cultures. *p<0.05, **p<0.01, ***p<0.001.**

4.4.2 Osteolytic activity of the LAP from three periodontopathogens

LAP from *A. actinomycetemcomitans*, *Por. gingivalis* and *Prev. intermedia* all showed similar and reproducible osteolytic dose responses over the concentration range 10 ng/ml to 10 µg/ml (figure. 4.2).

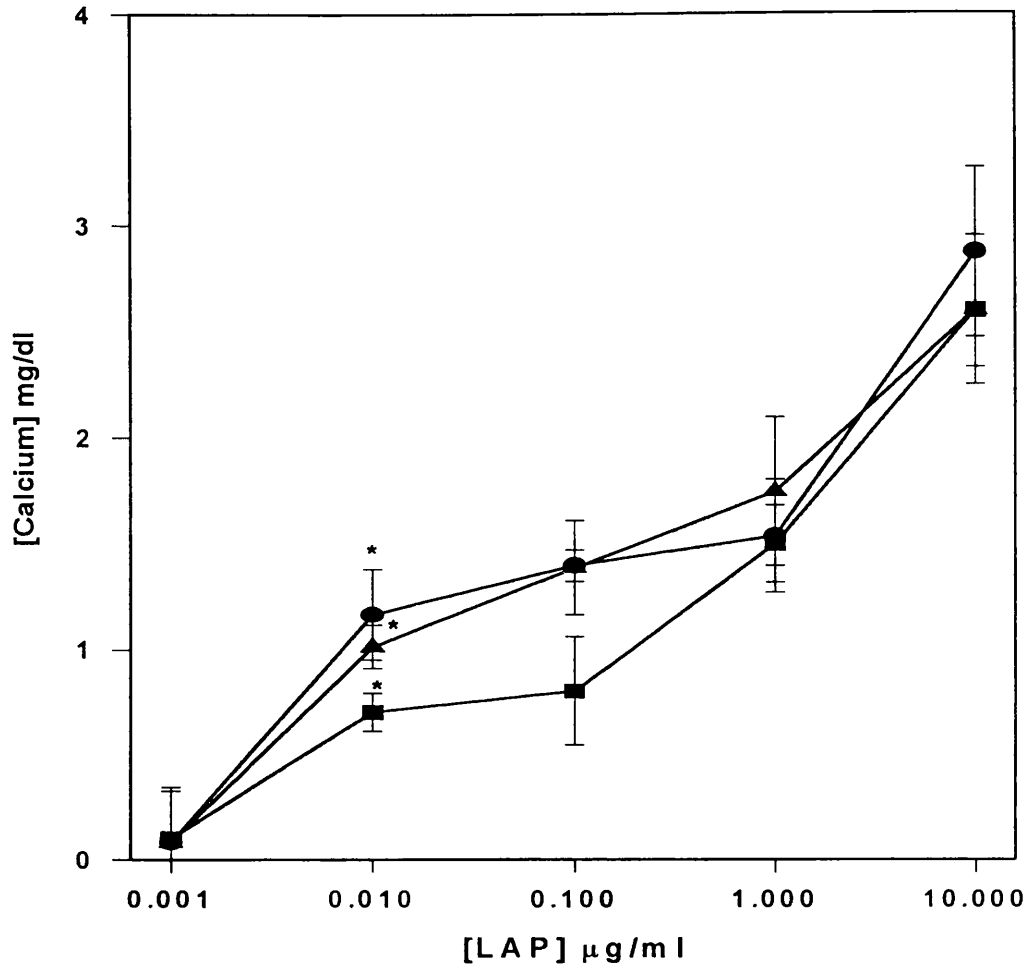


Figure 4.2 **The osteolytic dose response of the LAP from three oral bacteria over the concentration range 1 ng/ml to 10 µg/ml. (■) *Prev. intermedia;* (●) *A. actinomycetemcomitans;* (▲) and *Por. gingivalis.* All three LAPs showed similar and reproducible osteolytic dose responses over the concentration range measured. The results are expressed as the mean ± 2SD of five replicate cultures. *p<0.05.**

At 10 µg/ml (w/v) all three LAP preparations produced the same maximal response. Statistically significant osteolytic activity was observed at concentrations as low as 10 ng/ml (w/v) for all three preparations.

4.4.3 Osteolytic activity of the LPS from four periodontopathogens

The LPS from *A. actinomycetemcomitans*, *Por. gingivalis*, and *Prev. intermedia* were unable to stimulate calvarial bone resorption over the concentration range tested. In contrast, LPS from *Eik. corrodens* stimulated bone resorption in a

concentration-dependent manner, however, the activity was not as potent as the SAM from this organism.

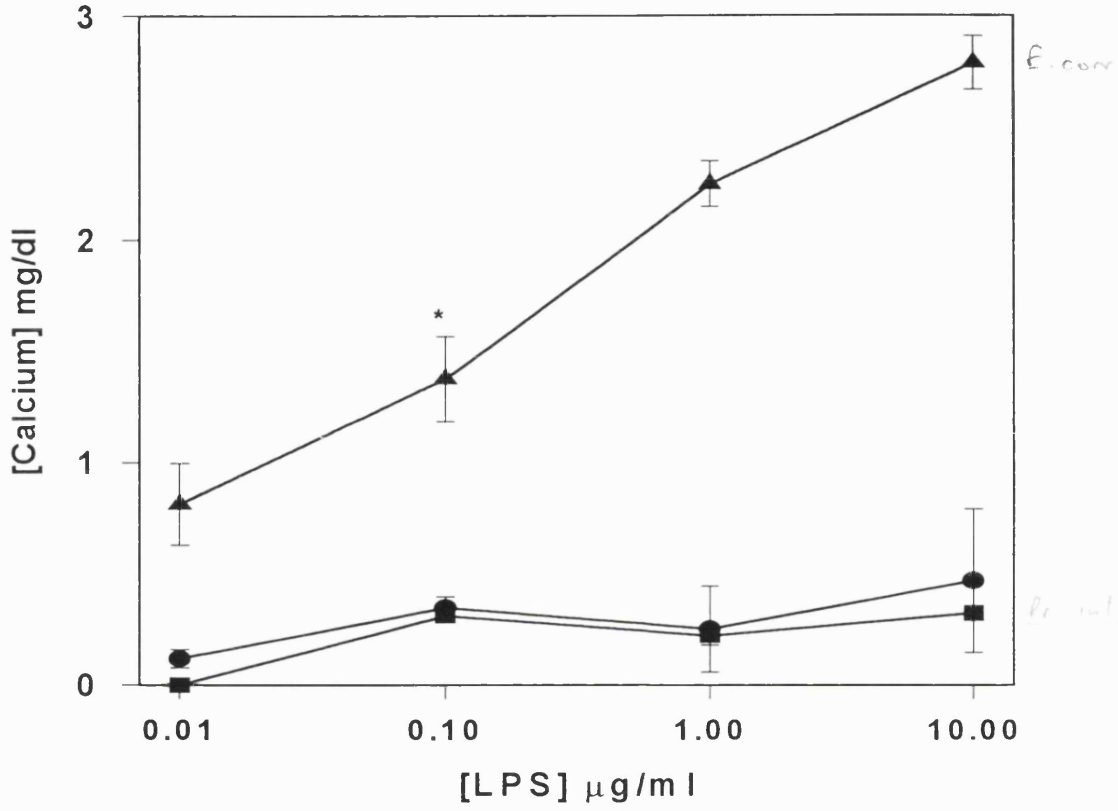


Figure 4.3 **The osteolytic activity of the LPS from four oral bacteria over the concentration range 10 ng/ml to 10 μg/ml. (▼) *Eik. corrodens*; (●) *A. actinomycetemcomitans*; (■) *Prev. intermedia* and (▲) *Por. gingivalis*. Only the LPS from *Eik. corrodens* possessed the ability to stimulate mouse calvarial bone resorption. The results are expressed as the mean ± 2SD of five replicate cultures. *p<0.05.**

4.4.4 Osteolytic activity of E. coli LPS

Unlike the LPS from the periodontopathogenic bacteria, highly purified *E. coli* LPS proved to be an extremely potent stimulator of calvarial bone resorption over the concentration range 10 pg/ml to 1 ng/ml. The dose response was reproducibly bell-shaped (figure 4.4) with a decrease in osteolytic activity at concentrations greater than 1 ng/ml.

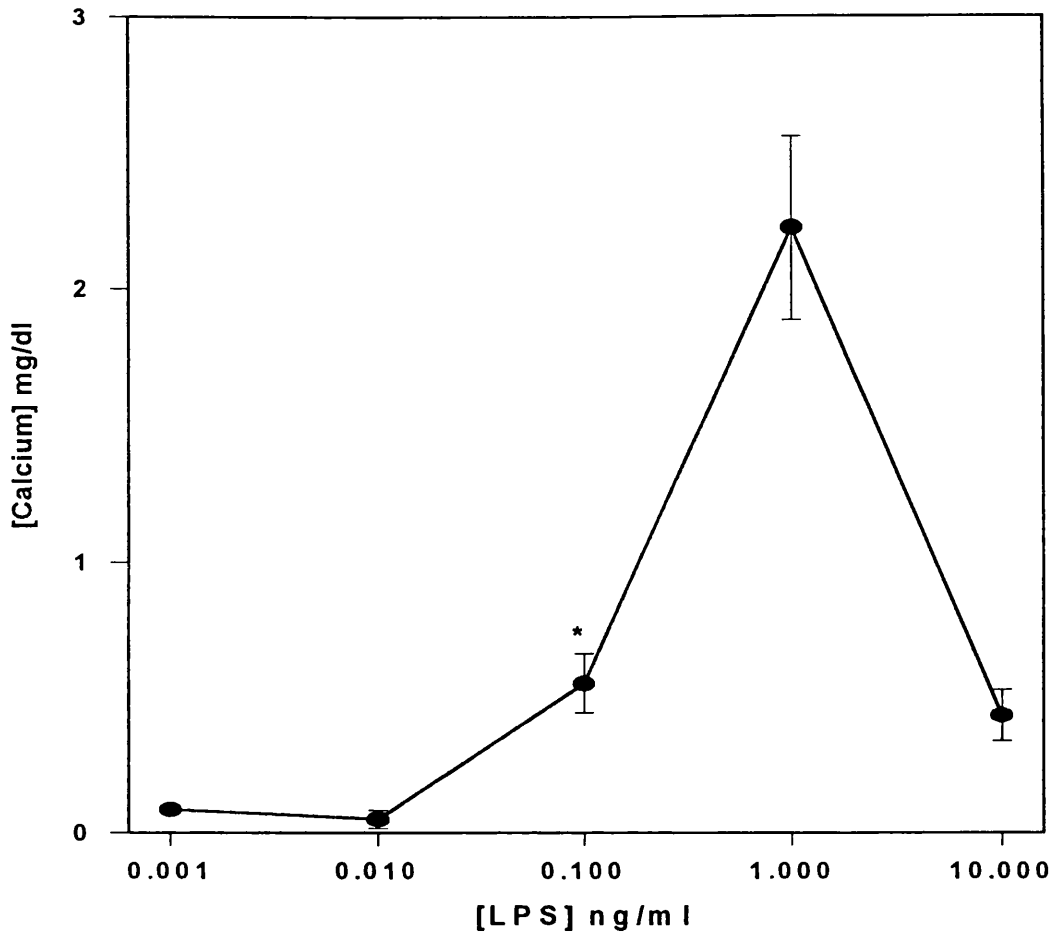


Figure 4.4 **The osteolytic dose response of *E. coli* LPS (NIBSC international standard 84/650) over the concentration range 1 pg/ml to 10 ng/ml. *E. coli* LPS possessed potent osteolytic activity at a concentration of only 1 ng/ml. The results are expressed as the mean ± 2SD of five replicate cultures. $p<0.05$.**

4.4.5 Heat and trypsin treatment

Heat- and trypsin-treatment of the SAM from all bacteria produced a significant loss of osteolytic activity, in the range of 70-100 % (table 4.1). In particular, the osteolytic activity of the SAMs from both *Prev. intermedia* and *C. rectus* was completely destroyed by both heat and trypsin treatment.

Heat- and trypsin-treatment of the LAP from *A. actinomycetemcomitans* and *Por. gingivalis* produced only a small decrease in activity. In contrast, the LAP from *Prev. intermedia* was more sensitive both to heat and trypsin showing a 30-40 % inhibition of activity.

Table 4.1 **Effect of heat (100^0 C for 1 h) or trypsin-treatment on the osteolytic activity of the five SAM preparations. The results are expressed as the mean of the net calcium release (mg/dl) ± 2SD of five replicate cultures. *p,0.05, **p<0.01, ***p<0.0001, ****p<0.0001.**

Periodontopathogen	Untreated	Heat-treated	Trypsin-treated
Eik. corrodens	2.5±0.17	*0.48 ± 0.13	*0.48 ± 0.14
A. actinomycetemcomitans	2.66±1.1	****0.28 ± 0.09	****0.57 ± 0.21
Por. gingivalis	1.15±0.3	*0.35 ± 0.1	*0.33 ± 0.09
Prev. intermedia	1.18 ±0.9	*0	*0
C. rectus	0.95 ±0.2	**0	***0
Prostaglandin E_2	3.39 ±1.2	-	-

4.5 Discussion

Resorption of the alveolar bone is the defining characteristic of periodontitis. The mechanism of resorption is not established but it is thought that components from Gram-negative bacteria are responsible.

In this part of the study the capacity of the SAM, LAP and LPS from five oral organisms implicated in the pathology of chronic periodontitis to stimulate bone resorption has been determined. The most potent inducer of calvarial bone resorption was the SAM from *Eik. corrodens* which, interestingly, showed a bell-shaped response with significant resorption of bone at a concentration of 1 ng/ml. A similar bell-shaped response was observed with highly purified *E. coli* LPS. This bell-shaped dose response may be due to desensitization or destruction of bone cell receptors, however, further investigation is required to confirm this.

A. actinomycetemcomitans also contained a potent osteolytic component in its SAM and demonstrated a similar maximum response to that produced by *Eik. corrodens*. In contrast, the other three periodontopathogenic bacteria contained SAMs which were either significantly less potent or efficacious than those just described. Efficacy is a parameter defined by Stephenson in 1956 which describes the 'strength' of a single agonist-receptor complex in evoking a response. Agonists can have similar potencies but different maximal

responses ie. efficacies. The SAM from *Por. gingivalis* was generally less potent than that from *A. actinomycetemcomitans* or *Eik. corrodens* and, as seen in figure 4.1, was less efficacious. The SAMs from *Prev. intermedia* and *C. rectus* showed both lower potency and efficacy in stimulating bone resorption. The SAM from the latter organism only demonstrated approximately 30 % of the response induced by the SAM from *A. actinomycetemcomitans*.

From the assessment of the depletion in osteolytic activity of the SAMs after heat or trypsin treatment it became obvious that bone destruction is due to the presence of protein(s) in these SAMs.

In spite of the significant differences between the potency and efficacy of the SAMs from the five bacteria, when the LAP from three bacteria were assayed they all showed very similar concentration response curves and all had the same degree of efficacy. The LAP from these bacteria were active at a concentration of 10 ng/ml. In agreement with the general findings of Hitchcock and colleagues (1984) heat and trypsin treatment of the LAPs had only a minor decrease in osteolytic activity. As the LAPs from these three oral bacteria have potent bone resorbing actions *in vitro*, they may be one of the mediators of the bone loss which accompanies periodontitis.

The ability of the LPS from *A. actinomycetemcomitans*, *Eik. corrodens*, *Por. gingivalis* and *Prev. intermedia* to resorb mouse calvaria was also determined. The LPS from *Por. gingivalis* and *Prev. intermedia* had very low osteolytic activity, similar to the SAMs from these organisms. The LPS from *A. actinomycetemcomitans* also possessed very little osteolytic activity, whereas the SAM from this organism was a potent stimulator of mouse calvarial resorption. The only LPS which caused significant bone resorption was that from *Eik. corrodens*, however, it was substantially less potent (3 log orders) than the SAM from this organism. The reason for the poor stimulation of bone resorption by LPS from these periodontopathogens is unknown. Many studies have been conducted on the structural requirements of endotoxin for bone resorption to occur (Hausmann *et al.*, 1975; Nishihara *et al.*, 1986). Hausmann and colleagues (1975) have compared the osteolytic potential of various forms

of the endotoxin from *Salmonella minnesota*. They studied the following :- (i). the smooth form which contains the O-specific polysaccharide, (ii). the polysaccharide core and the lipid A, (iii). the LPS from the mutant form, R-345, which is lacking the O-specific polysaccharide but contains most of the polysaccharide core and lipid A, and (iv). the LPS from the mutant from, R-595, which contains only 2 keto-3 deoxyoctonate (KDO) plus lipid A, and finally isolated lipid A. In addition they investigated the bone resorbing potential of lipoteichoic acid and compared it with a preparation free of fatty acids to determine whether LPS-stimulated bone resorption is mediated by prostaglandin synthesis. The reason for investigating this was because prostaglandins are fatty acid derivatives and are known to stimulate bone resorption. The conclusions of this work were that the preparations which contained the lipid A portion in their LPS were osteolytic. They also found that the preparations which contained the polysaccharide core also possessed some bone resorbing activity. It has been previously shown that fatty acids possess the ability to stimulate bone resorption, and as fatty acids are an essential feature of lipid A, Hausmann and colleagues have concluded that it is this part of the LPS which is responsible for the stimulation of bone resorption. This group postulated that endotoxins, lipoteichoic acids, free fatty acids and prostaglandins react with the same receptor on a cell to initiate resorption. This particular study was performed in 1975, however, some twenty years, on the mechanism of bone resorption induced by LPS has still not been resolved.

In 1980 Sveen and Skaug contradicted the findings of Hausmann and colleagues by demonstrating that the polysaccharide part of the LPS was far more active at stimulating bone resorption than the lipid A portion. This observation was particularly interesting as the LPS from *Bacteroides fragilis* does not appear to have a typical lipid A structure. As *Por. gingivalis* and *Prev. intermedia* are related to the *Bacteroides* genus it may be that these bacteria also do not posses a typical lipid A structure, hence being poor stimulators of bone resorption. In a recent study by Ni Eidhin and Mouton (1994) the LPSs of 44 strains of the former genus *Bacteroides* were screened and it was found that the profiles of this component were highly varied, however, it was thought that they may share antigenic elements at the lipid A-core level.

The majority of studies which have investigated the osteolytic potential of LPS have used very high concentrations of this component, usually 10 μg/ml and above. However, in this thesis the concentrations of LPS used were in the range of 10 ng/ml to 10 μg/ml. Therefore, the results obtained from this study cannot be compared with other investigations. Very few studies have looked at the bone resorbing potential of LPS (Iino and Hopps, 1984; Bon-Van Noorloos *et al.*, 1990; Nishihara *et al.*, 1994), however, even fewer have investigated the osteolytic activity of other bacterial components (Millar et al ., 1986; Gopalsami *et al.*, 1993). This is the first study in which the osteolytic activity of the SAM, LAP and LPS from periodontopathogens have been determined and compared.

4.6 Conclusion

In conclusion it has been demonstrated that the SAM from certain oral bacteria possess extremely potent osteolytic activity, in particular *Eik. corrodens* and *A. actinomycetemcomitans*. However, not all putative periodontopathogenic bacteria contain such active constituents in their SAM. For example, *C. rectus,* possessed very weak bone resorbing activity. It has also been demonstrated that with the exception of the LPS from *Eik. corrodens*, the LPS from the oral bacteria studies exhibited minimal osteolytic activity.

Part II Purification of the osteolytic component from the SAM of *A. actinomycetemcomitans*

4.7 Introduction

The SAM from *A. actinomycetemcomitans* has been of particular interest at the Eastman Dental Institute for the past decade. This SAM has been shown to have a number of biological activities relevant to periodontal pathology; for example it inhibits collagen synthesis (Meghji *et al.*, 1992b) and cellular proliferation (Meghji *et al.*, 1992c; White *et al.*, 1995). However, as demonstrated previously by Wilson and colleagues (1985) and in part I of this chapter, *A. actinomycetemcomitans* also has a potent osteolytic component within its SAM. The purification and characterisation of this osteolytic molecule would provide a means of investigating the mechanism by which the SAM from this organism stimulates bone resorption *in vitro*.

4.8 Aim

The aim of this part of the study was to purify the osteolytic component present in the SAM of *A. actinomycetemcomitans*.

4.9 Materials and Methods

The osteolytic component from the SAM from *A. actinomycetemcomitans* was isolated by using anion exchange HPLC and the fractions obtained screened using the mouse calvarial bone resorption assay as described in chapter 2. Briefly, an anion exchange MA7Q (Bio-Rad) HPLC column (5 cm x 0.78 cm) was equilibrated with 20 mM Tris buffer pH 8.5 (buffer A). Aliquots (500 μl) of 1 mg/ml SAM in buffer A were injected onto the column and eluted with 8 ml of buffer A, followed by a linear gradient from 0-50 % buffer B (buffer A + 2 M NaCl) in 30 ml, 50-100 % buffer B in 15 ml volume and finally 6 ml of buffer B. The flow rate was 1 ml/min and 1 ml fractions were collected in Eppendorf tubes with the protein absorbance being monitored at 280 nm. Fractions were dialysed against distilled water for 48 h, lyophilised and the biological activity of each fraction determined.

Statistical analysis

The data obtained are expressed as the mean ± 2SD, n=5. The students t-test was used to compare the data obtained for the calcium released by calvaria stimulated with SAM (and the fractionated SAM) with the control calvaria stimulated with media alone.

4.10 Results

4.10.1 Fractionation of the osteolytic activity from the SAM of A. actinomycetemcomitans

As the SAM from *A. actinomycetemcomitans* was shown not to bind to a cation exchange column (chapter 3), the first purification step used was anion exchange HPLC. The absorbance was monitored at 280 nm and a elution profile was obtained as shown in figure 4.5.

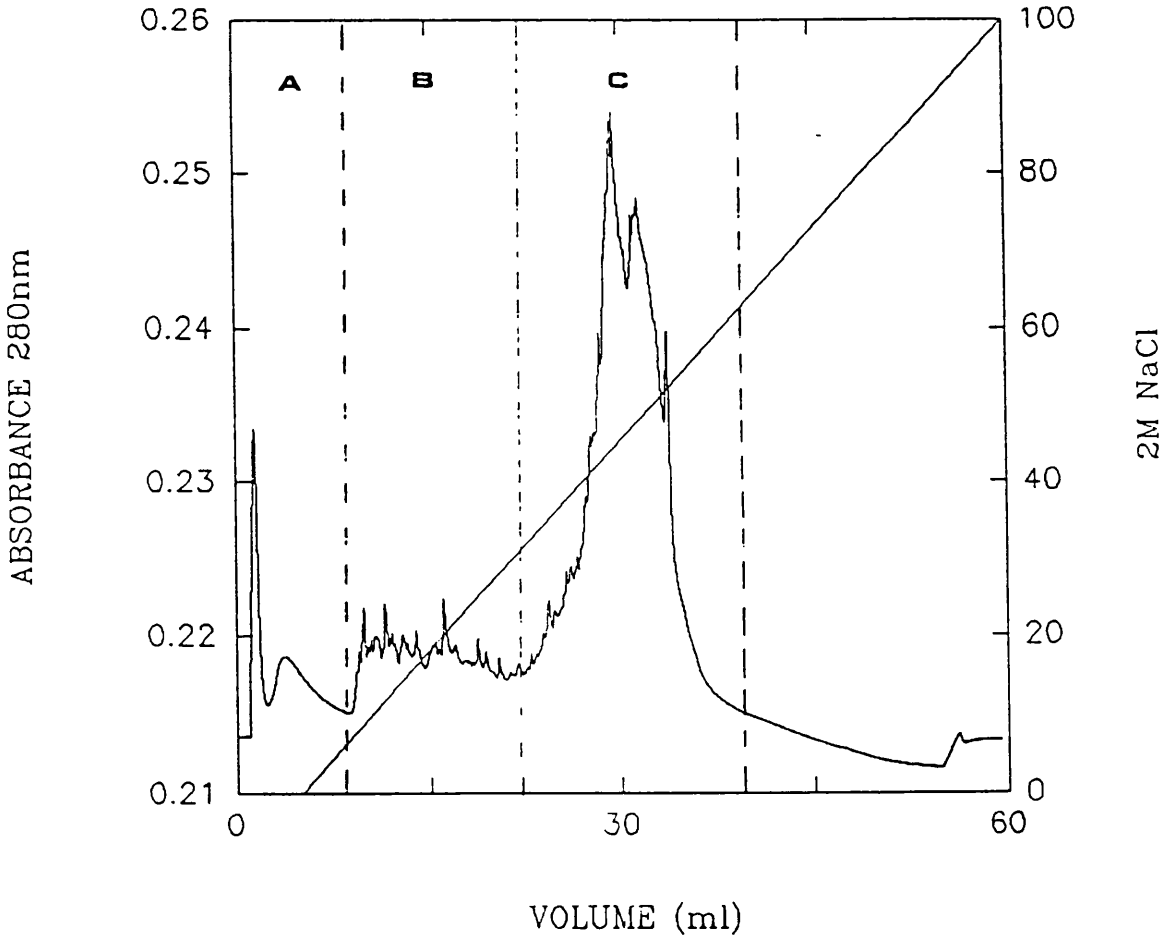


Figure 4.5 **Anion exchange HPLC of the SAM from *A. actinomycetemcomitans* showing the fractions (A, B, C) assessed for osteolytic activity in the mouse calvarial assay.**

As the mouse calvarial assay was very time-consuming and also very expensive, it was not possible to screen the osteolytic activity of all sixty fractions obtained by anion exchange HPLC. Hence the collected fractions were grouped together into three large fractions, A, B and C as shown in figure 4.5 and the osteolytic activity of each determined (figure 4.6).

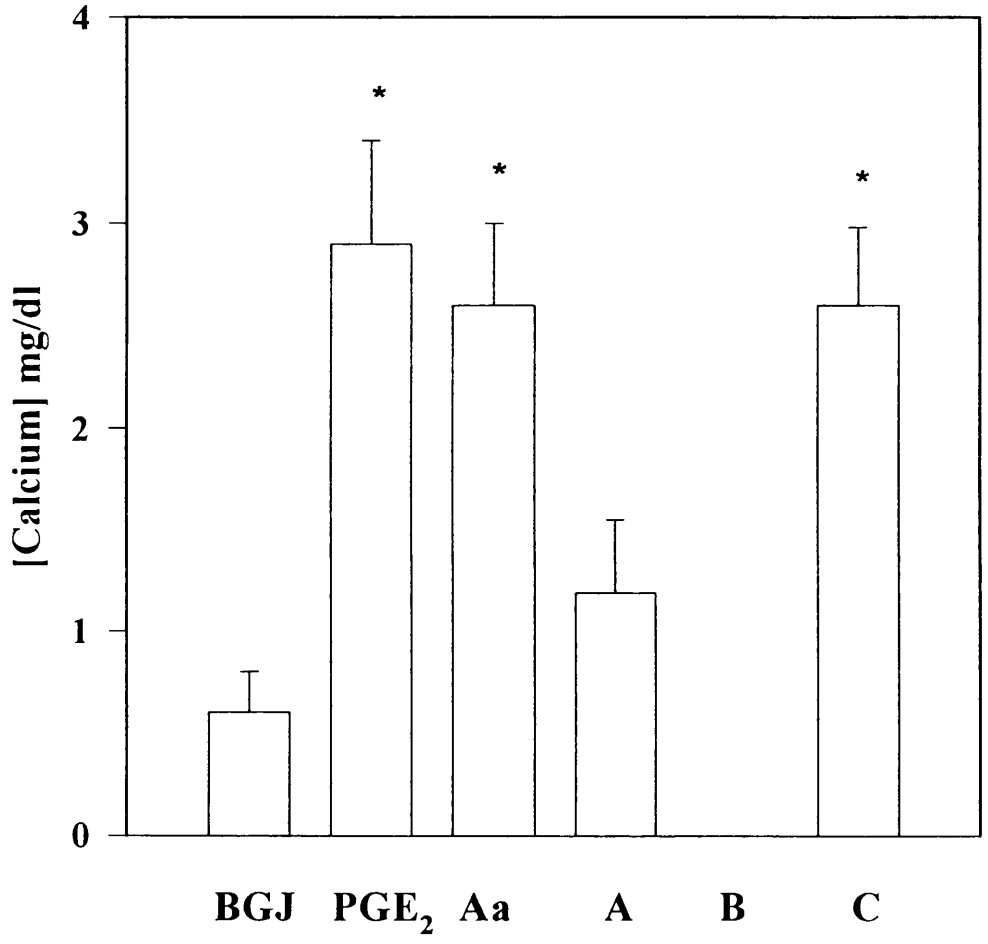


Figure 4.6 **Osteolytic activity of the SAM from *A. actinomycetemcomitans* and fractions A, B and C from anion exchange HPLC. BGJ- media control (basal release); PGE_2- Prostaglandin E_2. Both Prostaglandin E_2 and the SAM (10 μg/ml) were potent stimulators of mouse calvarial bone resorption. Fraction A-significantly stimulated mouse calvaria to release calcium, Fraction B-failed to stimulate mouse calvaria to release calcium and Fraction C- was a potent stimulator of calcium release by mouse calvaria. The results are expressed as the mean + 2SD of five replicate cultures. *$p<0.05$.**

Fraction B failed to stimulate mouse calvaria to release calcium and the group of peaks labeled fraction A did not possess significant osteolytic activity. However, the majority of the osteolytic activity resided in fraction C, therefore this group was further subdivided into fractions C1, C2 and C3 as shown in figure 4.7.

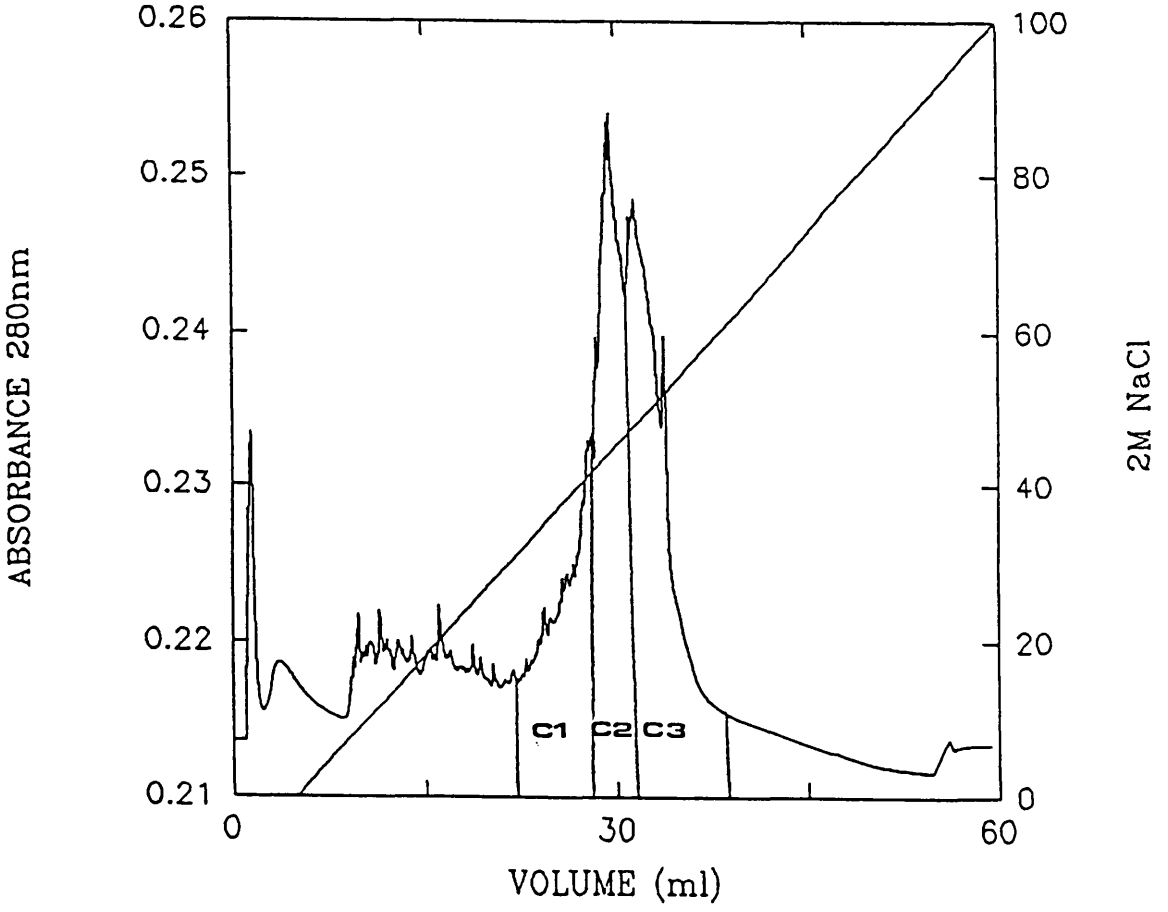


Figure 4.7 **Anion exchange HPLC of the SAM from *A. actinomycetemcomitans* showing the subfractions C1-C3.**

The fractions C1-C3 were each assayed for their osteolytic activity in a concentration-dependent manner from 5 ng/ml to 500 ng/ml (figure 4.8). Significant stimulation of mouse calvarial bone resorption was obtained by fractions C2 and C3 at a concentration of 5 ng/ml. These two fractions were not only more efficacious than the SAM from this organism but also ten times more potent at stimulating mouse calvarial bone resorption. The two fractions C2 and C3 needed to be purified further before the active osteolytic component from the SAM from *A. actinomycetemcomitans* could be isolated.

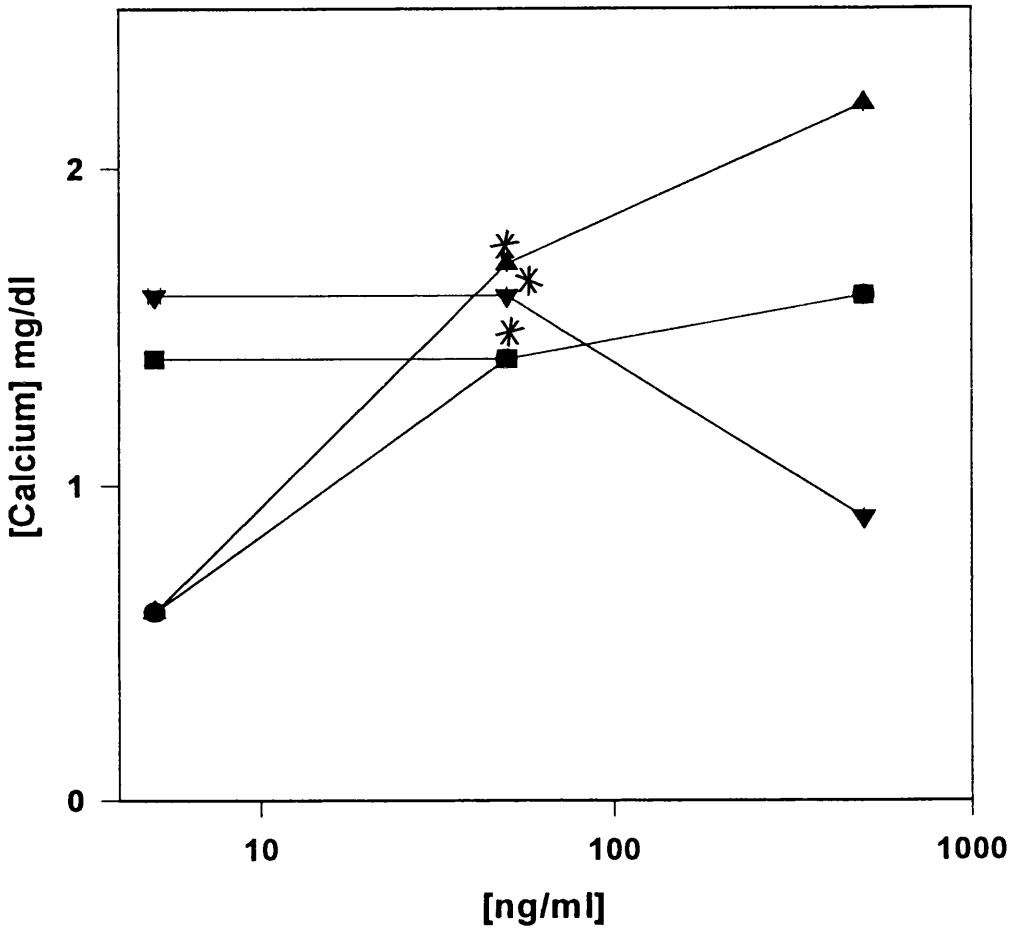


Figure 4.8 **Osteolytic activity of the subfractions C1-C3 over the concentration range 5 ng/ml to 500 ng/ml. (●)- SAM (starting material); (▲) C1; (▲) C2 and (▼) C3. The results are expressed as the mean ± 2SD of five replicate assays. **p<0.01.**

The osteolytic protein from the SAM of *A. actinomycetemcomitans* has since been isolated by Kirby and colleagues (1995) at the Eastman Dental Institute. This osteolytic component has been identified as chaperonin (cpn) 60, a member of the 60 kDa heat shock protein family.

4.11 Discussion

The crude SAM from *A. actinomycetemcomitans* was partially purified by ion-exchange chromatography. A number of protein peaks were obtained, of which the group of peaks labeled fraction C was found to contain most of the osteolytic activity. This group was subdivided, and subgroup C2 was found to contain most of the osteolytic activity. Based on this initial purification strategy, the osteolytic activity has now been purified by Kirby and colleagues (1995) at the Eastman Dental Institute. This osteolytic component was found to be a 62 kDa protein which has 99 % homology with that of *E. coli* GroEL over the first 36 residues of its N-terminal sequence. It was concluded that the SAM from *A. actinomycetemcomitans* contains a homologue of the *E. coli* chaperonin 60 or heat shock protein 60, groEL, and that this protein is responsible for the potent bone resorbing activity of the SAM.

Work at the Eastman Dental Institute by Dr. Sajeda Meghji has revealed that *E. coli* groEL is a potent stimulator of mouse calvarial bone resorption. Consequently, the mechanism by which SAM-induced and *E. coli* groEL-induced bone breakdown occurs is currently under investigation. In collaborative studies with Dr. Tim Arnett in the Department of Anatomy, University College London. Rat osteoclast cultures were incubated with *E. coli* LPS and also groEL from this microorgansim. I have shown that *E. coli* groEL is a very potent stimulator of osteclastogenesis at a concentration range of 1 ng/ml to 1μg/ml. Maximum stimulation occurred at 10 ng/ml of groEL after 24 h incubation. This was also coupled with an increase in the number of resorption pits indicating that the *E. coli* groEL was stimulating the osteoclasts to resorb bone. This work is currently being expanded to identify the mechanism by which SAM-induced bone breakdown occurs and also to determine the receptors involved.

4.12 Conclusion

In this part of chapter 4 it has been demonstrated that the osteolytic protein found in the SAM from *A. actinomycetemcomitans* could be partially isolated by anion exchange HPLC. Kirby and colleagues (1995) have now purified this component and found it to be chaperonin 60. In addition groEL from *E. coli* has been found to be a potent stimulator of calvarial bone resorption and osteoclast pit formation on dentine slices at a concentration as low as 1 ng/ml after 24 h.

Chapter 5

Pro-inflammatory-cytokine-stimulating activity of the SAMs of periodontopathogenic bacteria

5.1 Introduction

The simplest hypothesis to explain the pathology of periodontitis would be that bacterial components or products, released from the subgingival plaque, stimulate cells in the gingiva (both resident cells, such as fibroblasts, and inflitrating leukocytes) to synthesize and release pro-inflammatory cytokines. This leaves open the question as to which bacterial products are likely to contribute to cytokine production. In periodontitis a wide variety of inflammatory cytokines are thought to be stimulated in response to bacterial components, in particular IL-1 β, TNF-α, IL-6 and IL-8 (Bom-Van Noorloos *et al.*, 1990; Hanazawa *et al.*, 1991; 1993). In this context the most studied bacterial component is LPS. Many reports have shown that the LPS from *Por. gingivalis* and *Prev. intermedia* possess the ability to stimulate cytokine release by various cell types (Nair *et al.*, 1983; Bom-Van Noorloos *et al.*, 1990; Sismey-Durrant and Hopps, 1991; Takada *et al* ., 1991; Tamura *et al.*, 1992; Gemmell *et al.*, 1993). However very few reports have looked at the potential of other bacterial components to stimulate cytokine release by cells present in the periodontium (eg. monocytes/macrophages, fibroblasts, Langerhans cells and epithelial cells). To address this question the SAM from four periodontopathogens has been investigated to determine the capacity to induce cytokine synthesis.

5.2 Aims

As described in chapter 4, several of the SAMs from periodontopathogenic bacteria were shown to be potent stimulators of mouse calvarial bone resorption. In an attempt to elucidate the mechanism of bone destruction it was necessary to establish whether the bacterial components possessed the ability to stimulate cytokine release. Therefore the aim of this chapter was to investigate whether the SAMs from four oral bacteria possessed the ability to stimulate human PBMCs, HGFs and the myelomonocytic cell line Mono-Mac-6 cells to release cytokines.

The activities of these bacterial components were compared to the activity of the international standard *E. coli* LPS.

5.3 Materials and Methods

The methods were as described in chapter 2. The cells used in this part of the study were human PBMCs which were obtained from periodontally-healthy volunteers and HGFs from healthy gingival explants. The myelomonocytic Mono-Mac-6 cell line was a kind gift from Dr. Ziegler-Heitbrock and the cytokine assays were a generous gift from Dr. Stephen Poole (NIBSC). Cells were incubated with a range of concentrations of the SAM from 4 periodontopathogens and the release of IL-1β, TNF-α and IL-6 into the media was measured.

Statistical analysis

The data is expressed as the mean $\pm$ 2SD , n=6. The results obtained for the SAM preparations were compared with that of cells exposed to media only, by Students t-test.

5.4 Results

5.4.1 Stimulation of cytokine release from human gingival fibroblasts

Highly purified *E. coli* LPS was a potent stimulator of the release of IL-1β, IL-6 and TNF-α by HGFs with a linear concentration response over the range of 10 pg/ml to 1 ng/ml (figure 5.1).

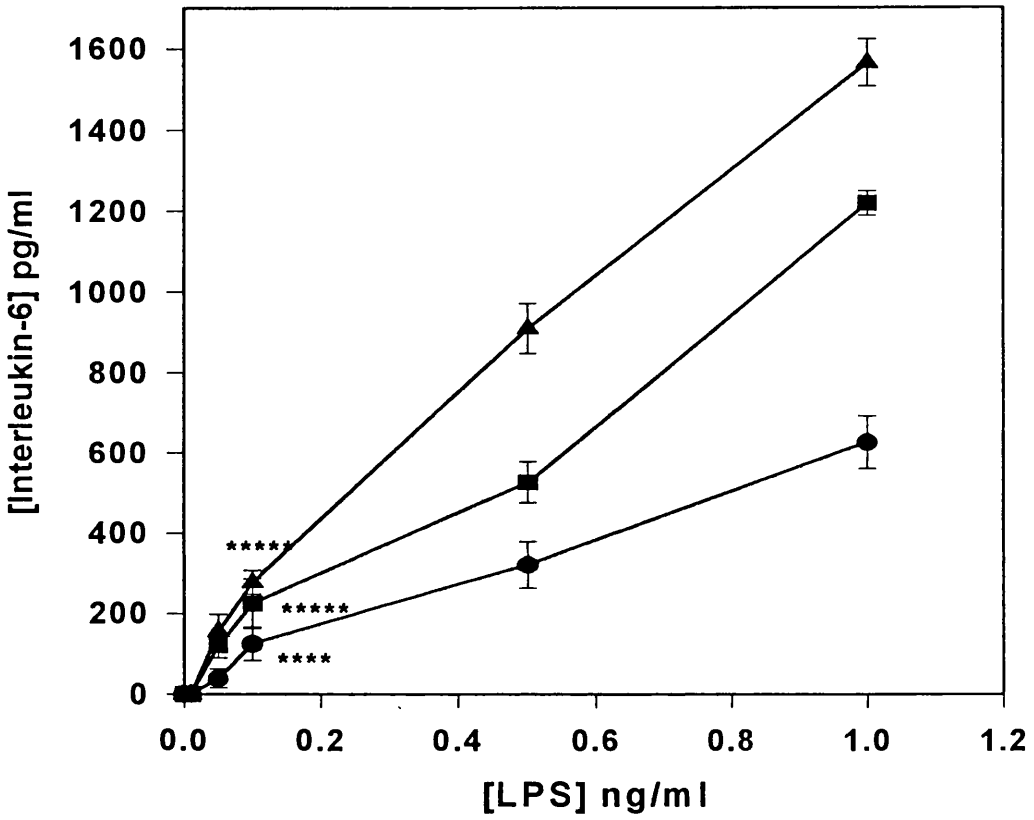


Figure 5.1 **Release of cytokines by HGFs stimulated with *E. coli* LPS (NIBSC 84/650). (▲) TNF-α; (■) IL-1 β; (●) IL-6. *E. coli* LPS was able to stimulate the release of all three cytokines over the concentration range measured. The results are expressed as the mean ± 2SD of six replicate cultures. ****p<0.0001, *****p<0.00001.**

While highly purified LPS was capable of stimulating the release of all three cytokines over the concentration range tested, there were differences in the levels of each cytokine released. TNF-α was the most abundantly produced cytokine and IL-6 the least.

5.4.1.1 IL-6 release

The SAM from *Eik. corrodens* and *Por. gingivalis* produced similar dose responses with significant amounts of IL-6 being released in response to 10 ng/ml of this extract. In contrast, the SAM from *C. rectus* and *Prev. intermedia* were substantially less potent and efficacious at stimulating HGF to release IL-6. Significant IL-6 release was only achieved at concentrations of 1-10 μg/ml with maximal response which was only a few percent of that of the other SAMs (figure 5.2).

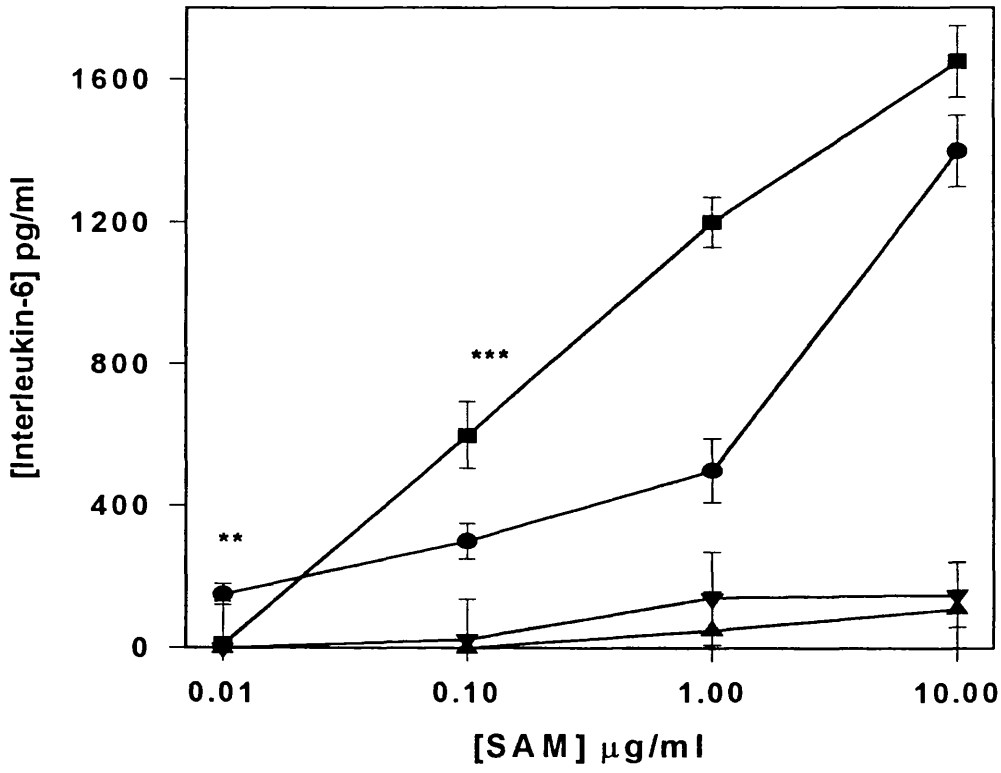


Figure 5.2 **IL-6 release by HGFs exposed to graded concentrations of the SAM from (●) *Eik. corrodens*; (■) *Por. gingivalis*; (▼) *C. rectus* and (▲) *Prev. intermedia*. The SAM from *Eik. corrodens* and *Por. gingivalis* produced similar concentration responses with significant amounts of IL-6 being released in response to 10 ng/ml of this extract. The results are expressed as the mean $\pm$ 2SD of 6 replicate cultures. * *p<0.01, ***p <0.001.**

5.4.1.1.1 Heat and trypsin-treatment of the SAM preparations

Exposure of all the SAMs to trypsin for 30 min completely inhibited cytokine-stimulating activity of the SAMs from *Pr. intermedia* and *C. rectus* (table 5.1).

Table 5.1 **IL-6 activity of the SAMs after treating with trypsin for 30 min. The results are expressed as the mean ± 2SD of six replicate cultures. ****p<0.0001,*****p<0.00001, *******p<0.0000001.**

SAMs	IL-6 release prior to trypsin treatment [pg/ml]	IL-6 release after trypsin treatment [pg/ml]
Eik. corrodens	1390 ± 78	*******9 ± 34
Por. gingivalis	1621 ± 250	*****125 ± 50
Prev. intermedia	110 ± 24	****0
C. rectus	150 ± 18	*****0

5.4.1.2 IL-1 β and TNF-α release

While *E. coli* LPS was able to stimulate HGFs to synthesize IL-1β and TNF-α over the concentration range 10 pg/ml to 1 ng/ml (figure 5.1) none of the SAMs were able to stimulate HGFs to release these cytokines into the culture media.

5.4.2 Stimulation of human PBMC cytokine release

5.4.2.1 IL-6 release

Of the four periodontopathogens studied, SAM from *Eik. corrodens* was the most potent stimulator of PBMC IL-6 release with maximal activity being demonstrated in the concentration range of 10-100 ng/ml. The least active of the four SAMs was that from *Prev. intermedia* which required concentrations of 1-10 μg/ml to achieve a significant release of IL-6. *E. coli* LPS was a potent stimulator of IL-6 release by these cells over the concentration range of 10 - 1000 pg/ml (figure 5.3).

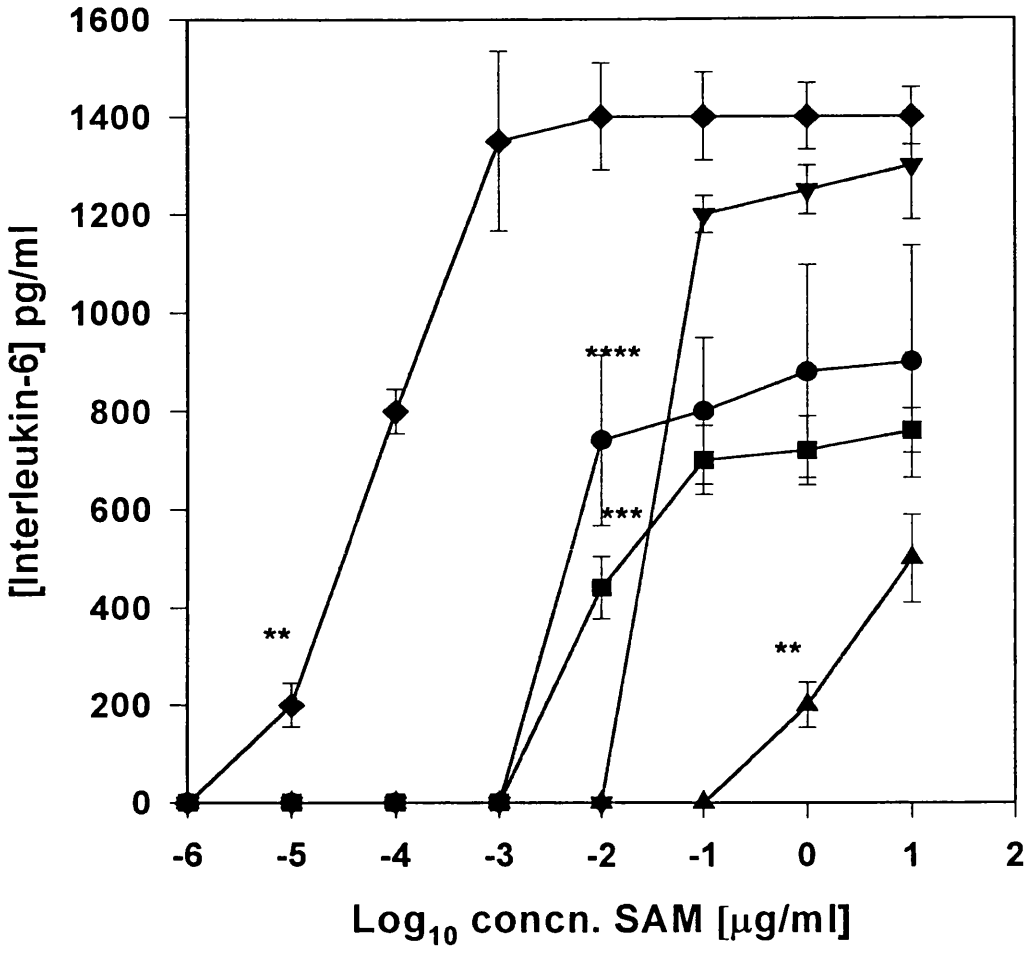


Figure 5.3 **IL-6 release from human PMBCs stimulated with increasing concentrations of LPS from (◆) *E. coli*; or the SAM from (●) *Eik. corrodens*; (■) *Por. gingivalis*; (▼) *C. rectus* or (▲) *Prev. intermedia*. The SAM from *Eik. corrodens* was the most potent stimulator of IL-6 release by PBMC. The results are expressed as the mean ± 2SD of 6 replicate cultures. **p<0.01, ***p<0.001, ****p<0.0001.**

5.4.2.3 IL-1 β release

In general the SAMs were poor stimulators of IL-1β from human PBMCs (figure 5.4). The maximum amount of IL-1β released by these cells was approximately 200 pg/ml when stimulated by 10 μg/ml of SAM from *C. rectus*. The SAMs from both *Eik. corrodens* and *Por. gingivalis* were less efficacious than the SAM from *C. rectus*, and the SAM from *Prev. intermedia* failed to elicit a response.

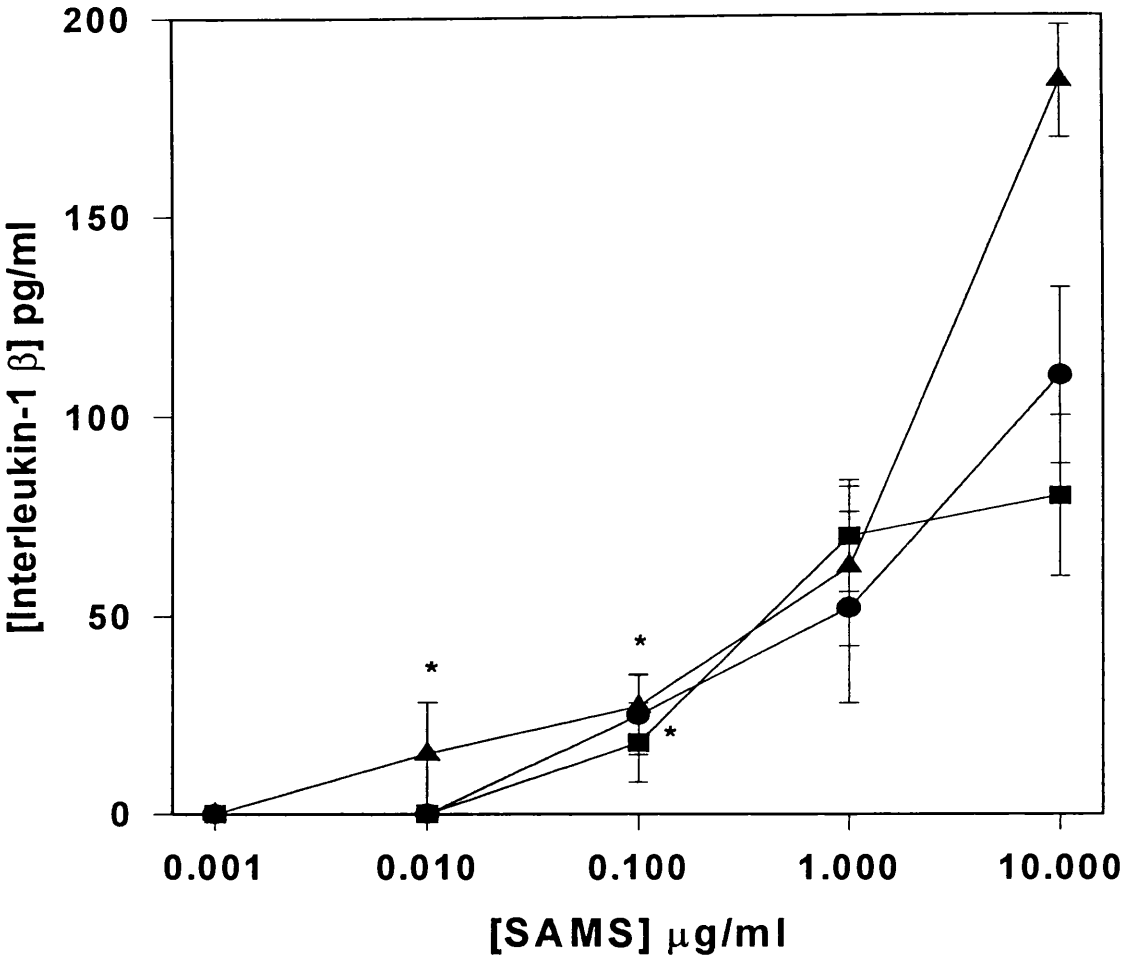


Figure 5.4 **IL-1β release by human PBMCs exposed to increasing concentrations of the SAM from (■) *E. corrodens*; (●) *Por. gingivalis*; or (▲) *C. rectus*. In general the SAMs were poor stimulators of IL-1β release from human PBMCs. The results are expressed as the mean ± 2SD of six replicate cultures. *p<0.05.**

5.4.2.3 TNF-α release

The potency of the SAMs in stimulating TNF-α release was generally lower than their capacity to stimulate IL-6 release, but was greater than their ability to induce IL-1 release. The SAM from *C. rectus* induced a maximal release at 100 ng/ml while the other two SAMs showed lower potency but higher efficacy. The SAM from *Prev. intermedia* was incapable of stimulating TNF-α release (figure 5.5).

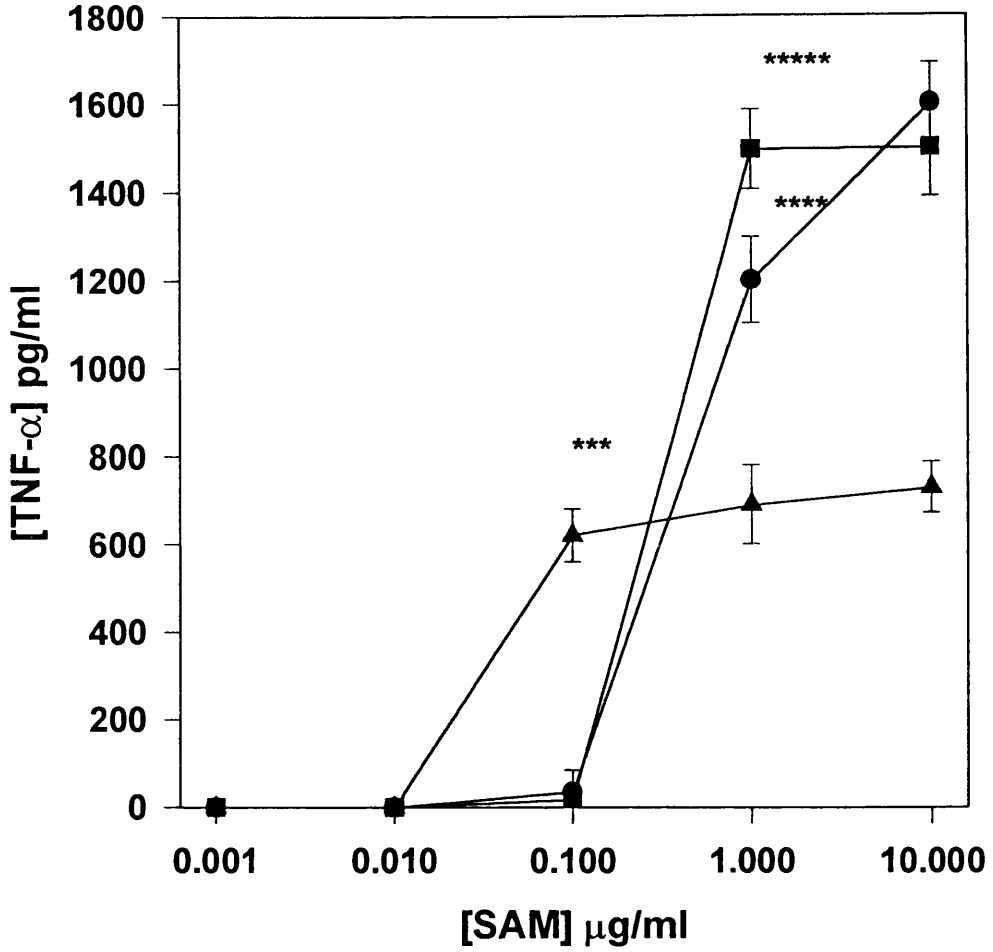


Figure 5.5 **TNF-α release by human PBMCs exposed to increasing concentrations of the SAM from (●) *Eik. corrodens*; (■) *Por. gingivalis* or *C. rectus* (▲). The potency of the SAMs in stimulating TNF-α release was generally lower than their capacity to stimulate IL-6 release but was greater than their ability to induce IL-1 release. The results are expressed as the mean ± 2SD of 6 replicate cultures. ***p<0.001, ****p<0.0001, *****p<0.00001.**

5.4.3 Stimulation of cytokine release from Mono-Mac-6 Cells

5.4.3.1 IL-6 release

The SAMs from *Eik. corrodens*, *Por. gingivalis* and *C. rectus* showed very similar dose responses (figure 5.6). However, the SAM from *Prev. intermedia* (100 ng/ml) was less potent than the others but was more efficacious at stimulating the release of IL-6 by these cells.

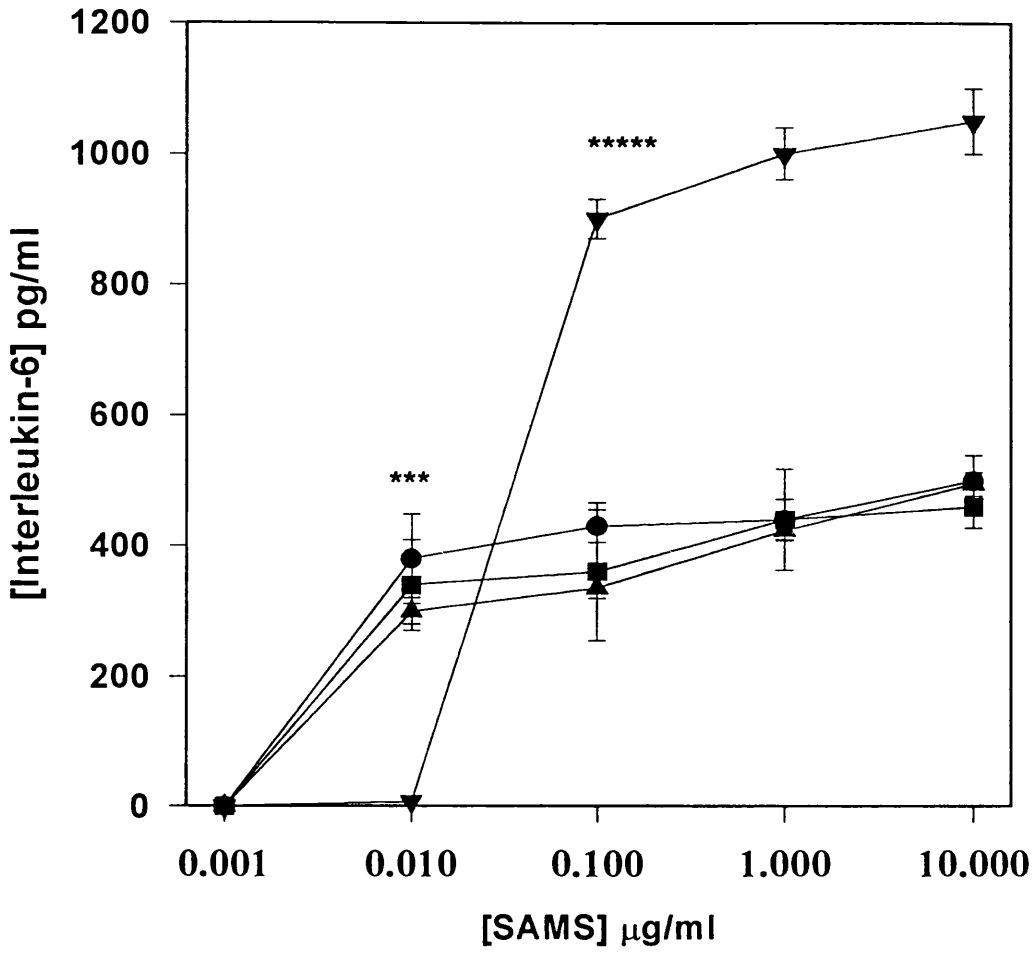


Figure 5.6 **IL-6 release by Mono-Mac-6 cells in response to increasing concentrations of the SAM from (●) *Eik. corrodens*; (■) *Por. gingivalis*; (▲) *C. rectus* or (▼) *Prev. intermedia*. The SAMs from *Eik. corrodens*, *Por. gingivalis* and *C. rectus* showed very similar dose responses. However, the SAM from *Prev. intermedia* was less potent than the others but was more efficacious at 100 ng/ml. The results are expressed as the mean ± 2SD of six replicate cultures. ***p<0.001, *****p<0.00001.**

5.4.3.2 IL-1β and TNF-α release

The Mono-Mac-6 cells were highly responsive to *E. coli* LPS giving a concentration-dependent release of IL-1 β and TNF-α over the dose-range of 10 pg/ml to 1 ng/ml. In contrast, the four SAMs failed to stimulate the release of either of these two cytokines from these cells even at concentrations as high as 10 μg/ml (figure 5.7).

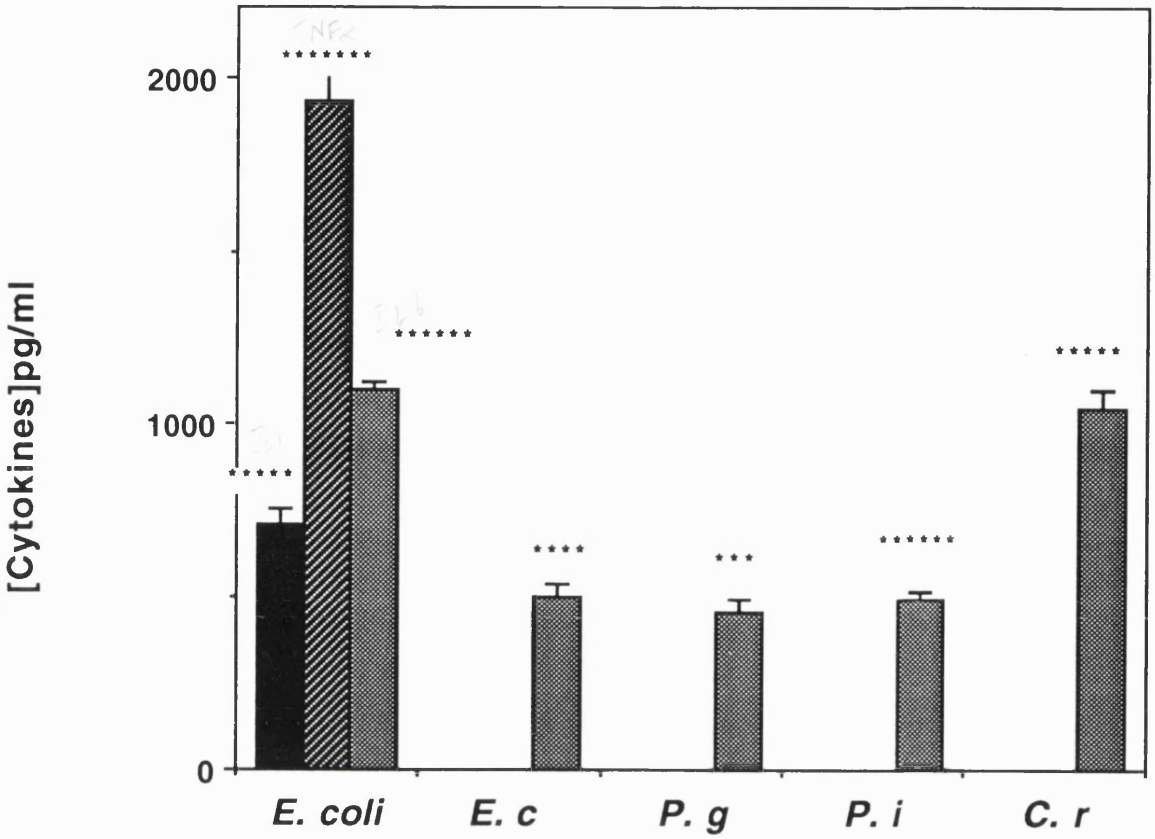


Figure 5.7 **The release of cytokines from Mono-Mac-6 cells exposed to either *E. coli* LPS or the SAMs from the 4 periodontopathogenic bacteria to show the pattern of cytokines induced. LPS was used at a concentration of 1 ng/ml and the SAMs at a concentration of 10 μg/ml. The results are expressed a as the mean + 2SD of six replicate cultures. The filled columns represent IL-1 β; the hatched columns TNFα and the dotted columns represents IL-6. ***p,0.001, ****p<0.0001, *****p<0.00001, ******p<0.000001, *******p<0.0000001.**

5.5 Discussion

Bacterial components in the periodontal pockets of patients suffering from periodontitis may elicit inflammation and tissue destruction by provoking the release of pro-inflammatory cytokines from various cell populations. This subsequently activates one or more of the degradative pathways discussed in chapter 1. Target cells for these mediators are not only inflammatory cells (PMNs and macrophages) but also the indigenous mesenchymal cell populations such as fibroblasts and osteoblasts. There is now a substantial body of evidence in support of the hypothesis that pro-inflammatory cytokines such as IL-1β and TNF-α are key mediators of chronic inflammatory disease associated with tissue destruction (Birkedal-Hansen, 1993b). The biological effects of IL-1 that are directly

relevant to periodontal attachment loss include induction of stromelysin and collagenase (Saito *et al.*, 1990a) and bone demineralisation (Gowen and Mundy, 1986). IL-1 activity has been identified in gingival crevicular fluid sampled from clinically inflamed sites in human subjects (Charon *et al.*, 1982) and in peripheral blood monocyte culture supernatants from periodontitis patients. In this thesis the release of the beta form of IL-1 was measured as opposed to the alpha form. IL-1 β has been detected in gingival crevicular fluid (GCF) in patients with moderate to advanced periodontitis (Masada *et al.*, 1990). This group found that IL-1β was secreted into GCF at 34 % more sites than IL-1α. In addition the IL-1 level in GCF reduced significantly following treatment which indicated that a relationship existed between active disease and IL-1 production. In a similar study by Kabashima and co-workers (1990) IL-1α was found to be the predominant form of IL-1 found in GCF. However, this appeared to depend on the type of assay employed to monitor the IL-1. If an ELISA was used then IL-1β was detected to a greater extent in GCF obtained from periodontally diseased sites. Jadinski and co-workers (1991) have found that the predominant form of IL-1 is the beta form and have localised it to the lamina propria which contains cells such as macrophages, fibroblasts and endothelial cells. It seems that the majority of studies have observed the beta form of IL-1 in GCF and in periodontal tissues, therefore IL-1 β was measured rather than to the alpha form in this study.

The role of IL-6 is more ambiguous as it may act to downregulate some of the actions of IL-1 and TNF (Akira and Kishimoto, 1992). However, in its own right IL-6 is a potent stimulator of B cell differentiation and growth (Akira and Kishimoto, 1992) and of osteoclast differentiation and bone resorption (Roodman, 1992). IL-6 is produced by activated T-cells in addition to IL-1 secreting cells such as activated monocytes/macrophages, endothelial cells, fibroblasts and bone cells (Walther *et al.*, 1988). Small amounts of IL-6 have also been shown to work synergistically with IL-1 to induce bone resorption *in vitro* (Ishimi *et al.*, 1990). It was found by Reinhardt and colleagues (1993) that the levels of IL-6 and IL-1 α and β were significantly elevated in refractory periodontitis subjects. It has also been found

that IL-6 levels are increased in diseases displaying bone resorption other than periodontitis, for instance, elevated IL-6 serum levels in rheumatoid arthritis.
In periodontitis IL-1 and IL-6 have found to be present locally in the gingival tissues and gingival crevicular fluid (Kjeldsen *et al.*, 1993). The major question which this finding raises is, what is the cause of such stimulation of cytokine gene transcription? The most obvious cause is the large number of Gram-negative bacteria present in the subgingival plaque which are considered to be the aetiological agents of these diseases. As these bacteria do not invade the periodontium, the stimulus for cytokine production must be their solubilised components or secretory products, which can diffuse into the gingiva and associated periodontal tissues and bind to autochthonous cells or infiltrating leukocytes.

A few studies have been carried out in which PMNs or gingival fibroblasts have been stimulated with various periodontopathogens and cytokine release measured (Yamazaki *et al.*, 1989; McFarlane *et al.*, 1990; Gemmel *et al.*, 1993), whereas other studies have merely concentrated on LPS-stimulated cytokine release (Reinhardt *et al.*, 1993). However, the present study is the first in which the ability of the surface material of periodontopathogens to stimulate cytokine release by various cell types has been investigated. This study has shown that SAM from *Eik. corrodens and Por. gingivalis* are potent stimulators of cytokine release by HGF, whereas SAM from all four pathogens apart from *Prev. intermedia* are capable of stimulating cytokine release by PBMC's. In general, similar results were obtained with both the monocytes and the myelomonocytic cell line Mono-Mac-6 cells. However, there was one difference, SAM from *Prev. intermedia* was a potent stimulator of IL-6 release by Mono-Mac-6 cells but was relatively inactive in the other cell types.

IL-1β and TNF-α were released by the SAM-stimulated PBMCs but not by the HGF. However, all SAM-stimulated cells released IL-6 in large quantities. This observation is of interest as in a recent study by Reinhardt *et al* (1993) it was found that IL-6 levels were significantly elevated in gingival crevicular fluid of patients with refractory periodontitis and it was suggested that IL-6 may play a key role in the pathogenesis of this form of the disease. As previously mentioned, IL-6

is a pleiotropic cytokine which, like IL-1, is known to mediate inflammatory reactions. IL-6 produced by calvarial osteoblasts has been shown to induce bone resorption, both alone and in concert with IL-1 (Ishimi *et al.*, 1990). In chapter 4 it was shown that SAM from *Eik. corrodens* was the most potent at stimulating mouse calvaria to resorb, followed by *Por. gingivalis* and *Prev. intermedia* respectively. SAM from *C. rectus* failed to show any bone resorbing activity over the concentration range measured. These data correspond with the observations in this part of the study where SAM from *Eik. corrodens* was the most potent at stimulating IL-6 release followed by the other SAM preparations, suggesting that the mechanism of bone resorption by SAM may be occurring via IL-6. IL-6 has been reported to stimulate osteoclast formation and bone resorption *in vivo* (Mundy, 1989). However the mechanism by which IL-6 is induced for osteoclast recruitment and bone resorption has not been established. It has been suggested that excess production of IL-6 and soluble IL-6 receptor induces osteoclast formation, by a mechanism involving the nonligand-binding, but signal transducing, 130-kDa glycoprotein chain gp130 (Tamura *et al.*, 1993). However, whether this mechanism of IL-6 induced osteoclastic bone resorption occurs in periodontitis needs to be established.

To determine which constituents in the SAM were contributing to the cytokine-inducing activity, this extract was exposed to heat or trypsin. Both treatments significantly inhibited the cytokine-stimulating activity of the SAM suggesting that the active components in all the SAMs were proteinaceous in nature.

The SAMs all had the capacity to stimulate one or more of the cell populations examined to produce cytokines. However, the potency and efficacy (maximal response obtainable) of the SAMs varied considerably. In these experiments the activity of the SAMs has been compared with that of a highly purified preparation of *E. coli* LPS which is used as an international standard in pyrogen assays (NIBSC 84/650). This LPS was a potent stimulator of HGFs, inducing the release of all the cytokines under investigation over the concentration range 10 pg/ml to 1 ng/ml. If comparison is made of the stimulation of IL-6 release by HGFs, then the SAM from *Eik. corrodens* and *Por. gingivalis* were roughly equipotent and all produced a similar maximum response. Significant increases in IL-6 release were seen with

some of these SAMs at a concentration of 10 ng/ml. In contrast, the SAM from *C. rectus* and *Prev. intermedia* required greater concentrations to stimulate IL-6 release (1-10 μg/ml) and a maximal response of only approximately 5-10 % of that of the other SAMs was achieved. *E. coli* LPS was also able to stimulate HGFs to secrete IL-1 β or TNF-α. It was therefore of interest to observe that, while all the SAMs had some capacity to stimulate gingival fibroblasts to release IL-6, they were all incapable of stimulating the same cells to release IL-1β or TNF-α even at concentrations as high as 10 μg/ml. The release of IL-6 from most cells normally requires prior IL-1 transcription which acts in a feedback fashion on the producing cell to induce the transcription of the IL-6 gene (Tosato *et al.*, 1990). The failure of the SAMs to stimulate HGF IL-1β or TNF-α transcription and/or release, suggests that the SAMs activate IL-6 gene transcription in these cells by a mechanism distinct from that of LPS. However, this needs to be investigated further.

The SAM from *Eik. corrodens* and *Por. gingivalis* also showed potent concentration responses with maximal activity reproducibly occurring at between 10 and 100 ng/ml. In contrast to their similar effects on fibroblasts, the SAM from the remaining two bacteria showed very different concentration response relationships with the PBMCs. Thus, the SAM from *C. rectus* showed a very steep dose response with maximal activity being observed at a concentration of 100 ng/ml. In contrast, the SAM from *Prev. intermedia* only showed stimulation of IL-6 release in the concentration range of 1-10 μg/ml. Thus there was a 5-log order difference in the potency of the SAMs in terms of their ability to induce human PBMCs to release IL-6. When culture media were assayed for the presence of IL-1β and TNF-α it was clear that, with the exception of *Prev. intermedia*, the SAMs also had the capacity to induce PBMCs to release these cytokines. Compared with their ability to stimulate IL-6 release, all the SAMs, with the exception of that from *Prev. intermedia* which was inactive, were less potent in stimulating TNF-α release.

The myelomonocytic cell line Mono-Mac-6 was also used to test the cytokine-stimulating activity of the SAMs. Again, all SAMs had the capacity to stimulate IL-6 release with roughly similar potencies. Surprisingly, given the fact that *E. coli* LPS

could stimulate this cell line to secrete IL-1β, IL-6 and TNF-α, the SAMs were only capable of stimulating IL-6 release.

5.6 Conclusion

The findings from this part of the study show that the SAM from four oral bacteria have the capacity to stimulate cells to release one or all of the pro-inflammatory cytokines IL-1β, IL-6 or TNF-α. There were marked differences in the potency and efficacy of these SAMs, but in general, those *from Eik. corrodens* and *Por. gingivalis* were the most potent and efficacious. The capacity to apparently directly stimulate HGF IL-6 synthesis without affecting the transcription of IL-1 or TNF synthesis is an interesting finding and, if this occurs *in vivo*, may account for some of the pathology of periodontitis. Therefore, a suggestion has been made that IL-6 may be a key cytokine leading to the pathology observed in periodontitis. Although much attention has focused on IL-1β as the causative cytokine leading to bone resorption in periodontal disease (Masada *et al.*, 1990; Tatakis *et al.*, 1993), more research needs to be undertaken with regard to the possibility that IL-6 plays a major role in initiating alveolar bone resorption in periodontitis.

Chapter 6

IL-6-inducing activity of lipid-A-associated proteins

6.1 Introduction

In the previous chapter it was shown that the SAM from four periodontopathogens possessed the ability to stimulate HGF and monocytic cells to release cytokines. As the majority of studies have investigated the potential role of LPS in the pathology of periodontal diseases, this part of the study investigates the ability of both the LPS and the LAP from *Por. gingivalis* and *Prev. intermedia* to stimulate the release of IL-6 by HGFs and Mono-Mac-6 cells.

The importance of LAP (Lipid-A-associated protein), also known as EAP (Endotoxin-associated protein) or EP (Endotoxin protein) have been of interest for many years. However, few reports have investigated the biological activity of these bacterial components. The biological activites ascribed to LAP are thought to be unique (Mangan *et al.*, 1992) in that they are a powerful mitogen for C3H/HeJ mice and human lymphocytes (Sultzer and Goodman, 1976). These C3H/HeJ mice are unresponsive to LPS therefore confirming that the response observed was due to the LAP. In a recent study by Mangan and colleagues (1992) it was found that the LAP from *Salmonella typhimurium* promoted the survival of monocytes by blocking programmed cell death (apoptosis) which was then found to lead to the enhancement of the production of IL-1. In this chapter, only IL-6 release in response to LPS or LAP has been investigated because, as demonstrated in the previous chapter, this cytokine was released by the target cells in the greatest amount. IL-6 has been detected in the gingival crevicular fluid of patients with chronic periodontitis (Reinhardt *et al.*, 1993). It is a multifunctional cytokine and exhibits a number of activities of relevance to the pathogenesis of chronic periodontitis including the ability to stimulate: (i). osteoclast formation (Lowik *et al.*, 1989; Roodman *et al.*, 1992; 1995); (ii). proliferation of murine thymocytes (Hodgkin *et al.*, 1988); (iii). polyclonal activation of B-cells (Kishimoto and Hirano, 1988). Such activities

may account for two of the characteristic features of periodontal lesions, namely loss of alveolar bone and infiltration of lymphocytes and plasma cells.

6.2 Aim

The aim of this study was to determine whether the LAP and LPS from *Por. gingivalis* and *Prev. intermedia* could induce IL-6 release from mammalian cells *in vitro*.

6.3 Materials and Methods

The methods were as described in chapter 2. In this part of the study HGF and Mono-Mac-6 cells were used. Cells were incubated with various concentrations (10 ng/ml-10 μg/ml) of the LAP and LPS from *Por. gingivalis* and *Prev. intermedia*. The IL-6 released into the media was then measured by an ELISA.

Statistical analysis

The results are expressed as the mean ± 2SD, n=6. The data obtained for the LAP and LPS stimulated cells were compared with the media only control by Students t-test.

6.4 Results

6.4.1 IL-6 release by HGF in response to the LAP or LPS from Por. gingivalis

The LAP from *Por. gingivalis* stimulated the release of IL-6 from HGFs in a dose-dependent manner over the concentration range 10 ng/ml - 10 μg/ml (figure 6.1). Statistically significant stimulation was found at concentrations as low as 10 ng/ml. LPS from this organism also stimulated the release of IL-6 from HGFs, although it was less potent than the LAP.

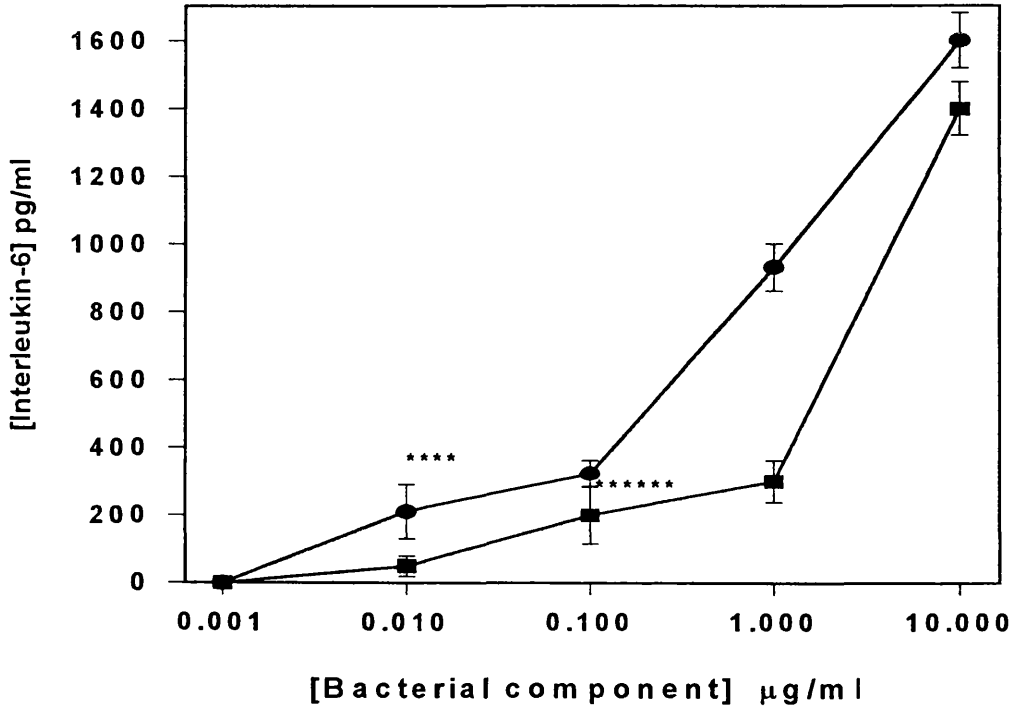


Figure 6.1 **IL-6 release from HGFs stimulated with the (●) LAP or (■) LPS from *Por. gingivalis*. The LAP from *Por. gingivalis* stimulated the release of IL-6 from HGFs in a dose-dependent manner. The LPS from this organism also stimulated the release of IL-6 although its potency was less than that of the LAP. The results are expressed as the mean ± 2SD of six replicate cultures. ****p<0.0001, ******p<0.000001.**

The LPS from *E. coli* stimulated HGF to release IL-6 over the concentration range of 50 pg/ml to 1 ng/ml. At a concentration of 0.1 ng/ml, LPS from *E. coli* stimulated the release of 280 pg/ml of IL-6 (results shown in chapter 5). The concentrations of the LAP and LPS from *Por. gingivalis* required to stimulate

the release of a similar amount of IL-6 were 10 ng/ml and 100 ng/ml respectively.

As there was a high percentage of lipid in the LAP from *Por. gingivalis* and *Prev. intermedia* (as determined in chapter 3), it was possible that it was capable of contributing to the IL-6-stimulating activity of the LAP. Therefore, chloroform/methanol extraction was performed to extract the lipids (which were then dried and weighed) and then an assay was performed with the extract as well as with the LAP prior to and after the extraction. After extraction with chloroform/methanol the cytokine-stimulating activity of the LAP was not reduced (figure 6.2) and the extract itself did not stimulate IL-6 release. This experiment showed that the IL-6 activity observed was not due to the chloroform-methanol extractable lipids in the LAP preparations.

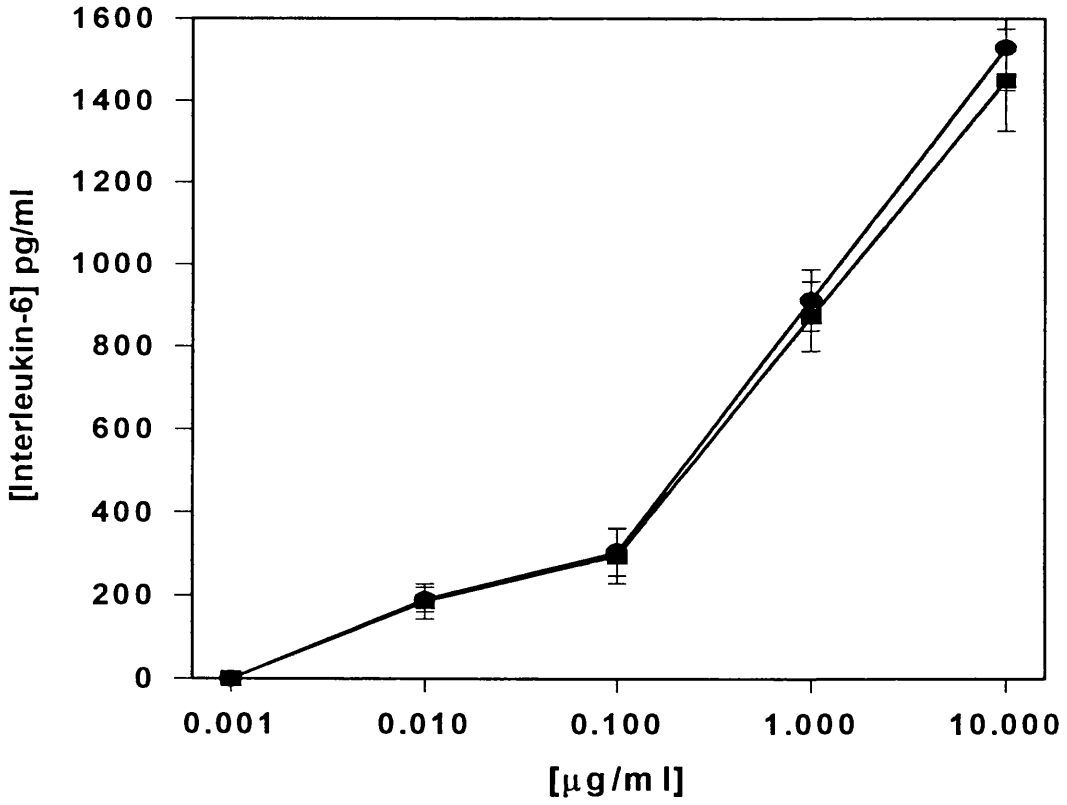


Figure 6.2 **IL-6 release by HGFs stimulated with the LAP from *Por. gingivalis* (●) prior to and (■) after chloroform/methanol extraction. After extraction with chloroform/methanol the cytokine-stimulating activity of the LAP was not significantly reduced and the extract itself did not stimulate IL-6 release. The results are expressed as the mean ± 2SD of six replicate cultures.**

6.4.1.2 *IL-6 release by HGF in response to the LAP or LPS from Prev. intermedia*

It contrast to the findings with *Por. gingivalis* the LAP and LPS from *Prev. intermedia* failed to stimulate HGFs to release IL-6 even at a maximum concentration of 10 μg/ml.

6.4.2 *IL-6 release by Mono-Mac-6 cells in response to the LAP or LPS from Por. gingivalis*

The LAP from *Por. gingivalis* was able to stimulate Mono-Mac-6 cells to release IL-6 in a dose-dependent manner over the concentration range 10 ng/ml to 10 μg/ml (figure 6.3). A similar pattern of IL-6 release was found in response to LPS from this organism which appeared to be equipotent to the LAP.

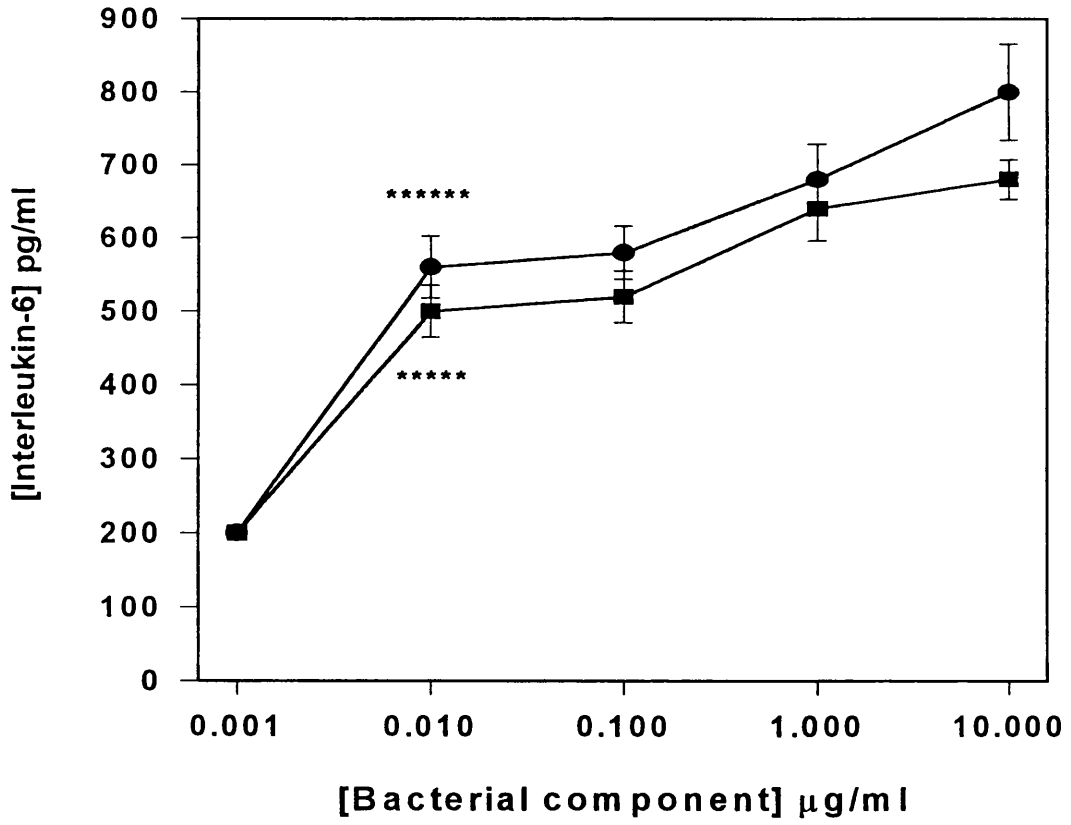


Figure 6.3 **IL-6 release from Mono-Mac-6 cells stimulated with the (●) LAP or (■) LPS from *Por. gingivalis*. The LAP from *Por. gingivalis* was able to stimulate Mono-Mac-6 cells to release IL-6 in a dose-dependent manner. A similar pattern of IL-6 release was found in response to the LPS from this organism which appeared to be equipotent to the LAP. The results are expressed as the mean ± 2SD of six replicate cultures. *****p<0.00001, ******p<0.000001.**

6.4.2.1 IL-6 release by Mono-Mac-6 cells in response to the LAP or LPS from Prev. intermedia

The LAP and LPS from *Prev. intermedia* were able to stimulate Mono-Mac-6 cells to release IL-6 in a dose-dependent manner over the concentration range 10 ng/ml to 10 μg/ml (figure 6.4).

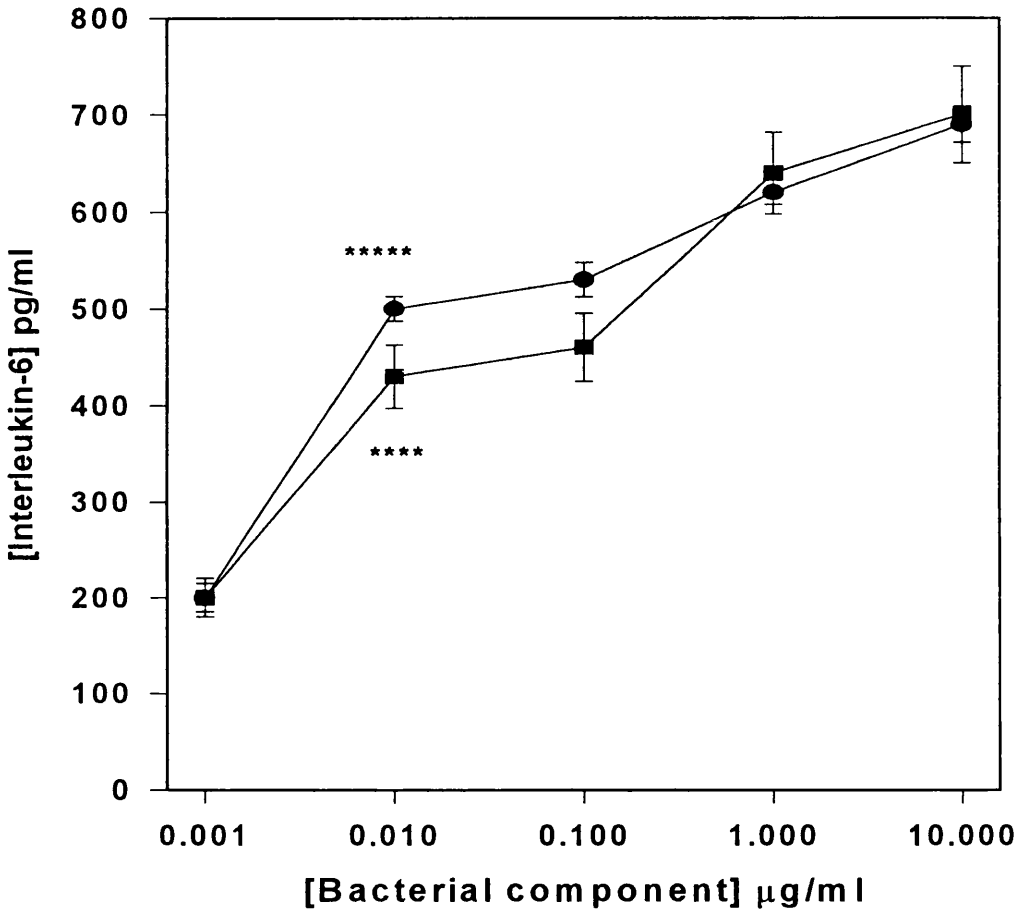


Figure 6.4 **IL-6 release from Mono-Mac 6 cells stimulated with the (●) LAP or (■) LPS from *Prev. intermedia*. The LAP and LPS from *Prev. intermedia* were able to stimulate Mono-Mac-6 cells to release IL-6 in a dose-dependent manner over the concentration range of 10 ng/ml to 10 μg/ml The results are expressed as the mean ± 2SD of six replicate cultures. ****p<0.0001, *****p<0.00001.**

As with the LAP from *Por. gingivalis*, the extractable lipid from the LAP of *Prev. intermedia* was removed and the assay performed using the LAP prior to and after lipid extraction as well as the extract itself. Extraction of the LAP with chloroform/methanol did not lead to any decrease in the cytokine-stimulating

activity of the LAP preparation, nor did the chloroform/methanol extract itself have any cytokine-stimulating activity (figure 6.5). This indicated that the component present in the LAP responsible for causing the IL-6 release was not lipidic.

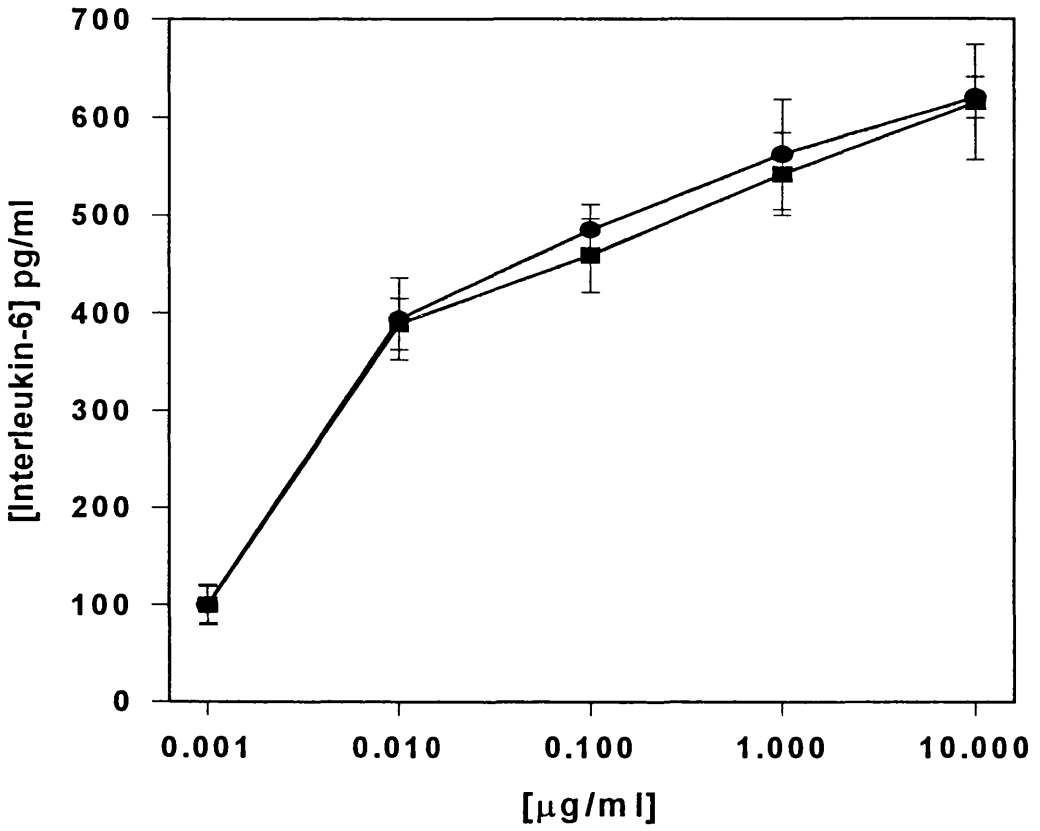


Figure 6.5 **IL-6 release from Mono-Mac-6 cells stimulated with the LAP from (●) *Prev. intermedia* and the residue from the LAP (■) after extraction with chloroform/methanol. Extraction of the LAP with chloroform/methanol did not lead to any significant decrease in the cytokine-stimulating activity of the LAP preparation. The chloroform/methanol extract itself have any cytokine-stimulating activity. The results are expressed as the mean ± 2SD of six replicate cultures.**

The LPS from *E. coli* stimulated Mono-Mac-6 cells to release IL-6 in a dose-dependent manner over the concentration range 10 pg/ml to 1 ng/ml. At a concentration of 5 pg/ml, LPS from this organism stimulated the release of 430 pg/ml of IL-6 from these cells. The concentrations of LAP and LPS from *Por. gingivalis* and *Prev. intermedia* necessary to stimulate the release of a similar amount of IL-6 was 10 ng/ml.

6.4.3 Heat- and trypsin-treatment of the LAP preparations

After heat-and trypsin treatment, the LAP from *Por. gingivalis* and *Prev. intermedia* failed to stimulate IL-6 release. This indicated that the component present in the LAP which was responsible for the stimulation of IL-6 release is likely to be proteinaceous in nature.

6.5 Discussion

Two distinctive histopathological features of the various forms of chronic periodontitis are alveolar bone loss and the presence of a high proportion of plasma cells. To date, lipopolysaccharides from the causative organisms have been considered to be the main agents responsible for initiation of periodontal tissue destruction, although the exact mechanisms involved and the likely role of cytokines have not been identified. Hence, LPS from *Por. gingivalis* and *Prev. intermedia* has been shown *in vitro* to be able to stimulate bone resorption (Iino and Hopps, 1984), inhibit mammalian cell proliferation (Slots and Genco, 1984) and stimulate cytokine release from a variety of mammalian cell types (Hamada *et al.*, 1990). The involvement of other surface components of periodontopathogenic bacteria in tissue destruction has received far less attention. However, there are increasing reports of the ability of such components to exhibit relevant *in vitro* biological activities.

The results from this study have shown that another surface component of periodontopathogenic bacteria, LAP, can affect mammalian cells in a way that may account for some of the pathological processes accompanying chronic periodontitis. Thus, the LAP was able to stimulate the release of IL-6 from HGFs and a monocytic cell line. However, there were differences in the spectrum of activity of the LAPs isolated from the two organisms under study. LAP and LPS from *Por. gingivalis* were able to elicit IL-6 release from both cell types while only monocytic cells responded in this way to LAP and LPS from *Prev. intermedia*. The potency of these preparations, were considerably lower than that of the LPS from *E. coli*.

Although there are no previous reports concerning the ability of LAP from periodontopathogenic organisms to stimulate cytokine release from mammalian cells, other investigators have demonstrated that LPS from *Por. gingivalis* and *Prev. intermedia* can induce IL-6 release from HGFs (Takada *et al.*, 1991). This group showed that 10 μg/ml of LPS from *Prev. intermedia* stimulated HGF to release approximately 900 Units/ml of IL-6. However, the results of the present study has shown that only the LPS from *Por. gingivalis* was able to stimulate HGF to release IL-6. Differences in methods of preparation of LPS (Wilson, 1993a) and/or the cytokine assay employed, may account for the different results obtained. Yamazaki and co-workers (1992) obtained similar results to those of this study in that they found that the LPS from *Por. gingivalis* stimulated HGF to release IL-6. Another interesting finding by this group was that the LPS from *Por. gingivalis* was able to stimulate IL-6 release by HGF both directly and indirectly (ie. through IL-1), although more IL-6 was found to be synthesized through the indirect route rather than the direct one.

The ability of the LAP from periodontopathogenic bacteria, particularly *Por. gingivalis*, to stimulate a number of key cells of the periodontium to produce IL-6 implies that this cell wall component may play an important role in mediating the inflammation and tissue destruction characteristic of chronic periodontitis. As the cytokine-stimulating ability of the LAP from both organisms was abrogated by heat and trypsin, it is likely that the active components of both preparations were proteins. This evidence is enhanced by the finding that after chloroforom/methanol extraction the IL-6 stimulating activity of the LAP preparations was not reduced.

In a study by Matsuki and colleagues (1992) it was found by *in situ* hybridisation that IL-6 mRNA-expressing cells were observed in both lympocytes and non-lymphoid cells in inflamed gingival tissues, but not in healthy gingival tissues. This finding indicates that IL-6 is produced in inflamed gingival tissues and is involved in the initiation or progression of periodontitis. In a recent study by Takahashi and co-workers (1994) it was found that there was no significant difference between subjects with periodontal disease and those with healthy gingival tissues either in serum IL-6 levels or in the amount

of IL-6 produced by PBMCs. This group suggested that non-lymphoid cells in inflamed gingival tissue may contribute to the pathogenesis of periodontal disease via IL-6 production, and that the IL-6 produced in gingival tissue may not reflect the IL-6 levels in the peripheral blood of these patients. As there is an obvious interest in the contribution of IL-6 to the progression of periodontal disease and in the expression of this cytokine in various cells, there is a need for further research to be performed to determine the molecular and cellular controls over periodontal IL-6 production.

Interleukin-11 (IL-11), an interesting cytokine which possesses a number of biofunctions overlapping that of IL-6 (Yin *et al.*, 1993; Elias *et al.*, 1995) make it an interesting cytokine to study in the periodontal diseases. In a recent study by Hughes and Howells (1993b) it was suggested that IL-11 may be an important inhibitor of bone formation and disease. Therefore, it would be interesting in the future to investigate whether GCF from periodontal patients contains IL-11 and also whether the SAM, LAP and LPS preparations described in this thesis could stimulate the release of this cytokine by mammalian cells.

6.6 Conclusion

The findings from this part of the study showed that the LAP and LPS from *Por. gingivalis* but not those from *Prev. intermedia* were capable of stimulating HGF to release IL-6. The LAP and LPS preparations from both bacteria were able to stimulate the monocytic cell line to release IL-6. The active component was proteinaceous in nature as heat and trypsin abrogated activity. This evidence was enhanced by the finding that after chloroform/methanol extraction the IL-6 stimulating activity of the LAP preparations was not reduced.

Chapter 7

Relative cytokine-stimulating activities of the surface components of *A. actinomycetemcomitans*

7.1 Introduction

The biological activities of the SAM from *A. actinomycetemcomitans* have been a major focus of interest at the Eastman Dental Institute for the past decade. As demonstrated in chapter 4 it has become apparent that not only the SAM from this organism, but also the LAP has certain biological activities *in vitro*, such as bone resorption. Although for a number of years it has been postulated that bacterial components are responsible for the pathology observed in periodontal diseases, very few studies have compared the biological activities of these individual constituents.

In general, since 1969 (Berglund and colleagues), LPS has been the component which has received the most attention in terms of its biological activity, in the study of periodontal disease research. However, there still remains some controversy as to whether this bacterial component can penetrate the gingival tissues because of its general insolubility. As the previous chapter demonstrated components of endotoxin such as the LAPs overlap with LPS in terms of biological activities. Therefore many of the studies reported in the literature of the biological actions of LPS probably used LAP-contaminated material.

The generation of cytokines by bacterial components is clearly a key process in the periodontal diseases. Comparative studies of the activities of key bacterial components are rare and so in this chapter such a comparison has been made.

7.2 Aims

In this study a comparison is made of the ability of the SAM, LAP and LPS from *A. actinomycetemcomitans* to stimulate HGFs, PBMCs and Mono-Mac-6 cells to synthesize the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IL-8. In

addition, the ability of these components to stimulate human neutrophils to release IL-8 has been determined.

7.3 Materials and Methods

The methods are as described in chapter 2. The cells used in this part of the study were human PBMCs, neutrophils, HGFs and the myelomonocytic cell line Mono-Mac-6 cells. The cytokine assays for IL-1β, IL-6 and TNF-α were a generous gift from Dr. Stephen Poole (NIBSC). The kit for the ELISA for IL-8 was obtained from R and D systems and the RIA for IL-8 was performed at the National Heart and Lung Institute under the supervision of Dr. Paul Collins. The cells were incubated with SAM, LAP and LPS from *A. actinomycetemcomitans* and the cytokines released into the media measured by an ELISA.

Statistical analysis

The results are expressed as the mean ± 2SD, n=6. The data obtained for the cells stimulated with SAM, LAP or LPS were compared with the control cells (ie. cells which received media alone) using Students t-test.

7.4 Results

7.4.1 Stimulation of HGFs, human PBMCs, Mono-Mac-6 cells and human neutrophils to release pro-inflammatory cytokines by *E. coli* LPS

As demonstrated in chapter 5 LPS from *E. coli* was shown to be an extremely potent stimulator of IL-6 synthesis by HGFs, PBMCs and Mono-Mac-6 cells. In this part of the study the ability of *E. coli* LPS to stimulate human PBMCs and Mono-Mac-6 cells to release both IL-1β and TNF-α is demonstrated in the following sections.

7.4.2 Stimulation of HGFs to release cytokines in response to SAM, LAP and LPS from *A. actinomycetemcomitans*

7.4.2.1 IL-1β and TNF-α release

In contrast to *E. coli* LPS, the SAM, LAP and LPS from *A. actinomycetemcomitans* failed to stimulate the release of these two cytokines over the dose range 10 ng/ml to 10 μg/ml.

7.4.2.2 IL-6 release

The SAM and LAP showed a consistent concentration-dependent stimulation of IL-6 release over the dose range 10 ng/ml to 10 μg/ml. The SAM and LAP were approximately equipotent but the latter stimulated a greater maximal response. In contrast, the LPS from this bacterium showed only minimal stimulation (figure 7.1) of IL-6 release. The release of IL-6 by HGFs in response to these components in the absence of IL-1β and TNF-α was a surprising finding as generally the prior synthesis of IL-1β is required before IL-6 is produced. This interesting finding is investigated and discussed in detail in chapter8.

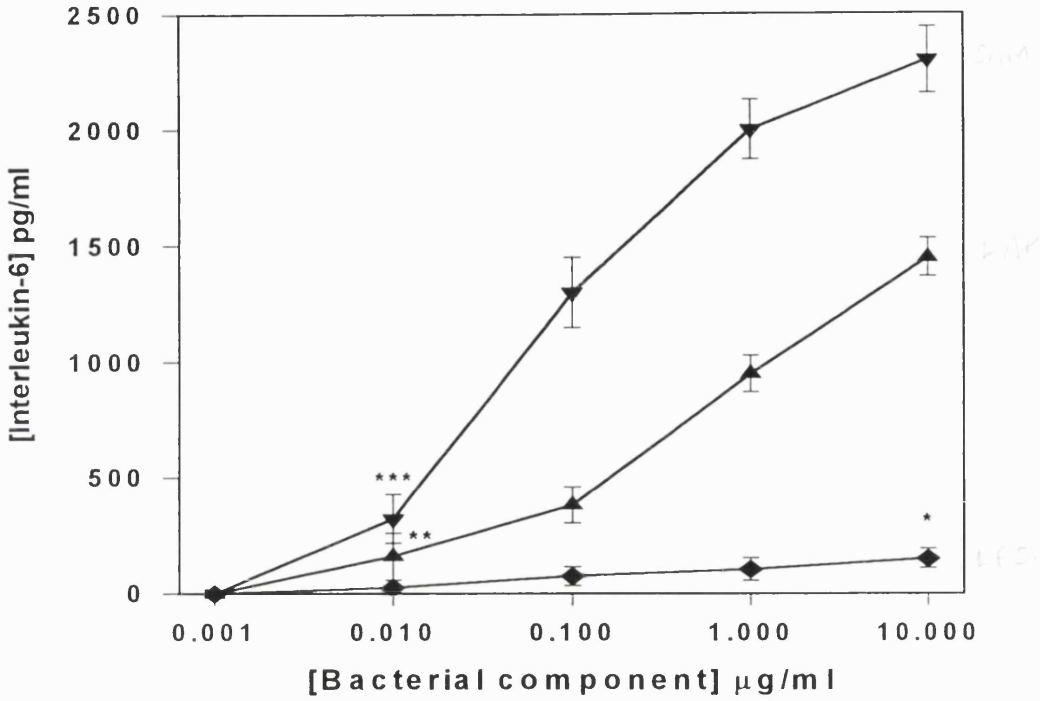


Figure 7.1 **IL-6 release by HGFs stimulated by the addition of various concentrations of (▲) SAM, (▼) LAP or (●) LPS over the dose range of 1 ng/ml to 10 μg/ml. The SAM and LAP from *A. actinomycetemcomitans* showed a consistent concentration-dependent stimulation of IL-6 release over the dose range of 10 ng/ml to 10 μg/ml. In contrast, the LPS from this bacterium showed only minimal stimulation of IL-6 release by these cells. The results are expressed as the mean ± 2SD of 6 replicate cultures. *p<0.05, **p<0.01,***p<0.001.**

7.4.3 Stimulation of human PBMC cytokine release in response to SAM, LAP and LPS from *A. actinomycetemcomitans*

7.4.3.1 IL-1β release

The SAM produced a linear dose-dependent stimulation of IL-1β release over the concentration range of 10 ng/ml to 10 μg/ml. The LAP and the LPS were much less potent and, in the experiment shown, required 2-3 log orders higher concentrations to stimulate IL-1β release (figure 7.2). *E. coli* LPS was found to be a potent stimulator of IL-1β release by these cells.

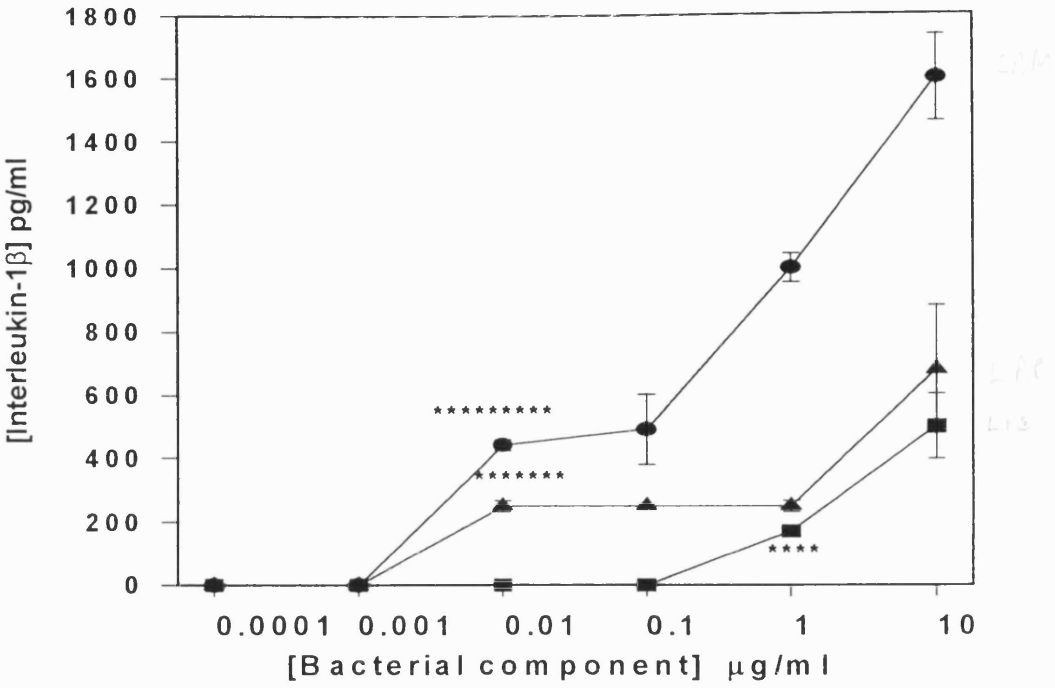


Figure 7.2 **The stimulation of IL-1β release from human PBMCs exposed to concentrations of (●) SAM, (▲) LAP or (■) LPS over the concentration range of 1 ng/ml to 10 μg/ml. The SAM produced a dose-dependent stimulation of IL-1β release over the concentration range measured. The LAP and the LPS were less potent (2-3 log orders) in stimulating IL-1β release. The results are expressed as the mean ± 2SD of 6 replicate c cultures. ****p<0.0001, *******p<0.0000001, *********p<0.000000001.**

7.4.3.2 TNF-α release

The LPS from *A. actinomycetemcomitans* was unable to stimulate TNF-α synthesis. The SAM was capable of stimulating TNF-α release only at a concentration of 1 μg/ml and the LAP was reproducibly one log order less active (figure 7.3). *E. coli* LPS, as expected, was a potent stimulator of TNF-α release.

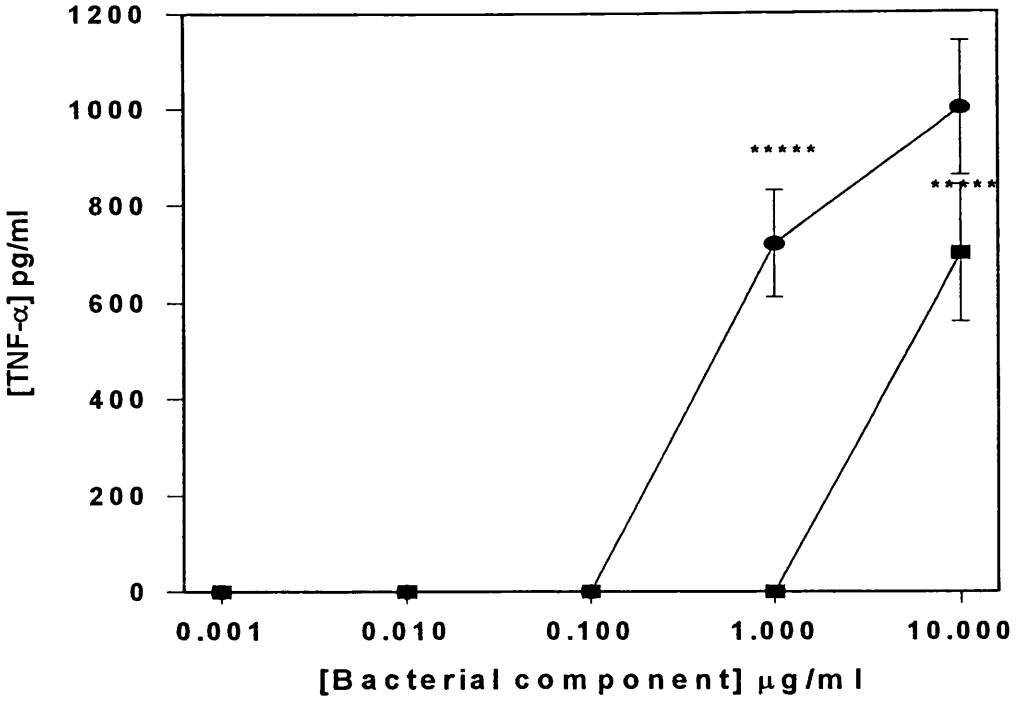


Figure 7.3 **Stimulation of TNF-α release from human PBMCs exposed to various concentrations of SAM (●) or LAP (■) from *A. actinomycetemcomitans*. LPS from this organism did not stimulate TNF-α release over the dose range tested. The results are expressed as the mean ± 2SD of 6 replicate cultures.*****p<0.00001.**

7.3.3.3 IL-6 release

E. coli LPS reproducibly stimulated IL-6 release at concentrations ranging from 10-1000 pg/ml. SAM from *A. actinomycetemcomitans* also reproducibly stimulated IL-6 synthesis over this concentration range though it required higher concentrations to stimulate maximal IL-6 synthesis. In comparison, the LAP and the LPS were 4 and 5 log orders, respectively, less potent than the SAM in stimulating IL-6 release (figure 7.4).

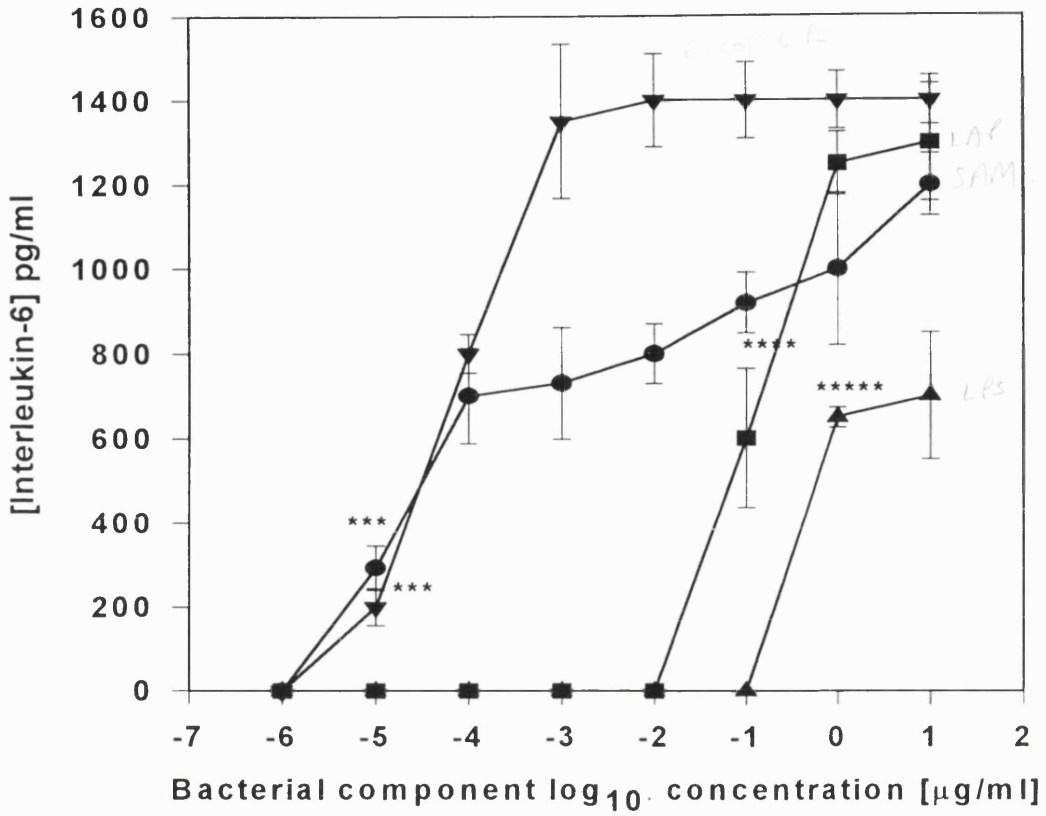


Figure 7.4 **The stimulation of IL-6 release by human PBMCs exposed to various concentrations of (▼) *E. coli* LPS, *A. actinomycetemcomitans* (▲) LPS, (■) LAP or (●) SAM. These components have been added over the dose range 1 pg/ml to 10 μg/ml. *E. coli* LPS and the SAM from *A. actinomycetemcomitans* reproducibly stimulated IL-6 release at concentrations ranging from 10-1000 pg/ml. The results are expressed as the mean ± 2SD of 6 replicate cultures. ***p<0.001, ****p<0.0001,*****p<0.00001.**

It was interesting to observe that although the SAM and LAP from *A. actinomycetemcomitans* did not stimulate IL-1β or TNF-α release by HGF, these components were able to stimulate PBMCs to release these two pro-inflammatory cytokines. This may indicate that IL-6 released by HGF in response to SAM and LAP may be via a different mechanism to the IL-6 released by PBMCs in response to these two components.

7.4.4 Stimulation of Mono-Mac-6 cells to release pro-inflammatory cytokines in response to SAM, LAP or LPS from *A. actinomycetemcomitans*

7.4.4.1 IL-1β and TNF-α release.

The LPS from *A. actinomycetmcomitans* failed to stimulate the release of IL-1β or TNF-α from this cell line. SAM stimulated IL-1β release at a concentration of 1 μg/ml while 10 μg/ml LAP was necessary to trigger release of this cytokine (figure 7.5). In contrast *E. coli LPS* was found to be a potent stimulator of the release of these two cytokines.

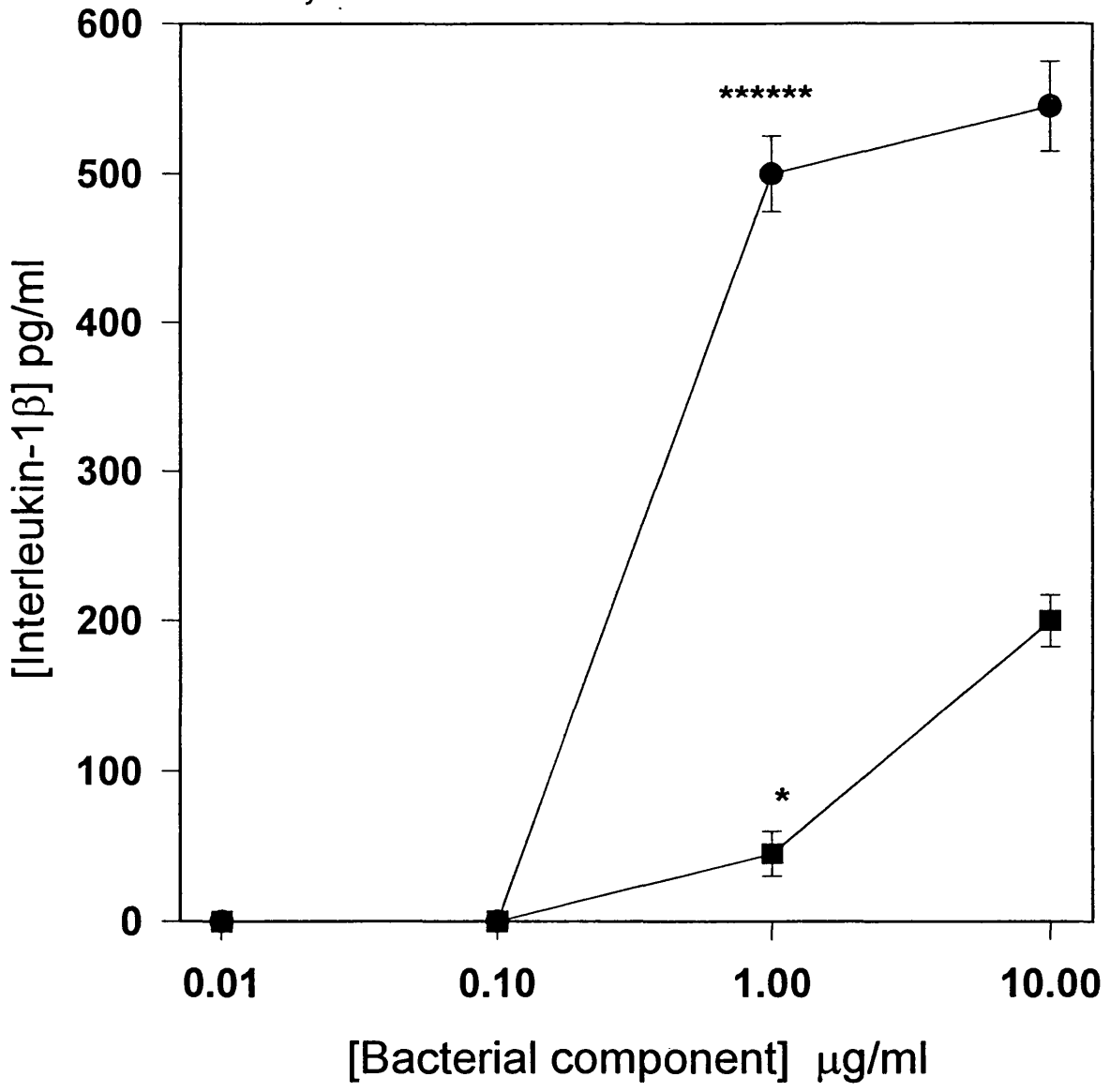


Figure 7.5 **Release of IL-1β by Mono-Mac-6 cells exposed to various concentrations of (■) LAP or (●) SAM. The LPS from *A. actinomycetmcomitans* failed to stimulate the release of IL-1β or TNF-α from this cell line. The results are expressed as the mean + 2SD of 6 replicate cultures. *p<0.05, ******p<0.000001.**

The LAP and SAM only stimulated minimal TNF-α release at the maximum concentrations (10 μg/ml) tested.

7.4.4.2 IL-6 release

E. coli LPS was a potent stimulator of cytokine synthesis by this cell line demonstrating a linear dose-dependent stimulation over the dose range 10 pg/ml to 1 ng/ml. The SAM induced IL-6 release from these myelomonocytic cells in a dose-dependent manner with significant levels of IL-6 being released at a concentration of 10 ng/ml with a plateau of production being reached at 100 ng/ml. In contrast, the LAP only stimulated the release of IL-6 at the maximal concentration used (10 μg/ml) and LPS failed to stimulate IL-6 release (figure 7.6).

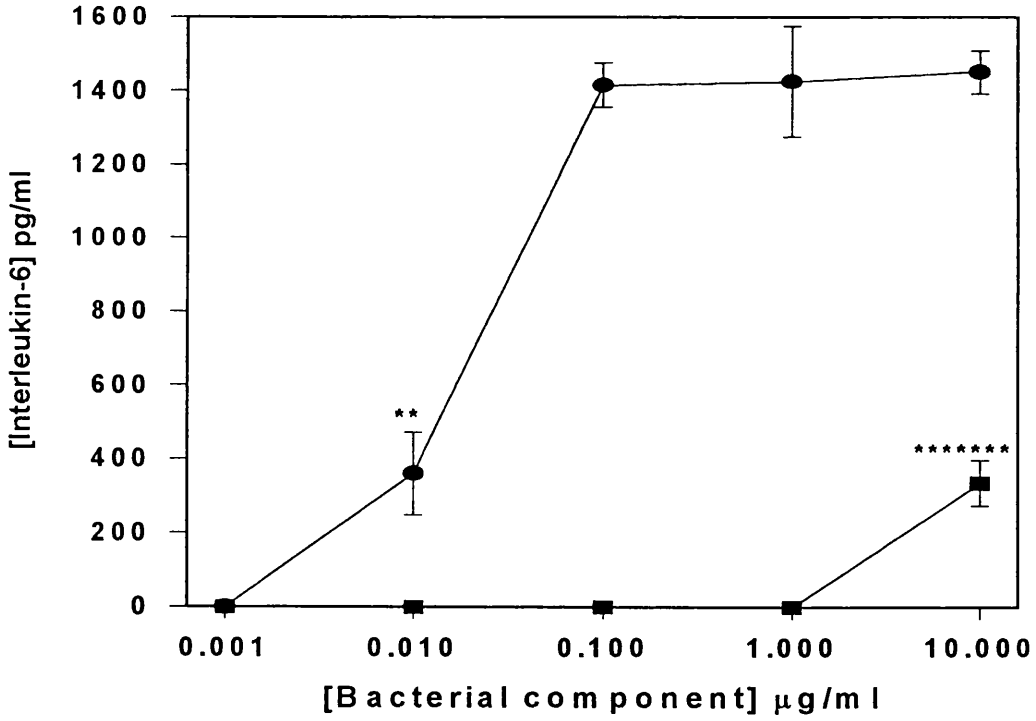


Figure 7.6 **Release of IL-6 from Mono-Mac-6 cells stimulated by various concentrations of (■) LAP or (●) SAM. LPS from *A. actinomycetemcomitans* did not stimulate IL-6 release over the dose range tested. The results are expressed as the mean ± 2SD of 6 replicate cultures. **p<0.01, *******p<0.0000001.**

7.4.5 Stimulation of IL-8 release from HGFs, human PBMCs and human neutrophils by the SAM of *A. actinomycetemcomitans*

The release of IL-8 by HGF and PBMCs in response to SAM was measured by ELISA and the release of IL-8 by human neutrophils stimulated by this component was measured by RIA.

7.4.5.1 IL-8 release by HGF

SAM from *A. actinomycetemcomitans* was a potent stimulator of IL-8, at a concentration as low as 10ng/ml, stimulating the release of approximately 1 μg/ml of IL-8 (figure 7.7). *E. coli* LPS was also a potent stimulator of IL-8 release by these cells (results not shown).

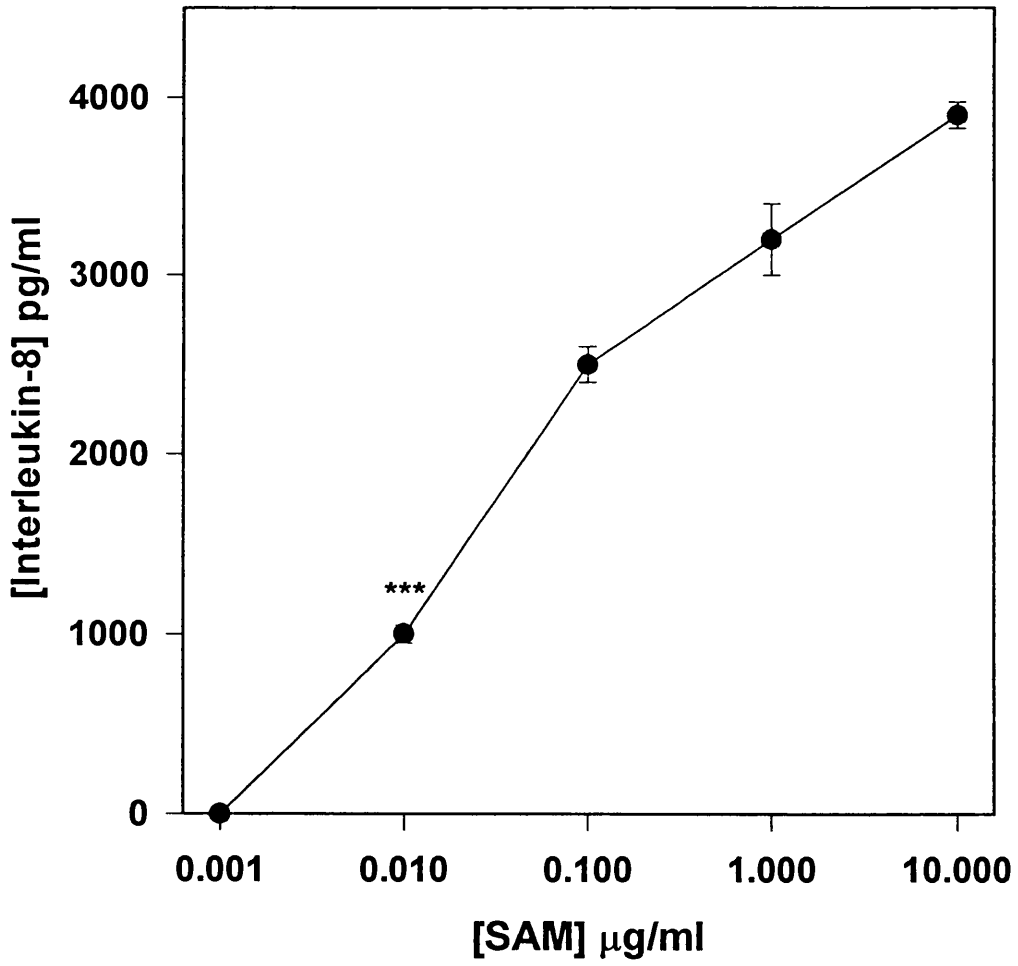


Figure 7.7 **Release of IL-8 by HGF in response to the SAM from *A. actinomycetemcomitans*. *E. coli* LPS was also a potent stimulator of IL-8 by these cells (results not shown). The results are expressed as the mean ± 2SD of six replicate cultures. ***p<0.001.**

7.4.5.2 *IL-8 release by human PBMCs after stimulation by the SAM from A. actinomycetemcomitans*

The SAM from *A. actinomycetemcomitans* stimulated human PBMCs at a minimum concentration of 10 ng/ml to release approximately 2000pg/ml of IL-8. The SAM was 1 log order more potent at stimulating PBMCs to release IL-8 compared with HGF (figure 7.8).

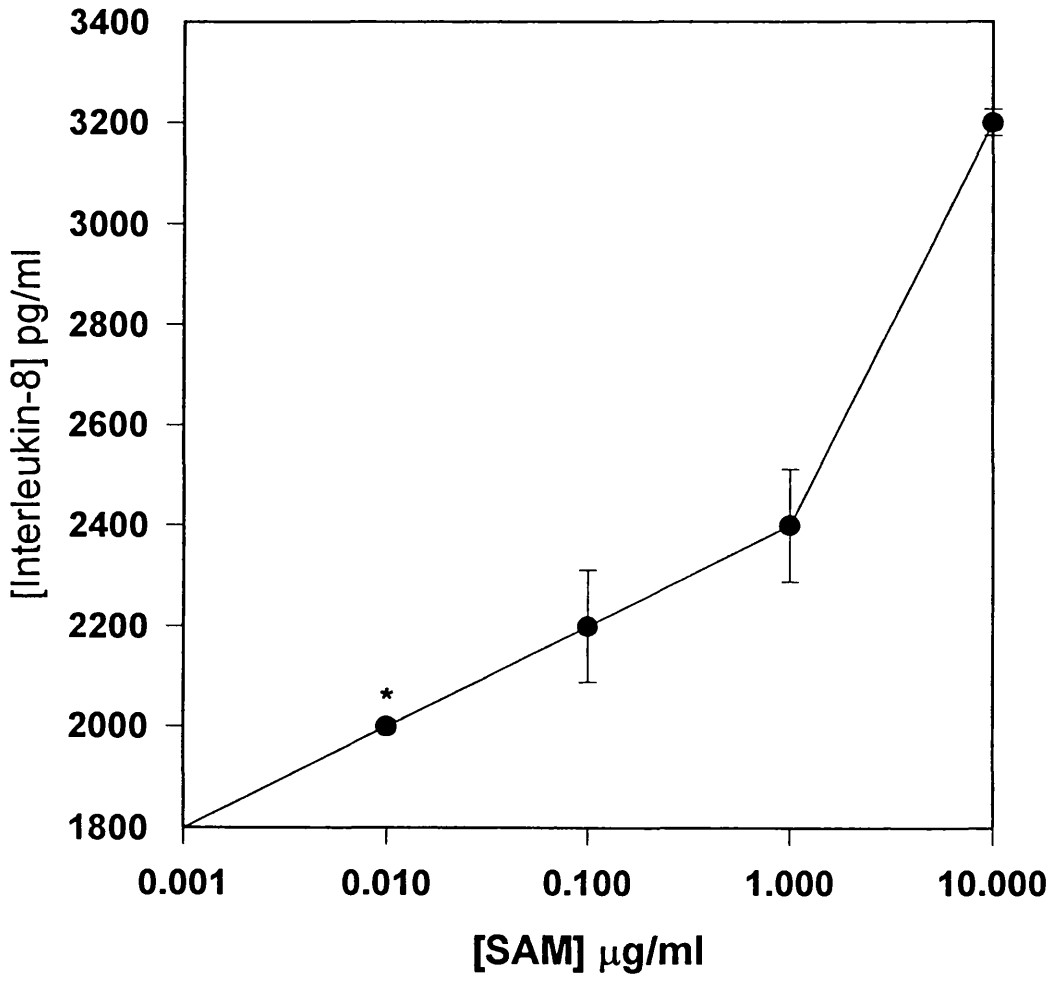


Figure 7.8 **IL-8 release by human PBMCs after stimulation by the SAM from *A. actinomycetemcomitans*. The SAM from *A. actinomycetemcomitans* stimulated human PBMCs at a minimum concentration of 10 ng/ml to release approximately 2000pg/ml of IL-8. The results are expressed as the mean ± 2SD of six replicate cultures. *$p<0.05$.**

7.4.5.3 IL-8 release by human neutrophils after stimulation with the SAM from A. actinomycetemcomitans

The SAM from *A. actinomycetemcomitans* was a potent stimulator of IL-8 release by human neutrophils, with 10 ng/ml of SAM causing the release of approximately 1000 pM of IL-8 by these cells. In contrast, the LPS from *A. actinomycetemcomitans* failed to stimulate human neutrophils to release IL-8 over the dose range measured (figure 7.9).

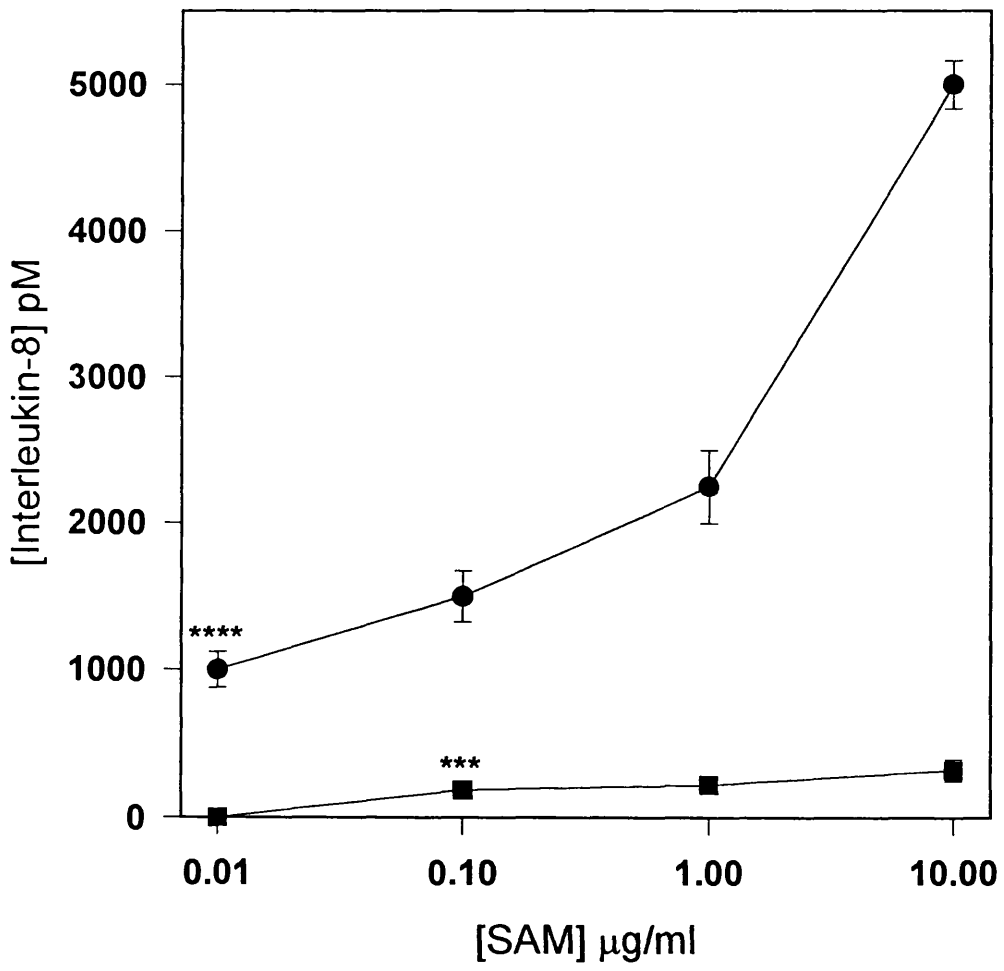


Figure 7.9 **IL-8 release by human neutrophils stimulated with (●) SAM and (■) LPS of *A. actinomycetemcomitans* over the concentration range of 10 ng/ml to 10 µg/ml. The SAM was a potent stimulator of IL-8 release by these cells. In contrast the LPS from this organism failed to significantly stimulate the release of IL-8 by these cells. The results are expressed as the mean ± 2SD of six replicate cultures. ***p<0.001, ****p<0.0001.**

The intracellular IL-8 level of human neutrophils after being stimulated by SAM and LPS from *A. actinomycetemcomitans* was also measured (figure 7.10).

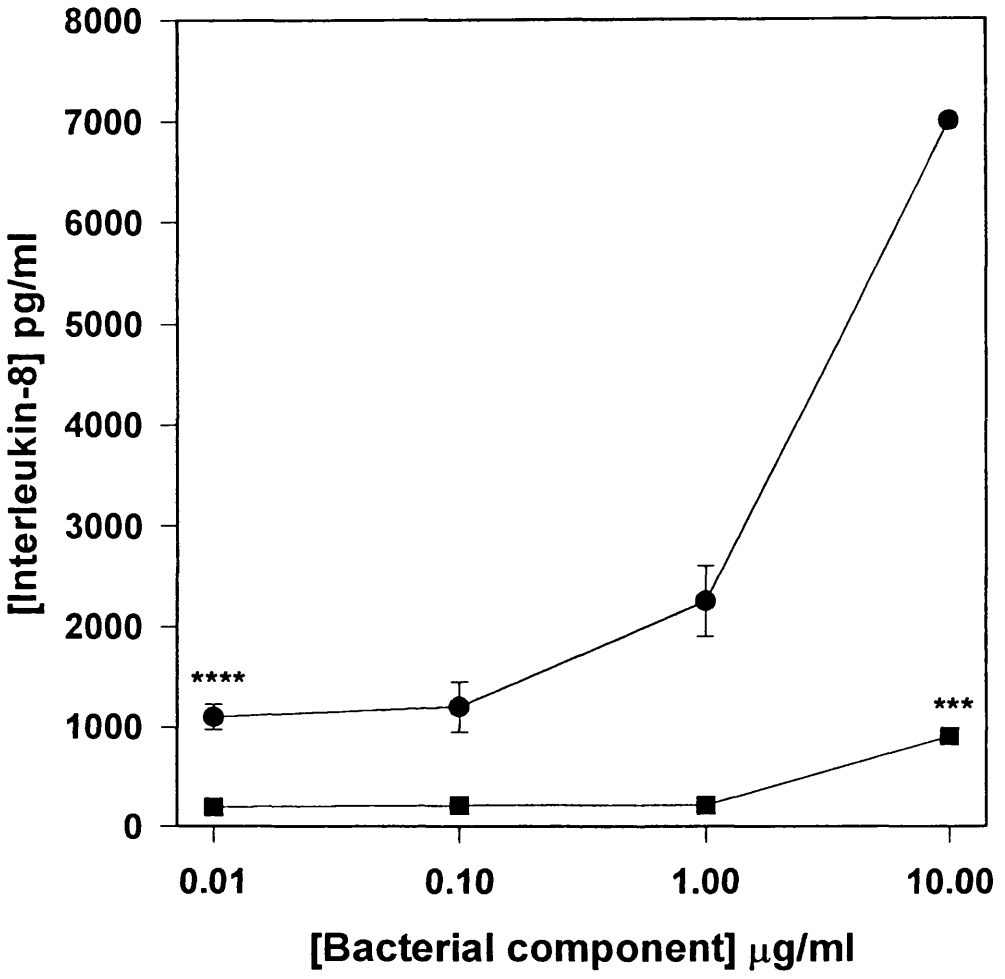


Figure 7.10 **Intracellular IL-8 levels of human neutrophils after stimulation with the (●) SAM and (■) LPS from *A. actinomycetemcomitans*. Large quantities of intracellular IL-8 was found in these cells after stimulating with the SAM from *A. actinomycetemcomitans*. Very little intracellular IL-8 was measured in response to 10 μg/ml of LPS from this organism in these cells. The results are expressed as the mean ± 2SD of six replicate cultures. ***p<0.001, ****p<0.0001.**

It was interesting to note that after stimulating human neutrophils with SAM from *A. actinomycetemcomitans*, high intracellular levels of IL-8 were found although IL-8 was also released by these cells in large quantities. In contrast, after stimulating these cells with LPS from this organism very little IL-8 was measured either intracellulary or extracellularly.

Although the LPS from *A. actinomycetemcomitans* did not significantly stimulate human neutrophils to synthesise IL-8, it was found that *E. coli* LPS stimulated both the synthesis and the release of IL-8 by these cells (figure 7.11). However, the SAM from *A. actinomycetemcomitans* was much more efficacious at stimulating the release of IL-8 by these cells.

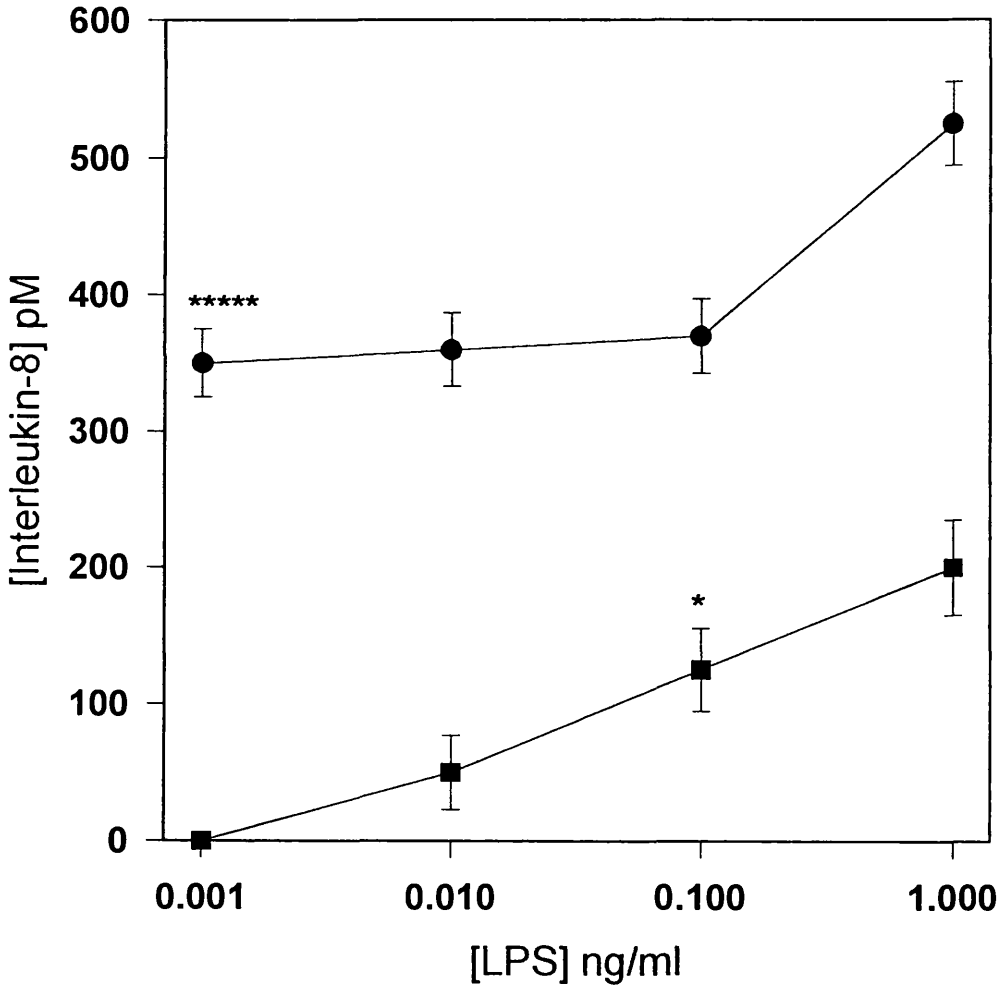


Figure 7.11 **Stimulation of IL-8 by human neutrophils in response to *E. coli* LPS showing both intracellular and extracellular IL-8 by these cells. (■) intracellular IL-8; (●) extracellular IL-8. The results are expressed as the mean ± 2SD of six replicate cultures. *****p<0.00001, *p<0.05.**

7.5 Discussion

Purified *E. coli* LPS (the international standard) was, as expected, a potent stimulator of IL-1β, TNF-α and IL-6 by HGF, PBMCs and Mono-Mac-6 cells

over the concentration range of 10 pg/ml to 1 ng /ml. However, in contrast, the LPS from this enteric bacterium was a poor stimulator of IL-8 synthesis and release by human neutrophils.

While LPS is generally considered to be a potent inducer of cytokine synthesis it is clear from these results that purified *A. actinomycetemcomitans* LPS is a very weak cytokine-stimulating agent when added to cultured human mesenchymal or myelomonocytic cells. This does not agree with the study by Saglie and colleagues (1990) where it was shown that the LPS from *A. actinomycetemcomitans* (Y4) was able to stimulate macrophages to produce IL-1 and TNF. The differences in results may be explained by observing the differences in the methodology used to obtain the LPS preparations. In the study of Saglie and co-workers the researchers failed to treat their LPS preparation with pronase thus their LPS preparations may be contaminated with the LAP from this organism. The differences in the potencies between *E. coli* LPS and the LPS from *A. actinomycetemcomitans* may be due to the differences in the structures of their lipid-A.

Addition of the LAP from *A. actinomycetemcomitans* to fibroblasts, monocytes and the myelomoncytic cell line Mono-Mac-6 revealed that this material had cytokine-stimulating activity. Comparison of the effects of the three separate bacterial preparations on HGFs revealed that the LAP reproducibly showed the greatest activity in terms of IL-6 release. However, the LAP was significantly less active than the SAM in stimulating cytokine synthesis by human PBMCs or the myelomonocytic cell line. This would appear to be the first demonstration of the ability of the LAP from *A. actinomycetemcomitans* to induce pro-inflammatory cytokine synthesis by both mesenchymal and myelomonocytic cells.

The most active cytokine-inducing extract of *A. actinomycetmcomitans* was the SAM. This difference in potency was particularly notable with the PBMCs and the monocyte cell line. The most significant finding was the ability of the SAM to stimulate IL-6 release from PBMCs at concentrations similar to those of LPS from *E. coli*, the most potent bacterial inducer of such synthesis yet described.

The SAM from *A. actinomycetemcomitans* was also a potent stimulator of the release of IL-8 by HGF, human monocytes and human neutrophils. In all three cell types 10 ng/ml of the SAM caused the release of 1000 pg/ml of IL-8. In contrast the LPS from this organism was a poor stimulator of IL-8 release. An interesting finding was that the level of intracellular IL-8 was equivalent to the level of IL-8 released by human neutrophils after stimulation with the SAM from *A. actinomycetemcomitans*. As described in chapter 1, IL-8 is a chemoattractant cytokine which has a distinct target specificity for the neutrophil. This cytokine is of importance in periodontal disease in which the majority of immigrant cells are neutrophils (Bickel, 1993). The presence of IL-8 in periodontal diseases is implied by the migration of the neutrophils to the gingival tissue and the release of granule enzymes. This may lead to the degradation of the connective tissues. Several investigations have also reported on the dysfunction of neutrophils in patients with periodontal diseases (Cianciola *et al.*, 1977; Clark *et al.*, 1977).

Cytokines are obviously involved in the pathogenesis of the CIPDs (Kjeldesen *et al.*, 1993). The finding that components present on the surface of *A. actinomycetemcomitans* can stimulate, at extremely low concentrations, human PBMCs, a human monocyte cell line and human gingival fibroblasts to release pro-inflammatory cytokines strengthens the argument that these components, and not LPS, are key factors in initiating the inflammation and tissue destruction characteristic of periodontitis. The difference in response to the SAM and LAP between the PBMCs and Mono-Mac-6 cells is difficult to explain as both respond in an equivalent manner to LPS from *E. coli*. The potent actions of the SAM on PBMCs may reflect interactions between the lymphocytes and monocytes which upregulate monocyte cytokine synthesis.

7.6 Conclusion

The incredible potency of enteric LPS as a stimulator of cytokine synthesis has dominated the thinking of cytokine biologists to the exclusion of a consideration of other possible immunomodulatory bacterial components. The results of this study have demonstrated that (i) the LPS of the non-enteric bacterium *A.*

actinomycetemcomitans is a very weak cytokine-stimulating agonist and (ii) that LPS-free preparations of LAP and SAM are capable of inducing cytokine release from mesenchymal and myelomonocytic cells. Indeed, in the case of PBMCs, the SAM was equipotent to LPS from *E. coli*, demonstrating activity at 10 pg/ml.

Chapter 8
Mechanism of the direct induction of IL-6 synthesis by the SAM of *A. actinomycetemcomitans*.

8.1 Introduction

In the previous chapters it has been demonstrated that the SAM from a number of periodontopathogens can stimulate HGFs to release IL-6 without inducing the release of IL-1 or TNF. The SAM from *A. actinomycetemcomitans* is a particularly potent inducer of fibroblast IL-6 synthesis, therefore the mechanism of its action is further investigated in this chapter.

IL-6 is now regarded as an example of a secondary cytokine in that it appears to require the action of primary mediators in particular IL-1 for its transcription (Tosato and Jones, 1990). The pro-inflammatory cytokine TNF-α and platelet-derived growth factor (PDGF) (Van Damme *et al.*, 1987; Littlewood *et al.*, 1991) as well as lipid mediators such as prostaglandins and leukotrienes (Rola-Pleszczynski and Stankova, 1992) have also been reported to induce IL-6 synthesis. Thus in this chapter the mechanism of SAM-induced IL-6 synthesis has been examined to determine if it is truly independent of IL-1 and TNF synthesis or whether it depends on the participation of other mediators such as those described.

8.2 Aim

The aim of this study was to investigate the mechanism by which SAM stimulated HGF to release IL-6.

8.3 Materials and Methods

The methods were as described in chapter 2. HGFs were pre-incubated for 1 h at 37 ^{0}C with an excess concentration of neutralising antibodies to human IL-1 (α or β) and/or to human TNF-α. The SAM (10 μg/ml) or *E. coli* LPS (1 ng/ml) were then added to the wells containing the neutralising antibodies. After an overnight incubation at 37 ^{0}C, the media were assayed for IL-6 release by ELISA. The neutralising antibodies were raised in sheep at NIBSC and were a generous gift from Dr. Stephen Poole. The same set of experiments were also

performed with recombinant IL-1 receptor antagonist (kind gift from Dr Bob Thompson, Synergen Inc) used at a concentration of 20 μg/ml. In addition a neutralising antiserum to PDGF (R and D systems) was used according to the manufacturers instructions (ie. at a concentration of 5 times the IC_{50} of the material to be neutralised). Experiments with 5-lipoxygenase inhibitor BW70C (Wellcome Foundation) and the 5-lipoxygenase activating protein (FLAP) inhibitor, MK886 (Merck Frosst) were also performed. These inhibitors were dissolved in DMSO and added to cell cultures in the concentration range of 10^{-9} to 10^{-6} M with appropriate solvent controls. Dexamethasone (Sigma) was added to the cells at a concentration of 10^{-9} M. Indomethacin (Sigma), an inhibitor of cyclooxygenase, was used at a concentration of 10^{-6} M.

The presence of intracellular IL-1 β in both *E. coli* LPS-stimulated HGF and SAM-stimulated HGF was also measured. The media were removed from the cells and distilled water added. After 3 freeze-thawing cycles the lysed cells were centrifuged at 10, 000 g for 30 min to pellet the cell debris and the supernatant assayed for IL-1 β.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) for cytokine mRNA: HGFs were grown to confluence in 3.5 cm culture dishes and incubated for 24 h in DMEM supplemented with 2 % FCS at 37 ^{0}C in 5 % CO2/air. After washing with PBS, cells were incubated in media containing LPS or SAM at 10 ng/ml and 1 μg/ml respectively. At various times, total RNA was extracted from cells as described by Chomczynski and Sacchi (1987). To improve the yield of cellular RNA, 10 μg of yeast tRNA (Sigma) was added as a carrier. For each time point, total RNA from two wells was pooled. Single stranded cDNA was generated using Superscript II reverse transcriptase (100U:Gibco/BRL) in a 20 μl reaction mixture containing reaction buffer (50 mM TrisHCl pH 8.3, 75 mM KCl, 3mM $MgCl_2$, 10 mM dithiothreitol), dNTP mix (dATP, dCTP, dGTP, dTTP) 0.5 mM of each, 0.5 μg Oligo $(dT)_{12\text{-}18}$, 10U rRNAsin (Promega) and 10 % of each pooled total RNA. The reaction was carried out for 1 h at 42 ^{0}C. Amplification of cDNA by PCR was performed using oligonucleotide primer pairs for IL-1β, IL-6 or TNF as described by Jung

and colleagues (1995). The reactions were performed in a thermal cycler (Eppendorf) in a 30 μl reaction volume containing reaction buffer (20 mM of each, 1.5 U of Taq polymerase (Gibco/BRL) and 2 μl of first strand reaction mix. Each primer was added to a final concentration of 0.1 μM. PCR was for 35 cycles, each cycle consisting of 1 min at 95 ^{0}C and annealing/extension at 60 ^{0}C for 2 min and 30 sec. The PCR products were separated on 1 % agarose gels containing 50 μg/ml ethidium bromide, visualised and photographed under ultraviolet light.

Statistical analysis

The results are expressed as the mean ± 2SD of 6 replicate cultures. The data were analysed using Students t-test by comparing the results obtained with the controls.

8.4 Results

8.4.1 Influence of neutralising antibodies to cytokines on the induction of IL-6 synthesis by HGF stimulated with E. coli LPS.

Purified LPS from *E. coli* stimulated HGFs to release IL-6 in a dose-dependent manner with maximal stimulation at between 1 and 10 ng/ml. This synthesis could be completely inhibited by neutralising antibodies to IL-1 (α and β), and/or by using a neutralising antibody to TNF-α or by the natural IL-1 receptor, IL-1 receptor antagonist (IL1ra, figure 8.1).

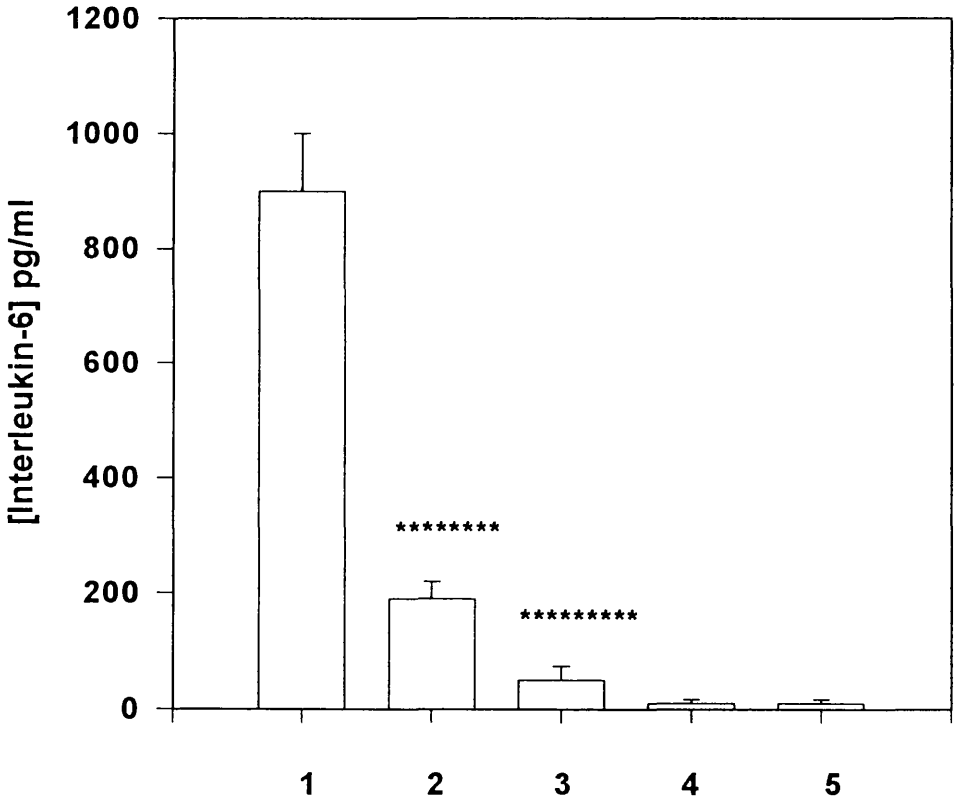


Figure 8.1 **IL-6 release by HGF stimulated by *E. coli* LPS in the presence of an excess concentration of neutralising antibodies to 1- IL-1 (both α and β), 2- TNF-α, 3- combination of anti IL-1 α, β and TNF-α antibodies and 4- IL-1ra. The stimulation of IL-6 release by LPS from *E. coli* could be completely inhibited by neutralising antibodies to IL-1 (α and β), and/or by using a neutralising antibody to TNF-α or by IL1ra. The results are expressed as the mean + 2SD of 6 replicate cultures. ********p<0.00000001, *********p<0.000000001.**

8.4.2 Influence of neutralising antibodies to cytokines on the induction of IL-6 release by HGF stimulated with SAM.

In contrast to *E. coli* LPS stimulated cells, the neutralisation of IL-1 and/or TNF-α, or both cytokines failed to inhibit IL-6 synthesis by the SAM-stimulated cells (figure 8.2).

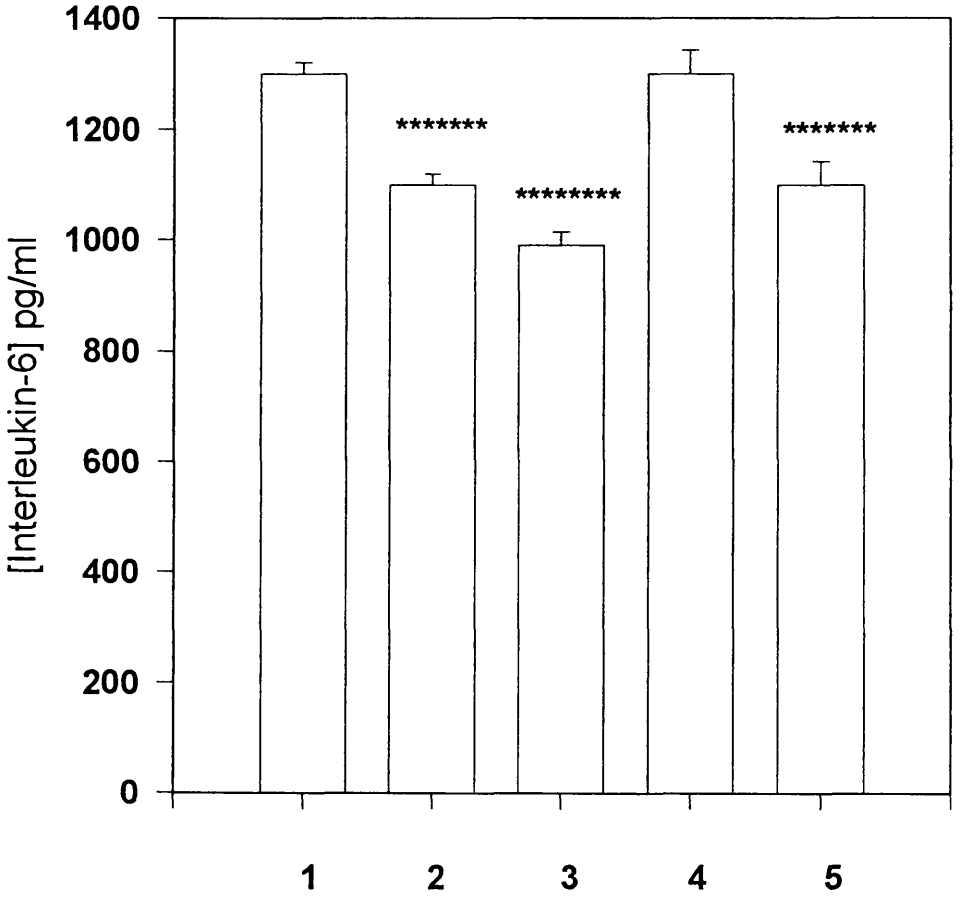


Figure 8.2 **IL-6 release by HGF stimulated with the SAM from *A. actinomycetemcomitans* alone in the presence of an excess concentration of neutralising antibodies to 1- IL-1 (both α and β), 2-TNF-α, 3- a combination of anti IL-1 α, β and TNF-α antibodies and 4- IL-1ra. The addition of neutralising antibodies failed to significantly inhibit IL-6 synthesis by the SAM-stimulated cells. The results are expressed as the mean + 2SD of six replicate cultures. *******p<0.0000001, ********p<0.00000001.**

8.4.3 Measurement of intracellular IL-1 β in the cell lysates of SAM-stimulated and E. coli LPS-stimulated HGF.

In chapter 5 it was demonstrated that *E. coli* LPS stimulated fibroblasts to release IL-1β. However, in contrast, HGF stimulated with SAM failed to release IL-1 β. Therefore, in this part of the study the presence of IL-1 β in the cell lysates of SAM- and *E. coli* LPS-stimulated cells was investigated. *E. coli* LPS-stimulated HGF was found to contain intracellular IL-1 β over the dose range of 100 pg/ml to 10 ng/ml. In contrast there was no IL-1 β measurable in the cell lysates of SAM-stimulated HGF over the dose range of 10 ng/ml to 10 μg/ml.

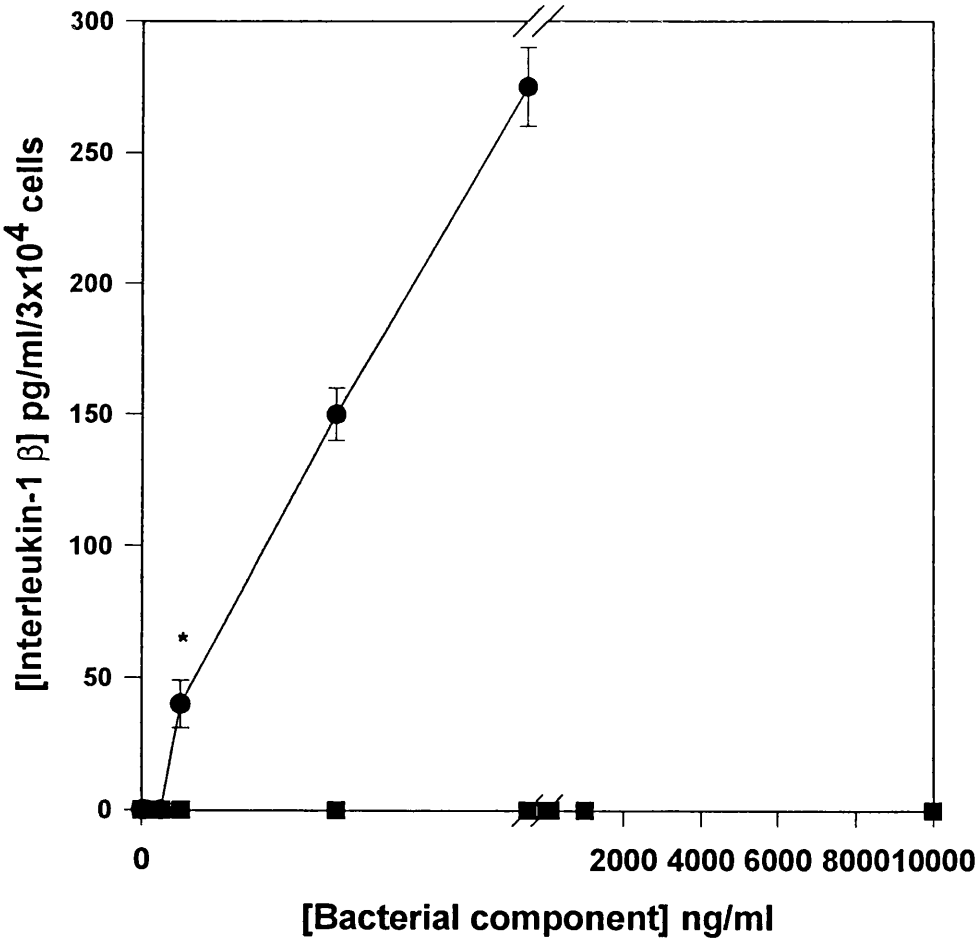


Figure 8.3 **Intracellular levels of IL-1 β in HGF after stimulation with (●) *E. coli* LPS or (■) SAM from *A. actinomycetemcomitans*. Intracellular IL-β was found in HGF stimulated with *E. coli* LPS over the concentration range of 100 pg/ml to 10 ng/ml. In contrast there was no IL-1 β measurable in the cell lysates of SAM-stimulated HGF over the concentration range 10 ng/ml to 10 μg/ml. The results are expressed as the mean ± 2SD of six replicate assays. *p<0.05.**

8.4.4 RT-PCR of the SAM-stimulated and E. coli LPS-stimulated HGFs

RT-PCR was used to amplify the mRNA for IL-6 in HGFs incubated with LPS or SAM. In LPS-treated cells IL-1 β message was amplifiable although levels appeared low. No message for IL-1 β mRNA was found in HGFs exposed to the SAM. In contrast both stimulants resulted in the rapid appearance of IL-6 mRNA. LPS-treated cells also showed amplifiable message for IL-1 α and TNF-α. The appearance of IL-6 mRNA can be compared to the kinetics of protein expression in figure 8.4. Clearly IL-6 is induced more rapidly by the SAM than by the LPS.

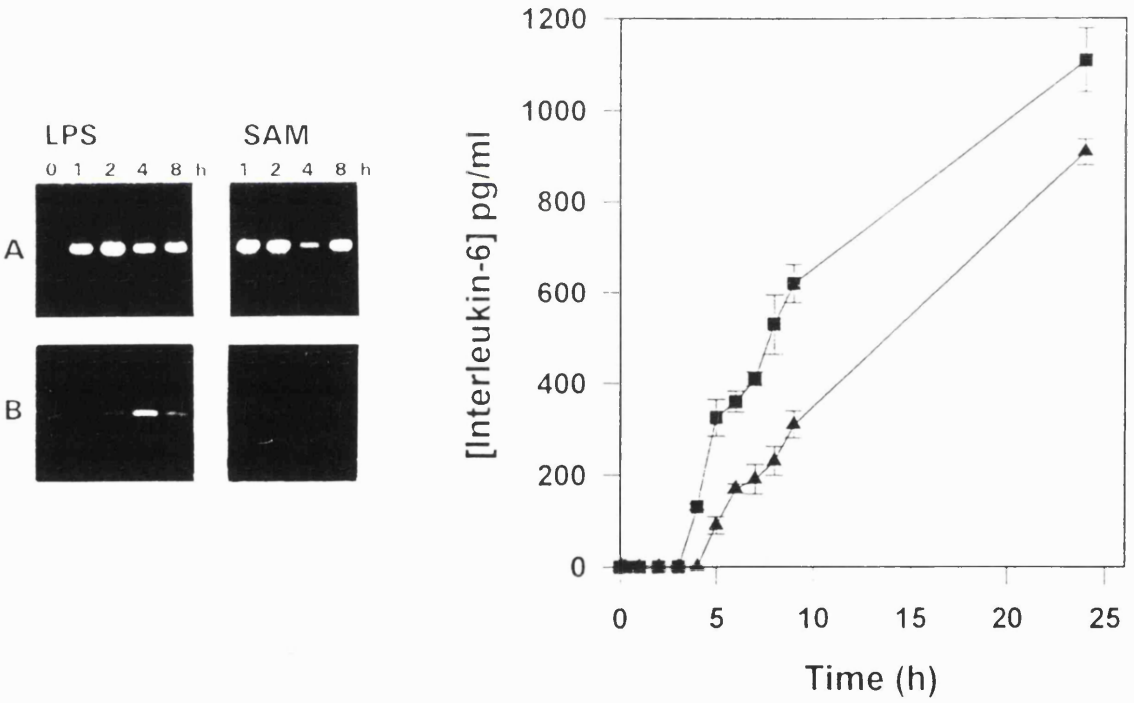


Figure 8.4 **RT-PCR of the IL-1 β and IL-6 mRNA in HGFs exposed to *E. coli* LPS or the SAM from *A. actinomycetemcomitans* for various times. A - PCR product using IL-6 primers, B- PCR product using IL-1 β primers. The associated graph shows the time course of IL-6 released in response to (▲) LPS or (■) SAM. The results are expressed as the mean and standard deviation of six replicate cultures.**

8.4.5 Influence of a neutralising antibody to PDGF on IL-6 release by HGF stimulated with E. coli LPS or SAM.

To determine whether PDGF was involved in the release of IL-6 in stimulated HGFs, the cells were pre-incubated with antibodies to human PDGF and then stimulated with *E. coli* LPS or SAM from *A. actinomycetemcomitans*. The IL-6 released into the medium was then measured as previously described. The results obtained are shown in figures 8.5 and 8.6 respectively.

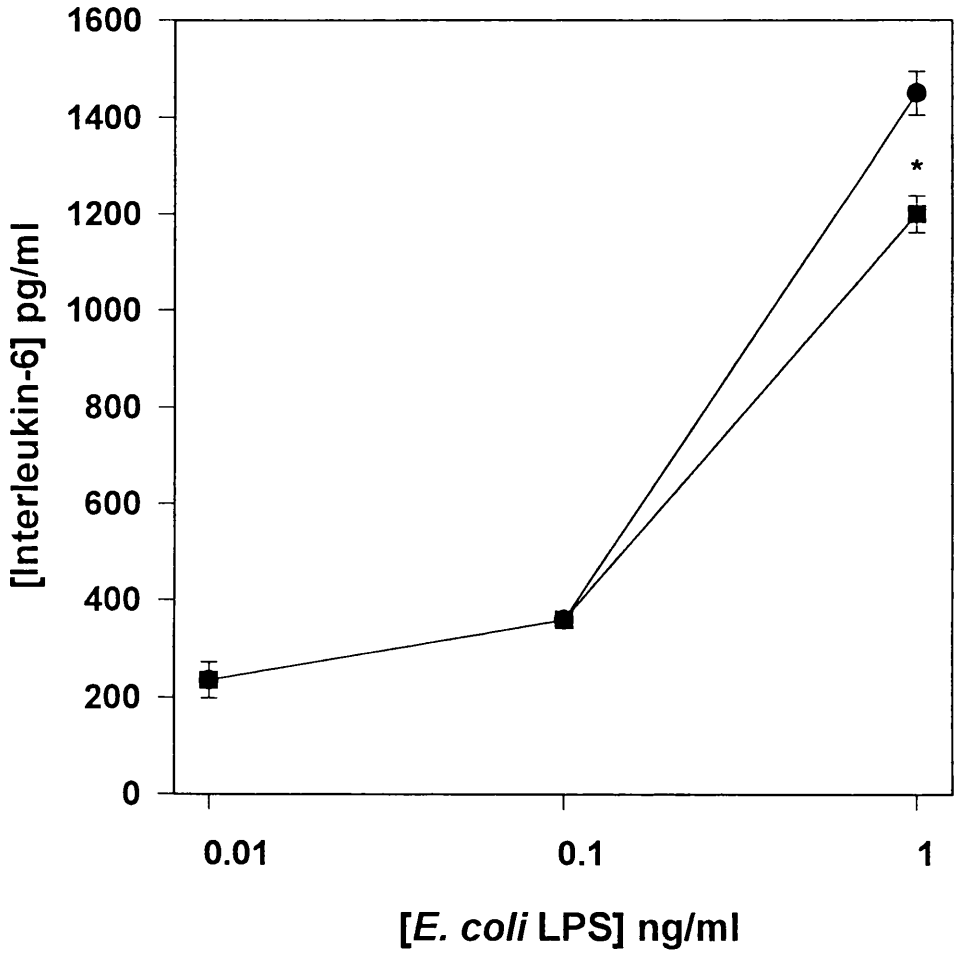


Figure 8.5 **IL-6 release by HGF after stimulating with *E. coli* LPS in the (●) absence and in the (■) presence of antibodies to PDGF. The neutralising antibody to PDGF failed to inhibit LPS-stimulated IL-6 release by these cells. The results are expressed as the mean ± 2SD of six replicate cultures. *p<0.05.**

A neutralising antiserum to PDGF did not completely inhibit either LPS- (figure 8.5) or SAM-stimulated (figure 8.6) IL-6 release, although significant inhibition of IL-6 release was observed at 1 ng/ml of *E. coli* LPS.

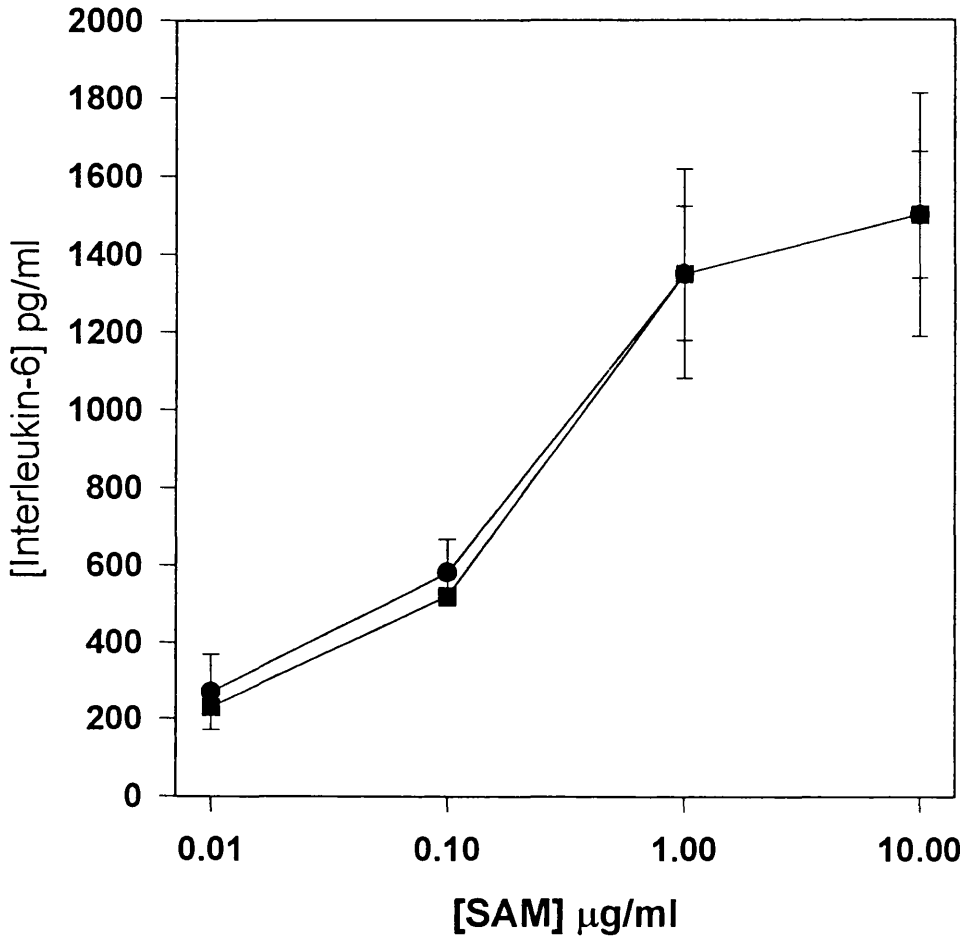


Figure 8.6 **IL-6 release by HGF stimulated with SAM in the (●) absence and in the (■) presence of antibodies to PDGF. The neutralising antibody to PDGF failed to inhibit the release of IL-6 by SAM-stimulated HGF. The results are expressed as the mean ± 2SD of six replicate cultures.**

8.4.6 Repression of IL-6 release

8.4.6.1 Repression of IL-6 release by dexamethasone in E. coli LPS-stimulated HGF

HGF were incubated with 10^{-9} M dexamethasone, stimulated with *E. coli* LPS or SAM and then the release of IL-6 monitored. It was found that dexamethasone completely inhibited the release of IL-6 from HGFs stimulated with LPS from *E. coli* (figure 8.7).

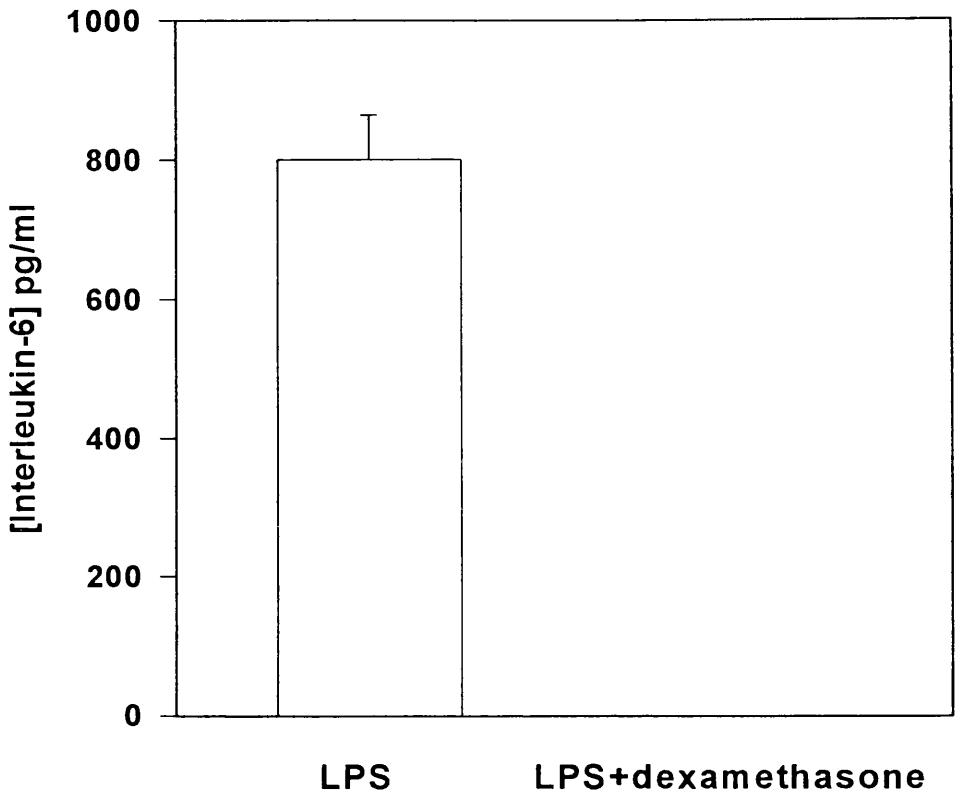


Figure 8.7 **IL-6 release by HGFs stimulated with *E. coli* LPS in the presence of 10^{-9} M dexamethasone. The dexamethasone completely inhibited the IL-6 release by *E. coli* LPS- stimulated HGF. The results are expressed as the mean + 2SD of six replicate cultures.**

8.4.6.2 Repression of IL-6 release by dexamethasone in SAM-stimulated HGF

In contrast to the effect of dexamethasone on IL-6-induced LPS release, this potent drug was only able to inhibit SAM-induced IL-6 synthesis by approximately 50 % (figure 8.8). The concentration of SAM chosen stimulated similar production of IL-6 as did the LPS.

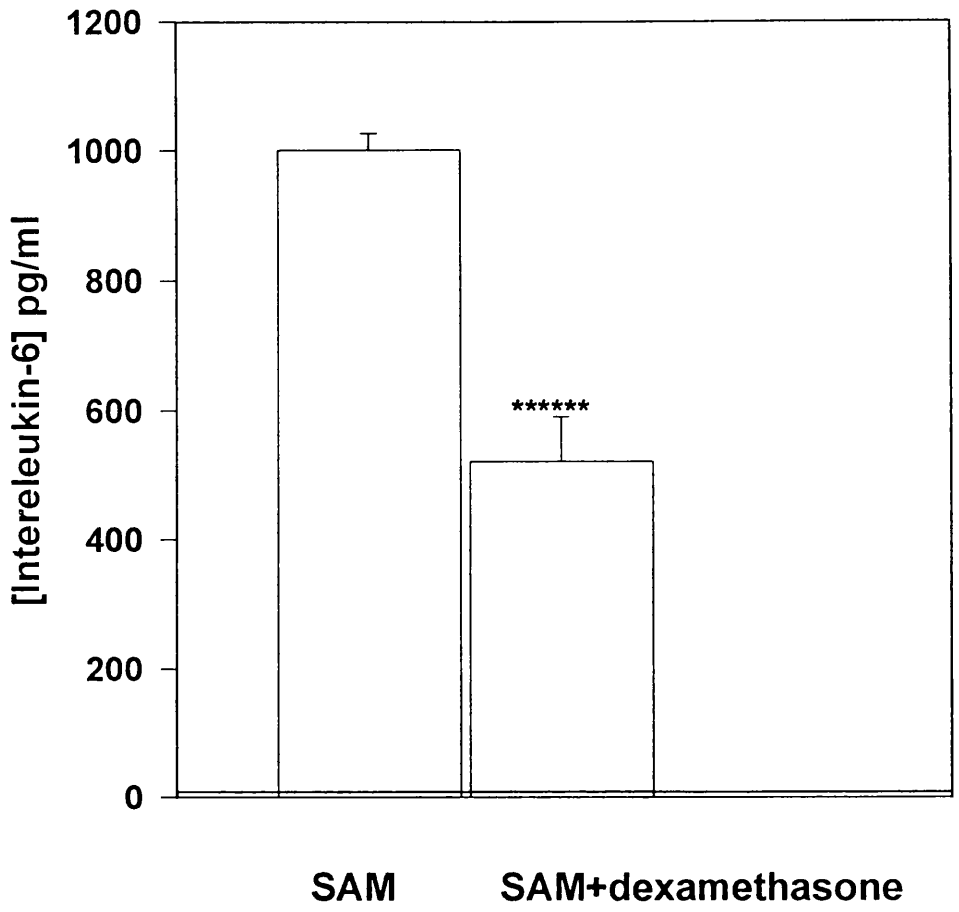


Figure 8.8 **IL-6 release by HGFs stimulated by SAM in the presence of 10^{-9} M dexamethasone. The dexamethasone only inhibited the synthesis of IL-6 by approximately 50 %. The results are expressed as the mean + 2SD of six replicate cultures. ******p<0.000001.**

8.4.6.3 Cyclooxygenase and Lipoxygenase pathways

To investigate whether the mechanism of cytokine release by SAM-stimulated HGF occurred via the cyclooxygenase or lipoxygenase pathway, indomethacin and 5-LO inhibitors (MK886 and BW70C) were used.

8.4.6.4 IL-6 release by E. coli LPS-stimulated HGF in the presence of indomethacin

Although 10^{-5} M indomethacin produced a slight but statistically significant

inhibition of IL-6 release (figure 8.9), in general there was very little inhibition ofIL-6 released by *E. coli* LPS stimulated HGF.

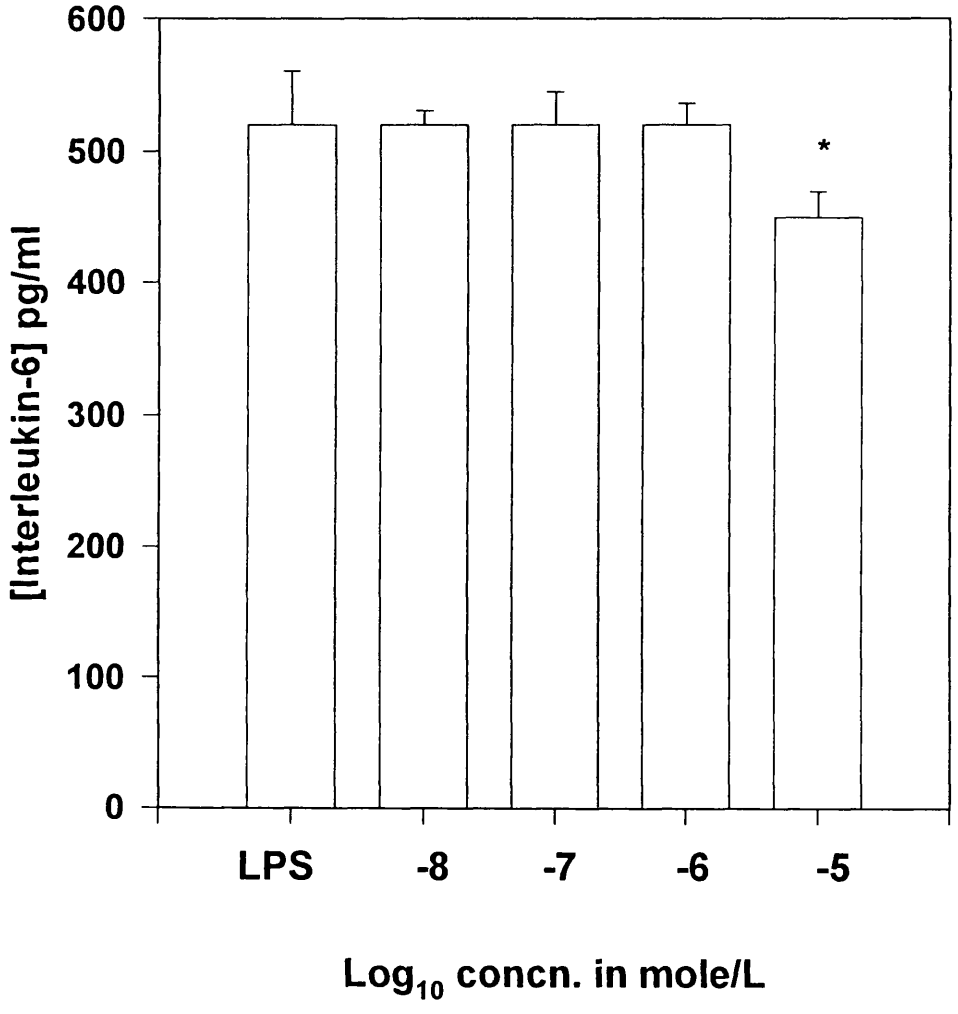


Figure 8.9 **IL-6 release by HGF stimulated with *E. coli* LPS in the presence of various concentrations of indomethacin. The IL-6 activity was not inhibited after adding 10^{-8} to 10^{-6} M concentrations of indomethacin to *E. coli* LPS-stimulated HGF. The results are expressed as the mean + 2SD of six replicate cultures. *p<0.05.**

8.4.6.5 IL-6 release by SAM-stimulated HGF in the presence of indomethacin

A similar pattern to the *E. coli* LPS was obtained with the SAM-stimulated HGF in the presence of various concentrations of indomethacin (figure 8.10). Although some inhibition of IL-6 release was observed in the presence of very

high, possibly toxic concentrations of indomethacin, total inhibition of IL-6 release did not occur. These results indicated that the mechanism of IL-6 released by HGF stimulated with *E. coli* LPS or SAM from *A. actinomycetemcomitans* did not occur via the cyclooxygenase pathway. Therefore, the possibility that IL-6 release was mediated by the lipoxygenase pathway was investigated

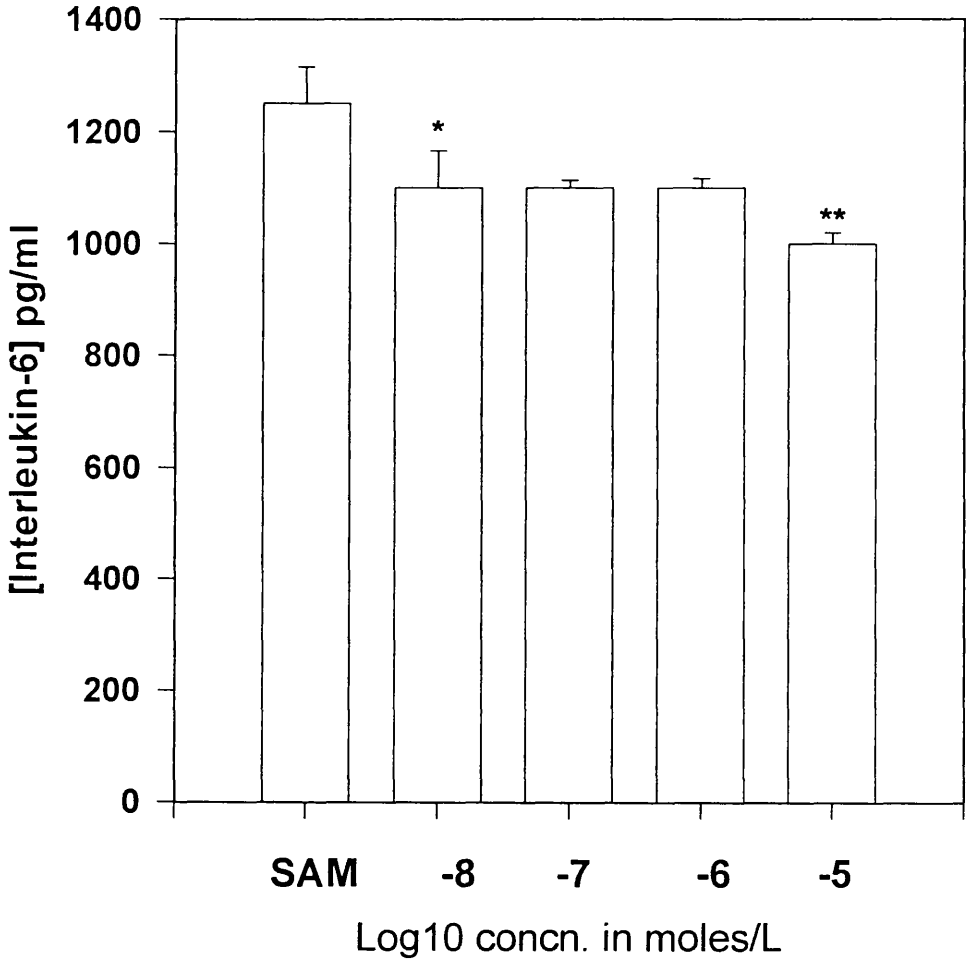


Figure 8.10 **IL-6 release by SAM-stimulated HGF in the presence of various concentrations of indomethacin. The IL-6 activity was not inhibited in the presence of indomethacin in SAM-stimulated cells. The results are expressed as the mean + 2SD of six replicate cultures. *p<0.05, **p<0.001.**

8.4.6.6 Lipoxygenase pathway

HGFs were pre-incubated with either MK886 or BW70C and then stimulated with *E. coli* LPS or SAM from *A. actinomycetemcomitans*.

8.4.6.7 IL-6 release by HGF stimulated with E. coli LPS in the presence of MK886

In the presence of MK886, there was no significant inhibition of IL-6 release by *E. coli* LPS-stimulated HGF (figure 8.11). A similar response was observed in the presence of BW70C (results not shown). MK886 and BW70C did not stimulate HGF to release IL-6.

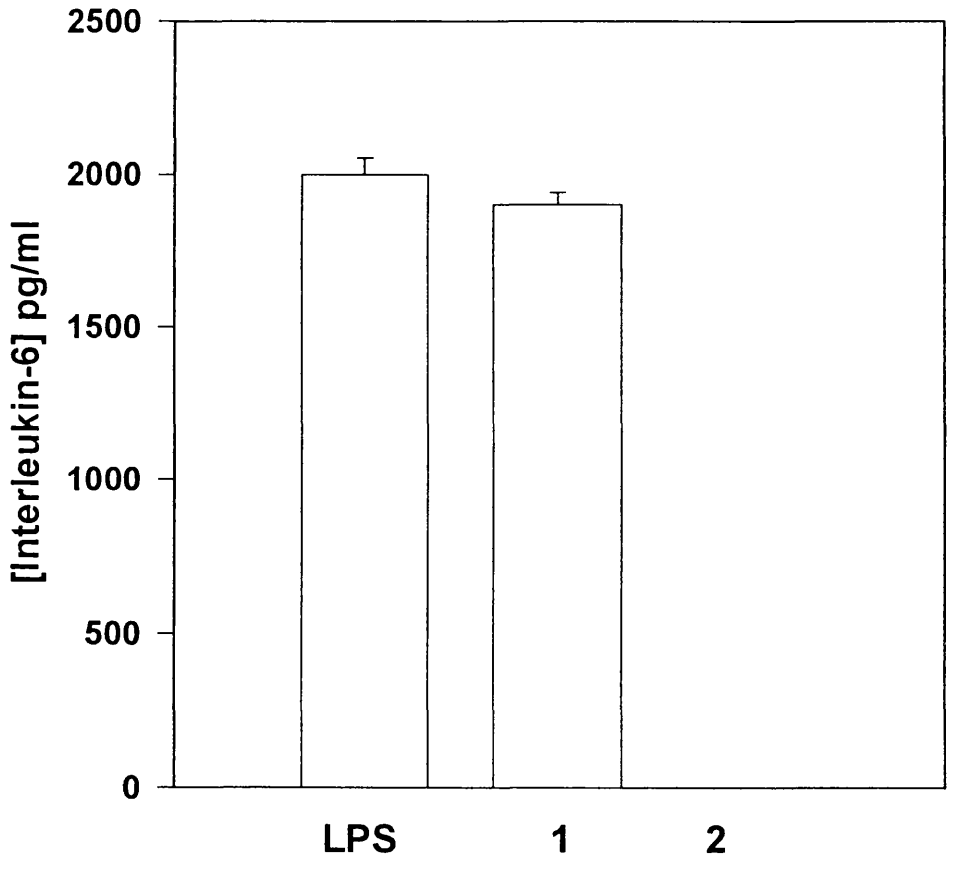


Figure 8.11 **HGFs stimulated with *E. coli* LPS or (1)- LPS plus MK886 or (2)- MK886 alone. The IL-6 activity was not inhibited by the presence of MK886 and MK886 did not induce IL-6 synthesis. The results are expressed as the mean + 2SD of six replicate cultures.**

8.4.6.8 IL-6 release by HGF stimulated with the SAM in the presence of MK886

There was some inhibition of IL-6 release by SAM-stimulated HGFs in the presence of MK886 however, total inhibition of IL-6 release did not occur (figure 8.12). A similar result was obtained with BW70C (results not shown).

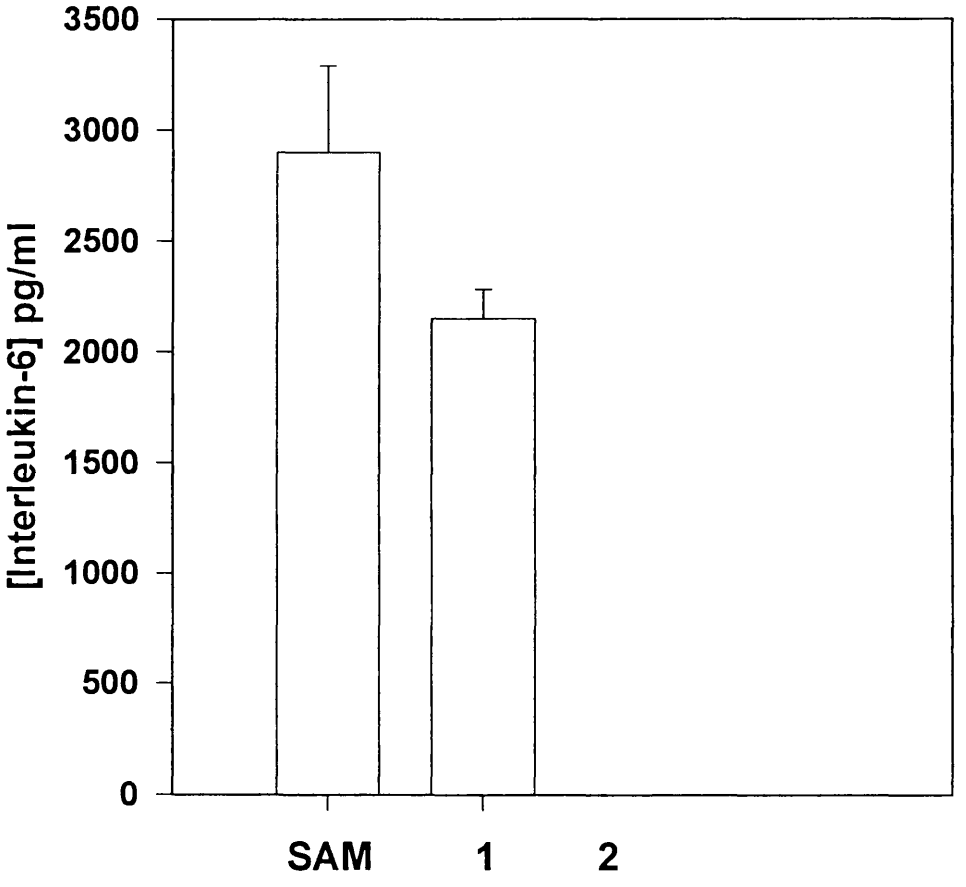


Figure 8.12 **HGFs stimulated with SAM or (1)- SAM plus MK886 or (2)- MK886 alone. There was some inhibition of IL-6 activity due to the addition of MK886 in SAM-stimulated HGF. However, total inhibition of activity did not occur. The results are expressed as the mean + 2SD of six replicate cultures.**

8.5 Discussion

The primary finding which stimulated the work described in this chapter was that SAM from periodontopathogenic bacteria can induce HGFs to release IL-6 in an IL-1 and TNF-independent manner. This finding was surprising as it has now been reported for a range of cell populations (monocytes (Tosato and Jones, 1990); endothelial cells (Jirik *et al.*, 1989); osteoblasts (Littlewood *et al.*, 1991); fibroblasts (Van Damme *et al.*, 1987) that, when exposed to LPS, IL-6 is released in an IL-1 or TNF-dependent manner. Indeed, the only other studies which suggests otherwise are (i). by Yamazaki and co-workers (1992) who found that LPS from *Porphyromonas gingivalis* could stimulate HGF to produce IL-6 both directly and indirectly and (ii). by Rosendal and colleagues (1995) who found that the potent mitogen-*Pasteurella multocida* toxin (PMT) stimulated fibroblast IL-6 synthesis without inducing IL-1 synthesis. However, the conclusions of this paper are suspect as the authors used the standard bioassay for measuring IL-6 which relies on the stimulation of proliferation of a cell line (B9). PMT is the most potent mitogen described and this inherent activity of PMT was not taken into account by the authors.

The first step in determining the mechanism of action of the SAM was to see if IL-6 synthesis was dependent on the prior synthesis of IL-1 or TNF. This was done by using neutralising antibodies to IL-1 α or β, to TNF α or to PDGF. In addition, the natural antagonist of the IL-1 receptor-IL-1ra was also used. As expected, fibroblasts exposed to *E. coli* LPS produced abundant amounts of IL-6 which was significantly, or completely, inhibited by the addition of saturating concentrations of neutralising antibodies to : (i) IL-1 (both α and β), (ii) TNF-α and (iii) to both IL-1 and TNF. IL-1ra also completely inhibited the LPS-induced IL-6 synthesis. However, in contrast, neutralisation of PDGF had no influence on the production of immunoreactive IL-6. Thus, this established that LPS-induced IL-6 synthesis was dependent on the prior synthesis of IL-1 and/or TNF. This was further demonstrated by the use of RT-PCR which the mRNA in the cell cytoplasm is converted by reverse transcriptase into cDNA which can then be amplified using specific cytokine primers to detect the presence of

particular cytokine gene products. This showed that HGFs exposed to LPS-contained mRNA for IL-1α, IL-1β, TNF-α and IL-6.

An identical set of experiments were run side-by-side using the SAM from *A. actinomycetemcomitans*. The results were completely at variance with that of the LPS, with none of the neutralising antibodies or the IL-1ra causing complete inhibition of IL-6 release. Examination of the intracellular levels of IL-1β in SAM-stimulated HGFs failed to find any immunoreactive cytokine. The final check using RT-PCR to examine intracellular cytokine, failed to amplify any signal for IL-1 α/β or TNF-α. There was, however, a large amplifiable signal for IL-6-mRNA. Neutralisation of PDGF had no effect on IL-6 production induced either by LPS or SAM.

Thus this establishes that the SAM, in contrast to LPS, fails to induce the transcription of the genes for IL-1 α or IL-1 β. Measurement was also made of immunoreactive IL-1ra in the culture media of SAM-stimulated HGFs and failed to detect any signal. Thus the SAM, or at least the active moiety in the SAM, is incapable of inducing the transcription of the genes for the IL-1 family. It is also incapable of inducing transcription of the TNF-α gene. Measuring the time course of IL-6 release from SAM- or LPS-treated HGFs revealed the earlier appearance of IL-6 in SAM-treated cells which is consistent with the lack of a requirement to induce IL-1 in order to upregulate IL-6 transcription. Thus, it appears that LPS rapidly induces IL-6 gene transcription of the gene for IL-1 β which then acts in a feedback manner to induce IL-6 gene transcription via nuclear factor kappa B (NFκB; see figure 8.13) or nuclear factor-IL-6 (NF-IL-6;Zhang *et al.*, 1994).

Many cytokine genes are exquisitely responsive to glucocorticoids such as dexamethasone, which prevent gene transcription, the IL-6 gene being one of these. It was therefore expected that both the LPS-and SAM-stimulated fibroblasts would be completely prevented from releasing IL-6 in the presence of dexamethasone. It was established, over a range of steroid concentrations from 10^{-4} to 10^{-9} M, that dexamethasone completely inhibited LPS-induced IL-6

synthesis. In contrast, over the same concentration range, dexamethasone was only able to inhibit SAM-induced IL-6 synthesis by approximately 50 %.

Three regulatory regions have been shown to be involved in the regulation of the IL-6 gene. In 1989 Ray and colleagues showed that a 23 base pair IL-6 multiresponse element (MRE) was responsible for the induction of IL-6 by IL-1, TNF, and serum (figure 8.13) as well as by the activators of protein kinase A (forskolin) and protein kinase C (phorbol ester). A 14 base pair palindromic sequence recognized by a nuclear factor (NF-IL6) was found to be present by Isshiki and colleagues in 1990. The third regulatory region was also discovered in 1990 by Shimizu and co-workers who found that an NF-κB binding site was present, which was responsible for the induction of IL-6 in response to IL-1 or TNF. In addition a number of responsive elements such as the glucocorticoid responsive element (GRE), cAMP responsive element (CRE) and the serum responsive element (SRE) have also been found.

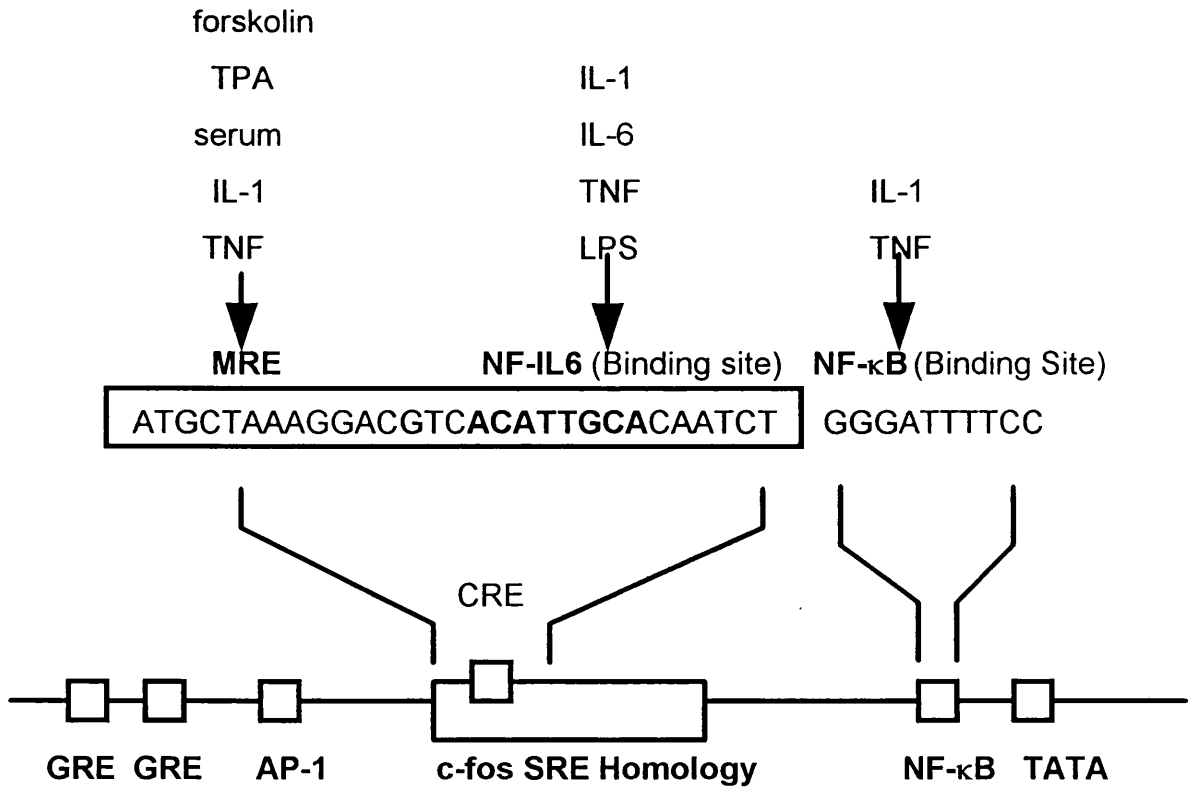


Figure 8.13 **Diagram representing the IL-6 promoter regions. GRE-glucocorticoid-response element; CRE-c-AMP-responsive element; c-fos SRE-serum-response element; AP-1- activator protein 1**

In 1990, NF-IL6, which is a nuclear factor responsible for the expression of the IL-6 gene, was cloned by Akira and colleagues. NF-IL6 was initially identified as a nuclear factor binding to a 14 base pair palindromic sequence (ACATTGCACAATCT) within an IL-1 responsive gene element in the human IL-6 gene (Ishiki *et al.*, 1990). It was found to be expressed at an undetectable or minor level in all normal tissues, but was drastically induced after stimulation with LPS, IL-1, TNF, or IL-6. NF-IL6 has been found to bind to the regulatory region of various genes including IL-8, G-CSF and IL-1, indicating that NF-IL6 may be a pleiotropic mediator of many inducible genes involved in acute-phase, immune and inflammatory responses (Akira and Kishimoto, 1992).

NF-κB was originally characterized as a kappa immunoglobulin enhancer DNA-binding protein. Binding sites for NF-κB are present in the regulatory regions of certain cytokine genes, including the TNF, IL-6, IL-8 and β-IFN genes. NF-κB is a complex of two proteins of 50 and 65 kDa which pre-exists in the cytoplasm of most cells in an inactive form, complexed to IκB. Stimulation by a number of agents such as phorbol ester, LPS, and TNF, results in the dissociation of the IκB-NF-κB complex, probably by phosphorylation of IκB. Subsequently the NF-kB heterodimer migrates to the nucleus, where it binds to the DNA binding sites and activates transcription.

As previously mentioned, it is known that glucocorticoids have an inhibitory effect on the production of many cytokines including IL-6, TNF, and IL-1. Several studies have demonstrated that glucocorticoids inhibit IL-6 production at the transcriptional level. Ray and co-workers (1991) have investigated the molecular basis for the repression of the IL-6 promoter by the glucocorticoid dexamethasone. Their data showed that the activated GRE binds to the inducible enhancers (MRE and NF-IL6 binding sites) as well as to the basal transcriptional regulatory regions (TATA box and RNA start sites) in the IL-6 promoter and its binding interferes with the binding of positive-acting inducible and basal transcription factors, resulting in the highly efficient repression of this gene by dexamethasone. The active moiety in the SAM failed to stimulate IL-1 or TNF gene transcription, but directly stimulated the transcription of the IL-6

gene by a mechanism which presumably does not involve steroid-sensitive transcription factors such as NFκB or NF-IL-6. Therefore it is concluded that the active constituent of the SAM induces IL-6 gene transcription by activating downstream transcriptional control elements of the IL-6 gene which differ from those activated by *E. coli* LPS. Of course as dexamethasone inhibited 50 % of the IL-6 gene transcription a number of transcriptional control sites including glucocorticoid sensitive ones could be activated by the SAM.

Chemical, mechanical or electrical stimuli of the cell membrane causes the release of arachidonic acid, which is then oxygenated to yield a variety of bioactive compounds including prostaglandins, thromboxanes, leukotrienes and lipoxins (Reviewed by Toh *et al.*, 1992; Kawaguchi *et al.*, 1995; Raisz, 1995). These compounds are known as 'eicosanoids' and act as critical mediators in inflammation, blood clotting, control of vascular tone, renal function, reproductive system function and cell differentiation. The pathway by which these eicosanoids are formed is called the 'arachidonic acid ' cascade (figure 8.14). There are suggestions that the lipid products of arachidonic acid oxidation can induce the synthesis of IL-6 by various cell populations including fibroblasts. The enzyme cyclooxygenase II (Cox II) is involved in the synthesis of prostaglandins and is upregulated by inflammatory stimuli such as LPS and cytokines (mainly IL-1 and TNF). The activity of Cox I (the constitutive form of the enzyme) and Cox II is blocked by a variety of inhibitors, referred to as non-steroidal anti-inflammatory drugs (NSAIDs), of which indomethacin is one.

The other major pathway of arachidonic acid oxidation is via the enzyme 5-lipoxygenase, which produces the leukotrienes. Leukotrienes are a class of metabolites of arachidonic acid that are synthesized mainly by leukocytes in response to inflammatory and immunological stimuli (Samuelsson, 1983). The biological effects of the leukotrienes, which include leukocyte chemotaxis and aggregation, airway smooth muscle contraction, and oedema formation, have implicated them as potential mediators of allergy and inflammation (Rouzer *et al.*, 1990a; 1990b). The first two steps of the leukotriene biosynthetic pathway are catalyzed by 5-lipoxygenase (Rouzer *et al.*, 1986) which is found primarily in cells of myeloid origin such as polymorphonuclear leukocytes and

eosinophils. These reactions involve the addition of molecular oxygen to carbon 5 of arachidonic acid to form 5(*S*)- hydroperoxyy-6,8,11,14-eicosatetraenoic acid (5-HPETE), and the further conversion of 5-HPETE to 5,6-oxido-7,9,11,14-eicosatetraenoic acid (Leukotriene A_4, (LTA_4) LTA_4 is then further metabolized by other enzymes to LTB_4 or LTC_4 depending on the cell type. The exact mechanism for 5-lipoxygenase activation remains unclear, however, the 5-LO enzyme is postulated to be normally in the dormant ferrous state (Fe^{2+}). This enzyme is found in the cytosol but is located away from the cell membrane where the arachidonic acid is situated. The 5-LO enzyme is converted to the active ferric form (Fe^{3+}) after activation by hydroperoxidase, ATP and calcium. This then translocates, possibly due to a change from a hydrophilic conformation into a hydrophobic conformation, to the cell membrane where it docks with a transmembrane protein FLAP (5-lipoxygenase activating protein). Then, as described above, 5-HPETE is formed and then LTA_4. The 5-LO inhibitor MK886 is a translocation inhibitor which prevents this docking of the 5-LO to FLAP. These inhibitors are the newest class of leukotriene synthesis inhibitors, and it has been shown in a double-blind clinical trial of asthma patients that oral administration of MK886 resulted in significant reduction of the antigen-induced early-phase reaction of this disease. The other 5-LO inhibitor used in this investigation was BW70C which is a hydroxamic acid class inhibitor. The mechanism of the inhibition of the 5-LO enzyme by such inhibitors is not established, but, it is thought that the mechanism of inhibition is via an antioxidant or free radical scavenging mechanism.

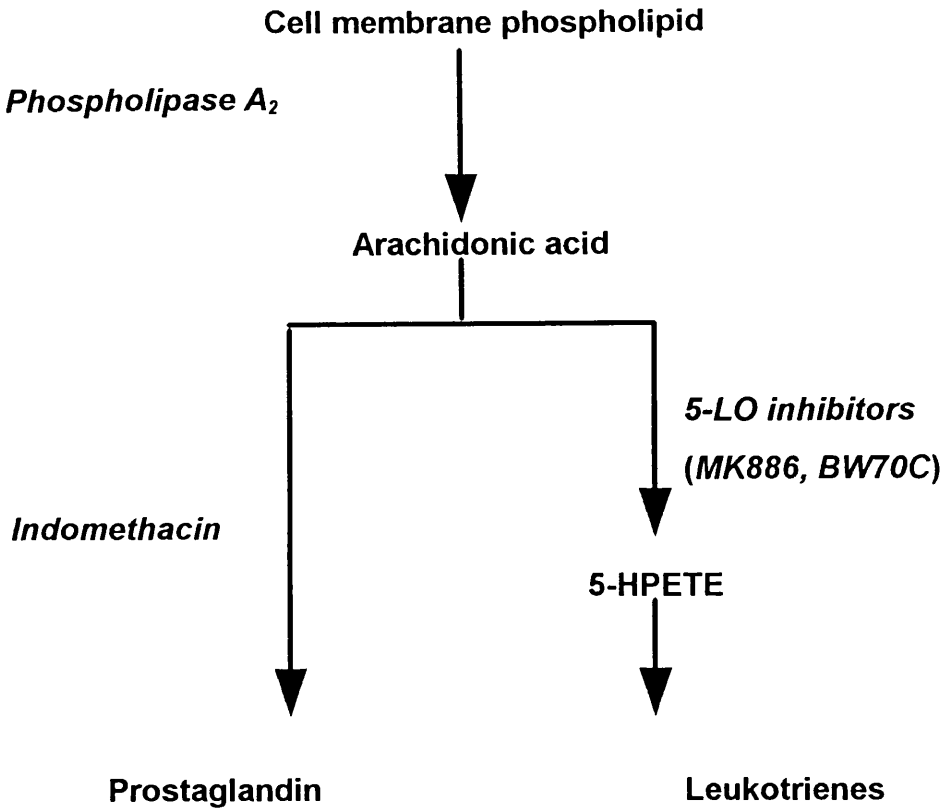


Figure 8.14 **Flow-chart showing the arachidonic acid cascade with particular reference to the inhibitors indomethacin, MK886 and BW70C**

Indomethacin had no biologically-significant influence on the synthesis of IL-6 by either LPS-or SAM-stimulated HGFs and thus this pathway appears not to be important in the transcriptional control of IL-6. In a similar manner inhibitors of leukotriene synthesis had no effect on IL-6 synthesis. Both of these pathways of arachidonic acid oxidation appear to play no significant role in the transcriptional regulation of IL-6 synthesis in HGFs exposed to these two particular bacterial components.

8.6 Conclusion

It is reasonable to conclude that the active IL-6-inducing moiety in the SAM promotes gene transcription by a mechanism distinct from that of LPS or other known IL-6 gene activators. The particular IL-6 gene regulatory elements being induced cannot be defined as yet and this will not be able to be determined until the active principle in the SAM is isolated.

Chapter 9

Isolation of the IL-6-stimulating protein from the SAM of *A. actinomycetemcomitans*

9.1 Introduction

The osteolytic protein from the SAM from this organism has been isolated and found to be a member of the chaperonin 60 family. The component in the SAM of *A. actinomycetemcomitans* responsible for stimulation of IL-6 synthesis has not been isolated. This molecule, because of its mechanism of action, is of inherent interest.

9.2 Aim

The aim of this part of the study was to isolate the protein from the SAM of *A. actinomycetemcomitans* responsible for the stimulation of IL-6 synthesis in HGFs.

9.3 Materials and methods

As described in chapter 3, the proteins present in the SAM from *A. actinomycetecomitans* were found to be anionic in nature, therefore the first purification step was anion exchange chromatography. An anion-exchange MA7Q (Bio-Rad) column (5 by 0.78 cm) was equilibrated with 20 mM Tris buffer, pH 8.5 (buffer A). One milliliter of a 4 mg/ml solution of SAM in buffer A was injected onto the column and eluted with 5 ml of buffer A followed by linear gradients from 0 to 50 % buffer B (buffer A plus 2 M NaCl) in 20 ml and 50 to 100 % buffer B in 5 ml. The flow rate was 1 ml/min, and 1 ml fractions were collected, with absorbance monitored at 280 nm. Fractions were dialysed against distilled water using a 2 kDa cutoff membrane (Sigma) for 48 h. The protein concentration of each fraction was measured using the Bio-Rad DC assay kit. Each fraction was assayed for its ability to stimulate HGFs to release IL-6 as described in chapter 2. One of the bio-active fractions was further purified on a C-4 (Vydac) reverse-phase column using a TFA/ acetonitrile gradient. The flow rate was 1 ml/min and 1 ml fractions were collected in

Eppendorfs. The fractions were dried on a Speedvac (Gyrovant) to remove the TFA /acetonitrile and then assayed for activity on HGFs. Further purification was performed by size exclusion HPLC. A Protein Pak 125 column (Waters) was equilibrated with 0.1 M sodium phosphate buffer, pH 6.7, and 20 µl samples of the active fraction (concentration 1 mg/ml) were injected. The flow rate was 1 ml/min, and 1 ml fractions were collected, with protein absorbances monitored at 280. Fractions were assayed for bio-activity on HGFs as previously mentioned. The active fraction was then further fractionated on a C-8 (Phenomenex) reverse phase column using a TFA/acetonitrile gradient. The flow rate was 1 ml/min and fractions were collected every minute in Eppendorfs. SDS-PAGE was performed on the starting material, the bio-active fraction obtained from anion exchange HPLC and the bio-active fraction obtained from reverse-phase HPLC as described in chapter 2.

Statistical analysis

The data are expressed as the mean $\pm$ 2SD, n=6. The activity of each fraction was compared with the control (media only) by Students t-test.

9.4 Results

9.4.1 Protein purification

Fractionation of the crude SAM by anion exchange chromatography on a Bio-Rad MA7Q column (5 cm x 0.78 cm) produced a complex chromatographic profile with the bulk of the biological activity eluting as two separate peaks at a NaCl concentration of between 0.3 M and 0.7 M (figure 9.1). After assaying each of the fractions for IL-6 stimulating it was found that two peaks of activity were obtained. SDS-PAGE of the most active fraction (ie. the first peak) revealed the presence of approximately 10 protein bands after staining with Coomassie Colloidal Blue (figure 9.1).

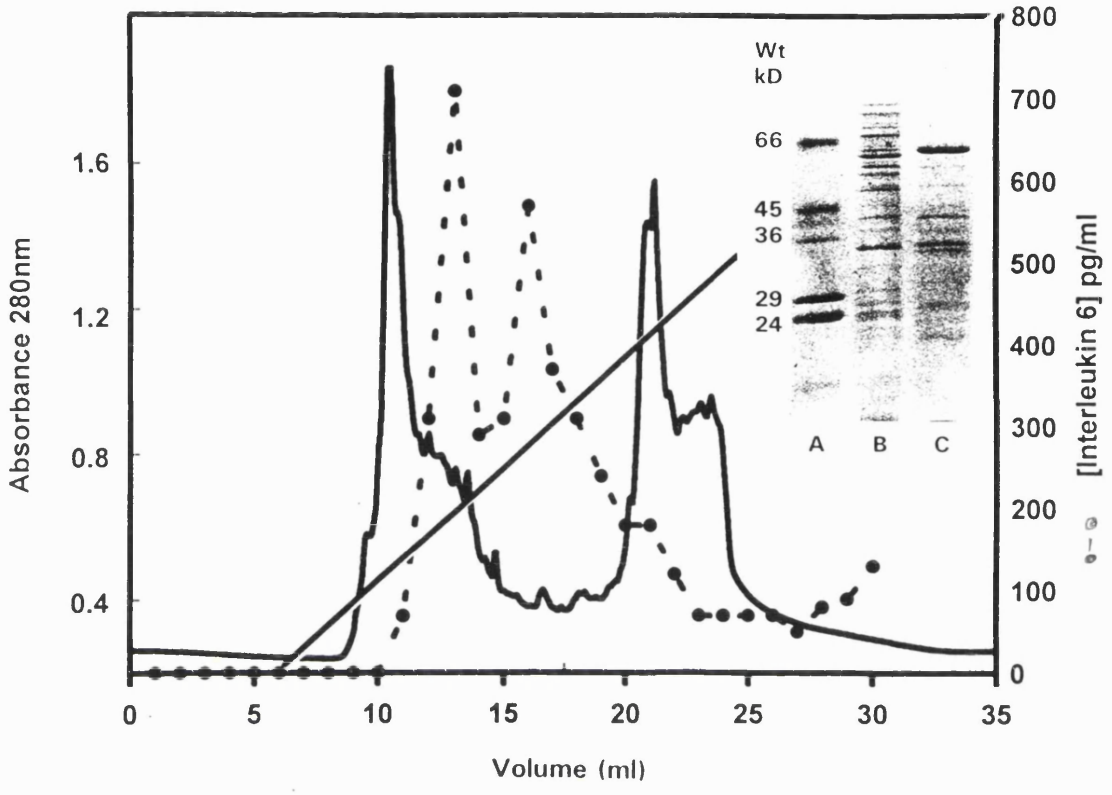


Figure 9.1 **HPLC of the SAM from *A. actinomycetemcomitans* (OD_{280}) on an MA7Q anion exchange column with a gradient of NaCl from 0-2M. (-----) shows the IL-6-stimulating activity of each fraction. The inset shows the SDS-PAGE of A- molecular weight markers, B- the starting material and C- the most bio-active fraction. Results are expressed as the mean and ± 2SD of six replicate cultures.**

The first peak of biologically active material was further fractionated on a Vydac C-4 column (15 cm x 0.46 cm) which resolved a number of protein species with only one peak demonstrating biological activity (figure 9.2). On a silver-stained SDS-PAGE gel this bio-active peak revealed the presence of only one band of low molecular mass.

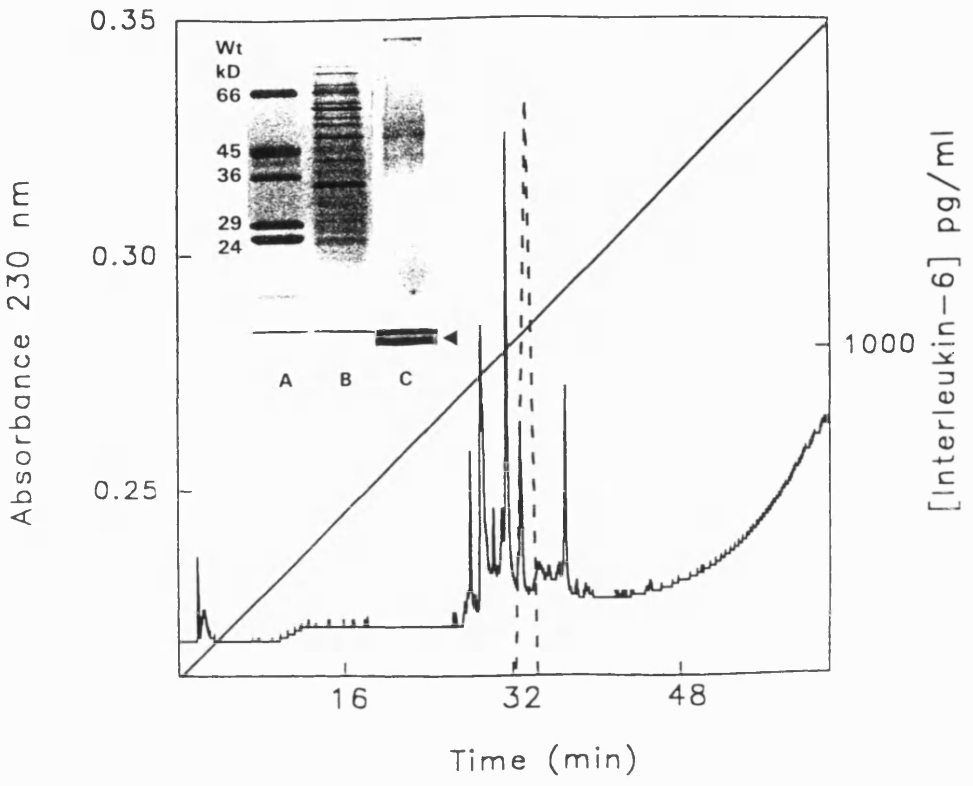


Figure 9.2 **Fractionation of the most biologically active anion exchange peak on a Vydac C-4 reverse phase column (Sigma; 15 cm x 0.46 cm) eluted with a TFA/acetonitrile gradient (0-100%). (-----) shows the IL-6-stimulating activity of the various fractions. Only one fraction possessed the ability to stimulate HGF to release IL-6. The inset shows the silver stained SDS-PAGE of A- molecular weight markers, B- the bio-active fraction obtained from anion exchange HPLC and C-the bio-active fraction from C-4 reverse-phase HPLC (protein band arrowed). Results are expressed as the mean ± 2SD of six replicate cultures.**

Further fractionation of this bio-active peak on a molecular weight marker calibrated Protein-Pak 125 column (30 cm x 0.75 cm) revealed the presence of only one peak with an apparent molecular weight of 2 kDa (figure 9.3).

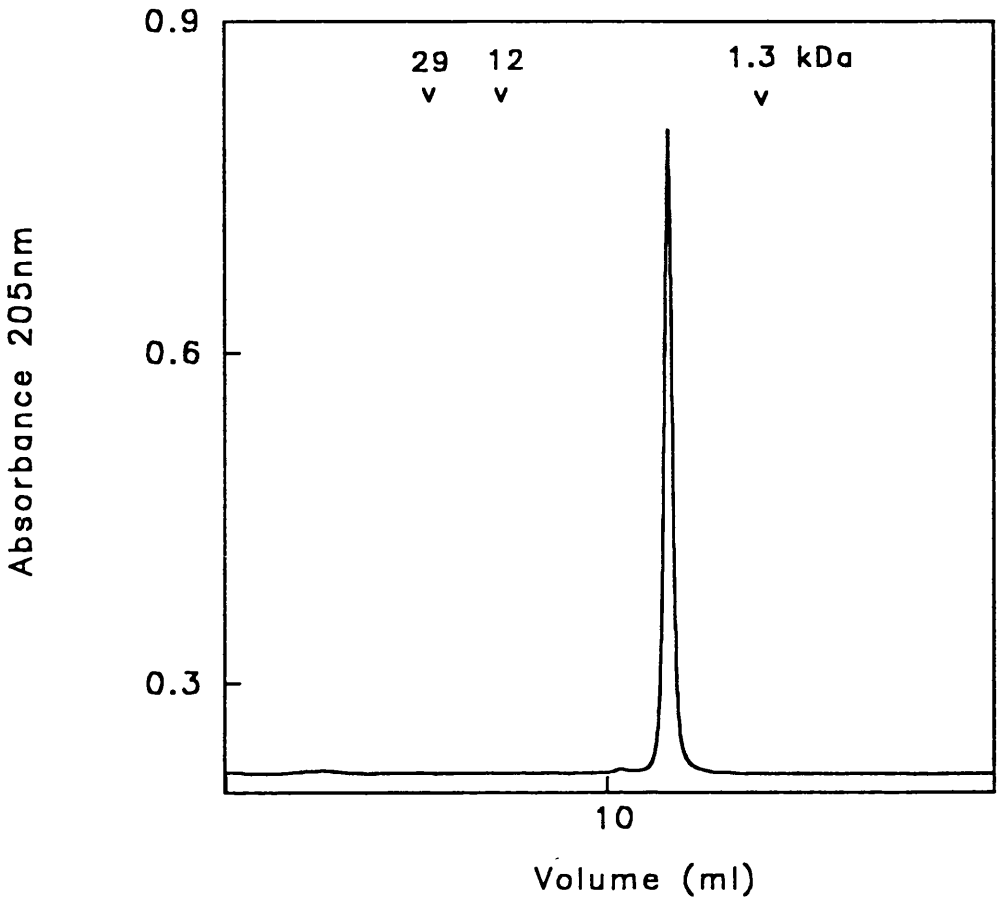


Figure 9.3 **Size exclusion HPLC of the active IL-6 stimulating peak from the C-4 column on a Protein-Pak 125 column. Only one peak with an apparent molecular mass of 2 kDa was eluted from this column.**

The material which eluted from the SEC column was re-fractionated on a C-8 reverse phase column (Phenomenex 25 cm x 0.46 cm) using an TFA/acetonitrile gradient which, revealed the presence of one major peak (figure 9.4).

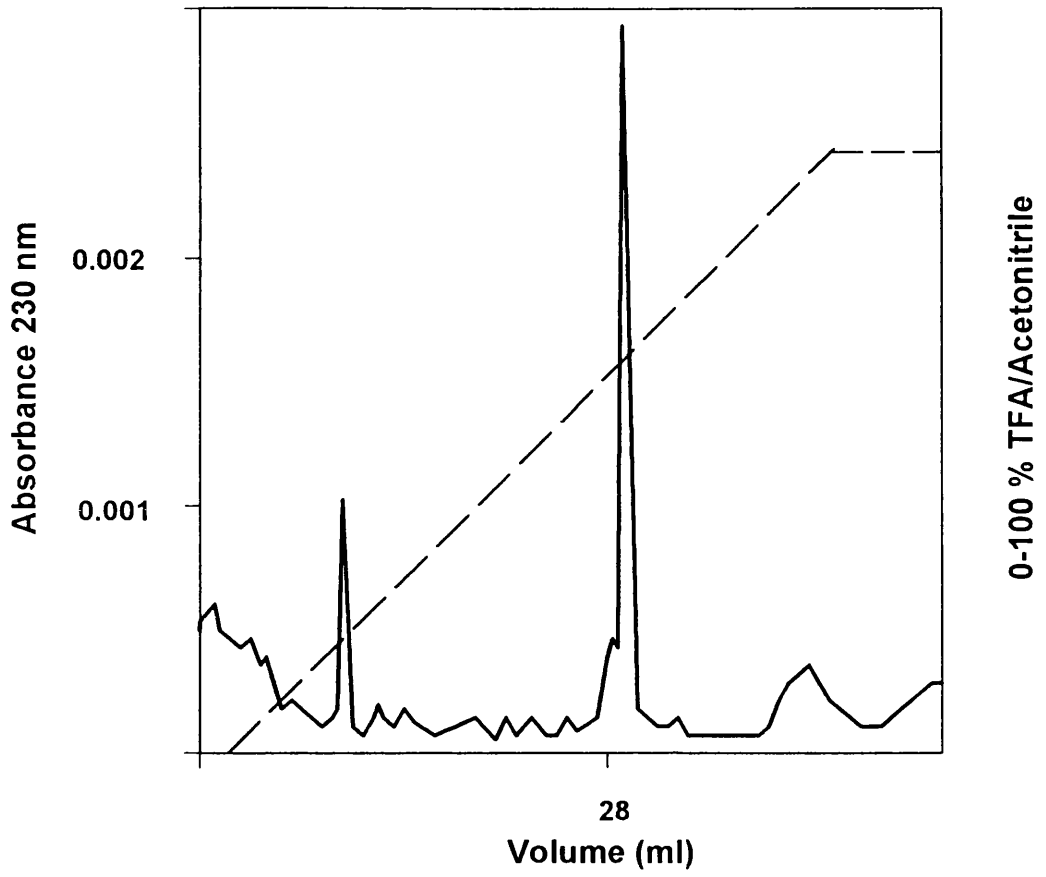


Figure 9.4 **Reverse-phase HPLC on a C-8 column of the peak obtained from the C-4 reverse phase column revealing the presence of only one major peak. (-----) shows the 0-100 % TFA/acetonitrile gradient.**

9.5 Discussion

A peptide from *A. actinomycetemcomitans* SAM has been isolated which has no bone-resorbing activity, but which directly stimulates gingival fibroblasts to synthesize and secrete IL-6. This surface-associated peptide may contribute to the pathology of localised juvenile periodontitis. IL-6 has a wide range of pro-inflammatory effects. Thus this peptide may contribute to the bone resorption seen in LJP by stimulating the synthesis of IL-6, a cytokine which has now been shown to stimulate the proliferation of osteoclast precursors (Roodman, 1992). IL-6 is a potent growth factor for B lymphocytes required for the maturation of B cells into immunoglobulin-secreting plasma cells (Kishimoto

and Hirano, 1988). This could account for the preponderance of blast cells and plasma cells in the gingiva in juvenile periodontitis.

Initial studies of the IL-6-stimulating activity revealed that it was sensitive both to heating and trypsinisation indicating that its biological activity was due to protein(s). Activity was not due to LPS contamination as purified LPS from *A. actinomycetemcomitans* was a weak inducer of IL-6. As demonstrated in chapter 3 the SAM from *A. actinomycetemcomitans* did not bind to the cation exchange column, therefore anion exchange HPLC was used as a first purification step. Elution of the SAM from this column revealed two peaks of activity. The first peak had the greatest IL-6 stimulating activity. Further purification was attempted by use of reverse phase HPLC (RP-HPLC). In this technique the matrix is composed of alkyl chains (of various lengths) covalently linked to porous silica beads. Proteins bind to the beads by hydrophobic interaction with the alkyl chains and elution (and purification) is achieved by using a gradient of acidified organic solvent such as acetonitrile. The main problem with reverse phase chromatography is that the organic solvents used can denature the proteins being isolated. Using a Vydac column with a short alkyl chain length (C-4) it proved possible to achieve excellent resolution of the first anion exchange fraction. Furthermore, when the fractions were tested for bio-activity one of them had IL-6-stimulating activity showing that not only had significant resolution been achieved but it had been done with the retention of biological activity. On a silver-stained SDS-PAGE gel this bio-active peak revealed the presence of only one band of low molecular mass which resolved below the dye front.

The active fraction from RP-HPLC was then run on a molecular mass-calibrated gel filtration column and only one sharp peak of molecular mass 2 kDa eluted. Given the SDS-PAGE and gel filtration data it was likely that the active peptide had been isolated. To confirm this the peak obtained after size exclusion HPLC was re-fractionated on a reverse phase column with C-8 alkyl chain length. Only one major peak eluted. Unfortunately, it had lost bio-activity. Thus it is concluded that the IL-6-stimulating activity of the SAM of *A. actinomycetemcomitans* is attributable to a 2 kDa peptide.

Attempts have been made to sequence this peptide but so far have not been successful. However, it is obvious that the next step in this project must be to obtain a total sequence of this peptide, produce a synthetic version and test its bio-activity. Once sufficient amounts of a bio-active peptide can be obtained then the mechanism by which it directly stimulates IL-6 transcription can be examined. This will also involve determining the nature of the membrane receptor and the intracellular signaling mechanisms involved in transmitting the signal to the nucleus.

9.6 Conclusion

The IL-6-stimulating activity of the SAM from *A. actinomycetemcomitans* is due to a peptide with an apparent molecular mass of 2 kDa. Work is currently underway to prepare sufficient quantities of this material for sequencing. However, this IL-6-stimulating peptide is present in very low concentrations and this will be a major undertaking.

Chapter 10

Conclusions and future investigations

Much attention has been devoted to endotoxin and its protein-free constituent, LPS, as mediators of periodontal pathology. However, the work presented in this thesis is based on the hypothesis that inflammation and bone loss in the periodontal diseases are due to soluble factors released from oral bacteria implicated in this disease process. It is hypothesised that this material, which is associated with the surface of the bacteria, is the first point of contact between the bacterium host cells. Therefore it is likely to contain biologically important molecules involved in signalling to host cells. Thus I have concentrated my attention on the surface fraction of periodontopathogenic bacteria with emphasis on the bone resorbing and cytokine-inducing activity.

10.1 Composition of the SAM

Characterisation of the SAM preparations revealed that a large amount of the dry mass was material associated with the bacterial surface. The other findings from this part of the investigation were that the SAM preparations contained large percentages of protein but low percentages of carbohydrate, lipid and nucleic acids. Furthermore, when SDS-PAGE was performed on the SAMs large numbers of protein bands were resolved in the molecular mass range <14 to >66 kDa. Thus these bacteria appear to have a very complex population of loosely-attached proteins on their surfaces. This obviously raises the question as to whether these results are specific to members of the oral microflora. However, gentle saline extraction of the commensal skin bacteria *Staphylococcus aureus* and *Staphylococcus epidermis* also elutes complex populations of proteins. It is not known if the enteric microflora also have such protein-rich extracellular matrices. In contrast, the composition of the LAP of these oral bacteria (with the exception of *A. actinomycetemcomitans*) shows a much more restricted population of molecules. Surprisingly, the LAP from *Por. gingivalis* had only one major protein band which may be responsible for the cytokine-inducing activity. It is planned to clone and express this protein.

The complexity of the surface-associated fractions of these bacteria makes the task of elucidating the molecules responsible for individual biological activities difficult. In this thesis the biological activity of the SAM has been examined in terms of its ability to induce bone resorption and to stimulate mesenchymal and myelomonocytic cells to produce pro-inflammatory cytokines. The activity of this outer surface fraction has, where possible, been compared to LPS. Given the extensiveness of the literature on LPS and endotoxin it was felt that, where possible, comparison should be made of the biological activities of the SAM the LPS and the LAP. This is the common motif flowing through this thesis.

10.2 Osteolytic activity of SAM, LAP and LPS

In spite of the commonly accepted view that LPS is a potent stimulator of bone resorption the findings from this investigation was that protein free LPS from *Por. gingivalis*, *Prev. intermedia* and *A. actinomycetemcomitans* were extremely weak activators of calvarial bone resorption. The only LPS tested which showed significant bone resorbing activity was the material from *Eik. corrodens*. In contrast the three LAP preparations tested all showed similar, and significant, bone resorbing activity. This suggests that the bone resorbing activity of the endotoxin from these three bacteria is due to the LAP. It is not clear why the osteolytic activity of the LPS from these oral bacteria is so much less than that of enteric LPS, but it may depend on the structure of the respective lipid As. Further work needs to be done to relate lipid A structure to osteolytic activity.

It is clear from the data presented in chapter 4 that the SAMs from the 5 bacteria examined have a wide range of osteolytic activity. The SAM from *Eik. corrodens* was a particularly potent stimulator of bone resorption, eliciting a response at a concentration as low as 1 ng/ml. A bell-shaped dose response was obtained indicating that maybe desensitisation or destruction of bone cell receptors had occurred. However, further investigation is required to confirm this. *A. actinomycetemcomitans* also contained a potent osteolytic component in its SAM and demonstrated a similar maximum response to that produced by *Eik. corrodens*. In contrast, the other three periodontopathogenic bacteria contained SAMs which were either less potent or efficacious than the former

two. Indeed, the possibility exists that if the osteolytic activity of the SAMs was due to the binding to the same receptor on the appropriate bone cell then some of the SAMs could be used as antagonists. This may be what is happening *in vivo* in the periodontal pocket, with the SAM from some bacteria interfering with the potent osteolytic activity of the SAMs from other organisms. The question which this raises is whether one could use this information for therapeutic purposes.

An extremely surprising result, and one which arose from the pilot studies reported in this thesis is that the osteolytic protein in the SAM of *A. actinomycetemcomitans* is chaperonin 60 (cpn60). In on-going studies, *E. coli* groEL has been found to be a potent stimulator of osteoclastogenesis as well as being able to stimulate the formation of resorption pits at a concentration as low as 1 ng/ml. The implications from the finding that *E. coli* groEL possesses such potent osteolytic activity are immense as all cells contain molecular chaperones. At present the mechanism by which the cpn60 from *A. actinomycetemcomitans* and *E. coli* stimulates bone resorption is being investigated. It is planned to identify the cpn60 receptor involved in the stimulation of bone resorption. If the receptors are identified and characterised then it may be possible to block them and hence inhibit bone resorption.

10.3 Stimulation of cytokine release by SAM, LAP and LPS

The pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF are believed to be key mediators of a range of inflammatory diseases and there is growing evidence for their involvement in the periodontal diseases. Most investigators have utilised endotoxin or LPS in cytokine studies. However, as has recently been reviewed, bacteria produce a wide range of molecules able to stimulate cytokine synthesis. Many of these molecules are as active, or more active, than enteric LPS preparations. In a similar manner to the bone resorption data, the findings on cyokine induction have revealed that the LPS from these oral bacteria are either weakly active or only as active as the LAP or the SAM. Thus the previously mentioned emphasis on LPS is not justified by the potency or efficacy of this bacterial component.

Recent studies by Brakenhoff and colleagues (1994) have investigated the presence and therapeutic potential of an IL-6 receptor antagonist (IL-6ra). It would be interesting to determine whether the SAM-induced IL-6 release by HGF could be inhibited by the IL-6ra. This may be particularly useful in the case of IL-6 release by HGF stimulated with the SAM from *A. actinomycetemcomitans*. As there was direct stimulation of IL-6 synthesis by this component, therapy for localised juvenile periodontitis could be directed towards blocking the receptors for IL-6.

The first generation of drugs being developed for inhibition of cytokine action are by the use of monoclonal antibodies (mAbs; Saito *et al.*, 1993), soluble receptors or IL-1ra (Poole, 1995; Chole *et al.*, 1995). This form of treatment is primarily being researched in the field of rheumatoid arthritis. In particular soluble receptors for TNF, IL-1 and drugs that interfere with the processing of TNF or IL-1 or with their signalling pathways are being investigated. However, recently a number of studies have looked at soluble receptors for IL-6 (Tamura *et al.*, 1993; Frieling *et al.*, 1994). While receptor antagonist and neutralising antibodies to the various cytokines may be therapeutically feasible a problem arises in that these are proteinaceous macromolecules and must therefore be administered parenterally which is a potential disadvantage in treating chronic inflammatory conditions (Lee *et al.*, 1993).

Returning to the biology of the IL-6-stimulating peptide. How can such a small molecule remain associated with the cell surface. It may be being produced in large amounts so that it appears to be on the surface. Alternatively, it may be associated with a carrier molecule at the cell surface. From preliminary experiments the latter hypothesis is favoured. When *A. actinomycetemcomitans* is grown in liquid culture the IL-6-stimulating activity elutes as a component with a large molecular mass. It appears that in cells grown on agar, less of this carrier molecule is needed. These results have not been included in this thesis, however, it clearly suggests that *A. actinomycetemcomitans* has a specific mechanism for coping with changes in its environment. This suggests that this peptide plays a key role in the bacterium's life-style which requires investigation.

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Comparison of the osteolytic activity of surface-associated proteins of bacteria implicated in periodontal disease

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OBJECTIVES: To compare the osteolytic activity of surface-associated material (SAM) and lipid A-associated proteins (LAPs) from periodontopathogenic bacteria.

MATERIALS AND METHODS: Surface-associated material was extracted from the surface and LAPs from the cell walls of a range of periodontopathic bacteria including *Actinobacillus actinomycetemcomitans* and *Eikenella corrodens*. These bacterial fractions were assayed to determine their composition and their capacity to induce bone resorption was determined by use of the neonatal murine calvarial bone resorption assay.

RESULTS: The SAMs from *E. corrodens* and *A. actinomycetemcomitans* demonstrated bone-resorbing capacity at concentrations as low as 1 ng ml^{-1} which, given the molecular weights of the active components, is in the picomolar range of activity. In contrast, the SAMs from the other three bacteria were significantly less potent and showed a lower efficacy. The LAPs all showed significant, and similar, capacities to induce bone breakdown.

CONCLUSIONS: This is the first demonstration that LAP from periodontopathic bacteria can stimulate bone degradation. The LAPs from diverse bacteria all produced similar levels of bone-resorbing activity. In contrast, the SAM showed significant differences in potency and in efficacy (maximal stimulation). This may mean that *in vivo* certain periodontopathic bacteria have significantly more bone-resorbing capacity than others and should be therapeutic targets.

Keywords: bone resorption; lipid A-associated protein; periodontal disease; surface-associated proteins

Introduction

A number of Gram-negative bacteria are implicated as causative agents of the gingival inflammation, connective tissue and bone destruction which characterizes the pathology of the chronic inflammatory periodontal diseases (CIPDs) (Socransky and Haffajee, 1991). Several bacterial species have been reported as frequent isolates from periodontal lesions. The most prevalent organisms include: *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Eikenella corrodens*, *Prevotella intermedia* and *Campylobacter rectus* (Socransky and Haffajee, 1990). The cellular mechanisms producing alveolar bone loss in chronic adult and localized juvenile periodontitis are still poorly understood. Bacteria are rarely found in the lesional periodontal tissues and it is therefore believed that bacterial components and/or products entering the periodontium from the subgingival plaque are the factors likely to stimulate the pathological bone resorption observed clinically (Liakoni *et al*, 1985).

We have been studying the biological actions of the loosely-adherent material associated with the outer cell wall of bacteria. This surface-associated material (SAM) may include the capsule, fimbriae, pili, fibrils and other loosely-adherent material. The SAM from three periodontopathic bacteria: *A. actinomycetemcomitans*, *E. corrodens* and *Por. gingivalis* have been assessed for their ability to stimulate bone resorption in the murine calvarial bone resorption assay (Wilson *et al*, 1985, 1993; Meghji *et al*, 1994). The former two organisms contain SAMs which have similar potencies, with significant stimulation of bone calcium release being observed at concentrations in the range 1–10 ng ml^{-1} (w/v) (Wilson *et al*, 1985, 1993). The SAM from *Por. gingivalis* is active only at concentrations greater than 1 μg ml^{-1} (Wilson *et al*, 1993). The potent activity of the SAM from these bacteria suggests that components present in it may be important in the bone damage which characterizes the CIPDs. However, this hypothesis is based on the examination of only three bacterial species. In the present study we have made comparison of the osteolytic activity of five Gram-negative bacterial species implicated in the pathology of CIPD. The potency of these SAMs turns out to be related to their capacity to induce cytokine synthesis (Reddi *et al*, 1994a). One other class of bacterial cell surface components which we have shown to be potent stimulators of pro-inflammatory cytokine synthesis are the lipid A-associated proteins (Reddi *et al*, 1994b). We have therefore also determined the osteolytic capacity of these

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proteins and report that they possess potent bone-resorbing activity.

Materials and methods

Bacterial cultivation
E. corrodens (NCTC 10596), *Por. gingivalis* (W50), *Pr. intermedia* (NCTC 9336) and *C. rectus* (NCTC 11489) were all cultured on Wilkins Chalgren agar (Oxoid, Hampshire, UK). *A. actinomycetemcomitans* (NCTC 9710) was cultured on Brain Heart Infusion agar (Oxoid). Both media were supplemented with 5% horse blood and all organisms were incubated at 37° C for 72 h under anaerobic conditions. All cultures were Gram-stained to check for contamination. Cells were harvested by gentle scraping and washed briefly with 0.85% NaCl prior to freeze-drying and extraction of the SAM.

Extraction of SAM
The procedure used was that described by Wilson *et al* (1985, 1993). Briefly, the lyophilized bacteria were suspended in 0.85% (w/v) NaCl and the SAM was removed from the bacteria by gentle stirring at 4° C for 1 h. The extraction process was repeated twice more. The supernatant was collected after centrifugation of the suspension at 3000 *g* for 1 h, and acetone (at −20° C) added to precipitate proteins. The SAM was centrifuged at 30 000 *g* for 1 h to collect the precipitate, exhaustively dialyzed (using benzoylated dialysis tubing with a 2-kDa cutoff: Sigma, Poole, Dorset, UK) against distilled water for 48 h at 4° C and lyophilized.

Extraction of LAP
LAP was obtained from SAM-extracted bacteria by the butanol extraction method of Morrison and Leive (1975). Briefly, cells were suspended in 0.85% NaCl at 4° C and an equal volume of butanol added, the suspension mixed thoroughly for 10 min and centrifuged for 20 min at 35 000 *g*. The aqueous phase was removed and the butanol, together with the insoluble residue, was re-extracted twice with half the initial volume of 0.85% NaCl. The combined aqueous phases were centrifuged at 35 000 *g* for 20 min to remove particulate matter and dialyzed against distilled water for 48 h. This crude preparation was then ultracentrifuged at 100 000 *g* for 1 h at 4° C and lyophilized. This material was then subjected to hot phenol extraction (Westphal and Jann, 1965) and purified by ultracentrifugation. The phenol layer, which contained the LAP, was removed, dialyzed against distilled water and lyophilized. For comparative purposes, *E. coli* LPS (NIBSC 84/650) was used in these studies as a known stimulator of calvarial bone resorption.

Composition of SAM and LAP
The protein content was measured by the method of Lowry *et al* (1951) and the carbohydrate content by the method of Dubois *et al* (1956) using glucose as a standard. The endotoxin content of preparations was measured by the LAL chromogenic assay using *E. coli* LPS (84/650) as standard. Lipid content was crudely estimated by extraction with methanol : chloroform (2 : 1), drying the extracted material and weighing.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
The SAM was separated on SDS-PAGE by the method of Laemmli (1970) using 12% gels which were stained with Coomassie blue. The loadings per lane for the SAMS were: *A. actinomycetemcomitans* 20 μg, *E. corrodens* 60 μg, *Por. gingivalis* 60 μg, *Pr. intermedia* 90 μg and *C. rectus* 120 μg. The LAP did not separate well probably because of its high lipid content.

Calvarial bone resorption assay
Bone resorption was assayed by measuring the release of calcium from 5-day-old mouse calvaria *in vitro* (Zenelli *et al*, 1969). Halved calvaria were cultured singly on stainless steel grids in 30-mm dishes (five per group) with 1.5 ml BGJ medium (ICN Flow, Oxon, UK) supplemented with 5% complement-inactivated rabbit serum (Gibco, Uxbridge, Middx, UK) and 50 μg ml^{-1} ascorbic acid (Sigma). After 24 h the media were replaced by fresh media containing various concentrations of bacterial extracts. Prostaglandin (PG)E_2 was added at 1 μM to five wells in each culture to act as a positive control for the responsivity of the bone. Replicates cultured in the presence of unsupplemented culture medium were used as a negative control. The calvaria were cultured for a further 48 h and the calcium content of the media was measured by automated colorimetric analysis (Gitelman, 1967). Each preparation of SAM or LAP was compared to the other preparations in at least two separate experiments and each individual SAM or LAP preparation was tested on a number of occasions. All preparations gave consistent results from experiment to experiment. The results presented are of single comparative experiments. To determine if the active moiety in the SAM from each bacterium was proteinaceous, 1 μg ml^{-1} solutions of each SAM were treated either by heating to 100° C for 1 h or by exposure to trypsin (0.25%) for 1 h at 37° C. After trypsin treatment, an excess of soya bean trypsin inhibitor (Sigma) was added to samples which were then cultured with the murine calvaria. To control for the effect of the inhibitor, calvaria were exposed to soya bean inhibitor alone; this showed no positive or negative effects on bone resorption.

Results

Composition of SAM and LAP
The protein, carbohydrate, lipid and endotoxin content of the SAM preparations is shown in Table 1 and of the LAP in Table 2. The endotoxin content of all preparations was low <0.001 IU ng^{-1}.

SDS-PAGE analysis
All preparations of SAM contained a relatively large number of protein bands with molecular weights in the range <14 kDa to >66 kDa (Figure 1).

Table 1 Composition of the SAM preparations expressed as a percentage of the dry weight of each extract

SAM	*% Protein*	*% CHO*	*% Lipid*	*Endotoxin*[a]
A. actinomycetemcomitans	70	13	12	0.008
Por. gingivalis	42	18	–	0.00002
Pr. intermedia	14	17	–	0.000003
E. corrodens	12	6	–	0.0006
C. rectus	3	1	–	0.0003

[a]Endotoxin content measured as IU ng^{-1} (there are 7000 IUs per μg *E. coli* LPS)

Table 2 Composition of the LAP preparations expressed as a percentage of the dry weight of each extract

LAP	*% Protein*	*% CHO*	*% Lipid*	*Endotoxin*[a]
A. actinomycetemcomitans	86	0	2.3	0.009
Por. gingivalis	4.1	0	85	0.000007
Pr. intermedia	4.0	0	70	0.0004

[a]Endotoxin measured in IU ng^{-1}

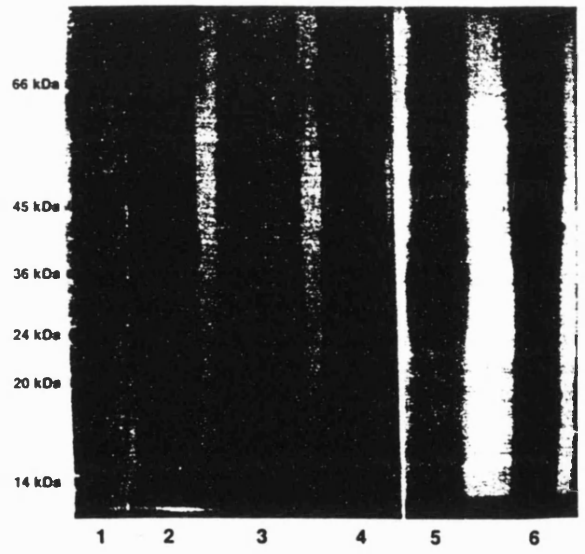

Figure 1 SDS-PAGE profiles of the proteins present in the SAM from the five periodontopathic bacteria. (1) MW markers; (2) *E. corrodens*; (3) *Por. gingivalis*; (4) *A. actinomycetemcomitans*; (5) *C. rectus*; (6) *Pr. intermedia*

Osteolytic activity of SAM

The SAM from *E. corrodens* proved to be the most potent showing the capacity to stimulate 50% of the maximum calcium release from mouse calvaria at a concentration of 1 ng ml^{-1}. There was a pronounced bell-shaped dose response with a loss of response at higher concentrations. The SAM from *A. actinomycetemcomitans* produced a linear dose response over the concentration range 1 ng ml^{-1} to 10 μg ml^{-1}. The SAMs from the other three bacteria reproducibly exhibited a dose response relationship which showed that this fraction from these organisms was both less potent and less efficacious than that from the other two bacteria (Figure 2). Heat or trypsinization of the SAMs caused 70–100% inhibition of the osteolytic activity (Table 3).

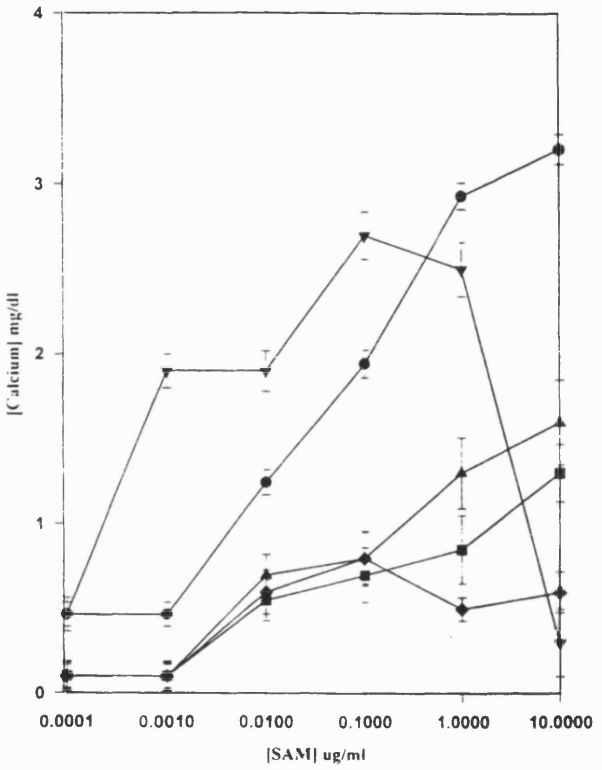

Figure 2 Osteolytic activity in the murine calvarial bone resorption assay of the SAM from the five oral bacteria over the dose range 1 ng ml^{-1} to 10 μg ml^{-1}. Results are expressed as the mean and standard deviation of five replicate cultures. (●) *A. actinomycetemcomitans*, (▼) *E. corrodens*, (■) *Pr. intermedia*, (▲) *Por. gingivalis*, (◆) *C. rectus*

Osteolytic activity of LAP

LAP was extracted from those bacteria which contained SAMs showing potent, moderate or poor osteolytic activity. When the LAP from *A. actinomycetemcomitans*, *Por. gingivalis* and *Pr. intermedia* were tested and compared for osteolytic activity, they all showed similar and reproducible dose responses with linear activity over the concentration

Table 3 Effect of heat (100° C for 1 h) and trypsin-treatment on the osteolytic activity of SAM preparations

	Osteolytic activity (Net calcium release (mg dl^{-1})		*Trypsin-treated*
	Untreated	*Heat-treated*	
E. corrodens	2.50	0.48	0.48
A. actinomycetemcomitans	2.66	0.28	0.57
Por. gingivalis	1.15	0.35	0.33
Pr. intermedia	1.18	0	0
C. rectus	0.95	0	0
Prostaglandin E_2	3.39	–	–

range 10 ng ml^{-1} to 10 μg ml^{-1} (Figure 3). All the LAPs produced the same maximal response which was at variance with the SAMs from the same organisms. Heat or trypsin treatment of the LAP from *A. actinomycetemcomitans* and *Por. gingivalis* produced only a small decrease in activity. In contrast, the LAP from *Pr. intermedia* was more sensitive both to heat and trypsin and 30–40% inhibition of activity was noted.

Osteolytic activity of E. coli *LPS*

Highly purified *E. coli* LPS proved to be an extremely potent stimulator of calvarial bone resorption over the dose range 10 pg ml^{-1} to 1 ng ml^{-1}. The dose response was reproducibly bell-shaped (Figure 4).

Discussion

Destruction of alveolar bone is the hallmark of the CIPDs. A number of Gram-negative bacteria are implicated in this process but it is still not clear how these bacteria achieve this or if they are all equally adept at stimulating bone resorption. Our group at the Eastman Dental Institute have been concentrating on the capacity of bacterial components, loosely associated with the cell wall, to induce bone resorption *in vitro*. We have shown that the SAM from *A. actinomycetemcomitans* is extremely active at promoting bone resorption with significant bone destruction being observed

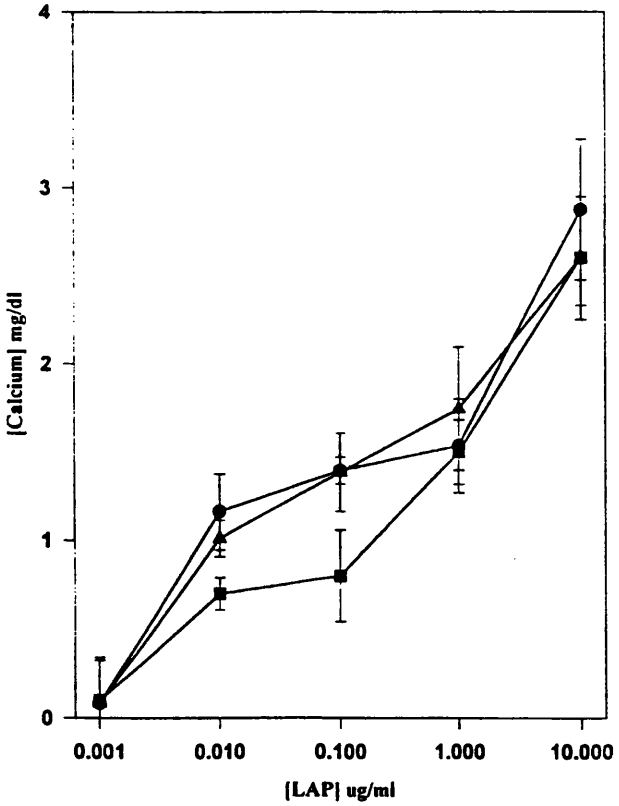

Figure 3 The osteolytic dose response of the LAP from three separate bacteria over the concentration range 10 ng ml^{-1} to 10 μg ml^{-1}. Results are expressed as the mean and standard deviation of five replicate cultures. (●) *A. actinomycetemcomitans*, (■) *Pr. intermedia*, (▲) *Por. gingivalis*

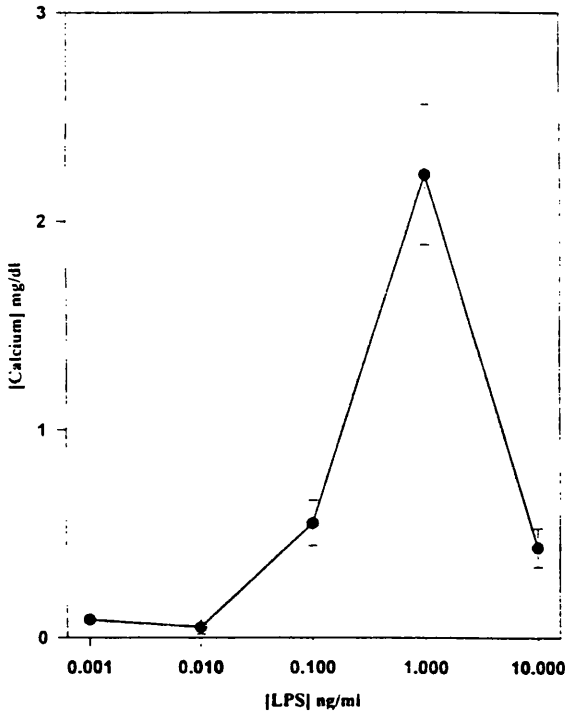

Figure 4 The osteolytic dose response of *E. coli* LPS in the calvarial bone resorption assay. Results are expressed as the mean and standard deviation of five replicate cultures

at 1–10 ng ml^{-1} in many experiments. In contrast, the LPS from this organism is a very weak bone-stimulating agonist only showing activity at concentrations of 1–10 μg ml^{-1} (Wilson *et al*, 1985). Other components of bacteria may play a role in bone resorption. The family of proteins known as the lipid A-associated proteins (LAPs) have only recently received attention as agents capable of stimulating cytokine synthesis (Johns *et al*, 1988; Porat *et al*, 1992). We have shown that the LAPs from several oral bacterial species can stimulate the synthesis of the osteolytic cytokines: interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)α (Reddi *et al*, 1994b and unpublished data). Given this capacity they may also be able to stimulate bone resorption.

In this study we have isolated the surface-associated material from five oral organisms and have also isolated the LAP from three of these bacteria. The osteolytic activity of these components has been compared to that of the highly purified international LPS standard 84/650 produced by the National Institute for Biological Standards and Control.

The isolated SAMs varied widely in their composition. The protein content of each SAM preparation was measured by the Lowry assay and a wide range of protein contents was found. The most proteinaceous material was from *A. actinomycetemcomitans* which had an average protein content of 70%, the remaining material being carbohydrate and lipid. In contrast, the SAMs from *C. rectus* appeared to contain very little protein, even less carbohydrate and little lipid. The remaining material was not nucleic acid on the basis of the 260/280 absorption of the SAM. However,

when these SAMs were run on SDS-PAGE they contained numerous protein bands. We conclude that the proteins in the SAMs of these organisms must be lacking in tyrosine, tryptophan and histidine which form complexes with the Folin–Ciocalteau reagent in the Lowry assay. The protein contents of the LAPs also differed widely. All preparations were assayed for LPS content and in all cases this was low and so would not have contributed to the biological activity observed.

It has long been established that LPS is a potent inducer of calvarial bone resorption and in our studies we have made use of the NIBSC international standard 84/650 which is a highly purified preparation of LPS. This material was an extremely potent stimulator of calvarial bone resorption showing a linear dose response over the concentration range 10–1000 pg ml^{-1}. The dose response showed a marked bell-shaped or biphasic phenomenon and at 10 ng ml^{-1} LPS the release of calcium was equal to that stimulated by 100 pg ml^{-1} LPS. A similar bell-shaped dose response was observed with the SAM from *E. corrodens*, the most potent of the bacterial SAMs. Such bell-shaped responses often denote receptor desensitization, however the nature of the receptors transducing the LPS and SAM signals have not been defined. Simple Amicon membrane filtration has demonstrated that the molecular weight of the active component of the *E. corrodens* SAM is >10 kDa (unpublished data). Thus the concentration of SAM required to stimulate a >50% response is less than 100 pM which is within the potency range of the most potent known osteolytic cytokine, IL-1 (Gowen and Mundy, 1986). Thus *E. corrodens* has an extremely potent osteolytic protein, or proteins, associated with its SAM which could play a major role in the bone destruction in the CIPDs. *A. actinomycetemcomitans* SAM was also, as previously described (Meghji *et al*, 1994), extremely potent at stimulating bone resorption, being active over the dose range 1 ng ml^{-1} to 10 μg ml^{-1} and showing similar maximum responses to that produced by *E. corrodens*. In contrast, the other three periodontopathic bacteria contain SAMs which were either significantly less potent or efficacious than those already described. The SAM from *Por. gingivalis* was generally less potent than that from *A. actinomycetemcomitans* or *E. corrodens* and, as seen in Figure 2, was less efficacious in this comparative study.

Efficacy is a parameter defined by Stephenson (1956) which describes the 'strength' of a single agonist-receptor complex in evoking a response. Agonists can have similar potencies but different maximal responses ie efficacies. The SAMs from *Pr. intermedia* and *C. rectus* showed both lower potency and significantly lower efficacies in stimulating bone resorption with the SAM from the latter organism only demonstrating approximately 30% of the response induced by the SAM from *A. actinomycetemcomitans*. This suggests that the active components in the SAM from these less efficacious organisms cannot stimulate a maximum agonist response when it binds to the triggering receptor on the appropriate bone cell population, eg the osteoblast. This failure to stimulate a full agonist response may be important in the progression of tissue damage. We do not know which receptors the active osteolytic components of the SAM stimulate. If they all stimulate bone breakdown via the same receptor then the possibility exists that the SAMs which fail to induce a full agonist-response relationship may act as partial agonists and could therefore act to down-regulate the activity of the more active SAMs. Work is currently underway to identify the receptors on bone cells which stimulate SAM-induced bone breakdown. From assessment of the osteolytic activity of the SAM after heat or trypsin treatment it is obvious that bone destruction was due to proteins in the SAM.

In spite of the significant differences between the potency and efficacy of the SAMs from the five bacteria, when the LAPs were assayed they all showed very similar dose responses and all had the same degree of efficacy. The LAPs from these bacteria were active at a concentration of 10 ng ml^{-1}. In agreement with the general findings with LAPs of other bacteria (reviewed by Hitchcock and Morrison, 1984), both heat and trypsin treatment had only a minor effect on the activity of these proteins. The stability of these proteins, particularly to proteinases, may result in their having a prolonged half-life in the inflamed periodontium and thus they may play a major role in inducing tissue inflammation and destruction. Again, the nature of the receptor(s) transducing this activity has not been defined but is an important question, as the LAP could represent a therapeutic target for the treatment of the CIPDs. This question is currently being addressed.

In conclusion, we have demonstrated that saline-extractable material from the surfaces of periodontopathogenic bacteria exhibit osteolytic activity which is as potent as the most potent osteolytic cytokine, IL-1. However, not all putative periodontopathic bacteria contain such active constituents in their SAM. Thus the SAM from the organism, *C. rectus* has very weak bone-resorbing activity. The identity of the components responsible for such activity is the subject of ongoing research in our laboratories. We have shown previously that LPSs from oral bacteria are fairly weak agonists in a number of assays (Wilson *et al*, 1985; Meghji *et al*, 1992). In the bacterial cell wall the LPS is complexed to the LAP. We now report, for the first time, that the LAPs from oral bacteria also have potent bone-resorbing actions *in vitro* and may therefore be key virulence factors, active in the pathogenesis of the CIPDs.

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Lipid A-associated proteins from periodontopathogenic bacteria induce interleukin-6 production by human gingival fibroblasts and monocytes

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Abstract

The aim of this study was to determine whether lipid A-associated proteins (LAP) from two periodontopathogenic species of bacteria were able to stimulate interleukin-6 (IL-6) release from human gingival fibroblasts and myelomonocytic cells. LAP and lipopolysaccharide (LPS) were extracted from *Porphyromonas gingivalis* and *Prevotella intermedia* and added to cultures of human gingival fibroblasts and mono-mac-6 monocytic cells. Release of IL-6 into the culture supernatants was determined by ELISA. LAP and LPS from *Por. gingivalis*, but not from *Prev. intermedia*, stimulated IL-6 release from both cell types in a dose-dependent manner although LPS was less potent than LAP in inducing IL-6 release from the fibroblasts. IL-6 was detectable in cultures of both cell types following stimulation with LAP from *Por. gingivalis* at a concentration as low as 10 ng/ml. In response to LAP from *Prev. intermedia*, IL-6 was produced by mono-mac-6 cells but not by fibroblasts. Our results show that bacterial cell wall components other than LPS can induce IL-6 release from cells of the periodontium in vitro. The production of such potent immunomodulatory agents in vivo may contribute to the connective tissue breakdown characteristic of chronic periodontitis.

Keywords: Lipid A-associated protein; Interleukin-6; Monocyte; Fibroblast; Periodontitis

1. Introduction

A limited number of organisms present in subgingival plaque are now considered to be the main aetiological agents of the various forms of chronic periodontitis. Such organisms include *Porphyromonas gingivalis* and *Prevotella intermedia* [1]. The tissue destruction characteristic of periodontitis is thought to result from the activities of various products and constituents of these organisms [2]. With regard to the latter, most attention has been devoted to the role of lipopolysaccharides (LPS) in the pathogenesis of the disease [3,4]. However, there are increasing reports of the ability of other cell surface components to display activities which, if

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operative in vivo, could contribute to the pathological changes observed. For example, LPS-free surface-associated material from *A. actinomycetemcomitans* has been shown to stimulate bone resorption [5], inhibit bone collagen synthesis [6] and inhibit fibroblast proliferation [7]. Furthermore, Takahashi et al. [8] reported that serotype-specific capsular polysaccharides from this organism induced interleukin (IL)-1 release from murine macrophages. Mihara et al. [9] have reported that an outer membrane protein from *Por. gingivalis* was able to stimulate human gingival fibroblast proliferation and resorption of rat long-bones while Hanazawa et al. [10] showed that fimbriae from this organism induced IL-1 production in murine macrophages.

For many years it has been recognised that the outer membrane proteins which co-extract with lipopolysaccharide (lipid A-associated proteins, LAP; endotoxin-associated proteins, EAP) have immunomodulatory activities which may be of relevance to the pathogenesis of infections caused by Gram-negative bacteria [11,12]. It has been suggested that these activities stem from the interaction of LAP with mononuclear phagocytes resulting in the release of immunoregulatory cytokines [13]. The purpose of this study was to determine whether LAP from two important periodontopathogenic species (*Por. gingivalis* and *Prev. intermedia*) could induce cytokine release from mammalian cells in vitro.

2. Materials and methods

2.1. Extraction of endotoxin

Porphyromonas gingivalis W50 and *Prevotella intermedia* NCTC 9336 were grown under anaerobic conditions on Wilkins-Chalgren (Oxoid Ltd.) agar plates containing 5% (v/v) horse blood for 72 h at 37° C.

All cultures were inspected visually for contamination, Gram-stained, removed from the plate with sterile saline and then centrifuged at 30 000 × *g* for 30 min at 4° C. Bacteria were then resuspended in saline, centrifuged and lyophilized. Extracellular material was removed by saline extraction as previously described [5]. Briefly, lyophilized bacteria were gently stirred at 4° C in sterile saline for 1 h and the cells sedimented by centrifugation. This was repeated twice and the cells lyophilised.

Endotoxin was obtained from the cells by the butanol extraction method of Morrison and Leive [14] as follows: cells were suspended in 0.85% (w/v) saline at 4° C and an equal volume of butanol added. The suspension was mixed thoroughly at 4° C for 10 min and centrifuged at 35 000 × *g* for 20 min. The aqueous phase was removed and the butanol, together with the insoluble residue, was re-extracted twice with approximately half of the initial volume of saline. The combined aqueous phases were centrifuged to remove particulate matter, dialysed against six changes of distilled water for 48 h and lyophilised. The crude endotoxin preparation was re-suspended in water and ultracentrifuged at 100 000 × *g* for 1 h. This procedure was repeated once more and the endotoxin lyophilised.

2.2. Preparation of lipid-A-associated proteins

LAP were obtained from each endotoxin by a hot phenol-water extraction [15]. Briefly, an aqueous suspension of the endotoxin was agitated with an equal volume of 90% (w/v) phenol at 68° C. The aqueous phase was removed and the phenol phase extracted twice more with water at 68° C. The phenol phase was then dialysed against distilled water for 96 h and the LAP lyophilized.

2.3. Preparation of lipopolysaccharide

The aqueous phases from the phenol/water extraction were pooled and dialysed against distilled water for 96 h. RNA, DNA and protein were removed as described previously and the LPS recovered by ultracentrifugation [5].

2.4. Analysis of LAP

Protein and carbohydrate determinations on each LAP preparation were carried out by the methods of Lowry et al. [16] and Dubois et al. [17] respectively. The total lipid content was determined after extraction three times with chloroform/methanol (2:1 vol/vol).

A chromogenic *Limulus* amoebocyte lysate assay was used to determine the lipopolysaccharide content of each LAP preparation.

Each LAP preparation was subjected to SDS-PAGE by the method of Laemmli [18] except SDS was used at a concentration of 16%. 15% gels were used in all cases and proteins visualised by silver staining (Sigma Ltd., Dorset, UK).

2.5. Stimulation of IL-6 synthesis by fibroblasts and myelomonocytic cells

Normal human gingival fibroblasts (HGFs) prepared from explants of gingiva removed at routine oral surgery, and used at between passage 6 and 12, were seeded into 24-well culture plates at 30000 cells/well and incubated overnight in DMEM at 37° C in 5% CO_2/air to adhere. Cells were then exposed to graded concentrations of each LAP preparation and to LPS from *Escherichia coli* (International Standard, preparation 84/650, NIBSC, UK), for 24 h. Control cells were incubated with unsupplemented media. At the end of the culture period the media were collected and stored at −70° C until assayed for IL-6.

The human myelomonocytic cell line mono-mac-6 was seeded into 24-well tissue culture plates at a cell density of 2×10^6 cells/ml in RPMI 1640 medium containing 2% FCS, 1 mM oxaloacetic acid, 9 μg/ml insulin and 1 mM sodium pyruvate. The cells were then exposed to graded concentrations of each LAP and to LPS from *Escherichia coli* for 24 h at 37° C in 5% CO_2/air. The medium was then collected and stored at −70° C until assayed for IL-6.

In both cases, additional experiments were carried out using LAP after heating to 100° C for 10 min, LAP after chloroform/methanol extraction, the chloroform/methanol extract and LAP which had been treated with 100 μg/ml trypsin for 24 h. Trypsin was inactivated with 10 μg/ml soyabean trypsin inhibitor.

2.6. Cytokine assays

Microtitre plates (Immunolon 4, Dynatech) were coated with immuno-affinity purified polyclonal goat anti-IL-6 antibodies at 1 μg/ml in phosphate buffered saline (pH 7.2) overnight at 4° C. Unbound coating antibody was removed before washing the wells with 0.01 M phosphate/0.05 M NaCl buffer (wash/dilution buffer) containing 0.1% Tween 20. IL-6 standards (human recombinant IL-6 standard, NIBSC preparation 88/514 in 100 μl), over the dose range 0–3 ng/ml, and supernatants to be tested, were added to wells. Plates were incubated for 2 h at room temperature before washing three times with wash/dilution buffer. Biotinylated affinity purified polyclonal goat anti-IL-6 (100 μl of a 0.014 μg/ml solution) was added to each of the wells and incubated for a further 1 h at room temperature. Plates were then washed three times and 100 μl of a 1:5000 dilution of avidin-HRP (Dako Ltd) added to each well. Plates were then incubated for 15 min at room temperature before washing three times with wash/dilution buffer. 100 μl of 0.2 mg/ml O-phenylenediamine (Sigma) in 0.1 M citric acid phosphate buffer, pH 5.0, with 0.4 μl/ml 30% H_2O_2 (Sigma) was then added to each well. The reaction was terminated by the addition of 150 μl of 1 M H_2SO_4 (Sigma) and the absorbance was measured at 492 nm on a Titertek Multiskan spectrophotometer (Flow).

3. Results

3.1. Composition of LAP

31.0 mg of endotoxin were obtained from 1.10 g of *Por. gingivalis* while 29.0 mg of endotoxin were obtained from 1.05 g of *Prev. intermedia*. The yields of LAP and LPS from *Por. gingivalis* were 15.0 mg (1.4% of the lyophilised cells) and 12.5 mg (1.1%) respectively. The yields of LAP and LPS from *Prev. intermedia* were 20.3 mg (1.9%) and 4.8 mg (0.5%) respectively.

LAP preparations from both organisms contained high proportions of material extractable by chloroform/methanol (85% and 70% for *Por. gingivalis* and *Prev. intermedia* respectively), low proportions of protein (4.1% and 4.0% for *Por. gingivalis* and *Prev. intermedia* respectively) and no carbohydrate (detectable by the method of Dubois et al. [17]). However, the values obtained for the protein content using the Lowry assay may be anomalous as the surface proteins of both organisms have been shown to have low contents of tyrosine [19]. The chloroform/methanol extract of the LAP from *Por. gingivalis* and *Prev. intermedia* contained 0.9% and 0% protein respectively. Both preparations contained

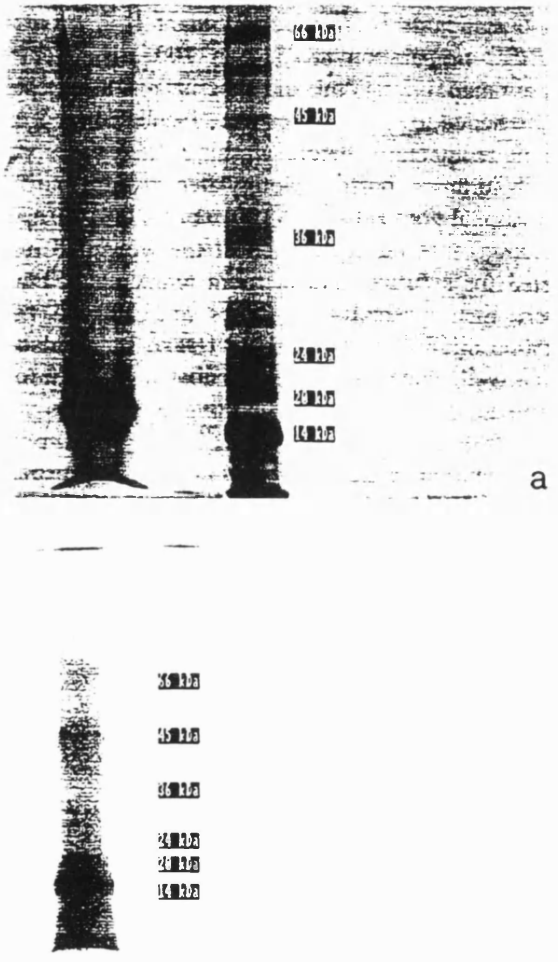

Fig. 1. SDS-PAGE analysis of LAP from (a) *Por. gingivalis* and (b) *Prev. intermedia*.

very low levels of LPS as detected in the *Limulus* amoebocyte lysate assay, 1.0 ng of each preparation having an activity equivalent to less than 3×10^{-4} ng of endotoxin from *Escherichia coli*.

SDS-PAGE analysis of the LAP from *Por. gingivalis* revealed the presence of one major band corresponding to a protein with a molecular weight of approximately 17 kDa together with one very feint band (Fig. 1a). The LAP from *Prev. intermedia* revealed 2 major (20 and 45 kDa) and 2 minor bands (Fig. 1b).

3.2. Cytokine-stimulating activity of LAP and LPS

Fibroblasts

LAP from *Por. gingivalis* stimulated IL-6 release from HGFs in a dose-dependent manner over the concentration range 10 ng/ml–10.0 μg/ml (Fig. 2). Statistically significant activity was demonstrated at a concentration as low as 10 ng/ml. After heating to 100° C for 10 min, the LAP (at a concentration of 10 μg/ml) failed to stimulate IL-6 release. 10 μg/ml LAP, after trypsin digestion, also failed to stimulate IL-6 release from these cells. The cytokine-stimulating activity of the LAP was not reduced by extraction with chloroform/methanol (Fig. 3) and the extract itself did not stimulate IL-6 release.

LPS from this organism also stimulated the release of IL-6 from HGFs, although its potency was less than that of LAP from the same organism (Fig. 2). LPS from *E. coli* stimulated IL-6 release in a dose-dependent manner over the concentration range 50 pg/ml–1.0 ng/ml. At a concentration of 0.1 ng/ml, LPS from this organism stimulated the release of 280 pg/ml of IL-6. The concentrations of LAP and LPS from *Por. gingivalis* required to stimulate the release of a similar amount of IL-6 were 10 ng/ml and 100 ng/ml, respectively.

Both LAP and LPS from *Prev. intermedia* failed

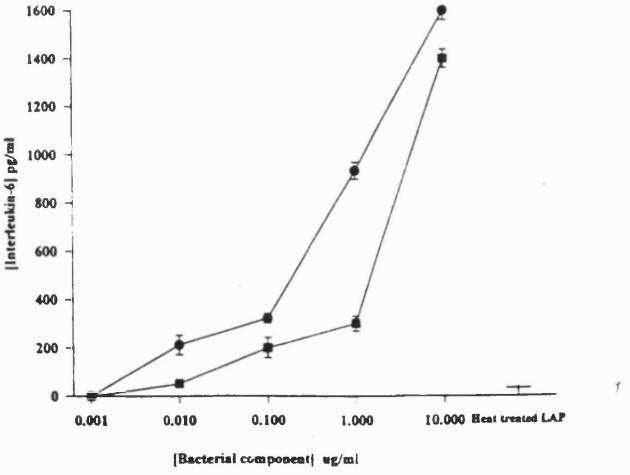

Fig. 2. IL-6 release from human gingival fibroblasts stimulated with LPS (■), LAP (●) and 10 μg/ml heat-treated LAP from *Por. gingivalis*. Vertical bars represent 95% confidence intervals.

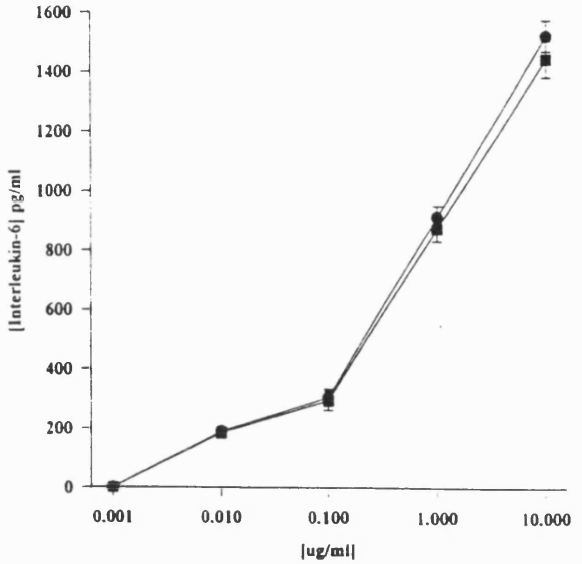

Fig. 3. IL-6 release from human gingival fibroblasts stimulated with LAP from *Por. gingivalis* (●) and the residue from the LAP after extraction with chloroform/methanol (■). Vertical bars represent 95% confidence intervals.

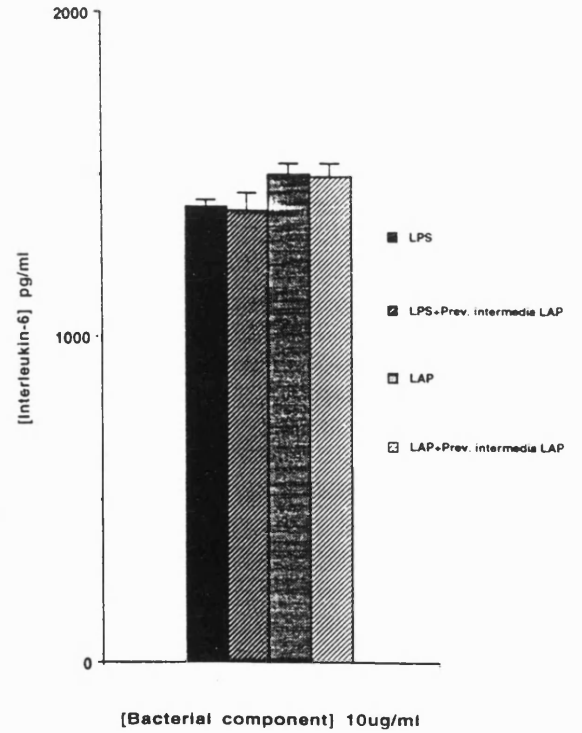

Fig. 4. IL-6 release from human gingival fibroblasts stimulated by LPS or LAP from *Por. gingivalis* in the presence of 10 μg/ml of LAP from *Prev. intermedia*.

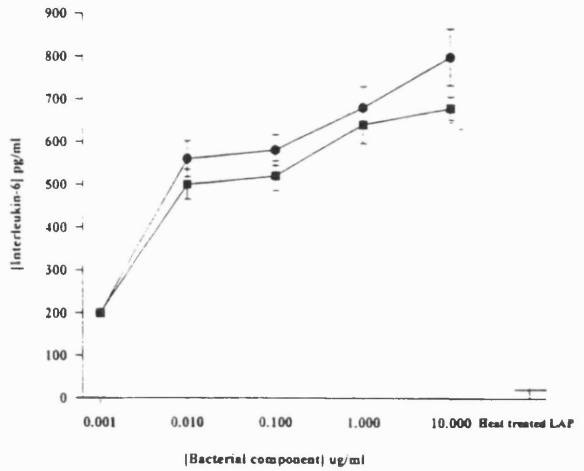

Fig. 5. IL-6 release from mono-mac-6 cells stimulated with LPS (■), LAP (●) and 10 μg/ml heat-treated LAP from *Por. gingivalis*. Vertical bars represent 95% confidence intervals.

to stimulate IL-6 release even at a concentration of 10 μg/ml.

As the LAP from *Prev. intermedia* failed to stimulate IL-6 release from HGF, its ability to block the cytokine-stimulating activity of the LAP and LPS from *Por. gingivalis* was determined. 10 μg/ml of LAP from *Prev. intermedia* together with 10 μg/ml LAP or LPS from *Por. gingivalis* were added to HGF cultures and IL-6 release quantified as described previously. The LAP from *Prev. intermedia* had no effect on IL-6 release from HGF stimulated by either the LAP or the LPS from *Por. gingivalis* (Fig. 4).

Monocytic cells

LAP from both *Por. gingivalis* and *Prev. intermedia* were able to stimulate IL-6 release from mono-mac-6 cells in a dose-dependent manner over the concentration range 10 ng/ml to 10 μg/ml (Figs. 5 and 6). Again, heat-treated LAP and trypsin-digested LAP from both organisms failed to stimulate IL-6 release from the mono-mac-6 cells. Extraction of the LAP with chloroform/methanol did not lead to any decrease in the cytokine-stimulating activity of the LAP preparation, nor did the chloroform/methanol extract itself have any cytokine-stimulating activity (Fig. 7).

A similar pattern of IL-6 release from mono-mac-6 cells was found in response to LPS from these organisms and the LAP and LPS appeared to be

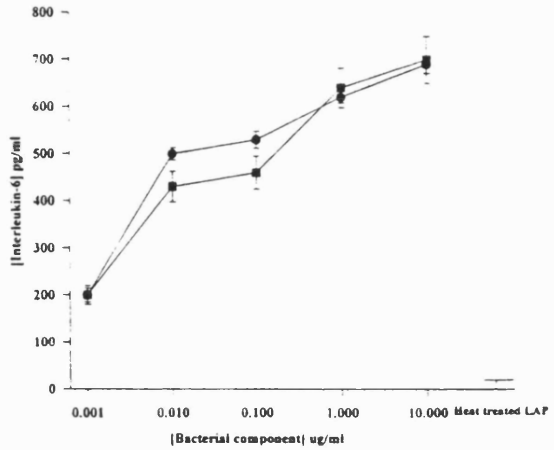

Fig. 6. IL-6 release from mono-mac-6 cells stimulated with LPS (■), LAP (●) and 10 μg/ml heat-treated LAP from *Prev. intermedia*. Vertical bars represent 95% confidence intervals.

equipotent (Figs. 5 and 6). LPS from *E. coli* stimulated IL-6 release from the monocytic cells in a dose-dependent manner over the concentration range 10 pg/ml–1.0 ng/ml. At a concentration of 5.0 pg/ml, LPS from this organism caused the release of 430 pg/ml of IL-6 from these cells. The concentrations of LAP and LPS from *Por. gingivalis* and

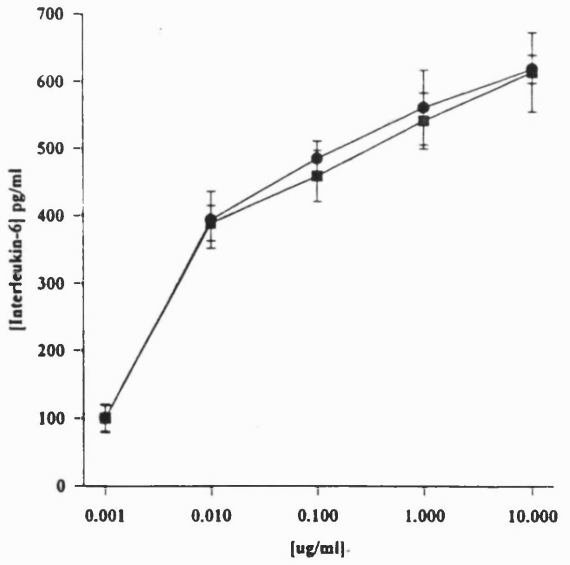

Fig. 7. IL-6 release from mono-mac-6 cells stimulated with LAP from *Prev. intermedia* (●) and the residue from the LAP after extraction with chloroform/methanol (■). Vertical bars represent 95% confidence intervals.

Prev. intermedia necessary to stimulate the release of a similar amount of IL-6 was 10 ng/ml in each case.

4. Discussion

Two distinctive histopathological features of the various forms of chronic periodontitis are alveolar bone loss and the presence of a high proportion of plasma cells. These pathological changes in the periodontium are thought to result from the activities of products or components of the bacteria associated with these diseases [20]. Bacterial products such as proteases are likely to have a direct effect on the integrity of periodontal tissues whereas bacterial components are more likely to exert their effects by stimulating the release of cytokines from host cells [21]. To date, lipopolysaccharides from the causative organisms have been considered to be the main agents responsible for initiation of periodontal tissue destruction, although the exact mechanisms involved and the possible role of cytokines have not been identified. Hence LPS from organisms such as *Por. gingivalis*, *Prev. intermedia* and *A. actinomycetemcomitans* have been shown in vitro to be able to stimulate bone resorption [22], inhibit bone formation [23], inhibit mammalian cell proliferation [2] and stimulate cytokine release from a variety of mammalian cell types [4]. The involvement of other surface components of periodontopathogenic bacteria in tissue destruction has received far less attention. However, there are increasing reports of the ability of such components to exhibit relevant in vitro biological activities. Hence, surface-associated material (other than LPS) from *A. actinomycetemcomitans* has been shown to be a potent bone resorbing agent [5], an inhibitor of mammalian cell proliferation [7], a potent inhibitor of bone formation [6] and an inducer of cytokine release from macrophages [8]. Outer membrane proteins and fimbrial proteins have also been shown to possess a number of relevant biological activities in vitro [9,10]. The results of our study show that other surface components of periodontopathogenic bacteria, LAP, can affect mammalian cells in a way that may account for some of the pathological processes accompanying chronic periodontitis. Hence, LAP were able to stimulate the

release of IL-6 from HGFs and a monocytic cell line. However, there were differences in the spectrum of activity between the two organisms. LAP from *Por. gingivalis* were able to elicit IL-6 release from both cell types while only monocytic cells responded in this way to LAP from *Prev. intermedia*. The potency of these preparations, however, was considerably less than that of LPS from *E. coli*.

Although there are no previous reports concerning the ability of LAP from periodontopathogenic organisms to stimulate cytokine release from mammalian cells, other investigators have demonstrated that LPS from *Por. gingivalis* and *Prev. intermedia* can induce IL-6 release from HGFs [24]. However, the results of the present study have shown that only the LPS from *Por. gingivalis* stimulated release of the cytokine. Differences in methods of preparation of LPS [25] and/or the cytokine assay employed, may account for the different results obtained.

IL-6 has been detected in the gingival crevicular fluid of patients with chronic periodontitis [26]. It is a multifunctional cytokine and exhibits a number of activities of relevance to the pathogenesis of chronic periodontitis including the ability to stimulate proliferation of murine thymocytes [28], polyclonal activation of B-cells [29] and stimulation of osteoclast formation [27,30]. Such activities may account for two characteristic features of periodontal lesions: loss of alveolar bone and infiltration by B-cells. The ability of LAP from periodontopathogenic bacteria, particularly *Por. gingivalis*, to stimulate a number of key cells of the periodontium to produce IL-6 implies that this cell wall component may play an important role in mediating the tissue destruction characteristic of chronic periodontitis. The LAP preparation from both *Por. gingivalis* and *Prev. intermedia* were found to be heterogeneous as has been reported by workers investigating other organisms [13,31]. Hence, Johns et al. [31] showed that a LAP preparation from *Salmonella minnesota* consisted mainly of 3 outer membrane proteins. As the cytokine-stimulating ability of the LAP from both organisms were abrogated by heat and trypsin, it is likely that the active components of both preparations were proteins. We now intend to determine which of the proteins in each LAP preparation is responsible for its ability to stimulate cytokine release from responsive cells.

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INTERLEUKIN 6 PRODUCTION BY LIPOPOLYSACCHARIDE-STIMULATED HUMAN FIBROBLASTS IS POTENTLY INHIBITED BY NAPHTHOQUINONE (VITAMIN K) COMPOUNDS

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Naphthoquinone vitamins (vitamins K) are widely recognized for their role in the γ-carboxylation of specific glutamyl residues in coagulation, anti-coagulation and extra-hepatic proteins. Recently, however, there have been reports that these compounds can exert actions other than those normally associated with protein γ-carboxylation. These observations suggest that naphthoquinones may have effects on the production of inflammatory mediators including cytokines. Fibroblasts are now recognized as a rich source of cytokines and we have examined the effect of various naphthoquinones on the production of interleukin 6 (IL-6) by lipopolysaccharide-stimulated human gingival fibroblasts. Compounds examined in this study include: phylloquinone (K1), menaquinone-4 (K2), menadione (K3), 2,3-dimethoxy-1,4-naphthoquinone (DMK) and a synthetic product of vitamin K catabolism, 2-methyl, 3-(2'methyl)-hexanoic acid-1,4-naphthoquinone (KCAT). All of these compounds are capable of inhibiting IL-6 production with a rank order of potency: KCAT>K3>DMK>K2>K1. The most potent compound, KCAT, inhibited IL-6 production with an IC_{50} of 3×10^{-7}M. The mechanism of action of these naphthoquinones on fibroblast IL-6 production is unknown. Given that K3 and KCAT are inactive in the γ-carboxylation reaction, we suggest that this activity is not essential for the inhibition of IL-6 production and that activity may be related to the redox capacity of these naphthoquinones.

Vitamin K is an essential micronutrient present at low levels in the blood,[1,2] but at much higher levels in various tissues including liver and bone.[3,4] There is an absolute requirement for vitamin K for the γ-carboxylation of specific glutamyl residues in several hepatic and extra-hepatic proteins.[5] The γ-carboxylation of these proteins confers a greater binding affinity for calcium, hydroxyapatite and, in the case of the coagulation factors, for phospholipids.[5]

Due to their structure, the naphthoquinones also have redox properties and, in studies in the 1970s, were shown to alter cellular metabolism in a manner which might confer anti-inflammatory properties.[6] In more recent years, reports have appeared to indicate that vitamin K compounds can have anti-inflammatory properties. In a rat paw oedema model it was found that vitamin K_1 and the seven carbon aliphatic carboxylic acid catabolic products from vitamin K were more potent than either phenylbutazone or Saridone[4] (a mixed analgesic preparation) in ameliorating inflammation and pain.[7,8] In a chronic antigen-induced rabbit model of arthritis the oral administration of menadione (vitamin K_3) proved to be active in inhibiting synovitis.[9] In cell culture studies, menaquinone-4, a member of the vitamin K_2 family of congeners, has been shown to be capable of modulating osteoblast proliferation, alkaline phosphatase activity[10] and cyclo-oxygenase activity.[11]

These various activities of vitamin K, both in vivo and in vitro, could be due, at least in part, to the modulation of pro-inflammatory cytokine synthesis. We have characterized the effect of LPS on human gingival fibroblast IL-6 production and have used this assay to determine if vitamin K analogues have any influence on the production of this cytokine.

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RESULTS

LPS-induced IL-6 synthesis

E. coli LPS is a potent stimulator of IL-6 synthesis by gingival fibroblasts and the production of IL-6

has been analysed over the concentration range 10 ng–2 μg (Fig. 1).

Inhibition of IL-6 production

All five naphthoquinone compounds tested in this study were capable of inhibiting human gingival fibroblasts from producing IL-6 (Fig. 2). The concentration-dependent inhibition of IL-6 production by the most potent compound, KCAT, is shown in Figure 3. The effects of indomethacin, and the lipoxygenase inhibitors MK 886 and BW70C on LPS stimulated production of IL-6 from fibroblasts is presented in Figure 4.

DISCUSSION

Cytokines such as IL-6, IL-1 and TNF are implicated in the pathology of a wide range of diseases including rheumatoid arthritis,[12] periodontitis,[13] osteoporosis (under estrogen control,[14]) and cancer.[15] The pharmacological control of the production of such molecules is therefore a pressing need.[16] There have been great efforts made to produce low molecular weight agents which inhibit cytokine synthesis without producing side-effects and a wide range of agents with some degree of activity have been described (reviewed in [16]). These compounds are generally active in the 1 to 100 micromolar range and, with the exception of the Sandoz compound IX 207 887, few have been tested in the clinic.

The possibility that naphthoquinone-based compounds could inhibit inflammation was first examined by Chayen and co-workers who were interested in the role of redox coupling systems in the control of cellular metabolism.[6,17] Using an organ culture system for maintaining small explants of non-rheumatoid and rheumatoid synovial lining they showed that menadione could downregulate the enhanced metabolic activity of rheumatoid synoviocytes.[6] These reports were strengthened by the findings that menadione epoxide could inhibit antigen-induced arthritis in the rabbit, a chronic model of rheumatoid arthritis.[18]

It is of interest that the putative antirheumatic compound RP 54745, which has been demonstrated to inhibit cytokine production, also, like vitamin K, inhibits cellular hexose monophosphate shunt activity.[19] It is tempting to suggest that these two compounds share common features in terms of their mechanism of action, though they are structurally distinct.

In this study we report that five vitamin K analogues can inhibit the production of IL-6 by human gingival fibroblasts stimulated by LPS. There is a rank potency of inhibition: KCAT>K3>DMK>K2>K1. It has been demonstrated that menadione is a potent inhibitor of lipoxygenase (Henderson & Higgs, unpub-

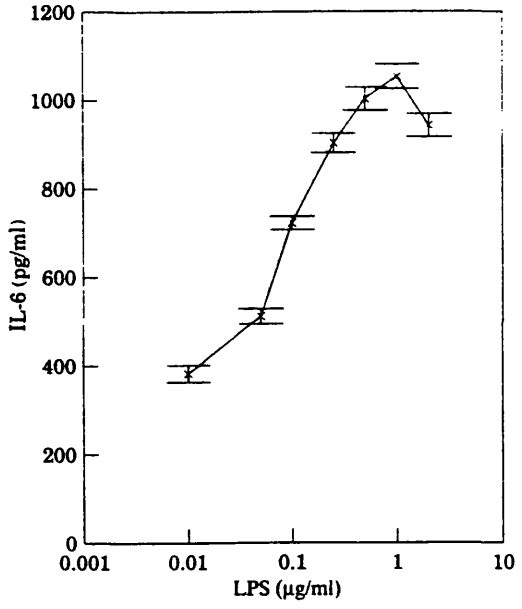

Figure 1. Concentration-dependent stimulation of IL-6 release from human gingival fibroblasts, by *E. coli* LPS (1 μg/ml).

Results are expressed as the mean ± SD of duplicates from four experiments.

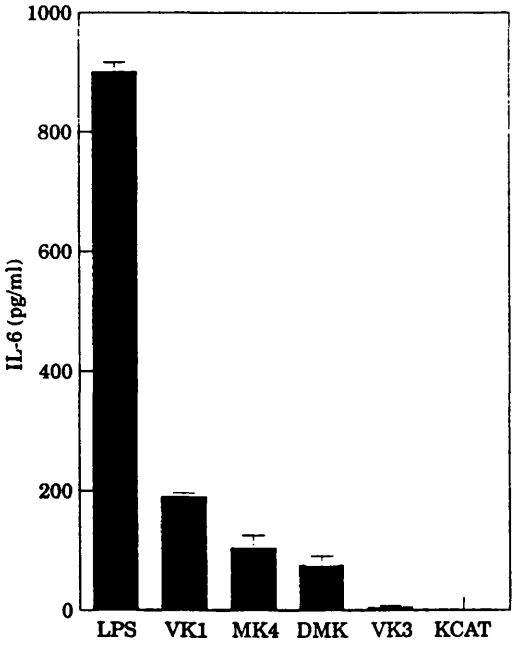

Figure 2. Inhibition of IL-6 release from human gingival fibroblasts by five naphthoquinone compounds (1 μg/ml) compared to the release of *E. coli* LPS (1 μg/ml).

Results are expressed as the mean ± SD of duplicates from three experiments.

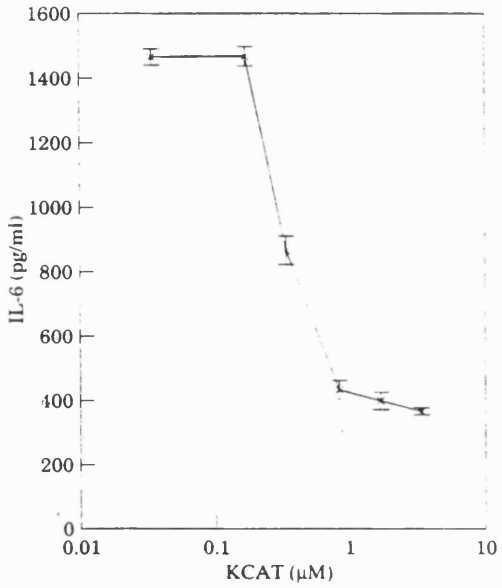

Figure 3. Concentration-dependent inhibition of *E. coli* LPS (1 μg/ml) stimulated IL-6 release by 2-methyl-3-(2'methyl)-hexanoic acid-1,4-naphthoquinone (KCAT).

Results are expressed as the mean ± SD of duplicates of three experiments.

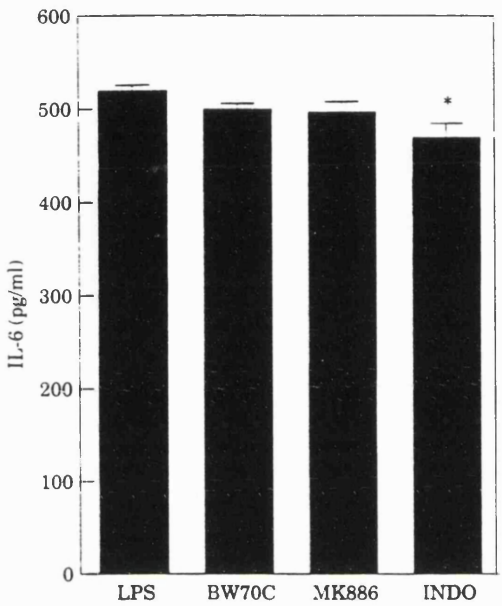

Figure 4. Effect of indomethacin and the lipoxygenase inhibitors MK886 and BW70C on *E. coli* LPS (1 μg/ml) stimulated production of IL-6 by human gingival fibroblasts.

Inhibitors were used over a concentration of 10^{-8} – 10^{-5}M; only the results from experiments using 10^{-5}M of each inhibitor are shown. Results are expressed as the mean ± standard deviation of duplicates of three experiments. *$P \leq 0.003$.

lished results) and as leukotriene B_4 has been shown to stimulate cellular IL-6 production[20] this could be the explanation for our findings. However, it is unlikely that the mechanism of the stimulation of IL-6 release from LPS-stimulated fibroblasts is through an arachidonic acid metabolite as both the cyclo-oxygenase and lipoxygenase inhibitors used in this study were unable to prevent IL-6 production. Interestingly, in vitro experiments have shown that RP 54745 is not an inhibitor of cyclo-oxygenase and only inhibits lipoxygenase at relatively high concentrations.[19] These observations are in contrast to an oxo-indole cytokine inhibitor, CP 66248,[21] which can inhibit both enzyme systems. In addition to possible similarities to RP 54745, the naphthoquinones may also act through similar pathways to the IL-1 inhibitor (Z)-3-(5-ethyl-4-hydroxy-3-methoxy-1-naphthalenyl)-2methyl-2-propenoic acid, which has been demonstrated to act at the transcriptional level without cyclo-oxygenase inhibitory activity.[22]

It is noteworthy that the most active IL-6 inhibiting naphthoquinone compounds are not active in the γ-carboxylation of glutamyl residues. Indeed, it is possible that the less active inhibitors of fibroblast IL-6 production, phylloquinone and menaquinone-4, must be metabolized into active forms to reveal their inhibitory potential. The mechanism of action of these compounds are the subject of further investigations and given that vitamin K_1 is non-toxic,[23] these compounds represent an interesting series for further investigation.

MATERIALS AND METHODS

Cell culture

Specimens of human gingivae were obtained during routine, minor oral surgery. The tissue was minced into small pieces and washed in Dulbecco's modified Eagles medium (DMEM, Sigma Chemical Co., UK). Gingival fibroblasts were grown from explant culture, the cultures being maintained in DMEM supplemented with 10% foetal calf serum (FCS, ICN Flow), 100 U/ml penicillin/streptomycin (Gibco Ltd.) and 2 mM glutamine (Sigma). The cells were subcultured at twice weekly intervals with 0.25% trypsin (Sigma) and used for experiments between passages 6 and 12. The fibroblasts were plated out at a density of 30 000 cells/well in 500 μl of DMEM containing 2% FCS in 24-well plates and incubated for 16 h at 37°C in a humidified atmosphere of 5% CO_2, 95% air. The concentration-response relationship for lipopolysaccharide (LPS, Sigma) stimulated fibroblast cultures was determined and from this result a concentration of LPS which exerted just supramaximal release of IL-6 into the media was estimated and used in all subsequent studies.

For the determination of the inhibitory capacity of the naphthoquinone analogues, 24-well plates were set up as described above and washed in Hank's balanced salt solution

(Sigma) before being incubated for a further 2 h in serial dilutions of vitamin K in 500 µl of DMEM. 2% FCS in groups of four wells per concentration. The cultures were then stimulated with 500 µl of LPS solution, to give a final concentration of 1 µg/ml, for 24 h at 37°C in 5% CO_2, 95% air. Control cultures received only fresh media. The role of arachidonic acid metabolism in LPS stimulated release of IL-6 was investigated using the cyclo-oxygenase inhibitor indomethacin (10^{-5}M-10^{-8}M) (Sigma) and the lipoxygenase inhibitors MK886 (10^{-5}M-10^{-8}M) (Merck Frosst, Canada) or BW70C (10^{-5}M-10^{-8}M) (The Wellcome Foundation, UK). The conditioned media were analysed for IL-6 by immunological assay.

ELISA for IL-6

Microtitre plates (Immulon 4, Dynatech) were coated with immunoaffinity purified G150 BM polyclonal goat anti-IL-6 (NIBSC) at 1 µg/ml, diluted in phosphate buffered saline (pH 7.2) and incubated at 4°C overnight. The wells were decanted and washed with 0.01M phosphate/0.05M NaCl buffer containing 0.1% Tween 20 (wash/dilution buffer, pH 7.2). To each well was added 100 µl of IL-6 standards (human recombinant standard 88/514: NIBSC) (concentration range 0–3 ng) or samples from the conditioned media. The plates were incubated at room temperature for 2 h before being rinsed in wash buffer. Biotinylated affinity purified polyclonal goat anti-IL-6 (100 µl at 0.014 µg/ml: NIBSC) was added to each well and incubated at room temperature for a further 1 h. The plates were rinsed in wash buffer and 100 µl of avidin-HRP (1:5000 dilution: Dako, Ltd.) and the plates incubated for 15 min at room temperature before a final rinse in wash buffer. The wells were developed with 20 µg orthophenylenediamine (Sigma Chemical Co.) in 100 µl critic acid (10 mM) – phosphate buffer (50mM, pH 5.0) containing 0.04 µl/ml 30% H_2O_2 (Sigma). The reaction was terminated by the addition of 150 µl 1M sulphuric acid. The IL-6 levels were quantified using the absorbance at 492 nm on a Titertek Multiscan plate reader.

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The Potent Bone-resorbing Mediator of *Actinobacillus actinomycetemcomitans* Is Homologous to the Molecular Chaperone GroEL

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Abstract

***Actinobacillus actinomycetemcomitans* is a Gram-negative bacterium implicated in the pathology of localized juvenile periodontitis, a condition involving rapid destruction of alveolar bone. We have established that gentle extraction of this bacterium in saline releases a proteinaceous fraction (which we have termed surface-associated material [SAM]) which has potent osteolytic activity in the murine calvarial bone resorption assay. Fractionation of the SAM has now revealed that activity is associated with a 62-kD protein. This bone-resorbing activity can be blocked by a monoclonal antibody (raised to the whole bacterium) that is claimed to recognize a protein homologous to the *Escherichia coli* molecular chaperone GroEL. Purification of this bone-resorbing protein to homogeneity has been achieved by a combination of anion exchange, gel filtration, and ATP-affinity chromatography and the NH_2-terminal sequence shows > 95% homology to *E. coli* GroEL. This GroEL homologue is found in the SAM of *A. actinomycetemcomitans* but is not found in the osteolytically active SAM from other Gram-negative or Gram-positive bacteria. The GroEL protein from *E. coli*, but not from *Mycobacterium tuberculosis* and *Mycobacterium leprae*, also showed activity in the bone resorption assay. We believe this to be the first observation that a molecular chaperone has the capacity to stimulate the breakdown of connective tissue. (*J. Clin. Invest.* 1995. 96:1185–1194.) Key words: periodontal disease • chaperonin 60 • heat shock proteins • bone resorption**

Introduction

The chronic inflammatory periodontal diseases (CIPDs)[1] are the most prevalent of the persistent inflammatory diseases of humans. They are characterized by inflammation of the gingivae and the resorption of the alveolar bone supporting the teeth, a process which can lead to tooth loss. Evidence strongly suggests that the presence of certain Gram-negative oral bacteria in periodontal pockets (which are formed between the gum and the tooth in this disease) is a major factor in the development of tissue pathology, and this relationship has been best documented with the bacterium *Actinobacillus actinomycetemcomitans* and its role in localized juvenile periodontitis (LJP) (1, 2). However, the precise mechanisms responsible for the resorption of alveolar bone in this disease remain to be established. Bacteria may invade the periodontal tissues (3), but the accepted paradigm is that the resorption mechanism involves factors released by the bacteria, which either directly stimulate bone breakdown or generate the synthesis and/or release of osteolytic mediators within host tissues (3, 4). These mediators may include prostanoids and/or certain proinflammatory cytokines such as IL-1 and TNFα, all of which have potent osteolytic activity.

Using a simple saline extraction procedure we have isolated a fraction from the surface (termed surface-associated material [SAM]) of a number of bacteria implicated in the pathology of the CIPDs. This fraction contains the capsule and other components loosely associated with the outer membrane of the bacteria, and electron microscopic examination of extracted organisms demonstrates the removal of extracellular material but fails to show evidence of cell lysis (5, 6). In the case of *A. actinomycetemcomitans* this highly soluble fraction consists largely of protein (> 60%) and an extremely small amount of LPS (6). There is now evidence that the SAM is shed by oral bacteria in situ, being found on the tooth roots of extracted teeth from patients with CIPD (7). The SAM from *A. actinomycetemcomitans*, has a number of biological activities including inhibition of cellular proliferation (8) and of collagen synthesis (9). However, its most potent action is the stimulation of murine calvarial bone breakdown, with activity being seen at concentrations as low as 1 ng/ml (5, 6). In this assay the SAM is 10–100 times more potent than the LPS prepared from the same organism (5). Thus, the active moiety in this fraction could be a major factor in the pathogenesis of bone resorption in LJP. A number of bacterial components including LPS, teichoic acids, muramyl dipeptide, and certain protein fractions have been shown to be capable of stimulating bone resorption (10–13). However, with the possible exception of a toxin from the bacterium *Pasteurella multocida* (14), the SAM from *A. actinomycetemcomitans* is the most potent bacterial osteolytic agent reported. As described in this paper we have established that the active moiety is a 62-kD protein and thus its molar potency is in the picomolar range, similar to that reported for potent osteolytic cytokines such as IL-1 or TNF.

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1. *Abbreviations used in this paper:* CIPD, chronic inflammatory periodontal disease; LJP, localized juvenile periodontitis; SAM, surface-associated material.

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We have now isolated this osteolytic protein by use of anion exchange, gel filtration, and affinity chromatography and have determined its NH_2-terminal sequence. Care has been taken to exclude the possibility that LPS is contributing to the osteolytic activity of this protein. Isolation was aided by the use of monoclonal antibodies which neutralize the bone-resorbing activity of the SAM. These studies have established that the osteolytically active protein of *A. actinomycetemcomitans* is a member of the GroEL family of molecular chaperones.

Methods

Growth of bacteria. A. actinomycetemcomitans NCTC 9710 was grown on brain-heart infusion agar (Oxoid, Basingstoke, United Kingdom) at 37°C in a CO_2-enriched atmosphere for 3 d. *Eikenella corrodens* NCTC 10596 and *Porphyromonas gingivalis* W50 were grown under anaerobic conditions at 37°C in a medium consisting of brain-heart infusion agar supplemented with 0.375 grams/liter cysteine-HCl (BDH), 0.25 grams/liter haemin (BDH), and 0.05 grams/liter menadione (BDH Poole, Dorset, United Kingdom). *Escherichia coli Y1090* was grown on Luria Bertani agar, consisting of 10 grams/liter Bacto-Tryptone (Difco Laboratories Inc., Detroit, MI), 5 grams/liter bacto-yeast extract (Difco), and 5 grams/liter NaCl (Sigma Immunochemicals, St. Louis, MO), for 24 h under aerobic conditions at 37°C. *Staphylococcus aureus* (Oxford strain NCTC 6571) was grown under aerobic conditions on brain-heart infusion agar at 37°C for 48 h.

Extraction of SAM. All cells were harvested in sterile saline, centrifuged, washed briefly in saline, and lyophilized. SAM was removed from the various bacteria by gentle saline extraction as described (5). Briefly, lyophilized bacteria were resuspended in sterile saline and stirred gently at 4°C for 1 h. The bacteria were removed by centrifugation and the soluble SAM was dialyzed extensively against distilled water and lyophilized. The protein content of the SAM was determined by the method of Lowry et al. (15), the carbohydrate content by the method of Dubois et al. (16), and the nucleic acid content by absorption at 260/280 nm. The LPS content was measured by use of a commercial chromogenic *Limulus* amebocyte lysate assay (Pyrogent, Byk-Mallinckrodt, London, United Kingdom) according to the manufacturer's instructions. In all studies the activity of the SAM was related to dry weight.

Monoclonal antibodies. Hybridomas were raised in Balb/c mice immunized with *A. actinomycetemcomitans* ATCC (#43718) whole cells (17). Briefly, spleen cells from intracutaneously immunized and intraperitoneally boosted mice were fused with SP2/0-Ag-14 myeloma cells. Hybridomas were screened by ELISA for immunoglobulin secretion and antibody producers were cloned by limiting dilution. Three of these, P1, P2, and P3, secreted antibodies which have been shown to be specific for 81-, 62-, and 62-kD proteins, respectively, and this was confirmed by Western blotting against SAM from *A. actinomycetemcomitans* NCTC 9710. Hybridoma cells secreting mAbs P1, P2, and P3 were grown in Iscoves modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY) containing 10% Seraclone FCS (Sera-Lab Ltd., Crawley Down, United Kingdom) at 37°C in an atmosphere of 5% CO_2/air. When cell death began to occur, the cells were removed by centrifugation and the supernatant was collected. This was filter-sterilized and stored at −20°C until needed. Monoclonal antibody A5 was raised against the SAM extracted from *A. actinomycetemcomitans* NCTC 9710. Balb/c mice were immunized subcutaneously and boosted intravenously. Hybridoma cells were produced as described above and screened by ELISA against SAM. mAb A5 has been shown to be specific for a 66-kD protein component of the SAM by Western blot. Hybridoma cells secreting A5 were cultured in RPMI 1640 medium (Gibco Laboratories) containing 10% FCS (Sera-Lab). Tissue culture supernatant was collected as described above. All mAbs were of the IgG1 subclass.

All four mAbs were purified from their respective tissue culture supernatants using a protein A column (Sepharose CL 4B–linked protein A; Bio Rad Laboratories, Hercules, CA) (18) and dialyzed extensively against PBS.

Murine calvarial bone resorption assay. Bone resorption was quantified by measuring calcium release from MF1 strain mouse calvaria in vitro (19). In some experiments the LPS-unresponsive strain C3H/HeJ was used. Calvaria were removed from 5-d-old mice and halved, with each half cultured separately on stainless steel grids. Calvaria were cultured in 30-mm dishes with 1.5 ml of BGJ medium (Flow Laboratories Inc., McLean, VA) containing 5% heat-inactivated rabbit serum (Gibco Laboratories) and 5 mg/100 ml ascorbic acid (Sigma Immunochemicals). The calvaria were treated in groups of five. After 24 h, the media were replaced with media containing the test substances. PGE_2 at 1 μM was used as a positive control in all assays (except those in which the C3H/HeJ strain mice were used, when IL-1α was used) to show that the bone was responsive to osteolytic mediators. Calvaria were cultured for a further 48 h before calcium release was measured by automated colorimetric analysis (20). The significance of the results was calculated using Student's *t*-test. Batches of SAM were assayed for bone resorbing activity over the concentration range 10 ng/ml to 10 μg/ml.

To assess the capacity of mAbs to inhibit the bone resorbing activity of the SAM, individual mAbs were added to calvarial cultures stimulated to resorb by the presence of 1 μg/ml SAM or 1 μg/ml LPS from *A. actinomycetemcomitans*. Each antibody was used at various dilutions to determine its inhibitory dose response. Nonspecific mouse immunoglobulin G, containing all four IgG subclasses (Sigma Immunochemicals), was used as a control in all the antibody studies. To determine if the mAbs were able to deplete the SAM of its osteolytic activity, solutions of the SAM were incubated with an excess (1:10 wt/wt) of antibody P3 or with nonspecific mouse IgG (Sigma Immunochemicals), or with mAb A5 overnight at 4°C with constant mixing. Antibody, along with any bound antigen, was then removed by the addition of *S. aureus* (Cowan strain) heat-killed/formalin-fixed whole cells (Sigma Immunochemicals) for 1 h at room temperature, again with constant mixing, followed by centrifugation and filter sterilization. The depleted fraction was added to the calvarial assay at 1 or 10 μg/ml and activity was compared with that of untreated SAM. SAM was also directly incubated with *S. aureus* at room temperature for 1 h to control for any possible nonspecific binding events between bacteria and SAM, this treated fraction being tested in the bone resorption assay.

To determine if LPS was contributing to the osteolytic activity of the SAM, the crude and purified GroEL preparations or LPS from *A. actinomycetemcomitans* were exposed to heat (100°C for 30 min) or trypsin (0.25% trypsin; Sigma Immunochemicals) for 1 h followed by excess (threefold molar excess) of soya-bean trypsin inhibitor (Sigma Immunochemicals). Control cultures exposed to the trypsin/trypsin inhibitor complex showed no increase in bone resorption, and the formed complex when added to bone cultures stimulated with PGE_2 did not inhibit osteolysis. Polymyxin B (20 μg/ml) was also added to bone cultures stimulated with either SAM or LPS from *A. actinomycetemcomitans* to determine if it could inhibit bone resorption.

SDS-PAGE. The components of the SAM were analyzed by SDS-PAGE using 12% gels according to the method of Laemmli (21). Samples were diluted 1:1 with sample buffer and boiled for 5 min before loading. Gels were run using a MiniProtean II system (Bio Rad Laboratories) and stained with Coomassie brilliant blue (Sigma Immunochemicals). The molecular weight markers used were Dalton VIIL (Sigma Immunochemicals). Gels were also silver stained using a commercial kit (Gelcode® silver stain kit; Pierce, Rockford, IL) to detect both the presence of protein and carbohydrate.

Two-dimensional PAGE. Two-dimensional PAGE gels were run according to the method of O'Farrell (22). Gels were run using a MiniProtean II system and stained with Coomassie blue, with similar molecular weight markers as above. The first dimension, isoelectric focusing, was over the pI range of 3–10. Second dimension separation was by molecular mass using a 12% SDS-PAGE gel.

Immunoblotting. Samples separated on one- or two-dimensional

SDS-PAGE were electroblotted onto Immobilon P polyvinyldifluoride membranes (Millipore Corp., Bedford, MA) overnight (23). Membranes were washed with PBS containing 0.1% Triton X-100 (Sigma Immunochemicals) (PBS-T) and blocked with PBS-T containing 2% FCS (blocking buffer) (Sera-Lab). Blocked membranes were then incubated with the test antibody (in blocking buffer) for 1 h and washed with PBS-T. Bound mouse IgG was detected using peroxidase-labeled goat anti-mouse IgG (γ-chain specific) (Sigma Immunochemicals) at 1:1,000 in PBS-T-2% FCS. After a final wash the blots were developed with a solution of 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma Immunochemicals) in 50 mM Tris (Sigma Immunochemicals), pH 7.6, containing 150 mM NaCl (BDH) and 0.05% hydrogen peroxide (Sigma Immunochemicals). Each reaction was terminated by extensive rinsing with distilled water.

Protein purification. Crude SAM was fractionated at 4°C on a Q-Sepharose anion exchange column (50 cm × 1.6 cm). The column was equilibrated in 20 mM Tris-HCl, pH 8.5 (buffer A), and the SAM (generally 100–400 mg) was loaded on in the same buffer. The column was washed with 500 ml of buffer A and then eluted with a 1,000-ml linear gradient of 0–2 M NaCl in buffer A. 10-ml fractions were collected, and the absorbance at 280 nm was monitored. The location of the osteolytic protein was determined by a combination of activity assay, SDS-PAGE, and Western blot analysis. Fractions containing osteolytic activity were dialyzed against deionized water to remove salt and lyophilized. The fraction with the highest specific activity and the least number of protein bands on SDS-PAGE was then further fractionated at room temperature on a second anion exchange column. There was some evidence of proteolytic clipping of the 62-kD protein and so aliquots of this fraction were dissolved at 1 mg/ml in 20 mM Tris, pH 7.2, containing proteinase inhibitors (1 mM PMSF, 1 mM EDTA, and 1 mM benzamidine) (buffer B) and fractionated on an EconoPak Q column (Bio Rad Laboratories) equilibrated in buffer B. Fractions were eluted by application of a gradient of 0–1.5 M NaCl in buffer B, and an absorbance profile at 280 nm was obtained. Fractions were again analyzed for osteolytic activity and Western immunoblotted with mAb P3 to confirm the presence of the immunogenic 62-kD protein. The purity of the fractions was again assessed visually by SDS-PAGE, and 100 μg of the cleanest fraction was dialyzed against 50 mM of Tris buffer, pH 7.6, containing 10 mM KCl and 10 mM $MgCl_2$ (buffer C). This sample was run on a 5-ml ATP-Sepharose (Sigma Immunochemicals) column. The column was washed with 10 column volumes of buffer C and bound protein eluted in 5 column volumes of 5 mM ATP (Sigma Immunochemicals), also in buffer C. Protein was located by SDS-PAGE and visualized using a silver stain kit (Sigma Immunochemicals). Gel filtration was used to determine the molecular mass range of the osteolytic protein isolated by ATP-affinity chromatography. This was done by running the purified protein on a Bio-Sil TSK250 (Bio Rad Laboratories) column in 0.1 M sodium phosphate buffer, pH 6.7, and measuring absorption at 205/280 nm.

Immunoaffinity purification. Affinity columns were prepared using mAb P2, mAb P3, and both P2 and P3 together. Briefly, in each case, 5 mg of antibody was linked to 3.5 ml of swollen cyanogen bromide–activated Sepharose 4B (Sigma Immunochemicals) in bicarbonate buffer at pH 8.3. After washing, the column was blocked with 1 M ethanolamine at pH 8.0, washed, and equilibrated in PBS. Crude SAM (5 mg) dissolved at 1 mg/ml in PBS was loaded on, the column was washed extensively, and bound protein was eluted using 0.1 M glycine, pH 2.5. Eluted fractions of 1 ml were concentrated by using Minicon microconcentrators with cutoff membranes of 10 kD (Amicon, Inc., Beverly, MA), to a volume of ~ 50 μl. These were analyzed by SDS-PAGE and immunoblotting and tested for osteolytic activity in the calvarial bone resorption assay.

Protein sequencing. Material eluting from the ATP-Sepharose column was run on a 10% SDS-PAGE gel according to the method of Laemmli (21) and electroblotted onto ProBlott membrane (Applied Biosystems Inc., Foster City, CA). The band of interest, at 62 kD, was excised and run on an ABI 470A protein sequencer (Applied Biosystems Inc.) for 40 cycles using an ABI "Blott" cartridge and an optimized program for electroblotted samples. Data were collected and analyzed using Waters Expert-Ease software (Millipore Corp.).

Table I. Composition of the SAM from the Various Bacteria Studied

Bacterium	Composition (%)			
	Protein	Carbohydrate	Nucleic acid	LPS
				IU/ng
A. actinomycetemcomitans	60–70*	12–15*	8–10*	9×10^{-3}‡
E. corrodens	60	6	14	6×10^{-4}
P. gingivalis	40–50*	18	3	2×10^{-5}
S. aureus	35–45*	5	5	+

* Range of estimates from 5 to 15 different preparations of SAM from each organism. ‡ 1 μg *E. coli* LPS contains 7,000 IU. +, Generally below limit of assay detection.

Heat shock proteins. Additional purified GroEL-like (chaperonin 60) proteins were obtained and tested for osteolytic activity in the calvarial bone resorption assay. GroEL-like proteins from *Mycobacterium leprae* and *Mycobacterium tuberculosis* were supplied by the World Health Organization antigen bank (Braunschweig, Germany). *E. coli* GroEL, was obtained commercially (Boehringer Mannheim, Mannheim, Germany). These proteins were separated by SDS-PAGE, Western blotted, and probed with antibody P3 to determine if they contained a cross-reactive epitope. Each specimen was also tested for osteolytic activity. Before testing, each sample was passed through a DeToxi-Gel column (Pierce), as per manufacturer's directions, to remove any LPS contamination. Removal of LPS was checked by running material before and after the DeToxi-Gel column on SDS-PAGE and silver staining the gels. Samples of the various LPS-free GroEL homologues were then diluted and tested over a limited concentration range to determine their potency and efficacy in the murine calvarial bone resorption assay.

Results

Composition of the SAM. The composition of the SAM from the various bacteria is shown in Table I. The SAM from *A. actinomycetemcomitans* when analyzed by two-dimensional PAGE demonstrated the presence of ~ 50 Coomassie blue–staining spots in the pI range of 3–10 and of molecular masses ranging from < 14 to > 66 kD (Fig. 1).

Activity of SAM in the murine calvarial bone resorption assay. The SAM extracted from *A. actinomycetemcomitans* showed a consistent profile of activity (Fig. 2) with calcium release increasing linearly over the concentration range from 0.01 to 10 μg/ml. With some batches of SAM, significant bone resorbing activity was found at a concentration of 1 ng/ml.

Q-Sepharose anion-exchange chromatography. Osteolytic activity was seen to elute at a salt concentration in the range of 0.9–0.92 M in four consecutive fractions (28–31) (Fig. 3). These bioactive fractions represented ~ 3% of the protein applied to the column. Active fractions contained a prominent 62-kD protein band on SDS-PAGE stained with Coomassie blue.

Neutralization of bone resorption by mAbs to A. actinomycetemcomitans. mAbs raised to whole *A. actinomycetemcomitans* were tested in the bone resorption assay to determine if they would have any effect on bone breakdown induced by

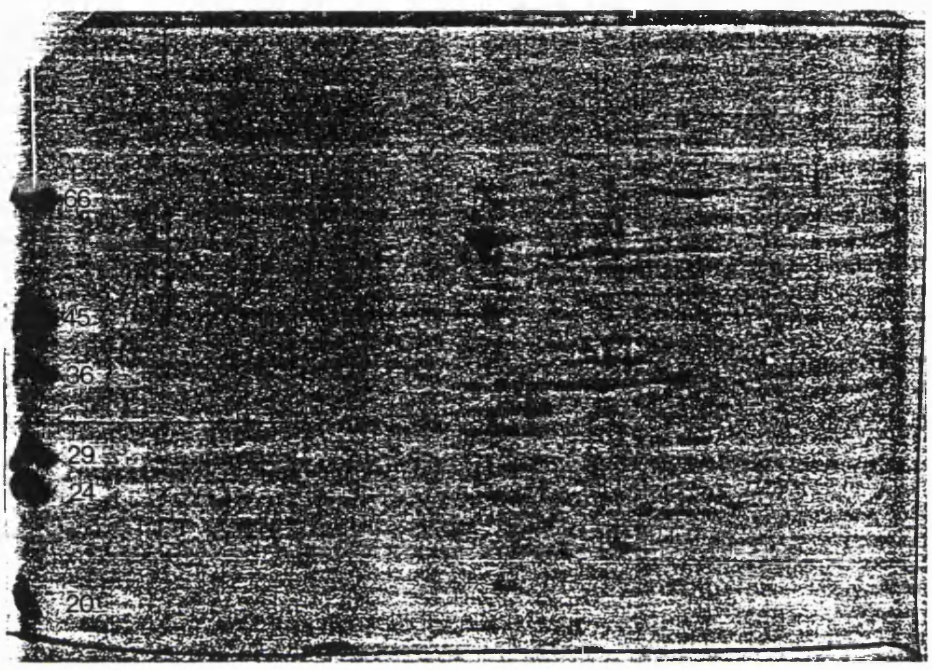

Figure 1. Determination of the protein composition of the SAM of *A. actinomycetemcomitans* by two-dimensional SDS-PAGE. Proteins have been separated in one dimension on the basis of their isoelectric point and in the other dimension on the basis of their molecular mass. The gel was stained with Coomassie blue to disclose proteins. The molecular mass markers are displayed on the left-hand side.

SAM. The inclusion of nonspecific mouse IgG or mAbs P1 or A5 had no noticeable effect on the bone resorbing activity of the SAM, even at concentrations as high as 100 μg/ml. In contrast, mAbs P2 and P3 inhibited calcium release. P3 completely inhibited the bone resorbing activity of the SAM when added at a concentration of 7 μg/ml (Fig. 4), whereas P2 only attained comparable inhibition at a concentration of 100 μg/ml. The antibodies had no effect on the bone resorption induced by the osteolytic agonist PGE_2. To confirm that antibody-mediated inhibition of bone resorption was associated with binding to components of the SAM, this material was incubated with antibody P3, and the antibody–antigen complexes produced were immunoprecipitated with heat-killed/formalin-fixed *S. aureus* (Sigma Immunochemicals). It was clear (Fig. 5) that immunoprecipitation with antibody P3, the most potent neutralizing antibody, reduced the osteolytic activity of *A. actinomycetemcomitans* SAM to background levels. This was seen to be the case even when the depleted fraction was added at a concentration of 10 μg/ml. Controls in which SAM was incubated with a nonspecific antibody, or with *S. aureus* alone, retained activity equal to the untreated SAM.

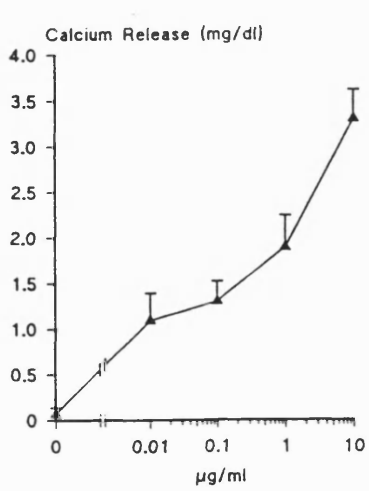

Figure 2. Dose response of the stimulation of calvarial bone resorption induced by the SAM from *A. actinomycetemcomitans*. Bone breakdown is measured as the release of calcium from the bone. Each point represents the mean and standard deviation of five separate cultures. The concentrations of SAM used ranged from 10 ng/ml to 10 μg/ml.

Specificity of mAbs P2 and P3 assessed by Western blotting. Using two-dimensional SDS-PAGE of *A. actinomycetemcomitans* SAM, many proteins or protein subunits were separated (Fig. 6 *a*). Immunoblotting these preparations using mAbs P2, P3 (Fig. 6 *b*), or a combination of both showed that both mAbs recognized the same protein, which had a molecular mass of 62 kD.

Affinity purification of the 62-kD osteolytic protein. Affinity columns containing mAbs P2, P3, or a combination of both antibodies, linked to Sepharose 4B, were used to try and achieve a one-pass purification of the active protein. Both antibodies appeared to bind very weakly to the 62-kD protein and it was only possible to isolate small quantities of this protein by this technique. The isolated protein was active in the bone resorption assay and reacted with antibodies P2 and P3 in Western blots.

In a prior study a small quantity of the 62-kD protein which bound to antibody P3 was used for sequencing. This suggested homology with the *E. coli* heat shock protein GroEL (24). It had been demonstrated that purification of this molecular chaperone can be achieved simply by affinity purification on an ATP column (25). When the anion exchange chromatography-purified fractions containing bone-resorbing activity were passed through an ATP-Sepharose column and the column was washed and then eluted with either ATP or magnesium-free buffer, a single protein was eluted as seen on a silver-stained SDS-PAGE gel (Fig. 7). This protein was active in the bone resorption assay and, when Western blotted, was recognized by mAbs P2 and P3 but not by mAbs P1 or A5 or by normal

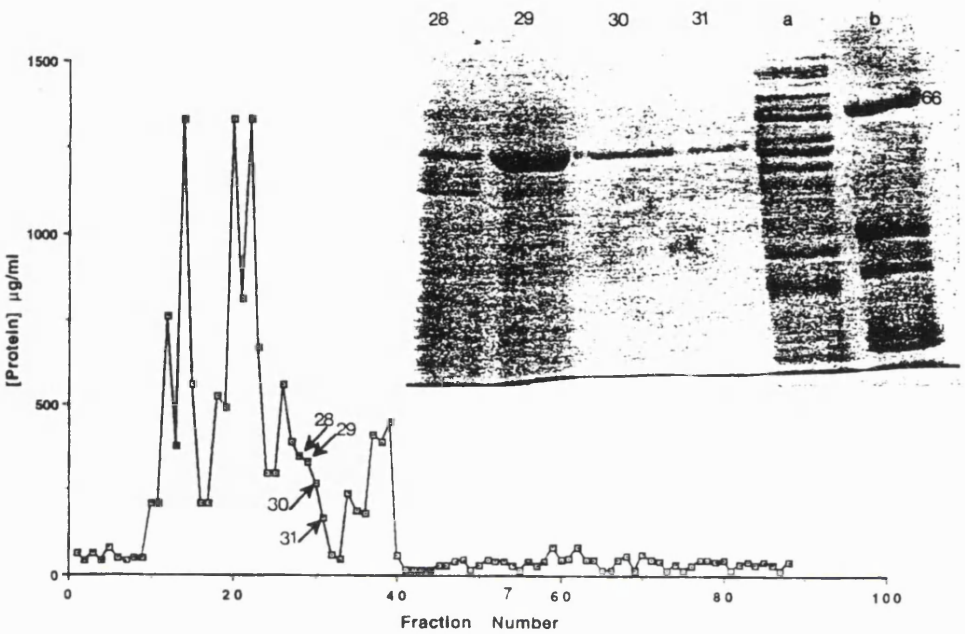

Figure 3. Elution profile of the SAM on Q-Sepharose anion exchange chromatography. Each fraction was assessed for protein content and for bone-resorbing activity in the murine calvarial bone resorption assay. The protein content of each fraction is shown and the fractions with osteolytic activity are arrowed. The SDS-PAGE profiles of bioactive fractions are shown in the inset. Lane *a* is the starting material and lane *b* contains the molecular mass markers with the 66-kD marker highlighted. Osteolytic activity is associated with the presence of a 62-kD protein in the fractions.

mouse IgG. When the protein isolated from the ATP column was fractionated by gel filtration on a TSK250 column, which has a molecular exclusion limit of 300 kD, osteolytic activity eluted in the void volumn fraction.

NH_2-terminal sequencing. NH_2-terminal sequencing of the 62-kD protein eluted from the ATP-affinity column produced a continuous sequence of 38 residues. Comparisons using the National Centre for Biotechnology Information Blast network service showed this sequence to have 100% homology to the GroEL protein of *E. coli* over the first 17 residues and to differ at only 2 of the 38 residues sequenced. The homology to other GroEl-like proteins such as those from mycobacterial species or the human P1 protein was in the order of 60–70% (Table II).

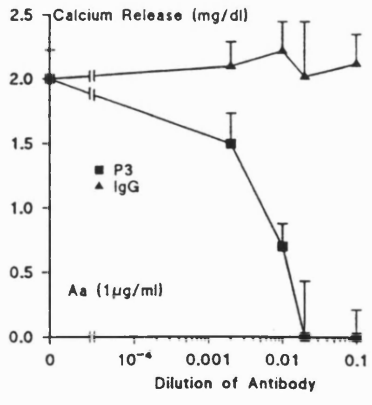

Figure 4. Dose-dependent inhibition of the bone resorption (measured as calcium release) induced by 1 µg/ml SAM from *A. actinomycetemcomitans* by addition of mAb P3 to the bone explants. The lack of effect of equivalent concentrations of mouse IgG is also seen. Results are expressed as the mean and standard deviation of five replicate cultures.

Cell surface expression of GroEL. Saline extracts of bacteria whose SAMs are known to stimulate bone resorption, *E. corrodens*, *P. gingivalis*, and *S. aureus*, and a crude extract of *E. coli*, were Western blotted and reacted with antibody P3. Only

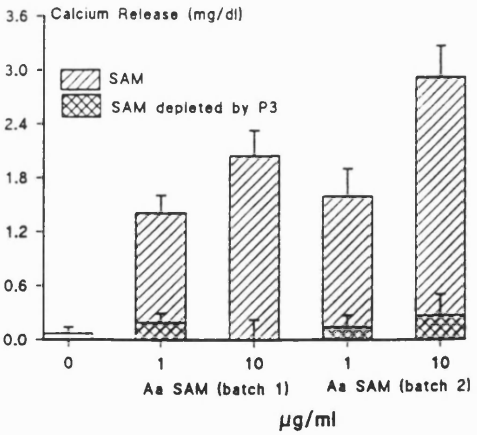

Figure 5. Effect of depletion of the SAM, by immunoabsorption with mAb P3, on the stimulation of murine calvarial bone resorption. Cultures were exposed to 1 or 10 µg/ml SAM which had either been depleted with mAb P3 or sham-depleted by incubation with heat-killed *S. aureus* (Cowan strain) in the absence of P3. Results are expressed as the mean and standard deviation of five replicate cultures. Two individual preparations (batches) of the SAM have been tested to show the batch-to-batch reproducibility. The column denoted 0 shows the amount of calcium released by unstimulated calvaria.

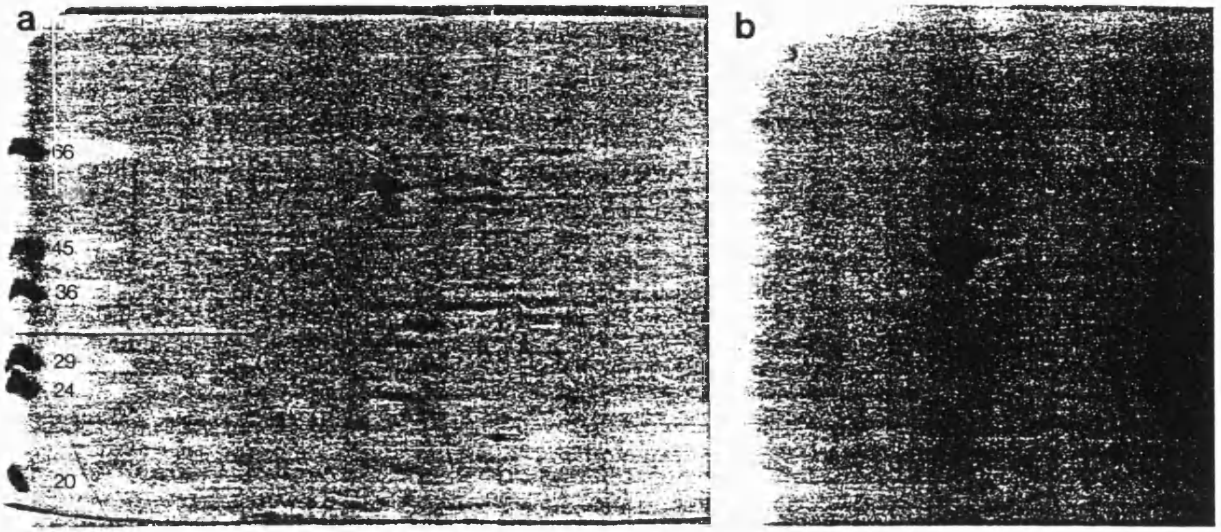

Figure 6. Western immunoblotting of *A. actinomycetemcomitans* SAM separated by two-dimensional SDS-PAGE with mAb P3. (*a*) Coomassie blue–stained two-dimensional gel with a prominent protein spot of 62 kD arrowed; (*b*) gel immunoblotted with antibody P3 to demonstrate that this antibody only binds to this 62-kD protein.

the SAM from *A. actinomycetemcomitans* showed the presence of the specific 62-kD antigen in this surface-associated fraction (Fig. 8).

Bone-resorbing activity of other bacterial GroEL-like proteins. The GroEL-like proteins from M. leprae and *M. tuberculosis* (i.e., hsp 60) and *E. coli* GroEL, when Western immunoblotted, reacted with antibody P3 (Fig. 8), showing that these proteins shared the epitope which P3 recognized and which was associated with the inhibition of the bone-resorbing activity. However, when these various purified proteins were tested for activity in the calvarial bone resorption assay only that from *E. coli* had the capacity to stimulate resorption after removal of associated LPS (Fig. 9). LPS removal was confirmed by running the GroEL preparations on overloaded SDS-PAGE and silver staining the gels to look for the characteristic LPS ladder pattern.

Role of LPS. The possibility that the osteolytic activity of the SAM was due to either LPS contamination or synergy between LPS and the GroEL homologue was addressed. The starting material (SAM) had low levels of endotoxin, and silver-stained SDS-PAGE gels overloaded with the purified GroEL homologue failed to show the ladder pattern characteristic of LPS (Fig. 10). Polymyxin B inhibited the bone-resorbing activity of the LPS from *A. actinomycetemcomitans* but failed to inhibit the osteolytic activity of the purified GroEL homologue from this bacterium (Table III). In contrast, antibody P3 inhibited the osteolytic activity of the GroEL homologue but failed to inhibit the activity of the LPS from *A. actinomycetemcomitans* (Table III). In addition, the osteolytic activity of the GroEL

Figure 7. Affinity purification of the 62-kD osteolytic protein on ATP-Sepharose. (*A*) Molecular weight standards; (*B*) starting material; (*C*) protein eluted from Q-Sepharose column; (*D*) material eluted from ATP-Sepharose column. The SDS-PAGE gel was stained with silver.

Table II. NH_2-terminal Amino Acid Sequences of GroEL and GroEL-like Proteins

	Amino acid residues
	1 17 19
A.a "GroEL"	AAKDVKFGNDARVKMLNGV
E. coli GroEL	R
M. leprae hsp60	M TIAYDEE RGLER L
Human P1 protein	Y A AL Q
	20 38
A.a "GroEL"	NILADAVKVTLGPKGRNVV
E. coli GroEL	V
M. leprae hsp60	S
Human P1 protein	DL A M T I

A.a, A. actinomycetemcomitans.

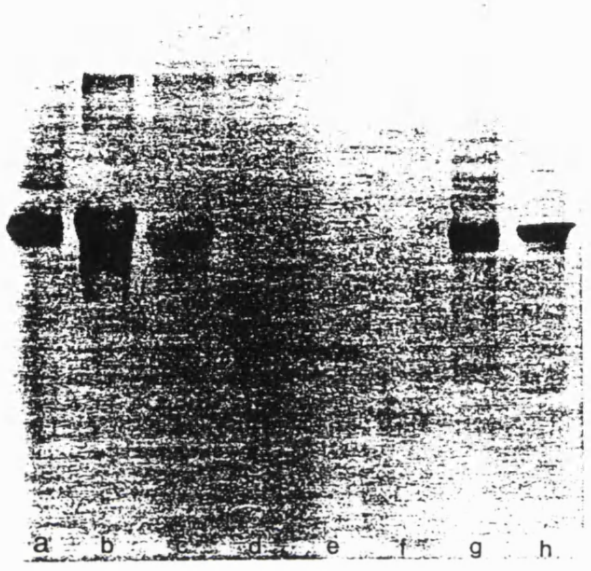

Figure 8. Western immunoblotting of bacterial components with antibody P3: (*a*) a crude lysate of *E. coli*; (*b*) *M. tuberculosis* hsp65; (*c*) *M. leprae* hsp65; (*d*) SAM from *S. aureus*; (*e*) SAM from *P. gingivalis*; (*f*) SAM from *E. corrodens*; (*g*) > 30-kD fraction of the SAM from *A. actinomycetemcomitans*; (*h*) crude SAM from *A. actinomycetemcomitans*.

homologue was sensitive both to heating and to trypsin (Table III). SAM and LPS from *A. actinomycetemcomitans* were also tested in the calvarial assay using C3H/HeJ mice which are unresponsive to LPS. The combined results from two separate experiments are shown in Table IV, which shows clearly that while the calvarial bone is responsive to the SAM it is unresponsive to the LPS from this organism.

To ascertain if LPS and GroEL interacted in a synergistic manner in the bone resorption assay, suboptimal concentrations of both components were added singly or together to calvaria and the amount of bone resorption was determined 48 h later. No evidence of synergistic interactions was noted, indeed

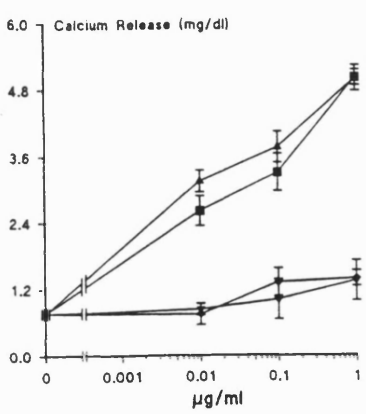

Figure 9. Stimulation of calvarial bone resorption by increasing concentrations of SAM from *A. actinomycetemcomitans* (■), purified *E. coli* GroEL (▲), or the GroEL-like proteins from *M. tuberculosis* (♦) or *M. leprae* (▼). Results are expressed as the mean and standard deviation of five replicate cultures. The concentration range is from 10 ng/ml to 1 μg/ml.

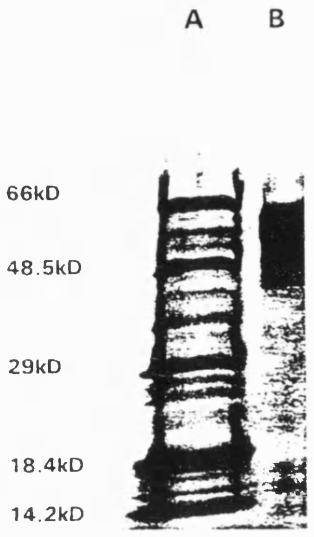

Figure 10. Silver-stained SDS-PAGE gel overloaded with the purified GroEL homologue (*B*) showing lack of a ladder pattern indicative of LPS contamination. The molecular mass markers are shown (*A*).

the two components failed to produce an additive response (Table V).

Discussion

LJP is characterized by the severe and rapid loss of alveolar bone on the approximal surfaces of first molar and/or incisor teeth and is generally associated with the presence of the Gram-negative bacterium *A. actinomycetemcomitans* (1, 2). Work from the Eastman Dental Institute has established that gentle saline extraction of this organism releases a proteinaceous fraction that is assumed to be associated with the external surface of the outer membrane. Electron microscopic examination of bacteria after extraction failed to show evidence of cell disruption (5, 6). The finding of extremely low LPS levels in the SAM is additional evidence of the lack of postextraction cell lysis. Of course it cannot be conclusively proven that all proteins in the SAM come from the cell surface and the term is therefore

Table III. Effect of Various Treatments on the Capacity of the A. actinomycetemcomitans GroEL Homologue or LPS to Stimulate Calvarial Bone Resorption

Treatment	Percentage of inhibition
GroEl homologue +	
Polymyxin B	3
100°C for 30 min	62
Trypsin for 60 min	79
mAb P3	81
A. actinomycetemcomitans LPS +	
Polymyxin B	79
100°C for 30 min	2
Trypsin for 60 min	3
mAb p3	6

The details of the methodology used are given in Methods.

Table IV. Bone Resorption of C3H/HeJ Strain Calvaria in Response to SAM or LPS from A. actinomycetemcomitans

Stimulant	Calcium release
	mg/dl
A.a SAM (μg/ml)	
10	3.6±0.7
1	1.6±0.3
0.1	0.8±0.2
0.01	0.3±0.2
A.a LPS (μg/ml)	
10	0.4±0.1
1	0.2±0.1
0.1	0.2±0.2
0.01	0.3±0.1
Media control (no stimulators)	0.2±0.1
rIL-1α (10 ng/ml)	4.2±0.9

Results are expressed as the mean and standard deviation of two separate experiments in which each concentration of agonist was tested in five replicate cultures. *A.a. A. actinomycetemcomitans.*

Table V. Interactions between LPS and GroEL in the Bone Resorption Assay

Combination	Calcium release
	mg/dl
Control	0.1±0.1
PGE_2 (maximal release)	4.1±0.7
A.a GroEL (100 ng/ml)	1.3±0.3
A.a LPS (1 μg/ml)	1.6±0.2
GroEL + LPS	1.8±0.2
E. coli GroEL (100 ng/ml)	0.9±0.2
E. coli LPS (10 ng/ml)	2.1±0.5
GroEL + LPS	2.2±0.1

Results show the release of calcium from murine calvaria exposed to either GroEL or LPS or to a combination of both agents. The control value is the calcium release from unstimulated bone. Maximal stimulation is induced by adding 1 μg/ml PGE_2. *A.a. A. actinomycetemcomitans.*

an operational one for material eluted when bacteria are stirred in isotonic saline. This surface-associated material has a range of biological actions, the most prominent being its ability to stimulate the breakdown of murine calvarial bone, an assay used to detect the activity of osteolytic agents. Significant stimulation of bone resorption can be induced with 1–10 ng/ml of this crude fraction. An interesting finding in contemporaneous experiments is that, in contrast to the SAM from *P. gingivalis* (26), *E. corrodens* (6), or *S. aureus* (27), the bone-resorbing activity of the SAM from *A. actinomycetemcomitans* cannot be inhibited by nonsteroidal antiinflammatory drugs or by inhibiting the actions of the potent osteolytic cytokines IL-1 or TNFα (6). Thus the active component in the SAM appears not to be able to induce key mediators normally associated with the induction of calvarial bone resorption (4). This suggests that the active moiety in the SAM is interacting directly with bone cells to induce resorption. Our preliminary studies suggest that the active protein can directly stimulate the recruitment of the primary bone-resorbing cell (the osteoclast), but we cannot rule out a direct effect on osteoblasts (6).

A substantial degree of purification of the osteolytic activity of the SAM was achieved by anion exchange chromatography and activity appeared to be associated with a 62-kD protein. Further purification, using anion exchange chromatography, failed to isolate the active protein to homogeneity. We had shown previously that sera from a proportion of patients with LJP could block the osteolytic activity of the SAM from *A. actinomycetemcomitans* (28) and we had begun a program to develop and test monoclonal antibodies to the SAM to both aid purification and to provide probes for studies of the biological activity of this material. Two antibodies were found that inhibited the SAM-induced resorption of bone, and one of these antibodies, P3, was able to remove the bone-resorbing activity by immunoabsorption. In contrast, normal mouse serum or mAbs P1 and A5 (which are of the same IgG1 subclass as mAbs P2 and P3) had no inhibitory activity. When antibodies P2 and P3 were used in Western blots to identify the antigen, they both recognized a 62-kD protein. Preliminary studies had suggested that these antibodies recognized a protein that had some homology with the *E. coli* molecular chaperone or heat shock protein GroEL (24). GroEL has ATP binding activity, and this activity provides a convenient method for its purification (25). On the basis of this information, we then used a previously described ATP-Sepharose chromatography technique (25) to isolate the 62-kD protein. The use of the ATP-column resulted in the isolation of a homogenous 62-kD protein, as assessed by silver-stained SDS-PAGE, with total recovery of bone-resorbing activity. NH_2-terminal sequencing showed that of the first 38 residues identified, 36 were identical to that of *E. coli* GroEL, and one was a conservative substitution. The homology to the GroEL-like proteins of mycobacterial species was in the region of 60–70% with similar homology to the mitochondrial P1 GroEL homologue (Table II). Electron microscopic examination of negatively stained preparations of this *A. actinomycetemcomitans* GroEL-like protein showed the characteristic double ring structure with sevenfold symmetry (25) (our unpublished data).

We conclude that the surface-associated material from the oral bacterium *A. actinomycetemcomitans* contains a homologue of the *E. coli* molecular chaperone or heat shock protein, GroEL, and that this protein is responsible for the potent bone-resorbing activity of this fraction. When the purified GroEL homologue was run through a TSK250 gel filtration column that had a molecular exclusion limit of 300 kD, the boneresorbing activity appeared in the void volume, implying that the GroEL multimer is the active moiety. The finding that a homologue of GroEL is osteolytic is surprising, not the least because the SAM fraction is assumed to contain only material associated with the bacterial outer surface and GroEL is generally assumed to be an intracellular protein. Even if this protein is not associated with the cell surface, it is rapidly removed from the cells by gentle washing in isotonic saline. We have shown that the SAM fraction from a number of oral bacteria, such as *P. gingivalis* (26), *E. corrodens* (6), and *S. aureus* (27), expresses potent osteolytic activity. This raised the possibility that the activity of these SAMs may have been due to the presence of GroEL-like proteins. However, no antigen cross-reactive with mAbs P2 or P3

was found in the SAM fractions isolated from these bacteria and their osteolytic activity could not be blocked by P3 (results not shown). Thus *A. actinomycetemcomitans*, at least in culture, appears to be unusual in that it may express GroEL on its surface although we have no evidence that this is the case in vivo. It should be noted that this is not a unique finding as surface expression of a GroEL homologue in *Helicobacter pylori* was also suggested by the fact that the protein could be removed by vortexing in distilled water and was capable of being labeled by surface-labeling techniques (29, 30). However, a direct pathogenic action of this *H. pylori* GroEL homologue has not been claimed. Heat shock proteins are major antigens in a variety of infectious and autoimmune diseases (31, 32). We have also established that: (*a*) the 62-kD protein of *A. actinomycetemcomitans* is an immunodominant antigen as assessed by Western blotting with sera from patients with LJP; and (*b*) that a proportion of patients with strong circulating antibody responses to the SAM has the capacity to neutralize its osteolytic activity and thus almost certainly contains antibodies directed against GroEL with similar specificities to P2 and P3 (28).

The GroEL homologue is a potent stimulator of bone resorption with some preparations of SAM demonstrating bone-resorbing activity at a concentration of 1 ng/ml. We have now established that the active protein has a molecular mass of 62 kD and that it represents ~ 2% of the dry mass of the SAM. Thus, this protein is capable of demonstrating bone-resorbing activity at a molar concentration of ~ 1 pM, a similar potency to that of the most active bone-stimulating agonist, IL-1 (33).

That the osteolytic activity was due to contaminating LPS, or to some interaction between LPS and the GroEL homologue, was a possibility that required investigation. The LPS content of the SAM and the GroEL has been determined by the *Limulus* assay and was low. One of the authors (T. Nishihara) has shown that the LPS from *A. actinomycetemcomitans* has comparable activity to that of *E. coli* LPS in the *Limulus* assay (34). *A. actinomycetemcomitans* LPS produces a classic ladder pattern on silver-stained SDS-PAGE gels (34). No such ladder pattern was seen on an overloaded silver-stained SDS-PAGE gel of the purified GroEL homologue. Thus, it would appear that LPS contamination is minimal. It was further shown that while the osteolytic activity of the LPS from *A. actinomycetemcomitans* could be inhibited by polymyxin B, that of the SAM or the purified GroEL homologue was unaffected. In contrast, the neutralizing mAb P3 had no inhibitory effect on the bone-resorbing activity of LPS. It was also demonstrated that calvaria from the LPS-unresponsive mouse strain C3H/HeJ would resorb in response to the SAM but not when stimulated with the LPS from *A. actinomycetemcomitans*, confirming that the osteolytic activity in the SAM was not due to LPS contamination. The biological effects of LPS are not sensitive to heat or trypsin, yet the bone-resorbing activity of the SAM and the GroEL was significantly inhibited by short-term heating or exposure to trypsin. These findings refute the hypothesis that the bone-resorbing activity is due to LPS. It was possible that residual LPS in the SAM could form a complex with the GroEL homologue that demonstrated the property of synergism. This possibility was tested by adding suboptimal concentrations of LPS or GroEL to bone. However, this showed no evidence of synergy and indeed suggested that both components could possibly interfere with each other in inducing bone resorption. Thus, we conclude that the osteolytic effects of the SAM are due to the activity of a GroEL-like chaperone protein and that LPS plays no part in the activity.

We have examined the bone-resorbing activity of GroEL from *E. coli* and GroEL homologues from *M. tuberculosis* and *M. leprae* and have found that the former does indeed demonstrate osteolytic activity but that the latter two (once the LPS has been removed) have little bone-resorbing activity. This difference in osteolytic activity may be due to structural differences in these GroEL proteins, and further studies are underway to clone and sequence the GroEL homologue from *A. actinomycetemcomitans*.

We therefore speculate that the GroEL homologue of *A. actinomycetemcomitans* functions both as a bone-resorbing virulence factor and as an immunogen. In a proportion of patients, the immune response to this protein is neutralizing, and we are currently setting up studies to determine if this neutralizing immune response plays any role in the clinical course of the bone destruction.

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Characterization of an Antiproliferative Surface-Associated Protein from *Actinobacillus actinomycetemcomitans* Which Can Be Neutralized by Sera from a Proportion of Patients with Localized Juvenile Periodontitis

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The gentle agitation of suspensions of *Actinobacillus actinomycetemcomitans* serotype a, b, or c in saline resulted in the release of a proteinaceous surface-associated material (SAM) which produced a dose-dependent inhibition of tritiated thymidine incorporation by the osteoblast-like cell line MG63 in culture. This cell line was sensitive to low concentrations of SAM (50% inhibitory concentration, 200 ng/ml for serotype c). Immunoglobulin G antibodies to constituents of the SAM were found in the blood of patients with localized juvenile periodontitis (LJP). Sera from 9 of 16 patients with LJP significantly neutralized the antiproliferative activity of the SAM, while sera from 15 controls, with no evidence of periodontal disease, were unable to neutralize this activity. Neutralization was not directly related to the patient's antibody titer to the whole SAM. Characterization of the antiproliferative activity in the SAM demonstrated that it was not cytotoxic and was heat and trypsin sensitive. The active component separated in a well-defined peak in anion-exchange high-performance liquid chromatography (HPLC) which, when further analyzed by size exclusion HPLC, revealed a single active peak, which had an apparent molecular mass of approximately 8 kDa. The lipopolysaccharide from *A. actinomycetemcomitans* was only weakly active. SAM from *Porphyromonas gingivalis* W50 and *Eikenella corrodens* NCTC 10596 did not exhibit any antiproliferative activity with this cell line, even at concentrations as high as 10 μg/ml. This study has shown that SAM from *A. actinomycetemcomitans* contains a potent antiproliferative protein whose activity can be neutralized by antibodies in the sera from some patients with LJP.

Actinobacillus actinomycetemcomitans is a gram-negative bacterium that has been strongly implicated in the etiology of localized juvenile periodontitis (LJP) (25, 26). The loss of the alveolar bone and periodontal ligament which support the tooth is one of the hallmarks of this disease and is the result of either the removal of the tissue by some destructive process or the failure to produce sufficient tissue to keep pace with the normal rate of tissue remodelling. It is widely assumed that the loss of the extracellular matrices of these connective tissues is a result of the local gingival inflammation. However, in LJP the afflicted gingivae are only mildly inflamed (15) and it is possible that other mechanisms play a role in tissue loss.

The alveolar bone and periodontal ligament are considered to be tissues with a rapid rate of remodelling (11). Agents which inhibit cellular proliferation could therefore adversely affect these tissues, resulting in the tissue loss associated with LJP. A number of antiproliferative factors from periodontopathogenic bacteria have been documented (3, 4, 13, 16, 17, 19), yet it remains unclear exactly what role these factors play in the pathogenesis of the disease. We have previously reported that surface-associated material (SAM) from *A. actinomycetemcomitans*, isolated by gentle saline extraction, contains antiproliferative activity (5, 9, 23). We now report the identification of an immunogenic protein which is responsible for this activity. Individuals with LJP have elevated serum antibodies to *A. actinomycetemcomitans* and to its SAM (2, 7, 14); however, the role that such antibodies play in the course of this disease remains unclear. Our results demonstrate that a number of patients with LJP have serum antibodies which can neutralize the activity of this potent antiproliferative protein, and the role of such neutralizing antibodies in disease is discussed.

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MATERIALS AND METHODS

Growth of bacteria. *A. actinomycetemcomitans* (NCTC 9710 serotype c and clinical isolates 286 and 670) were cultured at 37°C in a CO_2-enriched atmosphere on brain heart infusion agar (Oxoid) supplemented with 5% (vol/vol) horse blood. *A. actinomycetemcomitans* clinical isolates 286 and 670, representing serotypes a and b, respectively, were kindly donated by Maria Saarela, University of Helsinki (12). *Porphyromonas gingivalis* W50 was grown at 37°C under anaerobic conditions on a medium consisting of (per liter) 5 g of Trypticase (BBL), 5 g of Proteose Peptone (Oxoid), 2.5 g of glucose (BDH), 2.5 g of sodium chloride (BDH), 2.5 g of yeast extract (Oxoid), 0.0375 g of cysteine HCl (BDH), 0.25 g of hemin, and 0.05 g of menadione (Oxoid). *Eikenella corrodens* NCTC 10596 was grown at 37°C under anaerobic conditions on a medium consisting of brain heart infusion (37 g/liter; Oxoid), 0.375 g of cysteine HCl (BDH), 0.25 g of hemin, and 0.05 g of menadione (Oxoid). Bacteria were grown for 48 h, harvested with saline, and centrifuged at 3,000 × *g* for 20 min, and the pellet was stored at −70°C.

Extraction of SAM and LPS. SAM was extracted by a modification of the method of Wilson et al. (24). Briefly, bacteria were thawed on ice, gently stirred in 0.15 M saline for 1 h at 4°C, and centrifuged at 3,000 × *g*. The saline extraction was repeated, and the combined supernatants were dialyzed with benzoylated dialysis tubing with a 2-kDa cutoff (Sigma) against distilled water and lyophilized. The protein concentration of the SAM was determined by the Bio-Rad (Richmond, Va.) protein assay with bovine serum albumin as the standard. The carbohydrate content was determined by the method of Dubois et al. (1), and the content of lipopolysaccharide (LPS) was determined by a commercial chromogenic *Limulus* amoebocyte assay (Pyrogent, Byk-Mallinckrodt, United Kingdom). In one experiment SAM was also extracted with methanol-chloroform (2:1) to isolate lipidic materials, and the extract was lyophilized and weighed. LPS was extracted by the method of Westphal and Jann (22). The biological activity of extracted material was related to dry weight.

PAGE. The SAM and fractions from purification procedures were suspended

in buffer (0.06 M Tris, 10% glycerol, 1% sodium dodecyl sulfate [SDS], 2.5% 2-mercaptoethanol [pH 6.8]) and boiled for 4 min. Samples were then separated by either one-dimensional or two-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad mini-Protean II system (6, 10). Gels were stained with colloidal Coomassie blue (Sigma) or silver stained by using a commercial kit (Sigma). Molecular weight markers ranging from 14 to 66 kDa (Sigma) were run on all gels.

Fractionation of SAM from *A. actinomycetemcomitans*. (i) Amicon filtration. Crude SAM was separated by using Amicon YM30 membranes to isolate two fractions of <30 and >30 kDa and YM100 membranes to separate the >100-kDa from lower-molecular-mass material. Fractions were dialyzed, freeze-dried, and assayed for antiproliferative activity.

(ii) Anion-exchange chromatography. SAM was fractionated by anion-exchange high-performance liquid chromatography (HPLC) on a Bio-Rad HRLC system. An anion-exchange MA7Q (Bio-Rad) column (5 by 0.78 cm) was equilibrated with 20 mM Tris buffer, pH 8.5 (buffer A). One milliliter of a 4-mg/ml solution of SAM in buffer A was injected onto the column and eluted with 5 ml of buffer A followed by linear gradients from 0 to 50% buffer B (buffer A plus 2 M NaCl) in 20 ml and 50 to 100% buffer B in 5 ml. The flow rate was 1 ml/min, and 1-ml fractions were collected, with absorbance monitored at 280 and 205 nm. Fractions were dialyzed (Sigma dialysis tubing, 2-kDa cutoff) against distilled water for 48 h, and the protein concentration of each fraction was measured. Fractions were subsequently diluted 1,000 times to assess their antiproliferative activity on cultured MG63 cells.

Size exclusion chromatography. Bioactive fractions were further fractionated by size exclusion HPLC using a Protein Pak 125 column (Waters). The column was equilibrated with 0.1 M sodium phosphate buffer, pH 6.7, and 20-μl samples (concentration, 1 mg/ml) were injected. The flow rate was 1 ml/min, and 1-ml fractions were collected, with protein absorbance monitored at both 280 and 205 nm. Fractions were assayed for their ability to inhibit [^{3}H]thymidine incorporation by MG63 cells.

Osteoblast-like cell proliferation assay. The human osteoblast-like cell line MG63 (ATCC CRL 1427) was cultured at a density of 15,000 cells per well in 96-well plates and incubated overnight at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco) plus 10% fetal calf serum (FCS) (Sigma) in 5% CO_2-air. The medium was then removed, and the cells were washed twice with sterile Hanks solution (Sigma). To measure antiproliferative activity, various concentrations of the test materials were added to the cells in DMEM containing 2% FCS. To test the ability of human sera to neutralize the antiproliferative activity of the SAM from *A. actinomycetemcomitans*, 1:50 or 1:500 dilutions of sera were added to 500 ng (dry weight) of SAM per ml in DMEM containing 2% FCS and the mixtures were incubated at 37°C for 1 h before addition to the cells. The cells were incubated for 24 h at 37°C. During the last 6 h of culture, 0.05 μCi of [^{3}H]thymidine (Amersham) was added to the cells. Media were then removed, and the cells were fixed in 5% trichloroacetic acid. One hundred microliters of 0.5 M NaOH was used to lyse the cells; this was neutralized with an equal volume of 0.5 M HCl. Radioactivity was measured by scintillation spectrometry. The significance of the results was calculated by use of Student's *t* test.

Enzyme and heat treatments. SAM from *A. actinomycetemcomitans* was dissolved at 1 mg/ml in Tris buffer, pH 8.5. One hundred microliters of this solution was then mixed with 100 μl of trypsin (Sigma) dissolved at 100 μg/ml (100 BAEE units) in the same buffer and incubated for 1 or 24 h. One hundred microliters of soya bean trypsin inhibitor (Sigma) dissolved at 100 μg/ml was used to terminate the enzyme reaction (10 μg inhibits approximately 20 μg of trypsin with an activity of 100 BAEE units), and the samples were stored at 4°C. Control digestion mixtures contained no SAM but were otherwise identical. Thirty microliters from each digestion mixture was diluted into 970 μl of DMEM containing 2% FCS to a final SAM concentration of 10 μg/ml. All samples were tested for antiproliferative activity. SAM from *A. actinomycetemcomitans* was dissolved in saline at a concentration of 100 μg (dry weight) per ml and heated in water baths at various temperatures for 1 h. Samples were diluted 10 times in DMEM containing 2% FCS and assayed for antiproliferative activity.

Cytotoxicity assays. The cytotoxicity of the SAM was determined by lactate dehydrogenase (LDH) release measured by the CytoTox 96 nonradioactive cytotoxicity assay (Promega). Briefly, MG63 cells were cultured for 24 h in the presence of various concentrations of SAM ranging from 0.1 to 100 μg/ml. LDH levels in culture supernatants were measured with a 30-min coupled enzymatic assay which results in the conversion of a tetrazolium salt [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride] into a red formazan product. A_{492} was measured. The percent cell lysis was established by using a formula which correlates sample levels of LDH to the maximally induced level (by using Triton X-100 to lyse cells) and control LDH release.

Cytotoxicity was also monitored by acridine orange uptake after cells were incubated with the SAM at a concentration of 100 μg/ml for 24 h. The proportions of acridine orange-stained cells in control cultures and in those exposed to SAM were counted and compared with the incorporation of [^{3}H]thymidine into cells in parallel control cultures or cultures exposed to SAM.

Serum samples. Studies were performed with sera from 16 patients diagnosed as having LJP by standard criteria, including radiographic evidence of bone loss and first permanent molar or incisor pocket depths of 5 mm or more. Patients varied in age from 12 to 39 years. Samples were also obtained from 15 individuals judged to be periodontally normal, ranging in age from 13 to 41 years. Serum from clotted blood was harvested by centrifugation and stored at −20°C.

Enzyme-linked immunosorbent assay for serum antibody titers. Ninety-six-well microtiter plates (Immulon 4; Dynatech) were coated with *A. actinomycetemcomitans* SAM at 10 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C. Wells were washed three times with PBS containing 0.05% Tween (Sigma) (PBS-T) to remove any unbound antigen and blocked with PBS-T containing 1% nonfat milk powder (Safeway) (PBS-TM) for 1 h at 37°C. Sera were then incubated in the wells at dilutions ranging from 1:100 to 1:64,000 in PBS-TM for 1 h at 37°C. Plates were washed three times with PBS-T, and the bound antibody was detected with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (γ-chain specific) (Sigma) dissolved at a 1:1,000 dilution in PBS-TM, again incubated for 1 h at 37°C. Tetramethylbenzidine dihydrochloride (0.1 mg/ml) plus hydrogen peroxide (2 μl of fresh 30% solution per 10 ml) in 0.1 M citrate buffer (pH 5.1) was used as the enzyme substrate, and the reaction was terminated after 10 min by the addition of 1 M sulfuric acid. Plates were read at 450 nm with a Titertek Multiskan plate reader.

The relative binding of each serum at each dilution was calculated with reference to a 100% control (wells coated with excess human IgG [Sigma]) and a nonspecific binding control (antigen omitted), and from these results the serum titer giving 30% binding was determined. The significance of the results was tested by Wilcoxon's rank sum test.

Western blotting (immunoblotting). SAM separated on 15 and 12% gels by SDS-PAGE was electrophoretically transferred to nitrocellulose membranes as described by Towbin et al. (20). The membranes were washed for 5 min in PBS containing 1% Triton X-100 and then for a further 25 min in PBS containing 0.1% Triton X-100. Nitrocellulose membranes were then rinsed in blocking buffer (PBS containing 0.1% Triton X-100 and 2% FCS) for 1 h and incubated in human serum diluted 1:100 in blocking buffer for 1 h. Following a further wash, membranes were incubated for 1 h in goat anti-human IgG horseradish peroxidase conjugate (Sigma), diluted 1:1,000, and washed again. Membranes were placed in 3,3'-diaminobenzidine tetrahydrochloride solution (10 mg in 15 ml of Tris-buffered saline, pH 7.6) containing 12 μl of 30% hydrogen peroxide until bands were visualized.

Depletion of serum antibody using protein A. To determine if the neutralizing activity in the sera was due to antibody, the serum with the highest neutralizing activity (from patient 10) was diluted 1:10 in PBS, and Sepharose (CL-4B)-bound protein A (Sigma) was added and mixed for 2 h at 4°C. The protein A-Sepharose was then pelleted by centrifugation. Fifty microliters of this depleted serum was removed for analysis, and the remaining material was added to fresh protein A-Sepharose, which was again mixed for 2 h at 4°C. This process was repeated one more time. To demonstrate the removal of antibody, the washed protein A-Sepharose from each step was boiled in SDS-PAGE sample buffer and run on a 12% gel. This showed a progressive decline in antibody uptake onto the beads. Undepleted-serum or serum depleted once, twice, or three times was assayed for its ability to neutralize the antiproliferative activity of SAM (500 ng/ml) at a dilution of 1:500. To determine if the protein A-Sepharose had any effect on MG-63 proliferation or on the antiproliferative activity of the SAM, it was added to cells in the presence or absence of SAM.

RESULTS

Characterization of the SAM. The range of values for the composition of the SAM obtained from six separate batches was approximately 60 to 70% protein, with the remainder being carbohydrate and lipid. The LPS content of the SAM was low, generally on the order of 0.0001 to 0.001 IU/ng. On SDS-PAGE, the SAM showed a relatively large number of protein bands, and on two-dimensional gels over 40 Coomassie blue-stained spots were visible.

Antiproliferative activity of SAM. In the human osteoblast-like cell line MG63, the SAM from *A. actinomycetemcomitans* (NCTC 9710) caused a concentration-dependent inhibition of [^{3}H]thymidine incorporation with a reproducible 50% inhibitory concentration (IC_{50}) of approximately 200 ng/ml. The SAMs from two other periodontopathogenic bacteria, *P. gingivalis* W50 and *E. corrodens* NCTC 10596 were also tested but failed to show potent antiproliferative activity with this cell line (Fig. 1). In contrast to the SAM, the LPS from *A. actinomycetemcomitans* at a concentration of 10 μg/ml produced only 13% inhibition of proliferation (results not shown). Comparison of the antiproliferative activities of the SAMs from the three major serotypes (a, b, and c) of *A. actinomycetemcomitans* demonstrated that they all had the capacity to inhibit proliferation (Fig. 2). The methanol-chloroform extract of the SAM

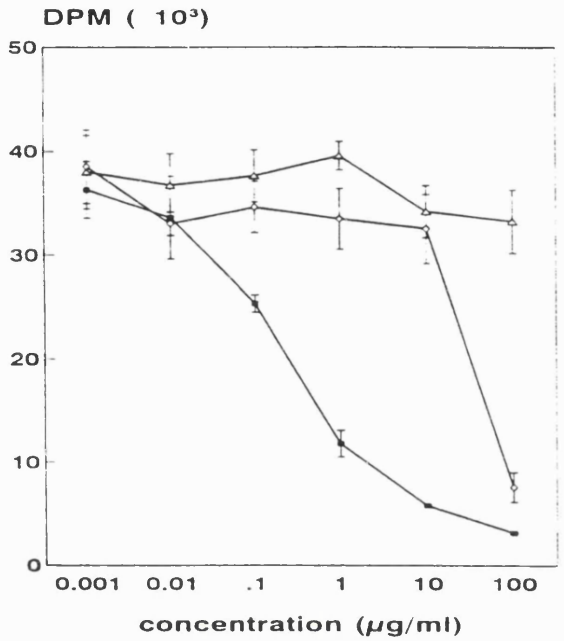

FIG. 1. Inhibitory effect of increasing concentrations of SAMs from *A. actinomycetemcomitans* (square), *P. gingivalis* (diamond), and *E. corrodens* (triangle) on DNA synthesis, measured as incorporation of [^{3}H]thymidine into DNA, by MG63 cells. The results are expressed as means and standard deviations for six replicate cultures.

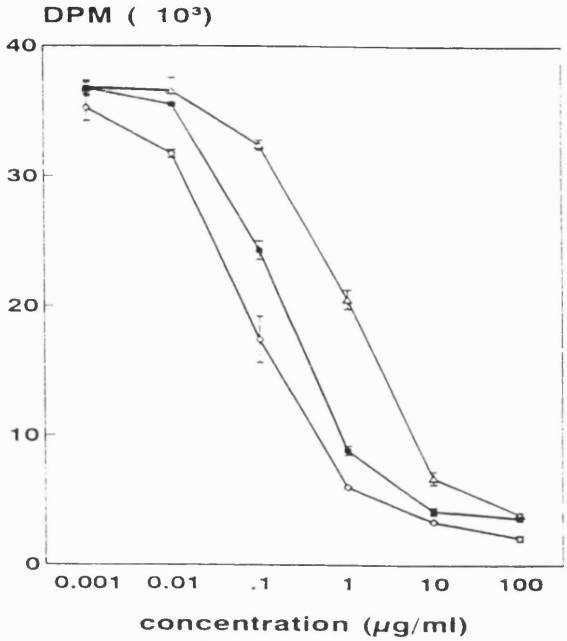

FIG. 2. Inhibitory effect of increasing concentrations of SAMs from *A. actinomycetemcomitans* serotypes a (triangle), b (diamond), and c (NCTC 9710) (square) on DNA synthesis, measured as incorporation of [^{3}H]thymidine into DNA, by MG63 cells. The results are expressed as means and standard deviations for six replicate cultures.

was also tested for antiproliferative activity but failed to show any activity at a concentration of 10 μg/ml.

Heat treatment of *A. actinomycetemcomitans* SAM demonstrated that at 56°C the SAM maintained its antiproliferative activity while at 78 and 100°C 81 and 95%, respectively, of the activity were lost (Fig. 3). Treatment with trypsin for 1 h destroyed over 70% of the antiproliferative activity, with no activity remaining after 24-h incubation (data not shown).

The SAM was not cytotoxic as assessed by a commercial assay for cytotoxicity based upon release of LDH which showed that cells exposed to the SAM released amounts of LDH (16% of maximum) that were similar to the amounts released by control cells. Use of a second measure of cytotoxicity, acridine orange uptake, also demonstrated that the SAM was not cytotoxic, as the number of nonviable cells in cultures incubated with SAM was not significantly different from that in the controls. However, at the concentration of SAM used, there was almost complete inhibition of [^{3}H]thymidine incorporation (Fig. 4).

Fractionation of the SAM. Amicon filtration of the crude SAM on YM30 membranes showed that all of the activity was in the >30-kDa fraction and that on YM100 cutoff membranes a large proportion of the antiproliferative activity was also retained. The crude SAM extract was separated by anion-exchange HPLC. The elution profile showed that the majority of the antiproliferative activity appeared in one well-defined peak (fraction 12) (Fig. 5). This bioactive material was further fractionated by HPLC gel filtration on a Protein Pak 125 column (Waters), and the antiproliferative activity was assessed (Fig. 6). Bioactivity was eluted as a broad peak and, on the basis of the retention time of standard markers, had a mean molecular mass of 8 kDa. Bioactive fractions were pooled, concentrated, and retested over a protein concentration range of 1 to 100 ng/ml. This semipurified material was 50 times more active than the crude material, with an IC_{50} of 4 ng/ml. This pooled fraction, analyzed by SDS-PAGE on a 15% gel stained with Coomassie blue, contained two proteins with molecular

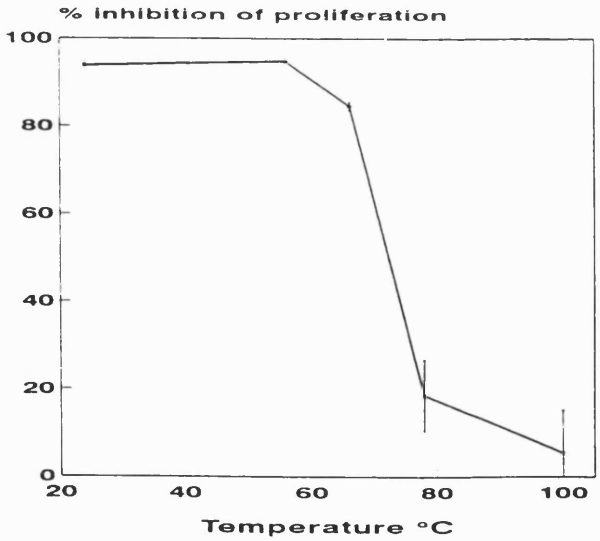

FIG. 3. Effect of temperature on the inhibitory activity of SAM from *A. actinomycetemcomitans* on DNA synthesis, measured as incorporation of [^{3}H]thymidine into DNA, by MG63 cells. Solutions of SAM were heated at various temperatures for 1 h and tested at a concentration of 10 μg/ml. The results are expressed as means and standard deviations for six replicate cultures.

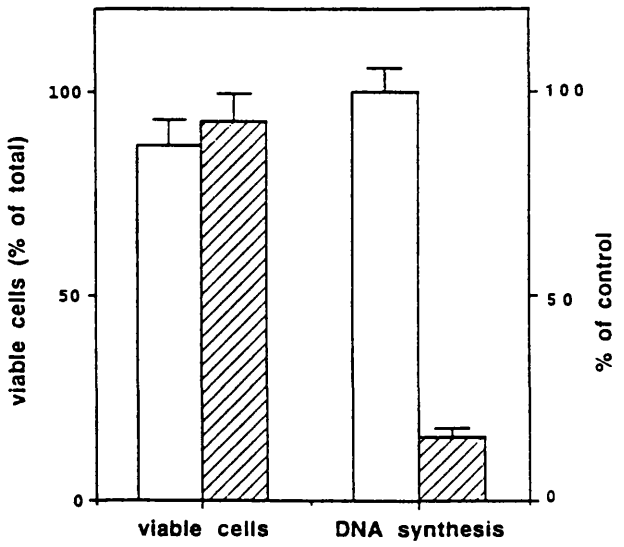

FIG. 4. Comparison of the effect of SAM on cell viability and on cellular replication, as measured by [^{3}H]thymidine incorporation into DNA. MG63 osteosarcoma cells were exposed to 100 μg of SAM per ml for 24 h. The control cultures, to which SAM was not added (open bars), and SAM-treated cultures (hatched bars) were pulsed with [^{3}H]thymidine to determine DNA synthesis (expressed as percentage of control) or tested for acridine orange uptake as a measure of cell viability (expressed as percentage of total cells). Results are expressed as means and standard deviations for three replicate cultures.

masses of less than 15 kDa and two other minor proteins of approximately 26 kDa (Fig. 7).

Serum antibody titers. Sera from 16 patients with LJP were assessed for titers of anti-SAM antibodies on microtiter plates coated with SAM. The titers of these sera and 15 representative controls are shown in Fig. 8. Sera from patients with LJP had titers that were significantly higher (median, 5,350) than those of the control sera (median, 620) ($P < 0.001$ [Wilcoxon's rank sum test]).

Serum inhibition of antiproliferative activity. Patients' sera had no effect on the proliferation of MG63 cells. Sera from 16 patients with LJP were added at a 1:50 or 1:500 dilution to MG63 cells incubated in the presence of 500 ng of SAM per ml to determine if they could neutralize the antiproliferative activity associated with the surface-associated protein. At 1:50 dilutions, 9 of the 16 sera significantly blocked the antiproliferative activity ($P < 0.001$) and sera from patients 7 and 10 almost completely neutralized this activity (Fig. 9). At 1:500 dilutions, five sera (patients 3, 7, 8, 10, and 16) were still able to significantly block the antiproliferative activity of the SAM ($P < 0.01$) (results not shown). High concentrations (1:50 dilutions) of sera from 15 individuals with no evidence of periodontal disease had no effect on the ability of SAM to inhibit cell proliferation (results not shown).

The serum which most potently blocked the antiproliferative effect of SAM (patient 10) was tested at various concentrations to establish whether the effect was concentration dependent. Figure 10 shows that sera from control, disease-free volunteers were unable to block the antiproliferative activity of 500 ng of SAM per ml. In contrast, serum from a patient with LJP (patient 10; titer, 1:9,200) was capable of inhibiting this antiproliferative activity in a concentration-dependent manner.

Removal of antibodies from the serum of patient 10 using protein A-Sepharose decreased the neutralizing ability after each treatment, reaching control levels (100% inhibition) after the third adsorption (Fig. 11). Protein A-Sepharose alone was not antiproliferative, nor did it effect the antiproliferative activity of the SAM.

Immunoblotting of sera. Immunoblot analysis of SDS-PAGE-separated SAM from *A. actinomycetemcomitans* was

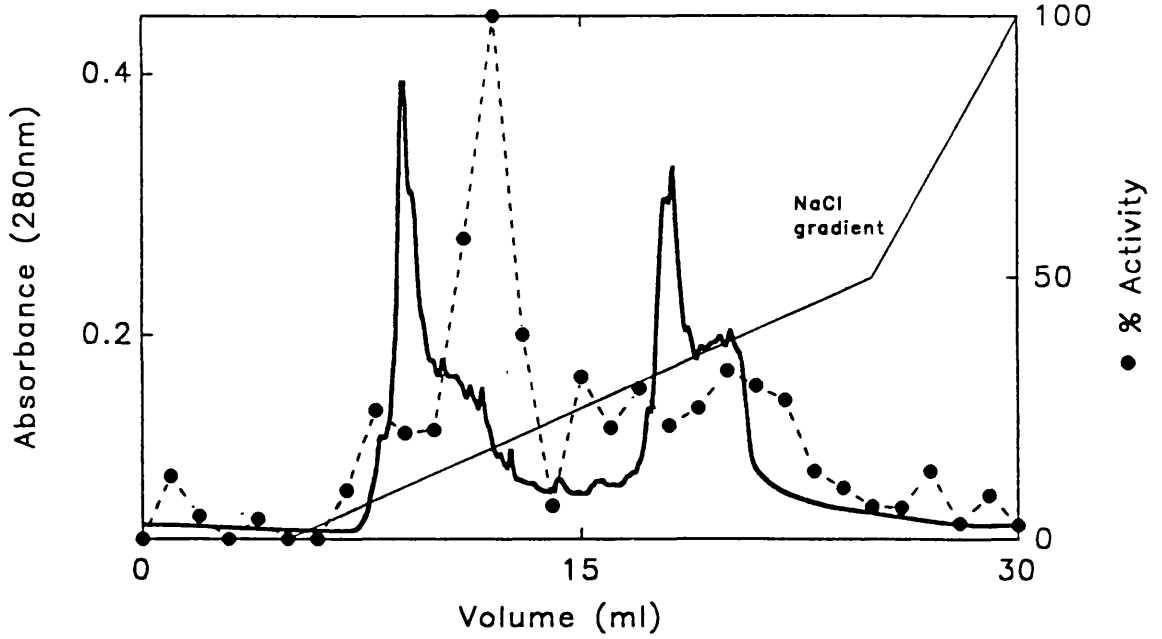

FIG. 5. Anion-exchange HPLC of SAM from *A. actinomycetemcomitans*. The protein elution profile is shown as A_{280} (solid line). Charged components were eluted with a 0 to 2 M NaCl gradient. Each fraction was assayed for inhibition of [^{3}H]thymidine incorporation into MG63 cells (dotted line), and percent activity was compared with the activity of the most active fraction (fraction 12), which was deemed 100% active.

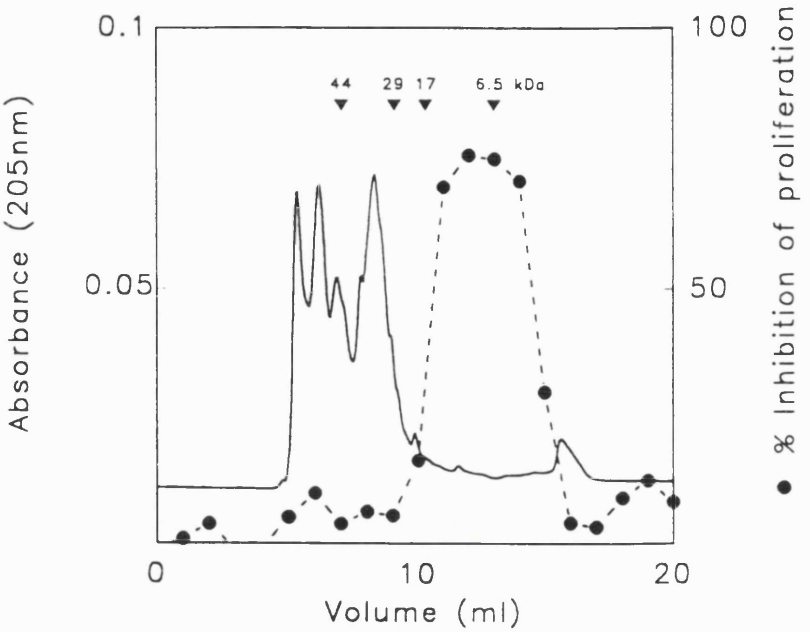

FIG. 6. Size exclusion HPLC of the most active fraction (fraction 12) from the HPLC anion-exchange column. The protein elution profile is shown as A_{205} (solid line). Each fraction was assayed for inhibition of [^{3}H]thymidine incorporation into MG63 cells (dotted line). Protein standards: aprotinin (6.5 kDa), myoglobin (17 kDa), carbonic anhydrase (29 kDa), and ovalbumin (44 kDa).

carried out with sera from all 16 LJP patients. Antibodies bound to a large number of the proteins ranging in molecular mass from >66 to <14 kDa. A representative immunoblot from patient 7 (Fig. 12) shows the pattern of antibody binding. Neutralizing and nonneutralizing sera showed similar patterns of IgG-binding antibodies, with dominant bands at 24 and 29 kDa.

DISCUSSION

Gentle saline extraction of *A. actinomycetemcomitans* (NCTC 9710) releases SAM which can potently inhibit [^{3}H]thymidine incorporation in numerous cell types including the fibroblast cell line L929, the monocyte cell line U937, keratinocytes, osteosarcoma cell line U2OS, gingival fibroblasts, periodontal fibroblasts, and primary osteoblasts (9, 11a, 22a). The component responsible may play a role in the pathogenesis of LJP by preventing the replication of cells in the alveolar bone and periodontal ligament, thus causing a de-

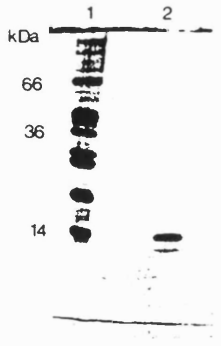

FIG. 7. SDS-PAGE of the biologically active fraction eluted from the size exclusion column, activity being found in fractions 10 to 14. Molecular weight markers are displayed in lane 1. Fractions 10 to 14 were pooled and concentrated, and the proteins present are shown in lane 2. The gel used contained 15% polyacrylamide and was stained with Coomassie blue to disclose protein bands.

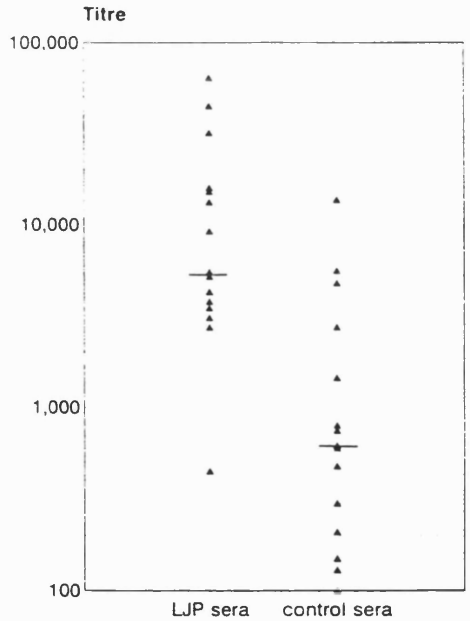

FIG. 8. Range of IgG antibody titers to *A. actinomycetemcomitans* SAM in the sera of individuals with LJP and individuals with no evidence of periodontal disease. Horizontal lines, median antibody titers. $P < 0.001$ (Wilcoxon's rank sum test).

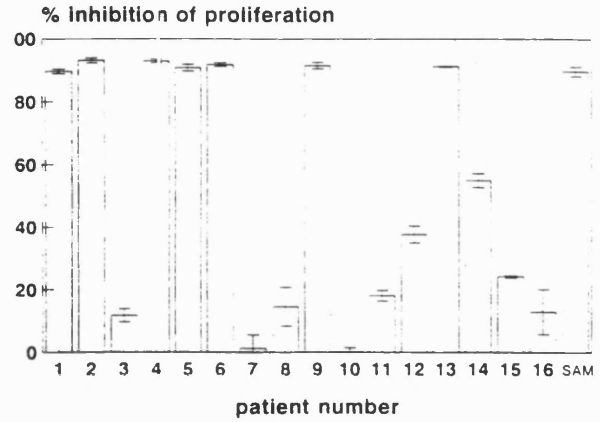

FIG. 9. Influence of LJP sera, used at a 1:50 dilution, on the antiproliferative ctivity of SAM from *A. actinomycetemcomitans* (incubated with cells at a con-entration of 500 ng/ml). Activity is measured as percent inhibition of [^{3}H]thy-idine incorporation relative to control cultures. The effect of SAM with no erum added is shown on the far right (SAM). The patient with the lowest titer f antibodies against the whole SAM is patient 1, and highest-titer patient is atient 16. Results are expressed as means and standard deviations for six eplicate cultures.

rease in the rate of replacement of these labile tissues. The onsequence of such inhibition of cellular proliferation could e the loss of connective tissue matrices and thus be equivalent o the loss by destructive processes, as is generally assumed to ccur.

We have now demonstrated that SAM from *A. actinomyce-emcomitans* (NCTC 9710) is an extremely potent inhibitor of

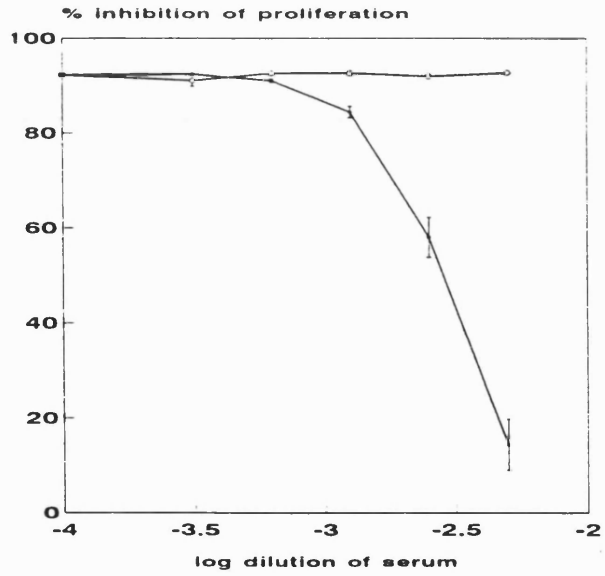

FIG. 10. Titration curves showing the effect of serum from patient 10 on the antiproliferative activity of 500 ng of SAM from *A. actinomycetemcomitans* per ml on MG63 cells. The graph shows that control serum from an individual with no evidence of periodontal disease was unable to block the antiproliferative activity (diamond). In contrast, serum from a patient with LJP was capable of inhibiting the antiproliferative activity of the SAM in a concentration-dependent manner. Results are expressed as means and standard deviations for six replicate cultures.

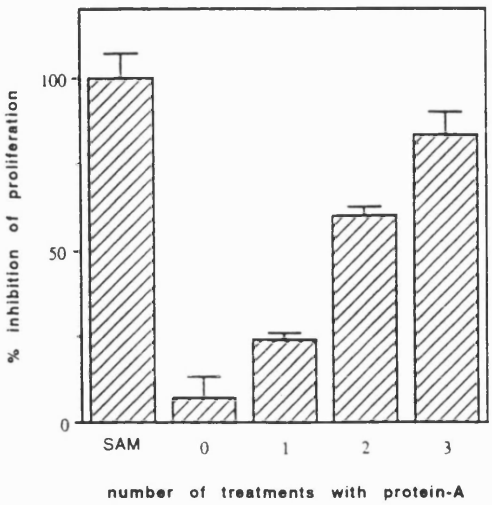

FIG. 11. Effect of sequentially adsorbing serum from patient 10 with protein A-Sepharose (to remove antibody) on the serum-mediated inhibition of the antiproliferative activity of SAM. The normalized percent inhibition of MG63 proliferation induced by SAM (500 ng/ml) is shown on the far left (SAM). 0, SAM plus unadsorbed serum; 1 to 3, inhibitory activities remaining after 1, 2, or 3 adsorptions of the serum with protein A. Results are expressed as means and standard deviations for six replicate cultures.

the proliferation of the human osteoblast-like cell line MG63, with an IC_{50} of approximately 200 ng/ml. SAMs from *A. actinomycetemcomitans* serotypes a and b also demonstrated antiproliferative activity. A concentration-dependent response parallel to that of serotype c was seen, although SAM from serotype a was not as active as SAMs from serotypes b and c, with an IC_{50} of approximately 1 μg/ml. The antiproliferative component is heat labile, trypsin sensitive, and noncytotoxic, elutes in a well-defined peak upon anion-exchange HPLC, and has an approximate molecular mass of 8 kDa as determined by size exclusion HPLC. Active fractions were pooled, concentrated, and separated by SDS-PAGE, revealing two major proteins of low molecular masses. It is conceivable that the smaller of these is the antiproliferative protein, although further purification is needed to confirm this. While size exclusion chromatography showed the activity to be of low molecular mass, on Amicon membrane filtration the antiproliferative activity was found exclusively in the >30-kDa fraction and approxi-

FIG. 12. Representative Western blot of SDS-PAGE-separated components of *A. actinomycetemcomitans* SAM. The blot was stained to show IgG binding of serum from a patient with LJP (patient 7). The molecular sizes of markers are indicated on the right.

mately half of the activity failed to pass through an Amicon 100-kDa cutoff membrane. Two possible explanations for these anomalous findings are (i) the active molecule is self-associating under particular circumstances and/or (ii) the active protein binds to some carrier protein. We have thus far failed to resolve this question. For example, treatment of the SAM with 6 M urea to dissociate complexes led to complete loss of antiproliferative activity. Further purification is continuing in order to isolate this potent antiproliferative protein. However, with the finding that sera from patients with LJP are able to neutralize this antiproliferative activity, we are now using an alternative strategy for isolation, namely, cloning of this protein by differential screening with nonneutralizing and neutralizing sera to identify positive clones.

The present study confirms earlier findings (7) that the SAM from *A. actinomycetemcomitans* is strongly immunogenic, and high titers of antibodies to this extract were found in the blood of patients with LJP. Antibodies in patients' sera reacted with many of the components in the SAM, and no differences in the binding pattern of neutralizing, compared with nonneutralizing, sera could be distinguished. This polyclonal response to the many components in the SAM is presumably why there was no relationship between antibody titer to SAM and neutralizing capacity of the sera. We have previously shown that the sera from a proportion of patients with LJP could block the bone-resorbing activity of the SAM from this organism (8). We now report that, in a similar manner, a proportion of patients with LJP have neutralizing antibodies to the antiproliferative component of SAM. At a dilution of 1:50 9 of the 16 sera could significantly block the antiproliferative action of the surface-associated protein, and at a 1:500 dilution 5 sera were still able to significantly block activity. The remaining sera, and sera from 15 individuals with no evidence of periodontal disease, failed to inhibit the antiproliferative activity. The neutralizing ability of sera could be removed by adsorbing the sera with protein A-bound Sepharose, indicating that the neutralizing capability of the sera was due to antibody.

The role played by high levels of circulating antibodies against periodontopathogenic bacteria is unclear, and the biological function of such antibodies has not been studied in detail. Tsai et al. (21) showed that 90% of sera from patients with juvenile periodontitis neutralized the leukotoxic activity of sonicated extracts of *A. actinomycetemcomitans* whereas most sera from periodontally healthy individuals and patients with adult periodontitis had no such activity. Taichman et al. (18) reported that a bacterial sonicate of *A. actinomycetemcomitans* was capable of inhibiting endothelial cell growth at a concentration of 10 μg/ml. In this study monoclonal antibodies which inhibited the leukotoxin from *A. actinomycetemcomitans* could not neutralize the endothelial cell inhibitor but sera from patients with juvenile periodontitis could.

Two unanswered questions remain to be elucidated: is the in vitro neutralization of the antiproliferative activity of *A. actinomycetemcomitans* repeated in vivo and do patients with the ability to block the antiproliferative activity have a less severe prognosis. These questions are now the focus of active research.

ACKNOWLEDGMENTS

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