BONE-MODULATING PROTEINS FROM *ACTINOBACILLUS ACTINOMYCETEMCOMITANS*: ISOLATION, CHARACTERIZATION AND CLONING

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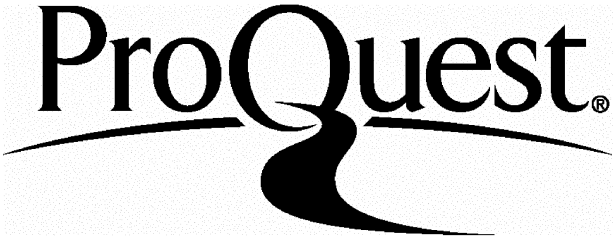

Dedicated to Parisa

None but those who have experienced them can conceive the enticements of science. In other studies you go as far as others have gone before you, and there is nothing more to know; but in scientific pursuit there is continual food for discovery and wonder.....Mary Shelly.

Whose revelation is science? Truth is its deity, proof is its state of grace, doubt is its reason to exist. The holder of many mysteries, beset with shadowy trails that lead to profound dangers, terrifying cliffs, and the deepest pits. Nothing to shed a light and illuminate the dark and murky way. Only ones frail judgement with which to choose the paths to safety.

Abstract

Actinobacillus actinomycetemcomitans is a Gram-negative coccobacillus which is responsible for a number of severe infections, including localized juvenile periodontitis (LJP). Surface-associated material (SAM) from this organism, isolated by a gentle saline extraction, has the capacity to modulate the complex process of bone remodelling. Specifically SAM could inhibit mammalian cell proliferation including the bone cell population known as osteoblasts and could stimulate bone destruction *in vitro.* This investigation was concerned with the isolation, characterization and cloning of these bioactive components.

SAM produced a dose-dependent inhibition of tritiated thymidine incorporation by numerous cell lines in culture, including the osteoblast-like cell line MG63. Characterization of the anti-proliferative activity in the SAM demonstrated that it was not cytotoxic and was heat- and trypsin-sensitive. A purification strategy was developed and the activity was fractionated using ammonium sulphate precipitation, anion exchange and size exclusion HPLC. Analysis of the active peak by SDS-PAGE and silver staining revealed a single protein with a molecular mass of approximately 8kDa. The mechanism by which this protein inhibits thymidine incorporation is unusual. Unlike direct inhibitors of DNA synthesis it has no effect on DNA, RNA or protein synthesis but acts by blocking mammalian cell cycle progression from the G_2 to the M phase. The anti-proliferative protein from *A. actinomycetemcomitans* has been termed *gapstatin.*

IgG antibodies to constituents of the SAM were found in the blood of patients with localized juvenile periodontitis (LJP). Sera from a proportion of patients with LJP significantly neutralized the anti-proliferative activity of the SAM, while control sera, from individuals with no evidence of periodontal disease, were unable to neutralize this activity. In order to clone the anti-proliferative protein, these sera were used to screen a genomic library of *A. actinomycetemcomitans* constructed by ligating *Sau3A*-digested and size-fractionated DNA into *BamH1*-cleaved pUC18. Although three clones expressing antigenic proteins were identified, no anti-proliferative activity was associated with them.

SAM from *A. actinomycetemcomitans* was found to stimulate bone resorption as assessed using the mouse calvarial bone resorption assay. A monoclonal antibody to whole *A. actinomycetemcomitans* was developed and found to inhibit SAM-induced bone resorption. This information has facilitated the isolation and N-terminus sequencing of the bone resorbing protein in the SAM of *A. actinomycetemcomitans*, defining it as member of the heat-shock 60 family of proteins. Attempts were made to clone the gene expressing this protein.

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Symbols and abbreviations

Symbol/abbreviation	Description
α	alpha
ATP	Adenosine triphosphate
β	beta
BSA	Bovine serum albumin
DAB	3,3′-Diaminobenzidine
DMEM	Dulbecco modified eagles medium
DNA	Deoxyribonucleic acid
bp	Base pair
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
FCM	Flow cytometry
FCS	Foetal calf serum
GJP	Generalized juvenile periodontitis
HGF	Human gingival fibroblast
HPLC	High performance liquid chromatography
IL-1	Interleukin-1
IL-6	interleukin-6
IPTG	Isopropyl β-D-thiogalactopyranoside
kb	Kilo base
kDa	Kilo dalton
LB	Luria-Bertani
LAP	Lipid-A associated protein
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LJP	Localized juvenile periodontitis
PAGE	Polyacrylamide-gel-electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leucocytes
SAM	Surface-associated material
SDS	Sodium-doecyl-sulphate
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TCA	Trichloroacetic acid
TEMED	N,N,N,'N'-Tetramethylethylenediamine
TE	Tris EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

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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 Maxillofacial Surgery Research Unit at the Eastman Dental Institute. The electron microscopy work in chapter 3 was performed by Ms. Pauline Barber of the EM Unit at the Eastman Dental Institute. The flow cytometry mentioned in chapter 9 was undertaken in conjunction with Ms. Mona Patel and I am grateful to Arnold Pizzey at the Department of Medical Haematology, University College Hospital, for his help.

Publications as a result of this thesis

Papers

White, PA, Wilson, M, Nair, SP, Kirby, AC, Reddi, K, Henderson, B. Characterization of an anti-proliferative surface-associated protein from *Actinobacillus actinomycetemcomitans* which can be neutralized by sera from a proportion of patients with localized juvenile periodontitis. *Infect. Immun.* (1995);**63**:2612-2618.

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Chapter 1
Introduction

The subject of this thesis is a group of extremely common diseases affecting the periodontium of man and in consequence termed the periodontal diseases. These conditions are believed to be caused by the build-up of certain Gram-negative anaerobic bacteria and the consequent release of bacterial virulence factors. The work described concentrates on one of the periodontopathogenic bacteria, *Actinobacillus actinomycetemcomitans*, and delineates the isolation, characterization and attempts to clone bone-modulating virulence factors which may play a major role in the periodontal pathology induced by this bacterium.

1.1. Structure and biology of the periodontium

The periodontium can be described as the structure which support the tooth, and includes the gingiva, periodontal ligament, cementum and alveolar bone. A brief description of these tissues is required to understand the basis of the work described in this thesis.

1.1.1. Gingiva

The gingiva is the fibrous mucosa which surrounds the teeth and covers the coronal portion of the alveolar process (figure 1.1.). In contrast to the deep red of the alveolar mucosa, the gingiva is pink in colour and is lined by keratinized stratified squamous epithelium. The gingiva is anatomically divided into:

(a) The free gingiva, consisting of the marginal part of the gingiva, which is not attached to the tooth surface. The epithelium of the free gingiva is divided into three types: (i) Oral epithelium, which is continuous with the epithelial lining of the attached gingiva and extends up to the gingival margin but not into the sulcus. (ii) Oral sulcular (crevicular) epithelium, this is continuous with the oral epithelium but is not keratinized and is closely related to the tooth surface but not attached to it. (iii) Junctional epithelium extends from the base of the

crevice to the junction between the tooth and the alveolar bone. Cell division in the junctional epithelium is very rapid, cells migrate coronally from the basal layer into the gingival sulcus in only a few days (Schroeder and Page, 1990).

(b) The attached gingiva extends from the apical border of the free gingiva to the mucogingival junction, which separates the attached gingiva from the alveolar mucosa.

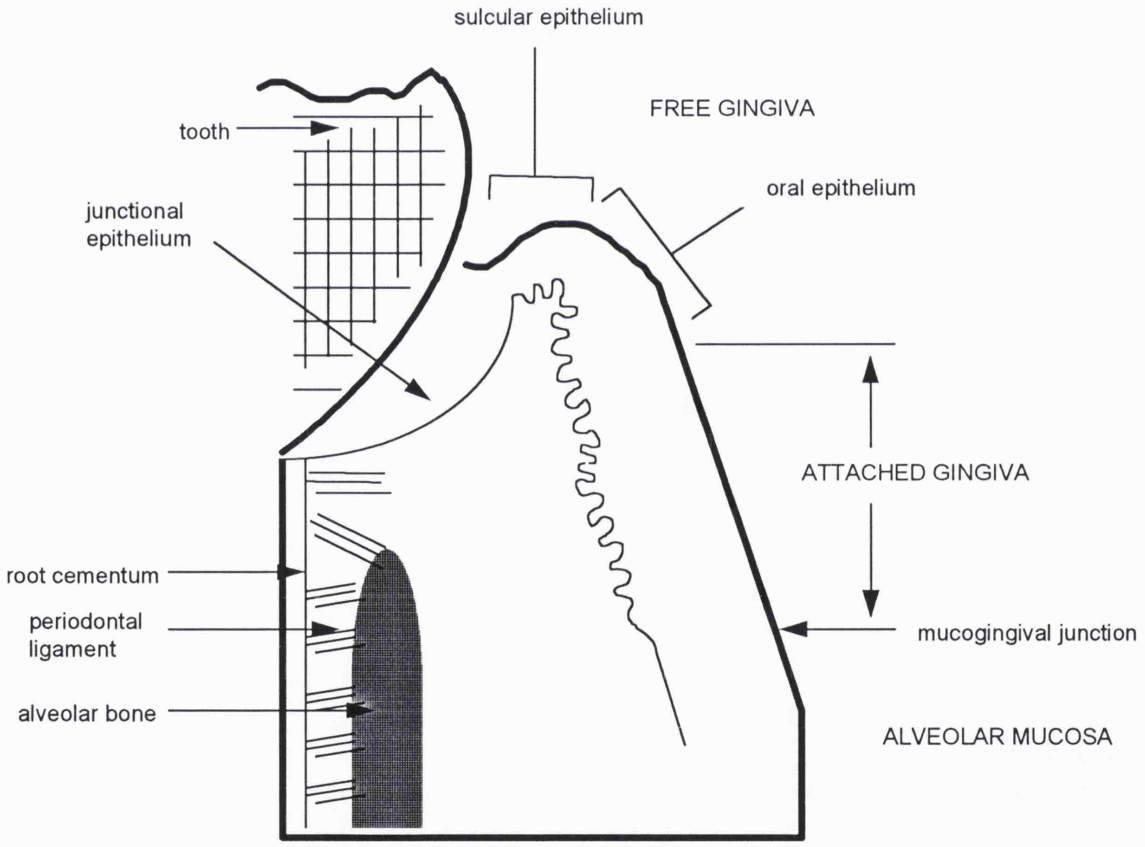


Figure 1.1. The structure of the marginal periodontium.

1.1.2. Connective tissue attachment

The connective tissue fibres of the periodontium provide the connection of the tooth to the alveolar bone as well as providing the support of each tooth to its neighbour. The attached gingiva is bound by dense bundles of collagen fibres which provide the gingiva with resilience and resistance to external forces. These fibres stabilize the position of individual teeth and unite the teeth of each arch into a functional entity.

1.1.3. Periodontal ligament

The periodontal ligament provides the attachment of the tooth to the alveolar bone and consists of collagen fibres between the bone and the tooth, these are made and arranged by fibroblasts. Osteoblasts and cementoblasts are also present on the bone surface and cementum respectively. The primary function of the periodontal ligament is to spread load and support the tooth during mastication. The ligament is metabolically active and capable of considerable remodelling and repair.

1.1.4. Alveolar bone

The alveolar process is the part of the jaw which supports the teeth. It consists of trabecular bone within facial and lingual cortical plates. The tooth socket is a thin plate of bone perforated in many places so that a large number of vascular and neural connections are made between the periodontal ligament and surrounding trabecular spaces. As a tissue there is no difference between alveolar bone and any other bone. It undergoes constant remodelling, and like all bone has two basic functions - to act as a mechanical framework and as a reservoir for inorganic ions. In health the remodelling process maintains the total volume of bone at the same level. Remodelling activity is slightly higher in alveolar bone than in the body of the mandible and is probably accounted for by the functional stress on this tissue.

1.1.5. Cementum

The entire root is covered in a layer of acellular cementum, while only the apical third has a further coating of cellular cementum. The cementum either abuts or overlaps the enamel. Cementum is similar in composition to bone, but is avascular and not innervated.

1.2. Cells of the periodontium

The periodontium is a dynamic structure that must respond continually to the stresses it tolerates. The cells normally present in the soft connective tissues of the periodontium and in the alveolar bone are capable of rapid turnover and replacement following trauma. This section describes the type of cells found in the periodontium and their function.

1.2.1. Cells of the periodontium

All types of blood cells are present, the great majority of them are located within the periodontal vasculature. The full range of leucocytic cells are also observed. Although neutrophils are rarely observed in healthy gingiva, they represent the first line of defence upon invasion of microbes, with the capability of moving towards microbes and engulfing them (phagocytosis), with subsequent killing. Plasma cells and lymphocytes (mostly T cells) are also commonly observed and thought to participate in the host response to minor insults. Other cells present in the healthy periodontium include mast cells, which can release potent vasoactive mediators such as heparin and histamine. Macrophages, which derive from blood monocytes, are common even in non-inflammed gingiva. They are capable of secreting powerful hydrolytic enzymes and their primary role is as scavengers, dispensing with bacteria, debris and toxic substances. If macrophages are eliminated as functioning cells the consequences on wound healing are detrimental (Hassell, 1993).

1.2.2. Fibroblasts

By far the most common cell in the periodontal connective tissue, and probably the most important functionally, is the fibroblast. Sixty five percent of the cells of the gingival connective tissue are fibroblasts (Hassell, 1993). Their function is to synthesize the connective tissue proteins, collagen and elastin, as well as the glycoproteins and glycosaminoglycans that comprise the extracellular matrix (ECM) of the periodontal ligament. Fibroblasts also secrete a family of matrix metalloproteinases, including collagenase which have the capacity to degrade the components of the extracellular matrix. Fibroblasts regulate the constitution and condition of the gingiva and maintain tissue integrity. When stimulated they can also synthesize a range of cytokines and lipid mediators.

1.2.3. Bone cells

Serving as a mechanical support and as a reservoir for calcium and phosphate, bone is continually remodelling to maintain a dynamic equilibrium. Remodelling is controlled by the opposite actions of the two main bone cell populations - the osteoblast and the osteoclast.

1.2.3.1. The osteoblast and bone formation

The osteoblast is the bone lining cell responsible for the production of the extracellular matrix of bone which consists mainly of collagen. It originates from a local mesenchymal stem cell (bone marrow stromal stem cell or connective tissue mesenchymal stem cell). These precursors, with the right stimulation, undergo proliferation and differentiate into pre-osteoblasts and then mature into osteoblasts. Osteoblasts never appear or function individually, but are always found in clusters of cuboid cells along the bone surface (100-400 cells per bone-forming site). Osteoblasts are normally found lining a layer of bone matrix that they are producing which is not yet calcified (osteoid tissue). The presence of the osteoid is due to a time lag between matrix formation and its subsequent calcification. Behind the osteoblast are usually found one or two

layers of cells, which consist of activated mesenchymal cells and pre-osteoblasts. Towards the end of the secreting period the osteoblast will become either a flat lining cell or an osteocyte.

1.2.3.2. The osteoclast and bone resorption

The osteoclast is a bone lining cell responsible for bone resorption. It is a giant multinucleated cell (containing 4-20 nuclei) usually found in contact with a calcified bone surface and within a pit (or lacuna) which is the result of its own resorptive activity. It is possible to find up to five osteoclasts in the resorptive site, although the norm is one or two. The most prominent feature of the osteoclast is the existence of deep foldings of the plasma membrane in the area facing the bone matrix and known as the ruffled border. Osteoclasts are derived from the same myeloid precursor cell as gives rise to monocytes.

Lysosomal enzymes are actively synthesized by the osteoclast and are secreted at the ruffled border along with non-lysosomal enzymes such as collagenase. The osteoclast seals off an area of bone, acidifies the extracellular area by secreting protons across the ruffled border membrane. Protons are supplied by the enzyme carbonic anhydrase. The extracellular bone resorbing compartment has the following features: i) a low pH, ii) the presence of lysosomal enzymes, and iii) the substrate. The low pH dissolves the hydroxyapatite crystals which give bone is rigidity and strength, exposing the matrix for subsequent degradation by enzymes.

1.2.3.3. Bone remodelling

Bone remodelling is a mechanism for keeping the skeleton "young" by a process of removal of old bone and replacement with new bone. In the process of bone remodelling, almost all osteoclastic activity is followed by osteoblastic activity. Excavated resorption lacunae are filled by waves of bone formation. That this sequence is locally regulated in a spatial and temporal way provides

one of the key observations in determining the mechanisms of the coupling of resorption/formation. The current belief is that osteoclasts release sequestered growth factors during the process of resorption. The factors then diffuse a short distance and stimulate mesenchymal cells to proliferate and ultimately differentiate into preosteoblasts and osteoblasts. These cells then reform the bone not only replacing the collagen and mineral but also the growth factors. This is one of the mechanisms by which the skeleton attempts to preserve its bone mass. Obviously interference with the resorption/formation process can lead to inappropriate regulation of bone mass. Thus factors which inhibit the proliferation of mesenchymal cells and osteoblasts following growth stimulation by local factors during bone remodelling, will result in reduced bone formation and ultimately could lead to bone loss. Most diseases of the skeleton are due to an imbalance between bone breakdown and formation, resulting in systemic or local bone loss. These include: osteoporosis, where resorption is normally increased and formation does not keep up with it; hyperparathyroidism, also associated with increased bone turnover; hypercalcemia of malignancy, where bone resorption increases to the point of elevating calcium in the blood; Paget's disease, which causes localized bone loss; metastatic disease in bone which produces osteolytic lesions; periarticular bone loss in rheumatoid arthritis; bone loss in immobilization due to the absence of mechanical stimuli, which maintain bone structure; bone loss associated with glucocorticoid treatment, which reduces bone formation; and, of course, local destruction of alveolar bone in periodontal disease.

1.3. Periodontal diseases

The periodontal diseases are the most prevalent of the chronic inflammatory, tissue destructive diseases of *Homo sapiens* (Liver *et al.*, 1991). No race is immune and the disease presents a worldwide health problem. The disease process may be restricted to the gingivae or may involve deeper periodontal structures. Inflammation of the gingivae is described as gingivitis, whilst

disease of the deeper periodontal tissues is termed periodontitis. Periodontal diseases are almost universally considered to be the result of microbial infection often resulting in an inflammatory response. The pathology of the periodontal tissues are subject to numerous forms of classification. However, the hallmark in all types of this disease, is soft tissue destruction and alveolar bone loss.

1.3.1. Juvenile periodontitis

One form of periodontal disease, juvenile periodontitis, is an uncommon, rapidly destructive, disease of the adolescent periodontium. Unlike other forms of periodontal disease, juvenile periodontitis is not related to poor oral hygiene. Often the disease goes undetected, as inflammation of the gingiva is not always apparent (Schluger *et al.*, 1990). The condition is usually brought to the patient's attention by the development of tooth mobility or migration, or the occurrence of a periodontal abscess - all signs of advanced destruction. Initially, the first permanent molars and/or permanent incisors are affected and is probably a reflection of their earlier eruption times and longer exposure to the oral environment. As the disease progresses it usually exhibits a roughly symmetrical pattern of tooth involvement. Ultimately the appearance may be that of generalized advanced attachment loss. Localised juvenile periodontitis (LJP) is used to describe juvenile periodontitis when only the incisors and first permanent molars are affected. Generalized juvenile periodontitis (GJP) describes the disease when it is more widespread. The two diseases share similar microbiological and host response characteristics. LJP is at least four times commoner than GJP. In Caucasians, the prevalence of juvenile periodontitis is roughly 0.13% compared to a prevalence rate of about 1-3% in ethnic Negroid populations (Loe and Brown, 1991).

1.4. *Actinobacillus actinomycetemcomitans*

1.4.1. Morphology and physiology

Actinobacillus actinomycetemcomitans is a Gram-negative, non-spore forming, non-motile, facultatively anaerobic coccobacillus. It was first described by the German microbiologist Klinger in 1912. At first it was called *Bacterium actinomycetum comitans,* which was changed to *Bacterium comitans* in 1921 and finally to *Actinobacillus actinomycetemcomitans* (Topley and Wilson, 1929). Apart from a brief spell in the late eighties when the organism was called *Haemophilus actinomycetemcomitans*, the name *Actinobacillus actinomycetemcomitans* has remained constant. *"Actinobacillus"* refers to the star shaped morphology observed when the organism is cultured on solid media and to the short rod shape of individual cells. *"Actinomycetemcomitans"* reflects its close association with *Actinomyces israelii* in actinomycotic lesions. When freshly isolated, colonies are small (0.1mm), convex, grey, translucent, smooth and non-haemolytic. The cells are straight or curved short rods with rounded ends (1.0-1.5μm by 0.4-0.5μm). Whilst *A. actinomycetemcomitans* grows well in 5% CO_2 or anaerobically, it grows poorly in air (Slots, 1982). Five different serotypes of *A. actinomycetemcomitans* have been identified (Saarela *et al.*, 1992), a,b,c,d and e.

1.4.2. *A. actinomycetemcomitans* is associated with localised juvenile periodontitis

Research over the past two decades has strongly implicated *A. actinomycetemcomitans* in the aetiology of localised juvenile periodontitis (Slots *et al.*, 1980; Hammond and Stevens, 1982; Slots *et al.*, 1982; Slots and Genco, 1984; Zambon, 1985; Moore, 1987; Zambon, 1988). Evidence for this association includes: (i) Studies have demonstrated that almost all LJP lesions contain *A. actinomycetemcomitans.* For example Zambon *et al.*, 1983, detected *A. actinomycetemcomitans* in 97% of LJP patients, whilst, Christersson reported that 95% of LJP patients harboured *A.*

actinomycetemcomitans (Christersson, 1993); (ii) patients with LJP develop high levels of serum, salivary and crevicular fluid antibodies against *A. actinomycetemcomitans* when compared to disease-free individuals (Ebersole *et al.*, 1987; Gunsolley *et al.* 1987; Ebersole *et al.*,1991; Zafiropoulos *et al.*, 1992; Saito *et al.*, 1993; Ebersole and Cappelli, 1994; Mooney and Kinane, 1994); (iii) *A. actinomycetemcomitans* produces a whole array of virulence factors which are likely to be involved in the pathogenesis of periodontitis (discussed in section 1.5.); (iv) periodontal treatment which results in the elimination of *A. actinomycetemcomitans* from subgingival sites, can be correlated with clinical improvement (Christersson *et al.*, 1983; Slots and Rosling, 1983). Although there is evidence for the role of *A. actinomycetemcomitans* in LJP, much of it is indirect. *A. actinomycetemcomitans* is also found commonly in individuals with no signs of periodontal disease and may not be the only cause of LJP. Indeed, other oral bacteria could also be involved in this disease. Alternatively, the disease could also be associated with a genetic mutation which makes certain people more susceptible to LJP. There are numerous theories to the causes of LJP, however as yet none have been proved. One particular study of interest by Haubek *et al.* (1995) showed that there was no association between any subpopulation of *A. actinomycetemcomitans* and the periodontal health status from the subject from whom the isolates originated. This suggests that the role of *A. actinomycetemcomitans* was largely opportunistic in the population examined.

1.4.3. Other diseases associated with *A. actinomycetemcomitans*

More recently, there is increasing evidence that *A. actinomycetemcomitans* is implicated in the aetiology of other forms of periodontal disease including adult periodontitis (Mombelli *et al.*, 1994a; Mombelli *et al.*, 1994b; Zambon, 1994). As a pathogen *A. actinomycetemcomitans* not only infects oral sites but has been isolated from extra-oral sites. There are many reported cases of endocarditis due to *A. actinomycetemcomitans* (Page and King, 1966; Pierce

et al.,1984; Gould *et al.*, 1985). This micro-organism can also cause thyroid gland abscesses (Burgher *et al.*, 1973), urinary tract infections (Townsend and Gillenwater, 1969), brain abscesses (Garner, 1979), and vertebral osteomyelitis (Muhle *et al.*, 1979).

1.4.4. Surface associated material from *A. actinomycetemcomitans*

There are few reports that *A. actinomycetemcomitans* invades host tissues, the considered opinion is that soluble virulence factors released by this organism are the more likely cause of tissue damage. Work at the Eastman has therefore concentrated on the easily solubilised material of *A. actinomycetemcomitans*. In this thesis the term surface-associated material (SAM) will be used to describe those components of the organism removed by a saline extraction at 4°C. SAM contains loosely adherent material from the bacterial cell surface and includes proteins, fimbriae, capsule and polysaccharides (figure 1.2.).

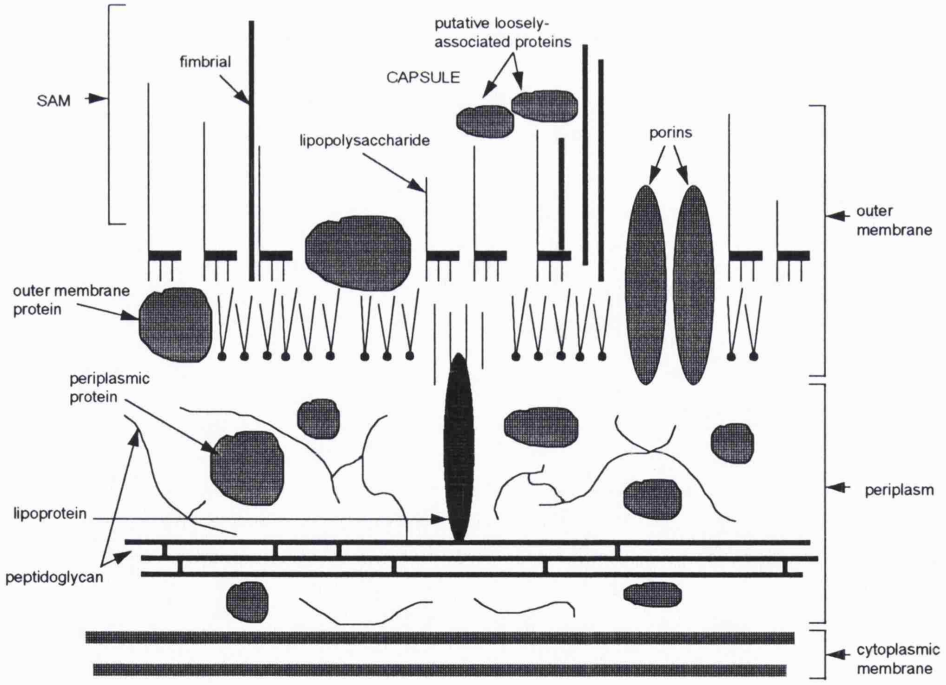


Figure 1.2. Diagram of a cell wall of a Gram-negative bacterium.

1.5. Virulence factors of *A. actinomycetemcomitans*

Now that *A. actinomycetemcomitans* has been recognised as a major periodontopathogenic organism, a relatively large amount of research has been undertaken in an attempt to identify and characterize those attributes of the organism which enable it to colonise the oral cavity, invade the periodontal tissues, overcome the host defences and induce tissue destruction (reviewed by Wilson and Henderson, 1995).

1.5.1. Adherence

The first stage of any infectious process involves adhesion of the micro-organism to host tissue. Recently, there have been a number of studies investigating the adherence of *A. actinomycetemcomitans* to epithelial cells. Mintz and Fives-Taylor (1994) have proposed the involvement of surface proteins in the adherence of *A. actinomycetemcomitans* as, following trypsin treatment, the number of bacteria able to adhere to epithelial cells was reduced by 50%. Meyer and Fives-Taylor (1994) demonstrated that proteinaceous extracellular material from one strain of this organism could increase the adherence of other weakly-adherent strains. Not surprisingly, the presence of fimbriae has been related to the adherence of *A. actinomycetemcomitans*. A fimbriated strain has been shown to have a greater ability to adhere to a mouse epithelial cell line than a non-fimbriated strain (Rosen *et al.*, 1988a). Other studies have proposed that the adherence of morphologically rough variants was associated with fimbriation, whereas nonfimbrial components, such as extracellular material, were probably involved in adherence of smooth strains (Meyer and Fives-Taylor 1993; Fives-Taylor *et al.*, 1995). Rosen *et al.* (1988b) also demonstrated that fimbriated clinical isolates exhibited greater ability to adhere to saliva-coated hydroxyapatite than non-fimbriated variants.

1.5.2. Invasion

Many bacteria use adhesins to attach to specific receptors on mucosal surfaces

during the early stage of infection. The epithelial cells on these surfaces are formidable barriers to further infection. Invasion through this barrier could provide a whole array of advantages to a bacterium; for example, protection from host defences and a nutritionally rich environment free of competing organisms. There is increasing evidence that *A. actinomycetemcomitans* can invade the tissues of the periodontium. Two studies have identified *A. actinomycetemcomitans* intracellularly using immunofluorescence (Saglie *et al.*, 1982; Christersson *et al.*, 1987). Furthermore, penetration of cultured epithelial cells and gingival fibroblasts by *A. actinomycetemcomitans* has now been clearly demonstrated (Meyer *et al.*, 1991; Sreenivasan *et al.*, 1993; Fives-Taylor *et al.*,1995).

1.5.3. Host defence interactions

A. actinomycetemcomitans has a considerable armoury with which to evade the host defence systems. Not only can this organism inhibit polymorphonuclear leucocyte (PMN) chemotaxis, (Van Dyke *et al.*, 1992; Ashkenazi *et al.*, 1992a) but it can inhibit the production of antibacterial agents by PMN (Ashkenazi *et al.*, 1992b). *A. actinomycetemcomitans* is also resistant to PMN-mediated killing; Sela (1983) showed that extracts of peripheral blood leucocytes were only partially successful at lysing *A. actinomycetemcomitans*, whilst Miyasaki *et al.* (1984) demonstrated its inherent resistance to H_2O_2. *A. actinomycetemcomitans* has also been shown to be resistant to antibacterial factors within neutrophil granules (Miyasaki *et al.*, 1990; Miyasaki *et al.*, 1991). Recently, *A. actinomycetemcomitans* has been shown to induce apoptosis in the murine macrophage cell line J774.1 (Kato *et al.*, 1995).

Phagocytosis of antibody-coated bacteria is an important mechanism for eliminating infecting bacteria and involves binding of the Fc region of the antibody to specific PMN receptors. Two reports demonstrate that *A. actinomycetemcomitans* expresses an Fc binding protein (Tolo and Helgeland, 1991; Mintz and Fives-Taylor, 1994), the latter report showed that this protein

had significant homology to the outer membrane protein A (OmpA) of *Escherichia coli* and other related OMP-like proteins from Gram-negative bacteria. The Fc binding protein could interfere with the phagocytic activity of myelomonocytic cells and block complement activation. Another report by Gregory *et al.* (1992) demonstrated that the supernatant from cultured *A. actinomycetemcomitans* could degrade immunoglobulins, and this could ultimately interfere with phagocytosis by myelomonocytic cells.

Of the numerous virulence factors of *A. actinomycetemcomitans* the leucotoxin has been the most widely studied. In 1979 Baehni and coworkers first showed that *A. actinomycetemcomitans* was cytotoxic for human PMNs. The purified toxin has a molecular mass of 115kDa (Tsai *et al.*, 1984) and destroys targets by osmotic lysis (Taichman *et al.*, 1991). Susceptible cells include human PMNs and peripheral blood monocytes (Taichman *et al.*, 1991), promyelocytic HL-60 cells (Zambon *et al.*, 1983), the monocytic cell line U937 and human T- and B-cell lines (Simpson *et al.*, 1988). Cells not susceptible include fibroblasts and human lymphocytes (Tsai *et al.*, 1984). Cloning and sequencing of the leucotoxin gene has since revealed that it exists in an operon structure which is similar to that of the *E. coli* hemolysin operon, consisting of four genes (Lally *et al.*, 1989; Kraig *et al.*, 1990). Non-leucotoxic strains (such as *A. actinomycetemcomitans* NCTC 9710, used in this study) appear to have a weak promoter (Brogan *et al.*,1994), thus resulting in lower expression of the toxin.

A. actinomycetemcomitans has been shown to exert an immunosuppressive effect *in vivo* by down-regulating serum IgG levels (Chen *et al.*, 1991) and by inhibiting T cell-dependent antibody responses (Kurita-Ochiai *et al.*, 1992). It has also been reported that patients with early on-set forms of periodontitis demonstrate low helper ($CD4^+$)-to-suppressor ($CD8^+$) T-cell ratios (Kinane *et al.*, 1989). Shenker *et al.*, (1990) isolated an immunosuppressive factor, with a molecular mass of 60kDa, capable of suppressing mitogen- and antigen-

induced DNA, RNA and protein synthesis as well as IgG and IgM synthesis in human T lymphocytes. The authors proposed that this molecule functions via the activation of a regulatory subpopulation of B lymphocytes, which in turn down-regulates both B- and T- cell responsiveness. In another study Rabie *et al.* (1988) demonstrated that the leucotoxin from *A. actinomycetemcomitans* was capable of impairing lymphocyte responsiveness.

1.5.4. Bone destruction

The major problem in LJP is the rapid loss of alveolar bone which supports the tooth (Aass *et al.*, 1992) (figure 1.3.). Studies using animal models orally inoculated with *A. actinomycetemcomitans* have confirmed the osteolytic potential of this organism (Taubman *et al.*, 1983; Wray and Grahame, 1992). Furthermore, the bone resorbing activity of *A. actinomycetemcomitans* lipopolysaccharide (LPS) has been demonstrated using an *in vitro* model (Kiley and Holt, 1980). Although, the lipid A-associated protein (LAP) which can be co-extracted with LPS, has been shown to be more potent than LPS, exhibiting activity at a concentration of just 10ng/ml (Reddi *et al.*, 1995c). Nowotny *et al.*, (1982) have identified a protease-sensitive osteolytic factor in the microvesicles of the organism active at 1μg/ml. There remains no doubt that this organism can induce bone resorption, however the osteolytic molecules responsible have, for the most part, been poorly defined. Work over several years at the Eastman Dental Institute has attempted to identify the osteolytic mechanism. In 1985, Wilson *et al.* demonstrated that a saline extract of *A. actinomycetemcomitans* containing surface-associated material (SAM) could stimulate bone resorption at concentration as low as 1ng/ml. Meghji *et al.*, (1994) showed that the osteolytic activity of the SAM from *A. actinomycetemcomitans* was heat- and trypsin-sensitive and was not due to LPS contamination. Moreover, the osteolytic activity did not appear to be mediated by prostanoids, or the pro-inflammatory cytokines interleukin 1 (IL-1) and tumour necrosis factor α (TNFα).

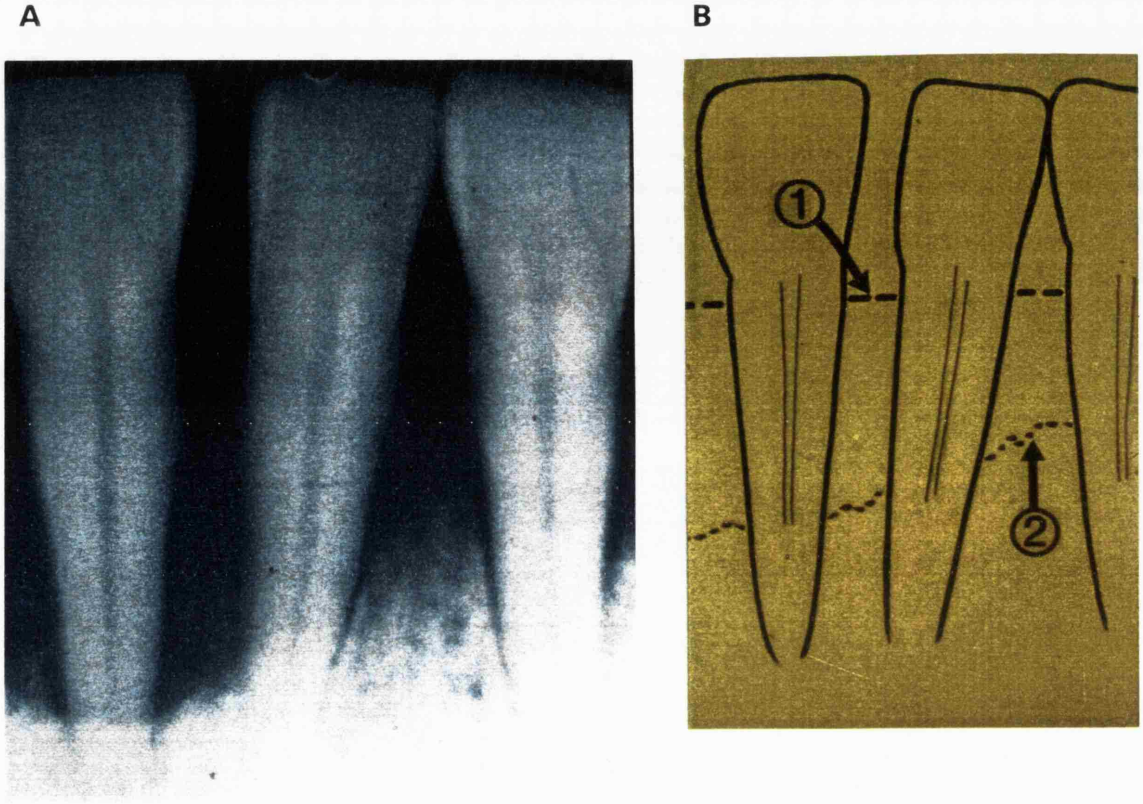


Figure 1.3. Panel A, a radiograph of a patient with LJP, showing extensive resorption of the alveolar bone. Panel B, shows the depth of the bone resorption (line 2), line 1 shows the position of the bone in a healthy individual.

1.5.5. Collagenase activity

The extracellular organic matrix of bone consists predominantly of type I collagen. In LJP the density of gingival collagen fibres are reduced, this could be accounted for by any collagenase activity of *A. actinomycetemcomitans*. Robertson *et al.*, (1982) demonstrated the ability of *A. actinomycetemcomitans* to degrade collagen, whilst, Suomalainen *et al.*, (1991) showed that 10 strains of *A. actinomycetemcomitans*, freshly isolated from the subgingival plaque of juvenile periodontitis patients, caused release of collagenase from PMNs *in vitro*.

1.5.6. Release of Inflammatory mediators

When discussing periodontal disease, often one hears the argument that it is not the bacteria which cause direct destruction of the host tissues, rather, it is an indirect effect initiated by bacterial virulence factors but exerted by the hosts own molecules. Of primary interest in this respect are the cytokines. Cytokines such as IL-1β, IL-6 and TNFα can all stimulate bone resorption (Bertolini *et al.*, 1986; Gowen and Mundy, 1986; Bataille and Klein, 1991). Whilst IL-1β and TNFα can stimulate the release of proteases and the prostanoid PGE_2 (itself a potent stimulator of bone resorption (Klein and Raisz, 1970) from fibroblasts (Mizel *et al.*, 1981; Dayer *et al.*, 1985; Elias *et al.*, 1987). See table 1.1. for cytokine stimulating activity of *A. actinomycetemcomitans*.

Table 1.1. The cytokine stimulating ability of *A. actinomycetemcomitans*.

Reference	date	bacterial component	cell type	cytokine or prostanoid
Lindemann & Economou	1988	whole cells	human monocytes	IL-1β TNFα
Lindemann *et al.*	1988	LPS	human monocytes	IL-1β TNFα
Ohmori *et al.*	1988	SE	murine macrophage	IL-1
McFarlane *et al.*	1990	LPS	human monocytes	IL-1β
Saglie *et al.*	1990	LPS	human macrophages	IL-1β TNFα
Garrison *et al.*	1991	LPS	human monocytes	PGE_2
Takahashi *et al.*	1991	SPA	murine macrophages	IL-1
Kjeldsen *et al.*	1995	whole cells	human monocytes	IL-1α TNFα IL-6
Reddi *et al.*	1995c	SAM	human monocytes	IL-1β TNFα IL-6
Reddi *et al.*	1995b	LAP	human monocytes	IL-1β TNFα
Reddi *et al.*	1995b	SAM	HGF	IL-6 IL-8
Reddi *et al.*	1995b	LAP	HGF	IL-6

HGF: human gingival fibroblasts
LAP: lipid-A associated protein
LPS: lipopolysaccharide
SAM: surface-associated material
SE: sonicated extract
SPA: serotype-specific polysaccharide antigens

1.5.7. Anti-proliferative activity

In 1982 Shenker *et al.*, first described the ability of *A. actinomycetemcomitans* to inhibit the proliferation of mammalian cells. Sonic extracts of *A. actinomycetemcomitans* were shown to cause a dose dependent inhibition of proliferation in both human foreskin fibroblasts and the murine fibroblast cell line L929, as assessed by measuring [^{3}H]-thymidine incorporation. The sonic extract demonstrated an IC_{50} value (concentration at which [^{3}H]-thymidine incorporation is inhibited by 50%) of approximately 10μg/ml. The active factor was heat labile, had a molecular mass of approximately 150kDa and was not cytotoxic. Later studies demonstrated the ability of sonicated *A. actinomycetemcomitans* to inhibit the proliferation of human keratinocytes (Kamen, 1983), human gingival fibroblasts (HGF) (Stevens *et al.*, 1983) and human endothelial cells (Taichman *et al.*, 1984) with IC_{50} values between 1 and 10μg/ml. In all three studies the sonic extract was inactivated when heated, was not cytotoxic to the cells, and caused an inhibition of proliferation when exposed to the cells for less than 30 min.

Studies at the Eastman Dental Institute of the anti-proliferative activity of *A. actinomycetemcomitans* have utilized the loosely adherent surface-associated material which can be easily removed by extracting the bacteria in saline. In 1986, Kamin *et al.* showed that SAM from *A. actinomycetemcomitans* inhibited the proliferation and collagen synthesis of HGF with an IC_{50} value of just 1μg/ml. The SAM was shown not to be cytotoxic and was approximately 100 times more active than the LPS from this organism. Interestingly this was the first study to report that there was an increased nuclear area in cells, following their inhibition of proliferation by *A. actinomycetemcomitans*. Later studies showed the SAM could inhibit the proliferation and collagen synthesizing activity of mouse calvarial osteoblasts (Meghji *et al.*, 1992a), and inhibit the proliferation of the human monocytic cell line U937, guinea pig keratinocytes and HGF (Meghji *et al.*, 1992b).

Helgeland and Nordby (1993), described the presence of a cell cycle-specific inhibitory activity in culture media supporting *A. actinomycetemcomitans.* In this study the authors found that cells accumulated in the G_2 phase of the cell cycle (the gap of time after DNA synthesis and before cell division), and the cells exhibited sheetlike protrusions and micronuclei. The activity was associated with an uncharacterized component of approximate molecular mass 50kDa. Kataoka *et al.*, (1992) reported the purification of a fibroblast inhibiting factor with an IC_{50} value of 0.15μg/ml and a molecular mass of 65kDa. See table 1.2. for anti-proliferative activity of *A. actinomycetemcomitans*.

Table 1.2. The anti-proliferative activity of *A. actinomycetemcomitans*.

Reference	Year	Extract	Cell type tested	IC_{50} (μg/ml)	Temp of inactivation	Minimum Exposure	Cyto-toxic	MW of activity
Shenker *et al.*	1982	SE	fibroblasts	10	75°C	3 h	No	150kDa
Kamen	1983	SE	keratinocytes	1-10	90°C	10 min	No	-
Stevens *et al.*	1983	SE	fibroblasts	2	80°C	10 min	No	>10kDa
Taichman *et al.*	1984	SE	endothelial	10	100°C	30 min	No	-
Kamin *et al.*	1986	SAM	fibroblasts	1	-	-	No	-
Kataoka *et al.*	1992	FIF	fibroblasts	0.15	-	-	No	65kDa
Meghji *et al.*	1992b	SAM	fibroblasts	1	-	-	No	-
			keratinocytes	1	-	-	No	-
			U937	0.30	-	-	No	-
Meghji *et al.*	1992a	SAM	osteoblasts	1	-	-	No	-
Helgeland	1993	CS	fibroblasts	10	-	-	No	50kDa

CS: culture supernatant
SE: sonic extract
FIF: fibroblast inhibitory factor
SAM: surface-associated material

The implications of an anti-proliferative activity in the pathogenesis of periodontal disease have as yet been poorly addressed. In this thesis the hypothesis proposed is that by inhibiting the proliferation of connective tissue cells and bone cells, tissue replacement would be greatly impaired. This could in itself lead to alveolar bone loss and periodontal tissue destruction, however, in combination with destructive processes caused by other bacterial virulence factors, anti-proliferative activity could greatly enhance the tissue damage observed in periodontal disease.

1.6. Aims of thesis

In periodontal disease elimination of the infecting bacteria is often difficult to achieve. To accomplish 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 hosts response to them. Only with this information can the successful treatment of this disease be realised.

The aims of this thesis were therefore:

(i) To extract and characterize the surface-associated material from *A. actinomycetemcomitans* when cultured in liquid and solid media.

(ii) To assess two bone-modulating activities of the SAM from *A. actinomycetemcomitans*, the first being the anti-proliferative activity and the second the osteolytic potential of this material. The anti-proliferative activity of *A. actinomycetemcomitans* was compared to the anti-proliferative activity of SAMs from two other periodontopathogenic bacteria, namely *Por. gingivalis* and *Eik. corrodens* using two key cell populations, the osteoblast and the gingival fibroblast. Furthermore the ability of the SAM from *A. actinomycetemcomitans* to inhibit the proliferation of other cell types was examined.

(iii) To isolate and clone the osteolytic component from the SAM of *A. actinomycetemcomitans*.

(iv) To test the ability of the sera from patients with LJP to neutralize the anti-proliferative component of the SAM and subsequently to use this sera to screen a genomic library of *A. actinomycetemcomitans* in order to clone surface antigens from this organism.

(v) To isolate, characterize and elucidate the mechanism of the anti-proliferative component of the SAM of *A. actinomycetemcomitans*.

Chapter 2
Materials and Methods

2.1. Bacterial strains and growth

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% (v/v) horse blood. *A. actinomycetemcomitans* clinical isolates 286 and 670, representing serotypes a and b respectively, were kindly donated by Maria Saarela, University of Helsinki (Saarela *et al.*, 1992). In some experiments *A. actinomycetemcomitans* NCTC 9710 was cultured in liquid media under anaerobic conditions in 0.5 litres of brain-heart infusion broth for 48h at 37°C. *Porphyromonas gingivalis* W50 and *Eikenella corrodens* NCTC 10596 were grown at 37°C under anaerobic conditions on Wilkins-Chalgren (Oxoid) agar plates containing 5% (v/v) horse blood. Bacteria were grown for 48h, harvested using saline, centrifuged at 3000 *g* for 20min and the pellet stored at -70°C.

Escherichia coli JM109 and *E. coli* JM105 were used for all cloning studies and were cultured on Luria-Bertani (LB) medium. When solid medium was needed, 1.2% w/v agar was added.

2.2. Extraction of bacterial components

2.2.1. Extraction of SAM

SAM was extracted by a modification of the method of Wilson *et al.* (1985). Briefly, bacteria were thawed on ice, gently stirred in 0.15M saline for 1 h at 4°C and centrifuged at 3,000 *g*. The saline extraction was repeated and the combined supernatant dialysed using benzoylated dialysis tubing with a 2kDa cut-off (Sigma) against distilled water and lyophilized.

2.2.2. Extraction of cell lysate from *A. actinomycetemcomitans*

Cells devoid of their surface-associated material were suspended in PBS and shaken with Ballotini beads (grade 12) for 20 min in a Mickle tissue disintegrator at 4°C. Bacterial debris was removed by centrifugation at 37,000 *g* for 15 min, the supernatants were dialysed against distilled water and lyophilized.

2.2.3. Extraction of lipopolysaccharide

LPS from *A. actinomycetemcomitans*, kindly donated by Dr. Kris Reddi, Eastman Dental Institute, was extracted by the method of Westphal and Jann (1965).

2.3. Electron microscopy

Cells were stained and examined by transmission electron microscopy before and after saline extraction. Electron microscopy was kindly performed by Mrs Pauline Barber, Eastman Dental Institute. A portion of each of the two specimens was fixed in 3% glutaraldehyde in 0.1M 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 contained 0.15% ruthenium red. All specimens were then centrifuged at low speed, the fixative was decanted, and the pellets washed in 0.1M cacodylate buffer. The control cells were then fixed in 1% osmium tetroxide in 0.1M 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% ruthenium red. The fixative was decanted after centrifugation and the pellets washed in 0.1M 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 in a JEOL 100CXII electron microscope.

2.4. Composition of SAM

The protein concentration of the SAM was determined by the Bio-Rad protein assay (Bio-Rad, Richmond, USA) with bovine serum albumin as the standard. The carbohydrate content was determined by the method of Dubois *et al.*, (1956), with glucose as a standard and the content of LPS determined by a commercial chromogenic *Limulus* amoebocyte assay (Pyrogent, Byk-Mallinckrodt, UK). The lipid content of the SAM was estimated by extracting in methanol:chloroform (2:1) to isolate lipidic material, lyophilizing the extract and weighing.

2.5. Polyacrylamide gel electrophoresis

2.5.1. Reagents used for SDS-PAGE

SDS	10% solution
Stacking gel buffer	0.5M Tris-HCl pH 6.8, stored at 4°C
Resolving gel buffer	1.5M Tris-HCl pH 8.8, stored at 4°C
Electrophoresis buffer (10x)	0.25M Tris, 1.92M Glycine, 1% SDS, pH 8.3
30% Acrylamide	29.2% Acrylamide, 0.8% Bis-acrylamide
Ammonium persulphate (APS)	10% solution, made fresh

Polymerization of the gels was induced by the addition of 10μl TEMED and 100μl of APS. The final composition of the gel was as follows:

Upper stacking gel	4.8% Acrylamide
	0.12M Tris-HCl, pH 6.8
	0.1% SDS
Lower resolving gel	7.5 - 15% Acrylamide, depending on pore size
	0.36M Tris-HCl, pH 8.8
	0.1% SDS

Samples were suspended in buffer (0.06M Tris, 10% glycerol, 1% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue, pH6.8) and boiled for 4min. SDS-binding imparts a strong negative charge to the protein, dominating its native charge. Thus, the charge to mass ratio becomes constant for most proteins and under an electric field the proteins separate according to their molecular mass.

2.5.2. Resolution of proteins by SDS-PAGE

The method of Laemmli (1970) was used for SDS-PAGE. This is a discontinuous system consisting of two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. Proteins are concentrated in the stacking gel and separated in the resolving gel. When the power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary is formed with the mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localized high voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack). On reaching the resolving gel, the glycinate ions become fully negatively charged and overtake the proteins, allowing the SDS-polypeptide complexes to separate on the basis of their molecular mass.

Samples were then separated at 30mA for the stacking gel and 15mA for the resolving gel, using a Bio-Rad mini-Protean II system. Electrophoresis was continued until the tracking dye (bromophenol blue) reached approximately 0.5cm from the bottom of the gel.

In some experiments commercial, pre-made 5-20% gradient (Bio-Rad) gels were used, samples being separated using a current of 30mA.

2.5.3. Reagents used for two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis is a technique that separates proteins initially, in the first dimension, according to their isoelectric point and in the second dimension according to their molecular mass (O'Farrell, 1975). This procedure was carried out using a Bio-Rad mini-Protean II system.

Reagents used for the first dimension

Triton X-100	10% solution
Upper chamber buffer	20mM NaOH, degassed
Lower chamber buffer	10mM H_3PO_4, degassed
Ammonium persulphate (APS)	10% solution, made fresh

Polymerization of the gels was induced by the addition of 10μl TEMED and 10μl of APS. The final composition of the gel was as follows:

First dimension gel	9.2M urea
	4% Acrylamide
	2% Triton X-100
	2.5% 5-7 ampholyte
	2.5% 3-10 ampholyte

The gel solution was degassed thoroughly for 15mins before polymerization. Samples were suspended in an equal volume of sample buffer (9.5M urea, 2% Triton X-100, 5% 2-mercaptoethanol, 1.6% 5-7 ampholyte, 0.4% 3-10 ampholyte) and incubated at room temperature for 15min.

2.5.4. Resolution of proteins by 2D-SDS-PAGE

The tube gels were equilibrated with 5μl of sample buffer for 15 min, then pre-electrophoresed by running the 2D cell at 200 V for 10min, 300 V for 15min and 400 V for 15min. The upper and lower chamber buffers were replaced and the pre-electrophoresis solutions removed from the tube gels. Samples were loaded carefully and overlaid with 20-40μl sample overlay buffer (9.0M urea, 0.8% 5-7 ampholyte, 0.2% 3-10 ampholyte, 0.05% bromophenol blue). The 2D cell was then run at 500 V for 10min, followed by 750 V for 3.5 h. Gels were carefully removed, equilibrated in SDS sample buffer and separated in the second dimension as described previously.

2.5.5. Gel staining

Gels were stained with colloidal Coomassie blue (Sigma) or silver-stained using a commercial kit (Pierce). Molecular weight markers ranging from 14kDa to 66kDa (Sigma) were run on all gels.

2.6. Cell and tissue culture

2.6.1. Maintenance of cells

The human osteoblast-like cell line MG63 was maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum (FCS) (Sigma), 2mM L-glutamine, 1000mg glucose/l, 27mmol/l $NaHCO_3$ and penicillin and streptomycin (each 100U/ml) and incubated at 37°C in 5% CO_2/air. Cells were subcultured at weekly intervals. Human gingival fibroblasts were obtained from explants of normal gingivae obtained during minor surgical procedures. Tissues were finely minced and incubated in 75mm^2 flasks to allow fibroblast outgrowth. Primary cultures were maintained in DMEM supplemented with 10% FCS (plus penicillin/streptomycin and 2mM glutamine). Cells were subcultured twice-weekly with cell viability being routinely monitored by Trypan blue exclusion. Fibroblasts were used from passages 6 to 14.

2.6.2. Proliferation assays

The proliferation of various cell types and lines was measured as described below (chapter 2, section 2.6.2.1.). Proliferation assays were performed on the following cell types and lines at the densities shown: Mouse osteoblasts, 5,000 cells per well; human osteoblast-like cell line U2OS, 15,000 cells per well; human periodontal ligament fibroblasts, 5,000 cells per well; human fetal lung fibroblasts, 5,000 cells per well; mouse fibroblast cell line L929 15,000 cells per well; human oral squamous cell carcinoma UPS2, 15,000 cells per well.

2.6.2.1. MG63 Cell proliferation assay

The human osteoblast-like cell line MG63 (ATCC CRL 1427) was used for the majority of experiments. Cells were cultured at a density of 15,000 cells/well in 96-well plates and incubated overnight at 37°C in DMEM (Gibco) plus 10% FCS (Sigma) in 5% CO_2/air. The medium was then removed and the cells washed twice with sterile Hanks solution (Sigma). To measure anti-proliferative activity, various concentrations of the test materials were added to the cells in DMEM containing 2% FCS. The cells were incubated for 24 h at 37°C. During the last 6 h of culture, 0.05μCi of [^{3}H]-thymidine (spec.act. 68Ci/mmole, 2.5TBa/mmole) (Amersham) was added to the cells. Media were then removed and the cells fixed in 5% TCA. 100μl of 0.5M NaOH was used to lyse the cells, this was neutralized with an equal volume of 0.5M HCl. Radioactivity was measured by scintillation spectrometry using a Wallac 1409 liquid scintillation counter. The significance of the results was determined by use of the Student's t-test.

2.6.3. Kinetics of DNA, RNA and protein synthesis

Cells were used for radioisotope incorporation studies between passages 6 and 12. To determine the effect of the surface-associated fraction on the kinetics of DNA, RNA and protein synthesis, fibroblasts were plated (at 5,000 cells/well) into 96 well plates (Nunc) and allowed to settle overnight at 37°C. The media was then removed and replaced with media containing various

concentrations of the surface-associated fraction, in DMEM containing 2% FCS plus 0.05μCi [^{3}H]-thymidine (Amersham, UK - specific activity 68Ci/mmol, 2.5TBa/mmole), or 0.05μCi [^{3}H]-uridine (Amersham, UK - specific activity 45Ci/mmol, 1.7TBa/mmole) or a tritiated amino acid mixture (Amersham, UK). At various times, over a 24 h period the media were removed, the plates washed 3 times with cold trichloroacetic acid and 100μl of 0.5M NaOH added to lyse fibroblasts. The cell lysate was neutralized by 100μl of 0.5M HCl and the contents of the wells were placed in scintillant and the radioactivity incorporated by cells measured by scintillation spectrometry.

2.7. Bone resorption

2.7.1. Murine calvarial bone resorption assay

The bone resorption assay measures the release of calcium from 5-day old mouse calvaria *in vitro*. This assay was kindly performed by Dr Sajeda Meghji, Eastman Dental Institute. In this procedure the calvaria were bisected and cultured in BGJ medium (Flow laboratories) containing 5% heat-inactivated rabbit serum (Gibco) and 5mg/100ml ascorbic acid (Sigma), on stainless steel grids in 30mm dishes. After 24 h the media were replaced with fresh media containing various concentrations of crude or partially purified SAM. PGE_2 at 1μM was used as a positive control in all assays to show that the bone was responsive to osteolytic mediators. The calvaria were cultured for a further 48 h and calcium concentrations in the media measured by automated colorimetric analysis (Gitelman, 1967).

2.7.2. Neutralization of bone resorption by mAb P3 to *A. actinomycetemcomitans*

This experiment was performed by Mr A. Kirby and Dr S. Meghji. To assess the ability of mAb P3 to inhibit the bone resorbing activity of SAM, P3 was added to calvarial cultures stimulated to resorb by the presence of 1μg/ml or 10μg/ml SAM. Nonspecific mouse immunoglobulin G, containing all four IgG subclasses

(Sigma), was used a control in all the antibody studies. To determine if P3 was able to deplete the SAM of its osteolytic activity, solutions of the SAM were incubated with an excess (1:10 wt/wt) of antibody or with nonspecific mouse IgG, 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) for 1h at room temperature, again with constant mixing, followed by centrifugation and filter sterilization. The depleted fraction was added to the assay at 1 or 10μg/ml and activity was compared with untreated SAM. SAM was also directly incubated with *S. aureus* at room temperature for 1h to control for any possible nonspecific binding events between bacteria and SAM, this treated fraction being tested in the bone resorption assay.

2.8. Characterization of anti-proliferative activity

2.8.1. Enzyme- and heat-treatment

SAM from *A. actinomycetemcomitans* was dissolved at 1 mg/ml in Tris buffer pH 8.5. 100μl of this solution was then mixed with 100μl of trypsin (Sigma) dissolved at 100μg/ml (100 BAEE units) (One BAEE unit = ΔA_{253} of 0.001 per min with BAEE as substrate at pH7.6 at 25°C) in the same buffer and incubated for 1h to 24h. 100μl of soya-bean trypsin inhibitor (Sigma) dissolved at 100μg/ml, was used to terminate the enzyme reaction (10μg inhibits approximately 20μg of trypsin with an activity of 100 BAEE units) and the samples were stored at 4°C. Control digestions contained no SAM but were otherwise identical. 30μl from each digestion was diluted into 970μl of DMEM containing 2% FCS, to give a final SAM concentration of 10μg/ml. All samples were tested for anti-proliferative activity. SAM from *A. actinomycetemcomitans* was dissolved in saline at a concentration of 100μg/ml (dry weight) and heated in water baths at various temperatures for 1 h. Samples were diluted 10 times in DMEM containing 2% FCS and assayed for anti-proliferative activity.

2.8.2. Cytotoxicity assays

The cytotoxicity of the SAM to both human gingival fibroblasts and MG63 cells was determined by lactate dehydrogenase (LDH) release using the CytoTox 96 non-radioactive cytotoxicity assay (Promega). Briefly, cells were cultured for 24 h or 48 h in the presence of various concentrations of SAM ranging from 0.1 to 100μg/ml. LDH levels in culture supernatants were measured with a 30min coupled enzymatic assay which results in the conversion of a tetrazolium salt (INT) into a red formazan product. Absorbance at 592nm was measured. The percentage cell lysis was established using a formula which correlates sample levels of LDH to maximally induced (using Triton X-100 to lyse cells) and control LDH release.

Cytotoxicity was also monitored by acridine orange uptake or trypan blue staining after incubating MG63 cells with the SAM at a concentration of 100μg/ml for 24h. The proportion of acridine orange or trypan blue-stained cells in control cultures and in those exposed to SAM was counted and compared to the incorporation of [^{3}H]-thymidine into cells in parallel control cultures or cultures exposed to SAM.

2.9. Cell cycle studies

The influence of the surface-associated fraction, or the purified anti-proliferative protein, on cell cycle progression was tested using human gingival fibroblasts and MG63 osteoblast-like cells cultured as described.

2.9.1. Cell cycle inhibitors

Hydroxyurea (1mM), colcemid (0.1μM) and cytosine β-D-arabinofuranoside (50μM), (all from Sigma) were used to block cell cycle progression in both human gingival fibroblasts and MG63 cells.

2.9.2. Flow cytometry

Aliquots of 10^6 fibroblasts or MG63 cells were fixed in 1ml ethanol at -20°C overnight. The cells were centrifuged and resuspended in 1ml of propidium iodide (PI) stain (5mg of RNase (Sigma) and 200μg PI dissolved in 10ml PBS) and incubated in the dark for 2h at room temperature. Flow cytometry (FCM) was carried out using a Coulter Epics Elite flow cytometer and data were analysed using the Lysis II analysis programme

2.9.3. Synchronized cell cycle studies

MG63 cells were cultured in either 96-well or 24-well plates and blocked at the G_1/S boundary by adding 1mM hydroxyurea to the culture media and maintaining for 24h. Release from hydroxyurea-induced S phase arrest was achieved by removal of the hydroxyurea and adding fresh media (Sinclair, 1967). Cell synchronization was checked by comparison to cell cultures where the addition of hydroxyurea was omitted. After hydroxurea-induced arrest, either 0.1μM of colcemid, 500ng/ml of purified anti-proliferative protein, or 10μg/ml of the surface associated-material were made up in DMEM containing 2% FCS and incubated with the cells for 12h at 37°C. Photographs of the cultures were taken every two hours and the proportion of mitotic cells measured and compared to control cultures.

2.9.4. Filming of cells after release from synchrony

MG63 cells were cultured in 25cm^2 flasks and arrested at the G_1/S boundary by adding 1mM hydroxyurea. Cells were incubated for 24 h after which either 500ng of semi-purified anti-proliferative protein dissolved in DMEM containing 2% FCS or DMEM containing 2% FCS were added. The number of cells passing through mitosis was compared to control cultures using an Olympus microscope connected to a Sony CCD-IRIS/RGB colour video camera.

2.9.5. Receptor binding studies

MG63 cells were cooled to 4°C and incubated with 1 μg/ml of SAM for various periods of time from 5 min to 18h. After each of these periods of exposure, the cells were washed 3 times with PBS and incubated for 24 h at 37°C. Proliferation was assessed by adding [^{3}H]-thymidine for the last 6 h as described. In another experiment, the depletion of the anti-proliferative protein from SAM dissolved in the culture media was measured. SAM (1 μg/ml) was incubated with a fresh MG63 monolayer for 2 h at 4°C, the supernatant medium was removed and subsequently incubated with fresh monolayers. This process was repeated a third time. The cells which had been incubated with the SAM for 2 h at 4°C were then re-cultured for 24 h at 37°C. DNA synthesis was measured by the incorporation of [^{3}H]-thymidine, and assessed relative to SAM-free control cultures.

2.10. Neutralization of anti-proliferative activity using patients' sera.

2.10.1. Use of sera to block the activity of SAM

This experiment was carried out as described previously (2.6.2.1.), with the following exception. To test the ability of human sera to neutralize the anti-proliferative activity of the SAM from *A. actinomycetemcomitans*, 1:50 or 1:500 dilutions of sera were added to 500ng/ml (dry weight) of SAM in DMEM containing 2% FCS and incubated at 37°C for 1h before addition to the cells.

2.10.2. 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 5mm 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.

2.10.3. ELISA for serum antibody titres

96-well microtitre plates (Immulon 4, Dynatech) were coated with *A. actinomycetemcomitans* SAM at 10μg/ml in PBS overnight at 4°C. Wells were washed three times with PBS containing 0.05% Tween (Sigma) (termed PBS-T) to remove any unbound antigen, and blocked with PBS-T containing 1% non-fat milk powder (Safeway) (termed PBS-TM) for 1h at 37°C. Sera were then incubated in the wells at dilutions ranging from 1:100 to 1:64000 in PBS-TM for 1h at 37°C. Plates were washed three times with PBS-T and the bound antibody was detected with horseradish peroxidase-conjugated goat anti-human IgG (gamma-chain specific) (Sigma) dissolved at a 1:1000 dilution in PBS-TM, again incubated for 1h at 37°C. Tetramethyl-benzidine dihydrochloride (0.1mg/ml) plus hydrogen peroxide (2μl of fresh 30% solution per 10ml) in 0.1M citrate buffer (pH 5.1) was used as the enzyme substrate and the reaction was terminated after 10min by the addition of 1M sulphuric acid. Plates were read at 450nm using 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 non-specific binding control (antigen omitted), and from these results the serum titre giving 30% binding value (ABT_{30}) was determined. The significance of the results was tested by Wilcoxon's rank sum test.

2.10.4. Western immunoblotting

SAM separated on 15% and 12% gels by SDS-PAGE was electrophoretically transferred overnight at 15V on to Immobilon-P PVDF membranes as described by Towbin *et al.,* (1979). The membranes were washed for 5min in PBS containing 1% Triton X-100, then for a further 25min 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% foetal calf serum) for 1h and incubated in human serum diluted 1:100 in blocking buffer for 1h. In some experiments, the anti-sera were pre-absorbed with 100μg/ml of *E.coli* JM109 whole cell lysate for 1 h in order to reduce cross-reactivity. Following a further

wash, membranes were incubated for 1h in goat anti-human IgG horseradish peroxidase-conjugate (Sigma), diluted 1:1000 and washed again. Membranes were placed in 3,3'-diaminobenzidine tetrahydrochloride solution (DAB) (10mg in 15ml Tris-buffered saline, pH 7.6) containing 12μl of 30% hydrogen peroxide until bands were visualised.

2.10.5. 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 (patient 10) was diluted 1:10 in PBS and Sepharose (CL-4B) bound protein-A (Sigma) was added and mixed for 2h at 4°C. The protein-A-Sepharose was then pelleted by centrifugation. 50μl of this depleted serum was removed for analysis and the remaining serum was added to fresh protein-A-Sepharose which was again mixed for 2h 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 anti-proliferative activity of SAM (500ng/ml) at a dilution of 1:500. To determine if the protein-A-Sepharose had any effect on MG63 proliferation, or on the anti-proliferative activity of the SAM, it was added to cells in the presence or absence of SAM.

2.11. Purification of the anti-proliferative component

2.11.1 Amicon filtration

SAM from *A. actinomycetemcomitans* was dissolved in distilled water at a concentration of 1mg/ml and 50ml passed through a 0.2μm filter (Whatman) to remove particulate matter. Amicon filtration membranes with molecular cut-off of 30kDa or 100kDa (YM30, YM100 ultrafiltation membranes, Amicon Ltd, Gloucestershire, U.K.) were treated according to the manufacturers instructions and placed in the Amicon cell. Filtration, using pressurized nitrogen (2 bar) at

4°C was continuous until 10ml of material remained in the cell. Samples were washed a further 3 times with 50ml of distilled water and the two fractions lyophilized.

2.11.2. Ammonium sulphate precipitation

2.11.2.1. Pilot study

SAM was dissloved at 5mg/ml in 5ml of distilled water and centrifuged at 10,000 *g* to remove any undissolved material. The solution was gently stirred at 4°C and protein was precipitated by the addition of sufficient ammonium sulphate to give a 30% solution. Residual protein in the supernatant was again precipitated by the addition of sufficient ammonium sulphate to give concentrations of 45%, 60% and 80%. The precipitates were collected at each stage, dissolved in a small amount of distilled water and dialysed against distilled water using benzoylated dialysis tubing. The protein concentration of each fraction was measured and anti-proliferative activity assessed at 1μg/ml.

2.11.2.2. Large scale precipitation

SAM was dissolved at 5mg/ml in 40ml of distilled water and centrifuged at 10,000 *g* to remove any undissolved material. The SAM was gently stirred at 4°C and protein was precipitated upon the addition of 30% ammonium sulphate. This fraction was redissolved in 5ml of distilled water, dialysed and lyophilized.

2.11.3. Anion exchange chromatography

2.11.3.1. Anion exchange HPLC

SAM from *A. actinomycetemcomitans* was fractionated by anion exchange high performance liquid chromatography (HPLC) using a Bio-Rad HRLC chromatography system. An anion exchange MA7Q (Bio-Rad) column (5cm x 0.78cm) was equilibrated using 20mM Tris buffer pH 8.5 (buffer A). 1ml of a 4mg/ml solution of SAM in buffer A was injected on to the column and eluted with either one of two gradients: (1) 5ml of buffer A followed by linear

gradients from 0-50% buffer B (buffer A + 2M NaCl) in 20ml and 50-100% buffer B in 5ml or (2) 5ml of buffer A followed by linear gradients from 0-50% buffer B in 30ml and 50-100% buffer B in 5ml. The flow rate was 1ml/min and 1ml fractions were collected with absorbance monitored at 280nm and 205nm. Fractions were dialysed (Sigma dialysis tubing 2kDa cut off) against distilled water for 48 h and the protein concentration of each fraction measured. Fractions were subsequently diluted 2000 times to assess their anti-proliferative activity.

2.11.3.2. Q-Sepharose anion exchange

Crude SAM was fractionated on a Q Sepharose anion exchange column (50cm x 1.6cm). The column was equilibrated in 20mM TrisHCl pH8.5 (buffer A) and the SAM (approximately 400mg) was loaded on in the same way. The column was washed with 500ml of buffer A and then eluted with a 1000ml linear gradient of 0 to 2M NaCl in buffer A. 10ml fractions were collected and the absorbance at 280nm monitored. All fractions were assayed for anti-proliferative activity or screened with monoclonal antibody P3.

2.11.4. Size-exclusion chromatography

2.11.4.1. Size exclusion HPLC

Bio-active fractions were fractionated by size-exclusion HPLC chromatography using either a Protein Pak 125 column (Waters) or a TSK-250 column (Bio-Rad). The column was equilibrated with 0.1M sodium phosphate buffer pH 6.7, containing 0.15M NaCl and 20μl sample volume injected. The flow rate was 1ml/min and 1ml fractions were collected, with protein absorbance monitored at both 280nm and 205nm. All fractions were assessed for anti-proliferative activity.

2.11.4.2. Manual size exclusion

Superdex S-200 or Sephadex G-100 were used to resolve samples by gel filtration. Columns (15cm x 1.5cm) were carefully poured and equilibrated in PBS. Samples were applied to the column. The column fractions were monitored for anti-proliferative activity and absorbance at 280nm. Gel filtration standards (Sigma) were used to determine the molecular weight of the samples.

2.11.5. Reverse phase HPLC

Samples were separated by reverse phase on a Resource™ 3ml column (Pharmacia Biotech). The column was equilibrated in buffer A (distilled water containing 0.1% TFA) and the sample loaded. A 0-100% linear gradient of buffer B (100% acetonitrile containing 0.8% TFA) in 30ml was used to elute the proteins. The flow rate was 1ml/min and absorbance was measured at 220nm. Fractions were dried using a Speed-vac vacuum drying apparatus and tested for anti-proliferative activity.

2.11.6. Dye binding chromatography

A selection of nine dye-binding columns (2.5ml bed volume) (Sigma) (Reactive green 19 and 5, Reactive brown 10, Reactive yellow 86 and 3, Cibacron blue 3GA, Reactive blue 72 and 4 and Reactive red 120, all covalently bound to agarose) were used to fractionate the SAM from *A. actinomycetemcomitans*. The columns were equilibrated with 10ml of buffer A (10mM Tris pH 8.0, 5mM $MgCl_2$) and 1ml of SAM solution (1mg/ml), dissolved in the same buffer, was applied to the column. Columns were eluted with 15ml of buffer A (fraction 1), followed by 15ml of buffer A + 2M NaCl (fraction 2). All fractions were assayed for anti-proliferative activity by diluting 1:100. In order to elute unrecovered activity, various other buffers were used including 6M urea, 0.1, 2 and 10% ethylene glycol, and 10mM tris buffer pH 8.0 containing 0.3M methyl mannoside. Controls to ensure that the buffers were not responsible for any reduction of activity consisted of an overnight incubation at 4°C of SAM

with the various buffers used. Fractions were dialysed and assayed for anti-proliferative activity.

2.11.7. Rotophor preparative IEF

SAM from *A. actinomycetemcomitans* was separated using a Rotophor preparative electrofocusing cell (Bio-Rad). 40mg of SAM from bacteria grown in liquid media and 50mg of SAM from bacteria grown on solid media were fractionated. SAM was dissolved at 1mg/ml in distilled water containing 10% (v/v) glycerol and 2% (w/v) ampholytes (pH 3.5 to 10; Bio-Lyte; Bio-Rad), and the volume made up to 55ml. The sample was centrifuged at 3000 *g* for 20min to remove any undissolved material. The electrolytes in the anode and cathode chambers were 0.1M H_3PO_4 and 0.1M NaOH, respectively. Isoelectric focusing (IEF) in the Rotophor cell was carried out at 12 W of constant power at 4°C for 4 h. Focusing continued for 30 min after the voltage had stabilized. Fractions were harvested, pH values measured and assayed for anti-proliferative activity. A blank run which contained no SAM was also assayed to ensure the ampholytes did not affect proliferation.

2.11.8. Protein sequencing

The purified 62kDa protein was run on a 10% SDS-PAGE gel and electroblotted onto Problott membrane (Applied Biosystems Inc.). The band of interest at 62kDa was excised and run on an ABI 470A protein sequencer (Applied biosystems Inc.) for 40 cycles using ABI "Blot" cartridge and an optimized program for electroblotted samples. Data were collected and analyzed using waters Expert-Ease software (Millipore Corp.). This work was kindly performed by Dr. A.C. Willis and Dr. R. Sim at the MRC Immunochemistry unit, Department of Biochemistry, University of Oxford, Oxford, U.K.

2.12. Cloning of proteins from *A. actinomycetemcomitans*

Basic molecular biological methods were based upon those given in Sambrook *et al.* (1989) unless otherwise stated.

2.12.1. Isolation of genomic DNA

Chromosomal DNA was extracted from the bacterial cells by a modification of a method described in Sambrook *et al.* (1989). 1.0g of bacterial paste (stored at -70°C) was resuspended in 25ml of TE buffer (10mM tris-HCL, pH 8.0, 1mM EDTA) and centrifuged at 3000 *g* for 10min at 4°C. The supernatant was discarded and the pellet resuspended in 3.2ml TE buffer. 0.6ml of freshly prepared lysozyme solution (20mg/ml) (Boehringer Mannheim) was added and the cells stored for 5min on ice. Cells were lysed by the addition of 0.6ml of 0.5M EDTA pH8.0, 0.5ml of 10% (w/v) SDS and 1ml of 1mg/ml proteinase K (Sigma) and incubated for 1 h at 60°C with gentle mixing. The samples were then extracted once using an equal volume of buffer-saturated phenol (5ml), then twice more with phenol-chloroform-isoamyl alcohol (25:24:1). 2.5ml of 7.5M ammonium acetate was added and the DNA precipitated by the addition of 10ml of absolute ethanol. DNA was "hooked out" with a bent pasteur pipette, rinsed in 70% ethanol and redissolved in 2.5ml of TE and dialysed against TE buffer overnight.

2.12.2. Construction of genomic library

A. actinomycetemcomitans chromosomal DNA was partially digested with various concentrations of the restriction endonuclease *Sau* 3A (Sigma) to determine the optimum concentration for the isolation of 2-10kb fragments of DNA. Using the conditions established, the partial digestion was scaled up for 100μg of DNA and the resulting DNA purified by phenol-chloroform extraction and ethanol precipitation. DNA was then size-fractionated on a 0-40% sucrose gradient. The gradient was established by freezing a 25% sucrose solution dissolved in 1M NaCl, 10mM tris-HCL, pH 8.0, 1mM EDTA at -70°C for 1 h and defrosting at 20°C and refreezing at -70°C and then defrosting slowly

overnight at 4°C. Fractions containing DNA in the size range 2-10kb, as determined by agarose gel electrophoresis, were ligated using T4 DNA ligase (Promega) into the cloning vector pUC18 (Pharmacia Biotech), which had previously been cleaved with the enzyme *Bam* HI and dephosphorylated with calf intestinal alkaline phosphatase to help prevent vector religation. All ligation reactions were purified using the Promega DNA clean-up™ system and checked by agarose gel electrophoresis.

2.12.3. Production of competent *E. coli*

Recombinant DNA was transformed into either *E. coli* JM105 (Pharmacia Biotech) or *E. coli* JM109 (Promega), which were made competent by a modification of the method of Hanahan (1983). Cells were grown overnight in 5ml of Luria-Bertani (LB) broth at 37°C. 500μl of the culture was used to inoculate 50ml of fresh LB broth, and incubated until the optical density (OD) at 550nm had reached between 0.4 and 0.5. Cells were centrifuged at 4°C in a pre-cooled rotor and resuspended in 25ml ice-cold, sterile 50mM $CaCl_2$. The bacteria, now competent, were centrifuged and resuspended in 5ml ice-cold, sterile 50mM $CaCl_2$ (these cells can be frozen in 50mM $CaCl_2$ containing 15% gycerol and stored at -70°C). In order to transform the competent *E. coli*, 50ng of ligated pUC18 was added to 0.5ml of the cells. The mixture was incubated on ice for 30min, and heat shocked at 42°C for 2min. LB broth (1ml) was added and the cells incubated at 37°C for 1 h. The cells were plated on LB agar containing 40μg/ml X-gal, 50μg/ml ampicillin and 250μM IPTG, to isolate clones containing antibiotic resistant plasmids with inserted DNA, then incubated for 24 h at 37°C.

2.12.4. Immunoscreening of library

Recombinant white colonies were replica-plated on to nylon Hybond-N™ membranes (Amersham). *A. actinomycetemcomitans* and *E. coli* JM109(pUC18) were also spotted on to each membrane as positive and negative controls respectively. Colonies were not lysed, to facilitate the isolation of recombinants

expressing surface-associated antigens. Instead, membranes were rinsed in PBS containing 1% Triton X-100 for 1h and then transferred into blocking buffer (PBS containing 0.1% Triton X-100 and 3% low-fat milk powder (Safeway)) for 1h. Membranes were incubated in serum from a patient with localized juvenile periodontitis (LJP) diluted 1:500 for 2 h. The antiserum had first been extensively adsorbed with 100μg/ml of *E. coli* JM109 whole cell lysate for 1 h. After washing, membranes were incubated for 1h in goat anti-human IgG horseradish peroxidase conjugate (Sigma), diluted 1:1000 and washed again. Membranes were developed in 3,3'-diaminobenzidine tetrahydrochloride solution (DAB) (10mg in 15ml Tris-buffered saline, pH 7.6) containing 12μl of 30% hydrogen peroxide. Positive clones were picked from the master plate and screened a second time using the same procedure in the presence and absence of IPTG.

2.12.5. Plasmid isolation

Plasmids were isolated from recombinant clones either by the alkaline lysis method (Birnboim and Doly 1979, Sambrook *et al.*, 1989) or using the Wizard™ plasmid isolation kit (Promega).

2.12.6. Restriction digests

Enzyme reactions were carried out under conditions recommended by the manufacturer (Promega). Briefly, DNA (1μg) was digested in 20μl volumes containing 1x of the supplied reaction buffer and 8-10 Units of restriction enzyme for 1-3 h at 37°C.

2.12.7. Electrophoretic techniques

2.12.7.1. SDS-PAGE

Positive clones were grown overnight on LB plates containing 100μg/ml ampicillin and harvested using saline. Bacterial lysates were made as follows: The bacteria were centrifuged, resuspended in 50mM Tris, pH 8.0, containing 2% SDS and boiled for 10min. Cell debris was removed by centrifugation and 10μl of the supernatant was mixed with 10μl of sample buffer and boiled for a further 2min. Samples were separated by SDS-PAGE on a 12% gel, using a Bio-Rad mini-Protean II system. Gels were either stained by colloidal Coomassie blue (Sigma) or immunoblotted (chapter 2, section 2.5.). Western blot immunoassays of lysates were performed by standard techniques, as described earlier (chapter 2, section 2.10.3.).

2.12.7.2. Agarose gel electrophoresis

DNA fragments were resolved by electrophoresis on 0.8% or 1% horizontal agarose gels in 1x TAE (40mM Tris-Acetate, 1mM EDTA), or 0.5x TBE (45mM Tris-Borate, 1mM EDTA) containing 0.5μg/ml of ethidium bromide. Bacteriophage lambda (Promega) was used as a size standard. Gels were run at 5 V cm^{-1} for 1-3 hours.

2.13. Cloning of the GroEL-like gene from *A. actinomycetemcomitans* using the polymerase chain reaction (PCR)

2.13.1 Polymerase chain reaction

PCR enables the 10 fold amplification of a specific region of DNA *in vitro*, in a matter of hours (Saiki et al. 1985). The process involves repeated cycles of heat denaturation, annealing of 2 oligonucleotide primers designed to define the fragment to be amplified and extension of the annealed primers by the enzyme DNA polymerase.

PCR reactions mixtures were as follows:

buffer (10x)	10μl (100mM Tris-HCl pH8.3, 50mMKCl)
15 mM Mg Cl_2	5μl
sterile distilled water	75μl
dNTPs	4μl (5 mM)
primer A (forward)	2μl (3.2μg)
primer B (reverse)	2μl (2.7μg)
DNA	1μl (200ng)
Taq polymerase	1μl (2U/μl)

All the reagents used were obtained from Promega, Southampton, UK, except where otherwise stated. The reagents were added to a 0.5 ml sterile microcentrifuge tube (Sigma, Poole, UK) in the order shown above. The oligonucleotide primers used in this study were synthesized by Pharmacia Biotech. The sequence for the forward primer (prime A) corresponded to the N-terminal of the groEL-like gene, calculated from the amino acid sequence of *A. actinomycetemcomitans* protein. The reverse primer was synthesized for a highly conserved region of the HSP60 family of proteins (Rusanganwa *et al.*, 1992). The forward (5'-GCNGCNAARGAYGTNAARTT-3') (where N = A, C, G or T, R = A or G and Y = C or T) and the reverse (5'-TCNCCRAANCCNGGNCGYTTNACNGC-3') were made to the amino acid sequences AAKDVKF and AVKAPGFGD, respectively. For these primers to work with DNA from different species, the third codon position in these was degenerate to allow for different codon usage. The total volume of reaction mixture was 100μl. Sixty μl of light weight mineral oil (Sigma, Poole, UK) was added to each reaction mixture and the microcentrifuge tubes pulsed for several seconds in the microcentrifuge. The microcentrifuge tubes were placed in a Mastercycler PCR machine (Eppendorf 5330) which was programmed as follows:

A 5 min denaturing step at 96°C, then 1 minute at 50°C. The Taq polymerase 1μl was added at this point (Faloona et al. 1990), followed by 1.5 min at 72°C. The thermal profile used included 30 cycles, denaturing at 96°C for 1 min, primer annealing at 48°C for 1 min, and nucleotide extension at 72°C for 1.25 min. Approximately 5μl of the reaction volume was then analysed by gel electrophoresis.

2.13.2. Cloning into the pCR^{TM}II vector

PCR products were cloned into pCR^{TM}II using the TA cloning kit (Invitrogen). This kit provides a quick, one-step strategy for the direct insertion of a PCR product into a plasmid vector. *Taq* polymerase has a non-template-dependent activity which adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized vector supplied in the kit has single 3′ deoxythymidine (T) residues, this allows PCR inserts to ligate efficiently with the vector. The kit was used as described in the manufacturers instructions.

Chapter 3
Characterization of SAM from *A. actinomycetemcomitans*

3.1. Introduction

Actinobacillus actinomycetemcomitans has been strongly implicated in the aetiology and pathology of localized juvenile periodontitis (LJP) (Zambon *et al.*, 1985,1988). A number of research groups have demonstrated that this organism contains loosely adherent extracellular material (Holt *et al.*, 1980; Meyer and Fives-Taylor, 1993, 1994; Scannapieco *et al.*, 1987). Work from the Eastman has shown that a gentle saline extraction of *A. actinomycetemcomitans* results in the release of a proteinaceous fraction, which we have termed surface-associated material (SAM). Surface layers may interfere with the humoral defense mechanisms and phagocytosis. They may also sequester a whole array of virulence factors. At present, the pathogenesis of *A. actinomycetemcomitans* is not fully understood, neither is it clear whether all the serotypes are equally virulent.

The aim of this section of the study was therefore to characterize the surface-associated material from this organism.

3.2. Results

3.2.1. Composition of SAM

Table 3.1. shows the relative composition of the SAM from *A. actinomycetemcomitans* when grown on solid or liquid media.

3.2.1.1. SAM produced from bacteria cultured on solid media

To enhance the production of surface-associated material *A. actinomycetemcomitans* was grown on a solid medium consisting of brain-heart infusion (BHI) agar supplemented with 5% (v/v) horse blood (chapter 2, section 2.1.). Typically, the SAM made up 15-20% of the dry weight of the cells (for

extraction method see chapter 2, section 2.2.1.). The majority of the SAM consisted of protein. The range of values for the composition of the SAM obtained from 6 separate batches was approximately 60-70% protein, the amount of lipid extracted using methanol:chloroform was assessed at 9% of the total material and the carbohydrate content measured at 4%. The lipopolysaccharide (LPS) content of the SAM was extremely low, generally of the order of 0.0001-0.001 IU/ng. Control plates containing no bacteria were harvested in saline and analysed by SDS-PAGE, this demonstrated that protein contamination by horse serum proteins was very low. Details of methods for determining the composition of SAM are given in chapter 2, section 2.4.

Table 3.1.

Composition of SAM produced from solid and liquid media

	% dry weight of bacteria	% protein	% carbohydrate	%lipid
solid media	15-20	60-70	4.0	9
liquid media	5.7	20	4.6	10

3.2.1.2. SAM produced from bacteria cultured in liquid media

As it is easier to handle large quantities of liquid media (as opposed to solid media) and as harvesting is also easier, the organism was also grown in BHI broth. 52 litres of media resulted in 14.4g (dry weight) of bacteria, which yielded 821mg of SAM, representing 5.7% of the dry weight of the bacteria. The composition was different from that of SAM produced from bacteria grown on solid media, protein was assessed at 20% of the total material with a carbohydrate content of 4.6%. The LPS levels were slightly higher, at 0.001-0.01IU/ng, when compared to SAM extracted from bacteria grown on BHI-agar.

3.2.2. Electron microscopy

Prior to the saline extraction, ruthenium red staining revealed the presence of a thick electron opaque layer external to the cell wall on bacteria grown on solid media (figure 3.1a) (chapter 2, section 2.3.). Saline extraction resulted in complete removal of this layer leaving intact cells (figure 3.1b).

a b

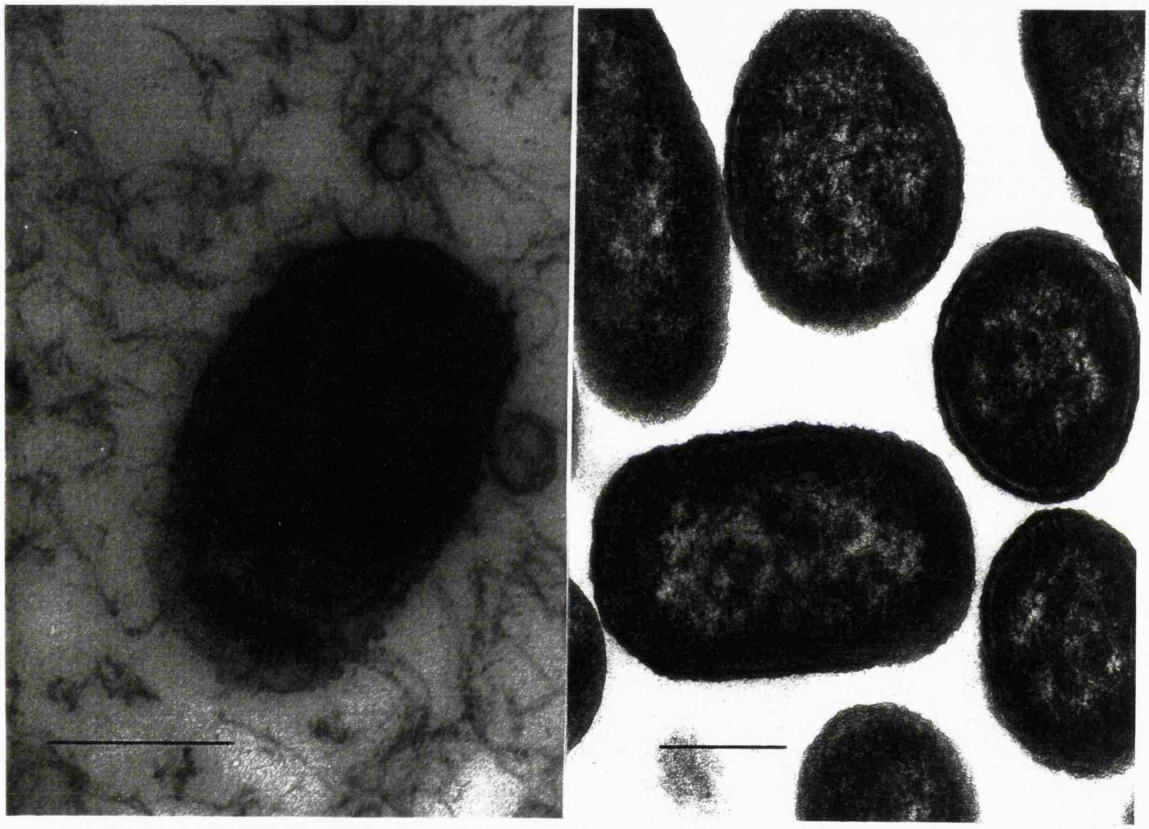


Figure 3.1. Thin sections of *A. actinomycetemcomitans* stained with ruthenium red before (a) and after (b) extraction with saline. Ruthenium red positive material is clearly visible in (a), but is absent in (b). a: bar = 0.25μm b: bar = 0.25μm.

3.2.3. SDS-PAGE analysis of SAM

3.2.3.1. One-dimensional SDS-PAGE

Comparison of the SAM from solid and liquid grown bacteria by SDS-PAGE analysis (chapter 2, section 2.5.) revealed some differences in the protein profiles, it was apparent that SAM originating from solid media supplemented with 5% horse blood contained considerably more proteins than SAM produced in liquid media. Figure 3.2. shows the banding patterns of the two types of SAM.

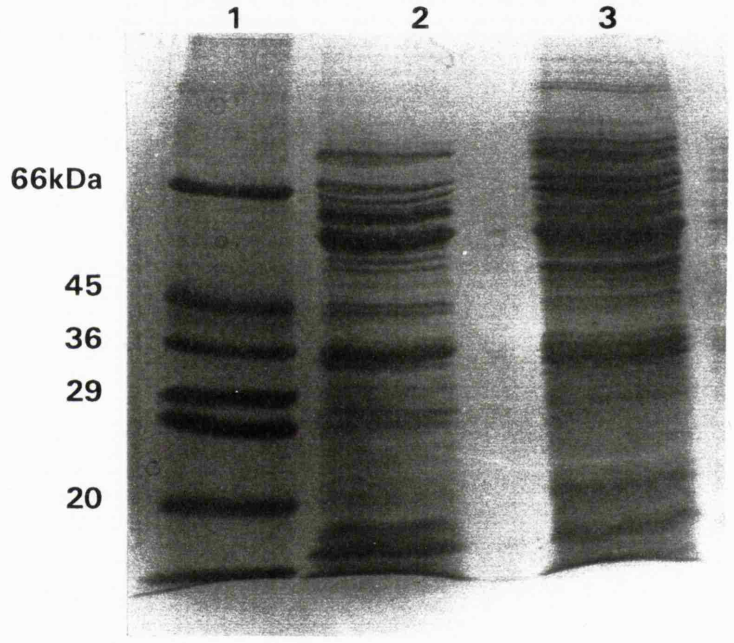


Figure 3.2. SDS-PAGE analysis of SAM isolated from *A. actinomycetemcomitans* grown on two different media, 20μg (dry weight) of each SAM was separated on a 12% gel and stained with Coomassie blue. Lane 1 shows the molecular weight markers. Lane 2 shows the SAM generated from bacteria cultured in liquid BHI. Lane 3 shows the SAM generated from bacteria produced from BHI agar supplemented with 5% horse blood.

3.2.3.2. Two dimensional SDS-PAGE

SAM extracted from agar-grown bacteria was further analyzed by 2D-SDS-PAGE (chapter 2, section 2.5.). Figure 3.3. shows the presence of approximately 50 Coomassie blue-stained spots in the pI range of 3-10 and of

molecular masses from < 14 to > 66kDa. Silver staining of the 2D-gel revealed a further 25 protein spots.

3.3. Discussion

Material external to the bacterial cell wall can act as a protective coat (Mozes *et al.*, 1991) but can also play a pivotal role in the interactions between the organism and mammalian cells. One of the main extracellular components of many bacteria is the capsule. Apart from their role in protection, capsules enable bacteria to adhere to substrata or to other bacterial species (Beveridge and Graham, 1991).

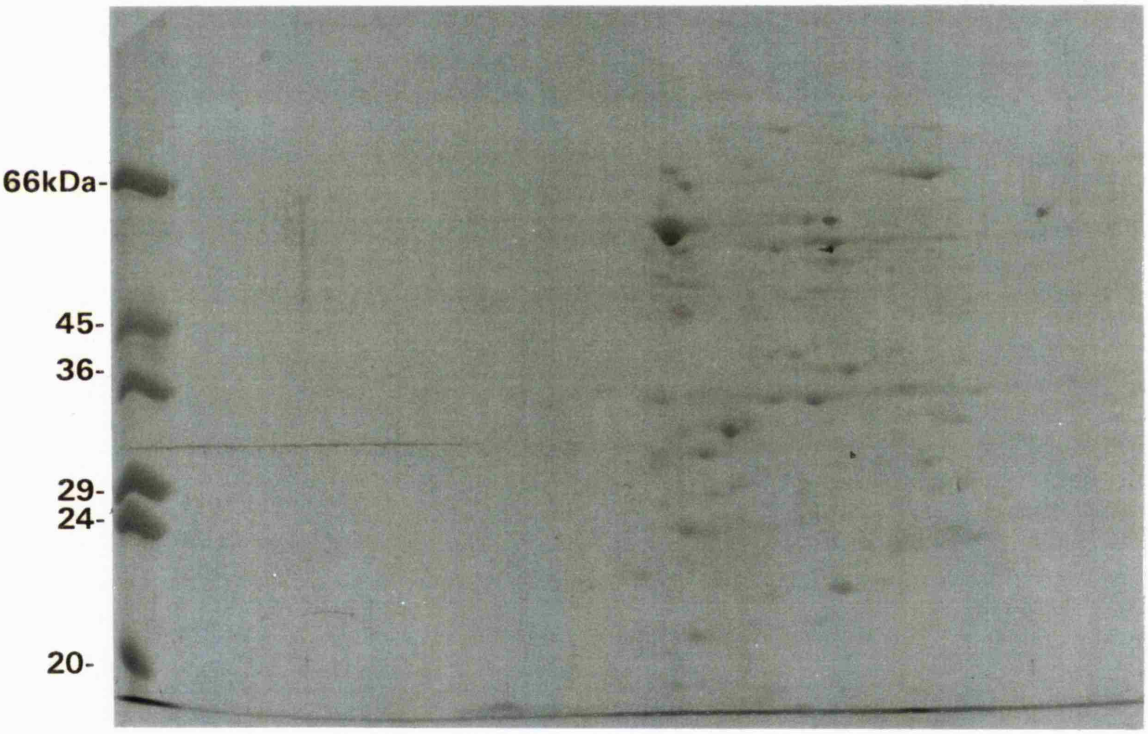


Figure 3.3. Determination of the protein composition of the SAM of *A. actinomycetemcomitans* by two-dimensional SDS-PAGE. Proteins were separated in one dimension on the basis of their iso-electric point and in the other dimension on the basis of their molecular mass. The gel was stained with Coomassie blue to disclose proteins. The molecular mass markers are displayed on the left- hand side.

Electron microscopic studies of the surface of *A. actinomycetemcomitans* by Holt *et al.*, (1980) and Scannapieco *et al.*, (1987) revealed that the surface of this bacterium was covered in large amounts of extracellular material. Other surface components identified in *A. actinomycetemcomitans* include microvesicles (Nowotny *et al.*, 1982), surface-associated fibrils (Holt *et al.*, 1980) and fimbriae (Scannapieco *et al.*, 1987). Gentle saline extraction of *A. actinomycetemcomitans* releases this surface-associated material. Our own Transmission electron microscopic demonstrated that this procedure removes the extracellular layer, leaving the bacteria intact, with no signs of damage (Meghji *et al.*, 1994; Wilson *et al.*, 1985). The amount of extracellular material of *A. actinomycetemcomitans* when viewed microscopically is quite considerable, indeed, this present study demonstrates that surface-associated material, removed using a simple saline extraction, accounts for 15-20% of the dry weight of *A. actinomycetemcomitans* when grown on solid media and 5.7% when grown in liquid culture. One reason for such a high yield of SAM could be due to cell lysis during the extraction. However microscopic studies clearly demonstrate that removal of the SAM has minimal effect on cell morphology, in that there is no sign of cell damage. Furthermore, the low levels of LPS contamination also suggests that the bacterial cells are not disrupted during the extraction.

Analysis of the composition of the SAM extracted from bacteria grown on agar or in liquid culture revealed differences in their composition. SAM produced from bacteria cultured on BHI agar plates supplemented with horse blood, tended to be more proteinaceous than SAM extracted from cells grown in BHI broth cultured under the same conditions; 60-70% compared with 20% protein. This finding was further demonstrated by SDS-PAGE analysis of the two SAMs, the SAM produced from agar-grown bacteria clearly contained more protein bands. Furthermore on a dry weight basis, bacteria grown on solid media yielded approximately three times as much SAM as cells grown in liquid media. In this respect the results of Holt *et al.*, (1980) are interesting, as they

showed that agar-grown *A. actinomycetemcomitans* produced more exopolymeric material and vesicles than cells grown on liquid culture. One reason for finding diminished amounts of extracellular material on cells grown in liquid culture, could be that in liquid culture, cells are subjected to a "trimming" effect, where material is washed from the bacteria into the culture media. Studies by two groups (Scannapieco *et al.*, 1987; Meyer and Fives-Taylor, 1994) demonstrated that liquid cultured *A. actinomycetemcomitans* were also devoid of fimbriae. Scannapieco *et al.*, showed that the protein content of bacterial surface-extracts from liquid-cultured bacteria were higher than bacteria grown on solid media, 37.6%, compared to 29.8%. Scannapieco *et al.* (1987) also showed that aerobically agar-grown bacteria were associated with a greater amount of extracellular material than organisms grown anaerobically. Of particular interest are the studies of Meyer and Fives-Taylor (1993), who showed that a phosphate buffered saline (PBS) wash of *A. actinomycetemcomitans* strain SUNY 75(S) readily removed extracellular amorphous material and reduced the adherence of the strain by 50%. Adherence of other *A. actinomycetemcomitans* strains was increased in the presence of the SUNY 75(S) extracellular amorphous material. This PBS extract was found to contain mainly protein with no carbohydrate detected. In a second study, (Meyer and Fives Taylor, 1994) the influence of growth conditions on adherence was investigated. Both the type of medium (broth or agar) and the anaerobicity influenced adherence levels and cell surface characteristics of three *A. actinomycetemcomitans* strains tested.

The literature suggests that capsules typically consist of branched or unbranched homo- or heteropolymer polysaccharides and are rarely proteinaceous (Beveridge and Graham, 1991). Therefore the high protein content and low carbohydrate content of the SAM from *A. actinomycetemcomitans* when grown on BHI-agar supplemented with 5% horse blood is somewhat surprising. Proteins are being recognized increasingly as important components of the cell surface of bacteria, their biological

importance as cell surface receptors cannot be doubted, as well as their role as virulence factors and in protection and adhesion. Considering these potential roles for bacterial proteins, it certainly seems feasible that secretion of these proteins would prove advantageous to *A. actinomycetemcomitans*.

These studies provide evidence that changes in environmental conditions alter the characteristics of the surface of *A. actinomycetemcomitans*. The ability of the organism to readily change its surface layer could be of great advantage in coping with environmental changes. This chapter has focused on the extracellular layer of *A. actinomycetemcomitans*, the proceeding chapter describes some of the potent biological activities found in this material.

Chapter 4

Bone-modulating activity of SAM from *A. actinomycetemcomitans*

4.1. Introduction

There is strong supporting evidence for the hypothesis that *Actinobacillus actinomycetemcomitans* is involved in the aetiology and pathology of LJP (Zambon *et al.*, 1985, 1988). The loss of alveolar bone and periodontal ligament are the hallmarks of LJP and thought to be a destructive process driven by soluble virulence factors released by *A. actinomycetemcomitans*. However, the periodontal tissues are well known to have a rapid rate of remodelling (Page and Ammons, 1974; Hassell, 1993). Therefore, an alternative mechanism to account for the loss of the alveolar bone and the periodontal ligament, is the failure to produce sufficient matrix components and cells to balance the loss of tissue and cells following host tissue destruction or possibly, during normal tissue remodelling. Thus, bacterial agents which inhibit cellular proliferation could adversely affect these tissues, resulting in the tissue loss associated with LJP. A combination of both direct destruction of the alveolar bone by bacterial factors followed by an inhibition of proliferation of cells which are needed to replace lost tissues probably more accurately describes the mechanisms involved with bone resorption in LJP.

Previous studies at the Eastman had revealed that the SAM from *A. actinomycetemcomitans* contained potent bone-modulating activity, with the ability to inhibit cell proliferation (Kamin *et al.*, 1986; Meghji *et al.*, 1992a; Meghji *et al.*, 1992b) and to directly stimulate bone resorption (Wilson *et al.*, 1985; Meghji *et al.*, 1994). In this chapter, the bone-modulating activity of *A. actinomycetemcomitans* SAM is examined. The nature of the anti-proliferative activity of *A. actinomycetemcomitans* SAM has been directly compared with the SAM from two other periodontopathogenic bacteria, *Por. gingivalis* W50

and *Eik. corrodens* NCTC 10596 using two key cell populations, the osteoblast and the gingival fibroblast. Furthermore the ability of the SAM from *A. actinomycetemcomitans* to inhibit the proliferation of other cell types is examined, as well as its potential osteolytic activity.

4.2. Results

4.2.1. The effect of SAM on the proliferation of MG63 cells

Using the human osteoblast-like cell line, MG63, the SAM from *A. actinomycetemcomitans* (NCTC 9710), extracted from bacteria grown on solid or in liquid media, caused a concentration-dependent inhibition of [^{3}H]-thymidine incorporation with a reproducible IC_{50} value (concentration that causes 50% inhibition) of approximately 100ng/ml (chapter 2, section 2.6.2.1.). The SAM from two other periodontopathogenic bacteria, *Por. gingivalis* W50 and *Eik. corrodens* NCTC 10596 were also tested but failed to show such potent anti-proliferative activity with this cell line at a concentration as high as 10 μg/ml (figure 4.1). In contrast to the SAM, the LPS from *A. actinomycetemcomitans* at a concentration of 10μg/ml only produced 13% ± 1% inhibition of [^{3}H]-thymidine incorporation. Comparison of the anti-proliferative activity of the SAM from *A. actinomycetemcomitans* (NCTC 9710, serotype c) with the two other major serotypes a and b (grown on solid media) demonstrated that they all had the capacity to inhibit proliferation, although it was apparent that serotype a was less active than serotypes b and c (figure 4.2.). The bacterial whole cell lysate was also tested and showed a similar activity profile to that of the SAM.

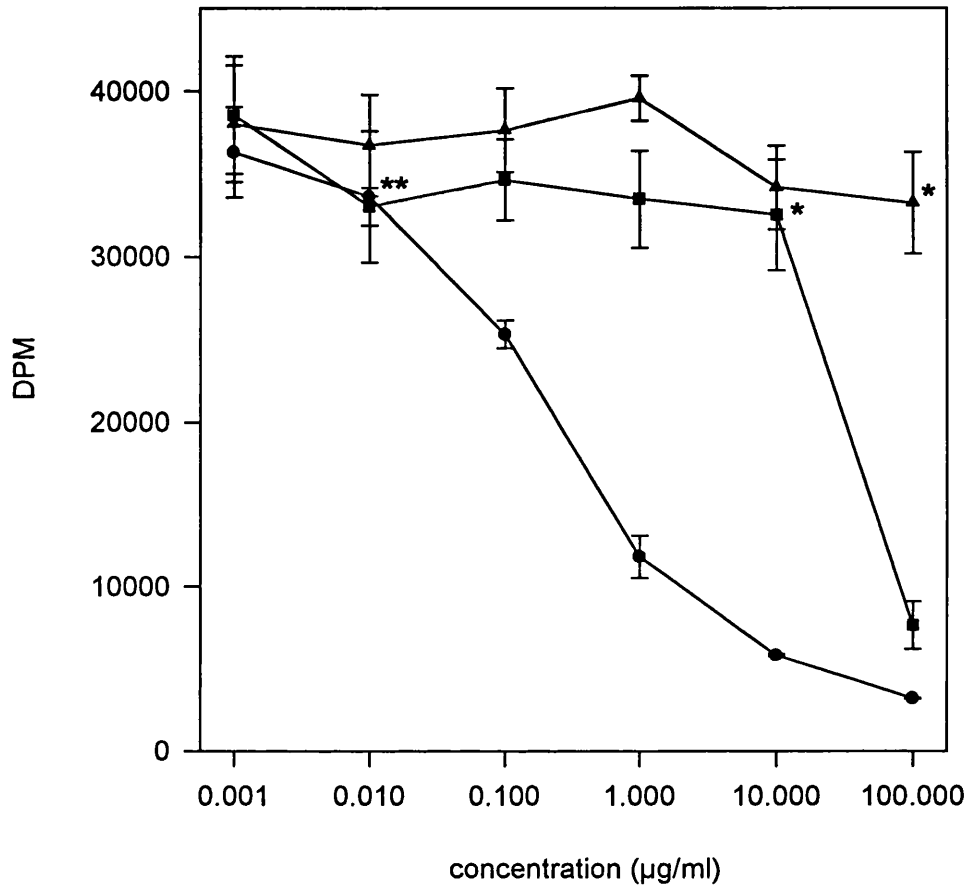


Figure 4.1. Inhibitory effect of increasing concentrations of SAM from *A. actinomycetemcomitans* (circle), *Por. gingivalis* (square) and *Eik. corrodens* (triangle) on DNA synthesis, measured as incorporation of [^{3}H]-thymidine into DNA, by MG63 cells. The results are expressed as mean and standard deviation (SD) of six replicate cultures.*$p<0.05$, **$p<0.01$.

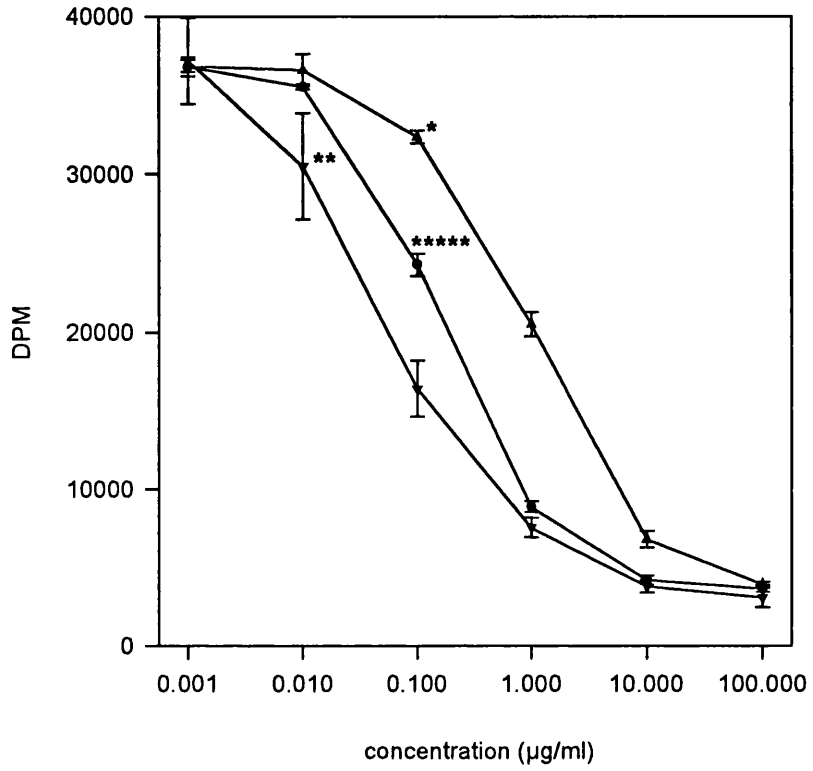


Figure 4.2. Inhibitory effect of increasing concentrations of SAM from *A. actinomycetemcomitans* serotypes a (triangle), b (inverted triangle) and c (NCTC 9710) (circle) on DNA synthesis, measured as incorporation of [^{3}H]-thymidine into DNA, by MG63 cells. The results are expressed as mean and SD of six replicate cultures. *$p<0.05$, **$p<0.01$, *****$p<0.00001$.

4.2.2. Effect of SAM on proliferation of human gingival fibroblasts

The proliferation of human gingival fibroblasts was examined following treatment with SAM from *A. actinomycetemcomitans*, *Por. gingivalis* and *Eik. corrodens* (chapter 2, section 2.6.3.) (figure 4.3.). Although the SAM from *Eik. corrodens* was not anti-proliferative to MG63 cells, it proved to be an extremely potent inhibitor of fibroblast proliferation with an IC_{50} value of approximately

100ng/ml. The SAM from *A. actinomycetemcomitans* also demonstrated anti-proliferative activity with an IC_{50} value of approximately 1μg/ml, in contrast, the SAM from *Por. gingivalis* was weakly active with an IC_{50} value of approximately 10μg/ml.

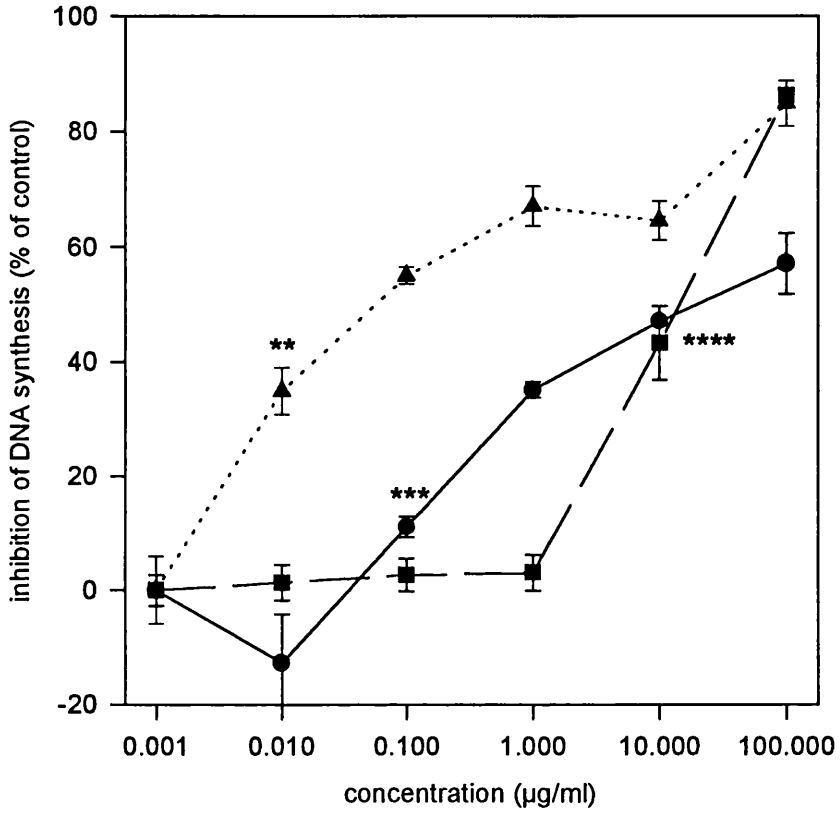


Figure 4.3. Inhibitory effect of increasing concentrations of SAM from *A. actinomycetemcomitans* (circle), *Por. gingivalis* (square) and *Eik. corrodens* (triangle) on DNA synthesis, measured as incorporation of [^{3}H]-thymidine into DNA, by human gingival fibroblasts. The results are expressed as mean and SD of six replicate cultures. **p<0.01, ***p<0.001, ****p<0.0001.

4.2.3. Effect of SAM from *A. actinomycetemcomitans* on the proliferation of other cell types

SAM from *A. actinomycetemcomitans* (NCTC 9710) grown on solid media was tested on various cell types and lines to assess its ability to inhibit mammalian cell proliferation. Table 4.1. shows the results of screening different cell types.

Table 4.1.

Inhibition of mammalian cell proliferation by surface-associated material of *A. actinomycetemcomitans*

cell type	IC_{50} value (μg/ml)
mouse primary osteoblasts	10.0
human osteoblast-like cell line MG63	0.1
human osteoblast-like cell line U2OS	0.1
human gingival fibroblasts	1.0
human periodontal ligament fibroblasts	1.0
human fetal lung fibroblasts	1.0
mouse fibroblast cell line L929	5.0
human oral squamous cell carcinoma UPS2	1.0

4.2.4. Characterization of the anti-proliferative component

4.2.4.1. Extraction of lipidic material from SAM

Lipidic material was extracted, using methanol:chloroform, from SAM produced from bacteria grown on BHI-agar supplemented with 5% horse blood. This material was tested for its ability to inhibit cell proliferation over a concentration range of 10ng/ml to 10μg/ml, but no anti-proliferative activity was detected.

4.2.4.2. Trypsin treatment

SAM from *A. actinomycetemcomitans* was dissolved at 1 mg/ml and incubated with trypsin for various periods ranging from 1h to 24h (chapter 2, section 2.8.1.). Soya-bean trypsin inhibitor was used to terminate the enzyme reaction and the samples were stored at 4°C. Control digestions contained no SAM but were otherwise identical. The treated SAM was then tested for anti-proliferative activity at a concentration of 10μg/ml. Treatment with trypsin for 1h destroyed over 70% of the anti-proliferative activity, with very little activity remaining after 24h of incubation (figure 4.4.).

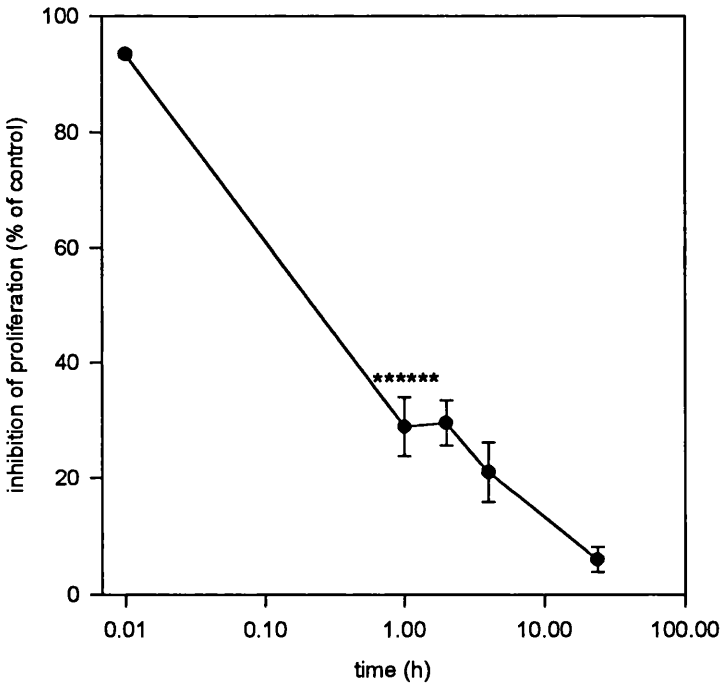


Figure 4.4. The effect of trypsin on the anti-proliferative activity of SAM from *A. actinomycetemcomitans*, measured as incorporation of [^{3}H]-thymidine into DNA by MG63 cells. Solutions of SAM were incubated with trypsin for various times and tested at a concentration of 10μg/ml. The results are expressed as mean and SD of six replicate cultures. ******$p<0.000001$.

4.2.4.3. Heat-treatment

SAM from *A. actinomycetemcomitans* was dissolved in saline and heated in water baths at various temperatures for 1h (chapter 2, section 2.8.1.). Samples were assayed for anti-proliferative activity at a concentration of 10μg/ml. At 56°C the SAM maintained its anti-proliferative activity, whilst at 78°C and 100°C, 81% and 95% respectively of the activity was lost (figure 4.5.).

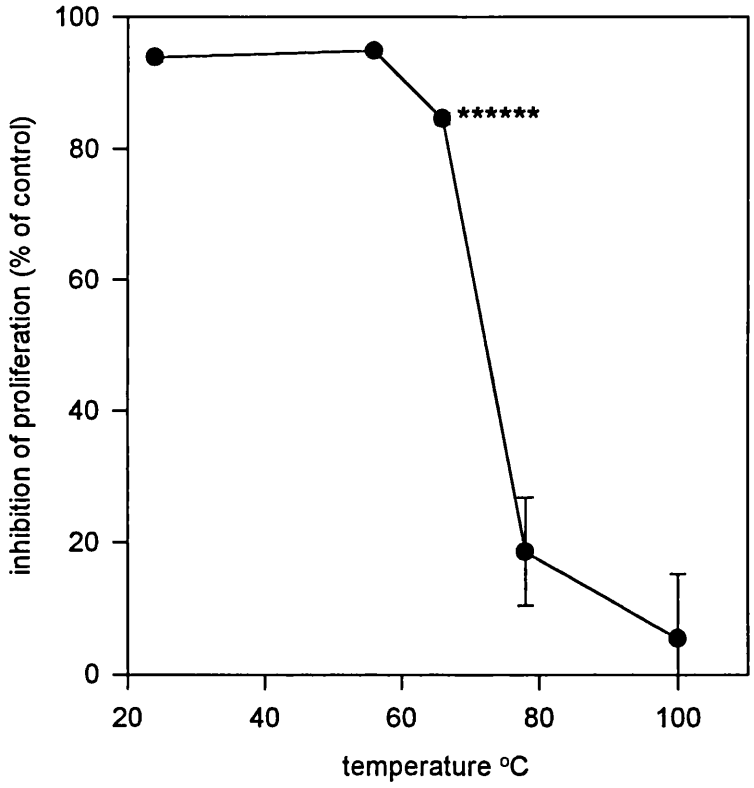


Figure 4.5. The effect of temperature on the anti-proliferative activity of SAM from *A. actinomycetemcomitans*, measured as incorporation of [^{3}H]-thymidine into DNA by MG63 cells. Solutions of SAM were heated at various temperatures for 1 h and tested at a concentration of 10μg/ml. The results are expressed as mean and SD of six replicate cultures. ******$p<0.000001$.

4.2.5. Osteolytic activity of SAM from *A. actinomycetemcomitans*

The ability of SAM from *A. actinomycetemcomitans* to cause bone destruction was tested using the mouse calvaria bone resorption assay. At 100ng/ml the SAM caused a release of 1.54 mg/dl of calcium and at 10μg/ml 2.74mg/dl of calcium were released. The lipidic material extracted from the SAM using methanol:chloroform showed no osteolytic activity even at 10μg/ml (figure 4.6.).

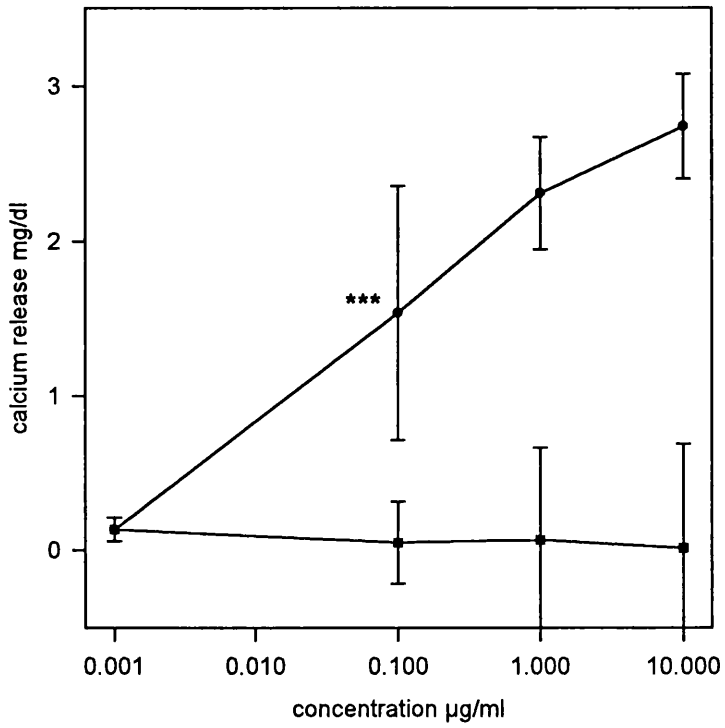


Figure 4.6. The bone resorbing activity of SAM from *A. actinomycetemcomitans* (circle) and the lipid extract of the SAM (square), measured as calcium release in mg/dl. The points represent the mean and standard deviation of five cultures. ***$p<0.001$.

4.3. Discussion

The anti-proliferative activity of SAM from *A. actinomycetemcomitans* NCTC 9710, *Por. gingivalis* W50 and *Eik. corrodens* NCTC 10596 was tested on the human osteoblast-like cell line, MG63 and human gingival fibroblasts. The SAM from *A. actinomycetemcomitans* potently inhibited the proliferation of MG63 cells and fibroblasts with IC_{50} values of approximately 0.1μg/ml and 1μg/ml, respectively. The SAM from *Eik. corrodens* was only weakly anti-proliferative against MG63 cells, although it potently inhibited the proliferation of human gingival fibroblasts with an IC_{50} value of approximately 100ng/ml. *Por. gingivalis* SAM showed anti-proliferative activity only at high concentrations with both cell types. Interestingly the SAM from *A. actinomycetemcomitans* was anti-proliferative against both cell types whilst the SAM from *Eik. corrodens* was extremely potent at inhibiting the proliferation of fibroblasts and demonstrated little anti-proliferative activity to osteoblast-like cells. These results could reflect the potent bone-modulating ability of *A. actinomycetemcomitans*, which is lacking in other periodontopathogenic bacteria.

To determine whether this anti-proliferative activity was present in other strains, the SAM was isolated from 3 strains of *A. actinomycetemcomitans* representing three serotypes, a, b and c. All strains exhibited anti-proliferative activity although serotype a was less active than b and c. SAM was extracted from *A. actinomycetemcomitans* NCTC 9710 grown on solid or in liquid culture and assayed for anti-proliferative activity. On a dry weight basis, the growth conditions did not appear to effect the anti-proliferative activity of the SAM from this organism.

Endotoxin or lipopolysaccharide (LPS) has been shown to have anti-proliferative activity (DeRenzis and Chen, 1983; Olson *et al.*, 1985; Layman and Diedrich, 1986). It was therefore important to establish that LPS contamination in the SAM was not responsible for the observed activity. Three findings suggest that

this is not the case: (i) LPS from *A. actinomycetemcomitans* was tested at 10μg/ml and at this concentration caused only 13% inhibition of proliferation (ii) the LPS content in the SAM was extremely low at 0.0001-0.001 IU/ng and (iii) The IC_{50} value of LPS on gingival fibroblasts is in the order of hundreds of μg/ml, where as the IC_{50} value of SAM is approximately 1μg/ml (Layman and Diedrich, 1986).

SAM from *A. actinomycetemcomitans* was tested on numerous cell types and lines and found to be anti-proliferative to all cells tested but with different potencies. The osteoblast-like cells MG63 and U2OS were most sensitive to the anti-proliferative effects of the SAM. Interestingly, both types of mouse cells tested were less sensitive to the inhibitory activity of the SAM than human cells tested. This could indicate that *A. actinomycetemcomitans* has honed this ability to its advantage, being more effective at inhibiting human cell growth in man, where it is more prevalent (McArthur and Clark, 1993).

The nature of the component in the SAM responsible for the anti-proliferative activity was unknown at the beginning of this study and the characterization of this activity was therefore considered a key initial step. To establish whether the activity was due to a lipidic molecule a methanol:chloroform extraction of the SAM was carried out. This extract, however, demonstrated no anti-proliferative activity. It was considered more likely that the anti-proliferative molecule was a protein. Evidence of the proteinaceous nature of the anti-proliferative activity was shown by its sensitivity to both heat- and trypsin-treatment. What advantage the bacteria gains by preventing the proliferation of osteoblasts and fibroblasts within the periodontal pocket is unclear. However, this activity could help to maintain a diseased site, whereby tissue repair processes are suppressed. This could result in the maintenance of a more conducive environment for the bacteria.

The SAM from *A. actinomycetemcomitans* was also shown to be osteolytic as well as anti-proliferative. Concentrations of 100ng/ml were able to stimulate the release of significant quantities of calcium when tested in the mouse calvaria bone resorption assay. The methanol:chloroform extract from the SAM was not active in this assay demonstrating that the activity is probably not caused by a lipidic molecule.

In conclusion, surface-associated material from *A. actinomycetemcomitans* contains potent bone-modulating activities demonstrating both anti-proliferative activity and osteolytic activity. The combination of destructive and inhibitory activities found in the extracellular layer of *A. actinomycetemcomitans* could account for the bone resorption and destruction of the periodontal ligament seen in LJP. If more information concerning the biological activities and nature of the component or components within the SAM responsible for these two activities is to be forthcoming, these components need to be isolated and characterized, this is the subject of proceeding chapters.

Chapter 5

Purification and cloning of the osteolytic component from *A. actinomycetemcomitans*

5.1. Introduction

One of the best documented associations between bacteria and periodontal disease is that of *A. actinomycetemcomitans* and LJP. Periodontal diseases are characterized by resorption of the alveolar bone which support the teeth, a process which can lead to tooth loss. The precise mechanisms responsible for the resorption of alveolar bone remain to be established although they are thought to involve soluble factors released by the bacteria. These either directly stimulate bone breakdown or induce the synthesis and release of osteolytic mediators within host tissues.

From the previous chapter, and other studies at the Eastman, the SAM from *A. actinomycetemcomitans* has been shown to have the ability to stimulate murine calvarial bone breakdown at concentrations as low as 10ng/ml (Wilson *et al.*, 1985; Meghji *et al.*, 1994). In this assay the SAM is 10-100 times more potent than the LPS prepared from the same organism (Wilson *et al.*, 1985). This chapter describes the isolation of the active component and the attempts made to clone the gene which expresses the osteolytic protein.

5.2. Results

5.2.1. Amicon Filtration

In order to obtain an estimate of the molecular mass of the osteolytic component an Amicon ultrafiltration cell was used (chapter 2, section 2.11.1). Initially 78mg of SAM (50mg of protein) was separated using a 30kDa cut-off membrane, which resulted in two fractions, one consisting of material less than 30kDa (<30kDa) and the other greater than 30kDa (>30kDa). Analysis of the <30kDa material by SDS-PAGE and silver staining, revealed eight proteins with molecular masses ranging from approximately 15kDa to 42kDa. The

fractions were lyophilized and tested at 10μg/ml for bone resorbing activity. The activity was found to reside entirely in the >30kDa fraction with no activity in the <30kDa fraction even at concentrations as high as 10μg/ml (figure 5.1).

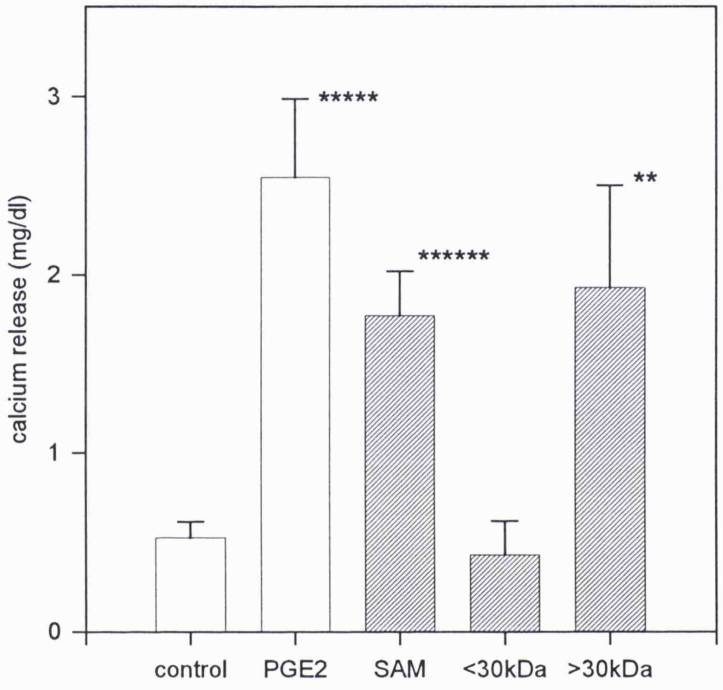


Figure 5.1. Osteolytic activity of 10μg/ml of SAM from *A. actinomycetemcomitans*, 10μg/ml of the <30kDa fraction and 10μg/ml of the >30kDa fraction, generated by Amicon filtration. The control represents the release of calcium in unstimulated cultures and PGE_2 is the positive control (1μM PGE_2). Each bar represents the mean and SD of 5 cultures. **p<0.01, *****p<0.00001, ******p<0.000001.

5.2.2. Neutralization of bone resorption by mAb P3 to *A. actinomycetemcomitans*

Work by Dr Tatsuji Nishihara and Dr Keisuke Nakashima (National Institute of Health, Tokyo, Japan) led to the development of a monoclonal antibody P3 (mAb P3) against *A. actinomycetemcomitans*. Mr Alun Kirby and Dr Sajeda Meghji tested this mAb in the bone resorption assay to determine if it would have any effect on bone breakdown induced by SAM (chapter 2, section 2.7.2.). SAM was incubated with antibody P3 and the antigen-antibody produced were immunoprecipitated with heat-killed formalin-fixed *S. aureus*. It was clear (figure 5.2.) that immunoprecipitation with antibody P3 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 concentrations of 10μg/ml. Controls in which SAM was incubated with nonspecific mouse antibody, or with *S. aureus* alone, retained activity equal to the untreated SAM.

5.2.3. Purification of the osteolytic component from *A. actinomycetemcomitans*

5.2.3.1. Q-Sepharose anion exchange chromatography

In order to isolate the osteolytic component, 400mg of SAM extracted from agar-grown bacteria were separated by Q-Sepharose anion exchange chromatography (chapter 2, section 2.11.3.2.). All fractions were Western blotted with monoclonal antibody P3 in order to identify the osteolytic component, which subsequently identified a 62kDa protein in fractions 26 to 30 (figure 5.3. panel A). Figure 5.3. panel B, shows the SDS-PAGE analysis of proteinaceous fractions 10 to 33, showing the prominent 62kDa protein band in fractions 26-33.

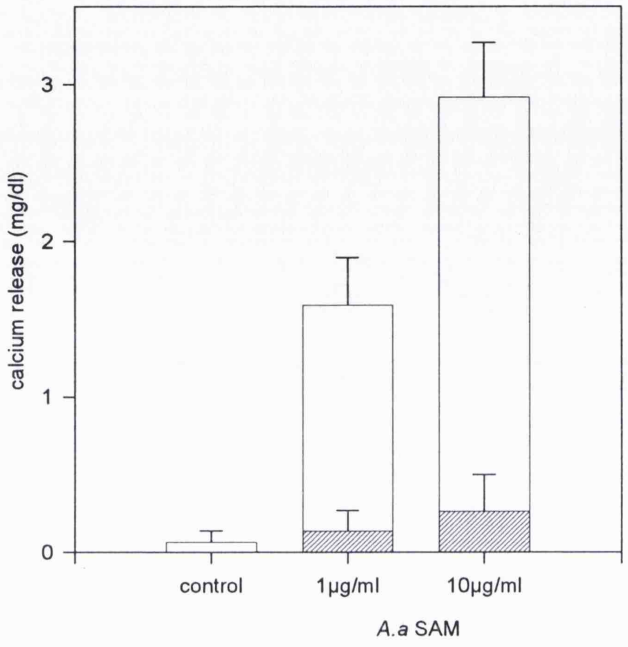


Figure 5.2. Effect of depletion of SAM, by immunoabsorption with mAb P3, on the stimulation of murine calvarial bone resorption. Cultures were exposed to 1 or 10μg/ml SAM which had either been depleted with mAb P3 (hatched bars) or sham-depleted with heat-killed *S. aureus* in the absence of P3 (open bars). Results are expressed as the mean and SD of five replicate cultures. The control shows the amount of calcium released by unstimulated calvaria.

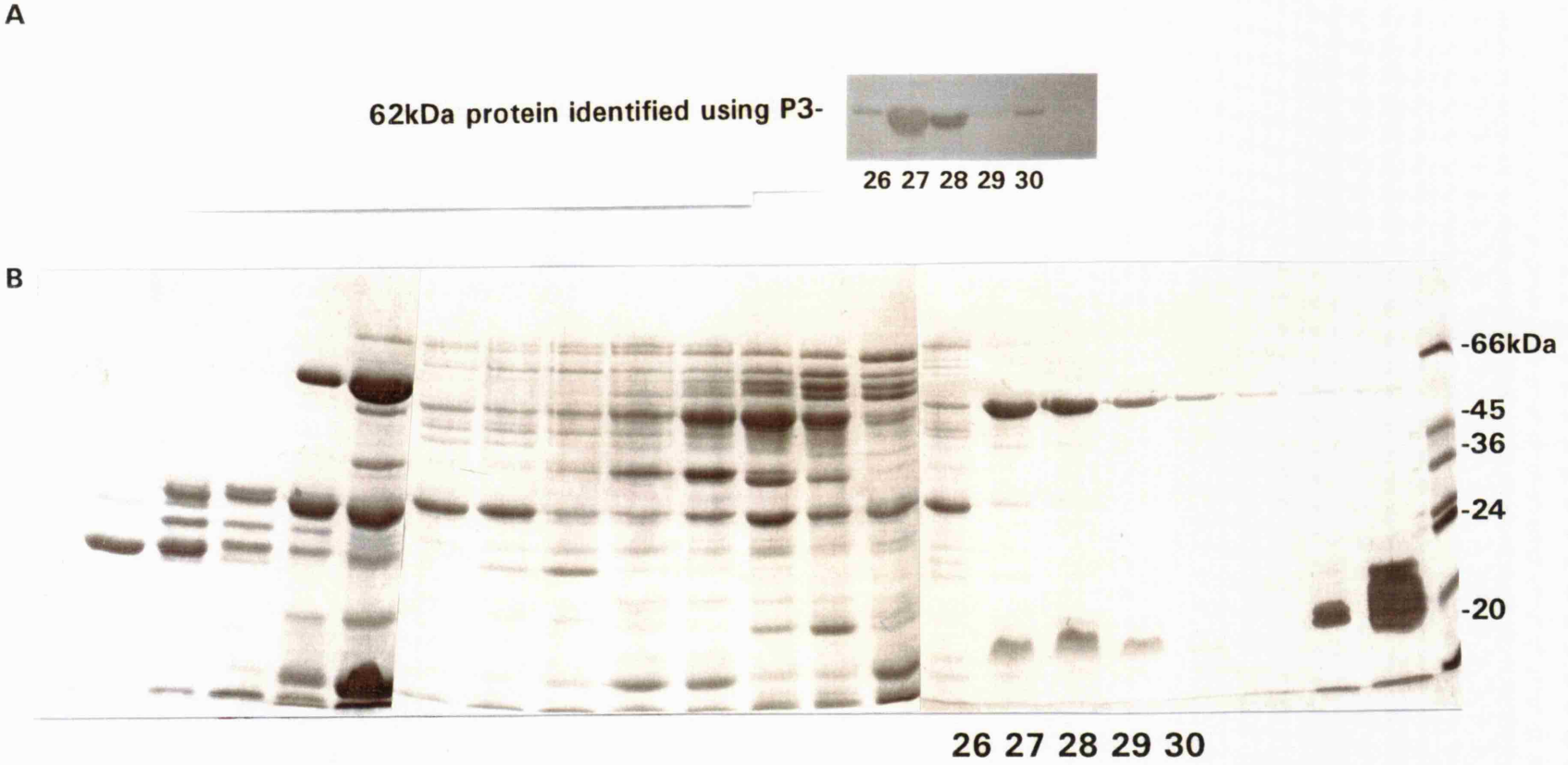


Figure 5.3. SDS-PAGE analysis and Western blot of fractions following Q-Sepharose anion exchange chromatography. Panel A, shows the 62kDa protein identified in fractions 26 to 30 using Antibody P3. Panel B, shows SDS-PAGE analysis of fractions 10-33 showing the prominent 62kDa protein band in fractions 26-33 following Coomassie blue staining.

5.2.3.2. Separation of anti-proliferative activity and osteolytic activity

In order to ascertain whether one component was responsible for the osteolytic activity and the anti-proliferative activity, or whether these activities were associated with separate molecules, all fractions were assayed for there ability to inhibit the incorporation of [^{3}H]-thymidine in to MG63 cells (chapter 2, section 2.6.2.1.). The anti-proliferative activity eluted in fractions 11 to 15, (see chapter 7, section 7.2.3.). Thus, the two bone-modulating activities eluted separately, indicating that each activity was due to a separate component.

5.2.3.3. Gel filtration chromatography

Fractions 26 to 30 were pooled and further purified using a Superdex 200 gel filtration column (15cm x 1.5cm) (chapter 2, section 2.11.4.2.). Figure 5.4. shows the SDS-PAGE analysis of fractions 19 to 36. Fractions 20 to 29 appeared to contained only the 62kDa protein.

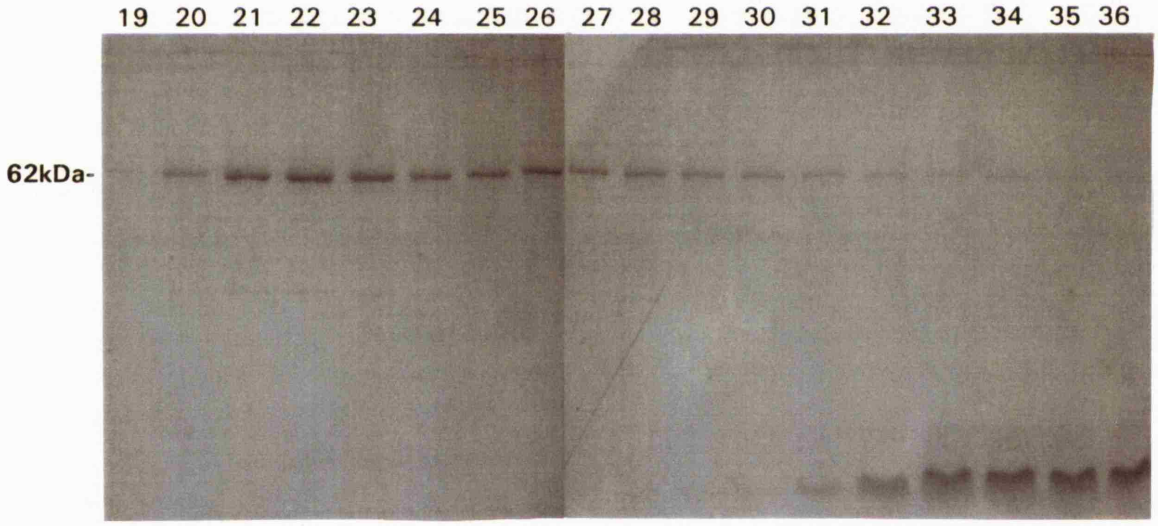


Figure 5.4. SDS-PAGE analysis of fractions 19 to 36 generated by Superdex 200 gel filtration chromatography. The 62kDa protein associated with the osteolytic activity was seen in fractions 20 to 29.

5.2.3.4. HPLC anion exchange

Fractions 20 to 29 were pooled and further purified by HPLC anion exchange using a MA7Q column, with absorbance monitored at 280nm (chapter 2, section 2.11.3.1.). The elution profile revealed the presence of two peaks, the larger of which was separated by SDS-PAGE and silver stained, revealing a single protein of 62kDa (figure 5.5). The purified protein was retested for bone resorbing activity over the concentration range 10ng/ml to 1 μg/ml and demonstrated osteolytic activity at concentrations as low as 10ng/ml.

Figure 5.5. SDS-PAGE analysis of GroEL homologue purified by HPLC anion exchange using a MA7Q column. Molecular weight markers are displayed in lane 1. The purified protein is shown in lane 2. The gel was silver-stained.

5.2.4. NH_2-terminal sequencing

NH_2-terminal sequencing of the purified 62kDa protein produced a continuous sequence of 38 amino acid residues. Comparisons using the NCBI Blast network service showed this sequence to have 100% homology to the groEL protein of *E. coli* over the first 17 amino acid residues and to differ at only 2 of the 38 residues (figure 5.6.).

		17	21
A.a "groEL"	AAKDVKFGNDARVKML	N	GVN I LADAVKVTLGPKGRNVV
E. coli groEL		R	V

Figure 5.6. NH_2-terminal amino acid sequences of *E. coli* groEL and *A. actinomycetemcomitans* groEL-like protein.

5.2.5. Cloning of the groEL homologue of *A. actinomycetemcomitans*

5.2.5.1. Use of P3 to screen a genomic library of *A. actinomycetemcomitans*

The monoclonal antibody P3 was used to screen a genomic library constructed using the cloning vector pUC18 (chapter 2, section 2.12.2.). Approximately 200 colonies were screened, however all the colonies screened gave a positive signal to this antibody.

5.2.5.2. Use of PCR to amplify the groEL-like gene from *A. actinomycetemcomitans*

An alternative method was employed in order to clone the groEL-like gene from *A. actinomycetemcomitans*. The oligonucleotide primers used in this study were as follows: The sequence for the forward primer (prime A) corresponded to the N-terminal of the groEL-like gene, calculated from the amino acid sequence of the purified *A. actinomycetemcomitans* protein. The reverse primer was synthesized for a highly conserved region of the HSP60 family of proteins (Rusanganwa *et al.*, 1992). The forward (5'-GCNGCNAARGAYGTNAARTT-3') (where N = A, C, G or T, R = A or G and Y = C or T) and the reverse (5'-TCNCCRAANCCNGGNCGYTTNACNGC-3') were made to the amino acid sequences AAKDVKF and AVKAPGFGD, respectively. For these primers to work with DNA from different species, the third codon position in these was degenerate to allow for different codon usage. Other published sequences of GroEL-like genes indicated that using these specific primers, the resulting PCR product should be approximately 762bp in size, which is essentially the first half of the gene (Hemmingsen *et al.*, 1988; Rusanganwa *et al.*, 1992).

PCR amplification was carried out as described in chapter 2, section 2.13.1. When these primers were used in the PCR experiments using *A. actinomycetemcomitans* DNA, a fragment of about 750bp was amplified along with a much smaller fragment of approximately 300bp (figure 5.7.). The larger fragment, being the putative groEL-like gene fragment, was excised from the gel, purified and subsequently subcloned into the pCR™II vector (chapter 2, section 2.13.2.) ready for sequencing. At this point of the study a paper was published by Nakano *et al.*, (1995) describing the complete sequence of the groEL-like gene from *A. actinomycetemcomitans*, this gene demonstrated 87% homology to the groEL gene from *E. coli*. Thus, my study was abandoned.

Figure 5.7. Agarose gel electrophoresis of the PCR reaction product. PCR amplification using specific primers was carried out as described in chapter 2, section 2.13.1. After 30 cycles 1/10th of the reaction products were analysed on a 1% agarose gel. Lane 1, shows the markers; lane 2 shows the PCR product using DNA from *A. actinomycetemcomitans* as a template.

5.3. Discussion

LJP is characterized by the severe and rapid loss of alveolar bone on the approximal surfaces of the first molar and/or incisor teeth and is generally associated with the presence of *A. actinomycetemcomitans* (Slots and Genco 1984; Zambon, 1985). The previous chapter demonstrated that the SAM from this organism contained potent osteolytic activity at concentrations as low as 100ng/ml and Amicon filtration suggested that the activity was associated with a component with a molecular mass greater than 30kDa.

Previously, work at the Eastman had shown that sera from a proportion of patients with LJP could block the osteolytic activity of the SAM from *A. actinomycetemcomitans* (Meghji *et al.*, 1993). We therefore tested a range of monoclonal antibodies, and found that one antibody, designated P3, raised to whole cells of *A. actinomycetemcomitans*, was able to neutralize SAM-induced bone breakdown directly and remove activity by immunoabsorption. In contrast, nonspecific mouse antibody had no inhibitory or adsorptive activity. Antibody P3 was subsequently used to identify the osteolytic molecule, by fractionating the SAM using Q-sepharose anion exchange chromatography and Western blotting all resultant fractions. This experiment demonstrated that mAb P3 recognized a 62kDa protein located in fractions 26 to 30. The 62kDa protein was purified to homogeneity, using gel filtration and HPLC anion exchange chromatography, as assessed by silver-staining. NH_2-terminal sequencing showed that of the first 38 residues identified, 36 were identical to that of the *E. coli* chaperonin protein 60 (cpn60) termed groEL.

This study demonstrates that the surface-associated material from the oral bacterium *A. actinomycetemcomitans* contains a homologue of the *E. coli* chaperonin 60 or heat shock protein, groEL, and that this protein is responsible for the potent bone resorbing activity of this material. 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, this study has shown that it is rapidly removed from the cells by gentle washing in isotonic saline.

E. coli groEL has also been shown to be osteolytic using the mouse calvarial bone resorption assay (Kirby *et al.*, 1995) and has more recently been shown to stimulate rat osteoclasts to cause resorption of dentine slices at concentrations as low as 1ng/ml (Reddi *et al.*, 1996). Other molecular chaperones have now been shown to exhibit osteolytic activity, these include

members of the mammalian molecular chaperone families, hsp90, hsp70 and hsp27, with the exception of the hsp47 (Nair *et al.*, 1996).

The cloning of the chaperonin 60 gene from *A. actinomycetemcomitans* was attempted using two approaches. Initially P3 was used to immunoscreen a genomic library constructed in pUC18. At first, it came as a great surprise that all colonies screened gave a positive signal. However, the subsequent purification and N-terminal sequence analysis identified the 62kDa protein as a member of the heat shock family of proteins, with 95% homology to *E. coli* groEL over the first 38 amino acid residues. Thus, during the immunoscreening, P3 was recognizing the *E.coli* protein rather than a recombinant *A. actinomycetemcomitans* protein. Therefore a PCR based cloning approach was attempted, based on the N-terminal information provided. This resulted in the amplification of a 750bp fragment and a much smaller fragment, which was probably due to non-specific amplification. The putative GroEL-like gene fragment was sub-cloned into the pCRII vector ready for sequencing. Unfortunately at this time a paper was published by Nakano *et al.*, (1995) describing the complete sequence of the GroEL-like gene from *A. actinomycetemcomitans*, this gene demonstrated 87% homology to the GroEL gene from *E. coli*.

The members of the heat shock protein 60 (hsp60) family of proteins are ubiquitously present in all species ranging from bacteria and plants to humans. As well as playing an important "molecular chaperone" function in the proper folding of polypeptide chains, their assembly into oligomeric proteins, and in the intracellular transport of various proteins to various locations (Hemmingsen *et al.*, 1988), it has become clear that these proteins are immunodominant antigens of numerous pathogenic bacteria. These include *Mycobacterium leprae, Coxiella burnetti, Trepenoma paladium, Legionella pneumophilia, Chlamydia trachomatis, Borrelia burgdorferi,* etc. responsible for many of the common infectious diseases (e.g. tuberculosis, leprosy, Q fever, syphilis,

Legionnaires disease, Lyme disease, etc.) (Young, 1992; Shinnick, 1991). In view of their strongly antigenic nature and high degree of sequence conservation, antigenic mimicry between the bacterial HSP60 and its human homologue could possibly elicit an autoimmune response in the infected host. Indeed autoimmune response to bacterial HSP60 has been suspected to play an important role in the pathogenesis of immune arthritis and insulin dependent diabetes in both human and animal studies (Minowada and Welch, 1990). The strong antigenic nature of these molecules, perhaps, indicates that, under the right circumstances, they could play a much larger role in infections than originally thought. Considering the present study, it is advantageous to a host to raise neutralizing antibodies against potentially destructive molecules expressed by infecting bacteria. The sinister activity of what would appear to be a harmless molecule, cunningly diguised as an evolutionarily conserved protein, raises questions to their role in other infections.

Chapter 6

Neutralization of the anti-proliferative activity of SAM using sera from LJP patients

6.1. Introduction

A significant amount of work over the past two decades has concentrated on the antibody response to infecting microorganisms both in humans with periodontal diseases and in animal models of periodontal disease. It is well established that individuals with LJP and other forms of periodontitis such as adult periodontitis have elevated levels of serum antibodies to *A. actinomycetemcomitans* (Ebersole *et al.*, 1987 and 1991; Gunsolley *et al.* 1987; Zafiropoulos *et al.*, 1992; Saito *et al.*, 1993; Ebersole and Cappelli, 1994; Mooney and Kinane, 1994). Work at the Eastman Dental Institute has also demonstrated that the sera of patients with LJP contain high levels of antibodies to the surface-associated material from this organism (Meghji *et al.*, 1993, 1995). The role that antibodies play in the course of this disease, however, remains unclear. Antibodies which block a functional characteristic, a virulence factor, or render the bacteria susceptible to leucocyte-mediated killing would inhibit its ability to damage the host. For example an anti-fimbrial antibody could prevent adherence and colonization, or an antibody against the amino acids in the biologically-active portion of a toxin may either neutralize it directly or inhibit its binding to its cell surface receptor.

Therefore, the aim of this study was to determine whether the anti-proliferative activity of SAM could be neutralized by antibodies present in the sera of patients with LJP.

6.2. Results

6.2.1. Serum antibody titres

Sixteen patients diagnosed as having LJP by standard criteria, including radiographic evidence of bone loss and first permanent molar or incisor pocket depths of 5mm or more and 15 individuals, judged to be periodontally normal, were assessed for titres of anti-SAM antibodies on microtitre plates coated with SAM (chapter 2, section 2.10.2.). The titres of these sera and 15 representative controls are shown in figure 6.1. Patients with LJP had significantly higher titres (median of 5,350), compared to that of the control sera (median of 620), ($p<0.001$ using Wilcoxon's rank sum test).

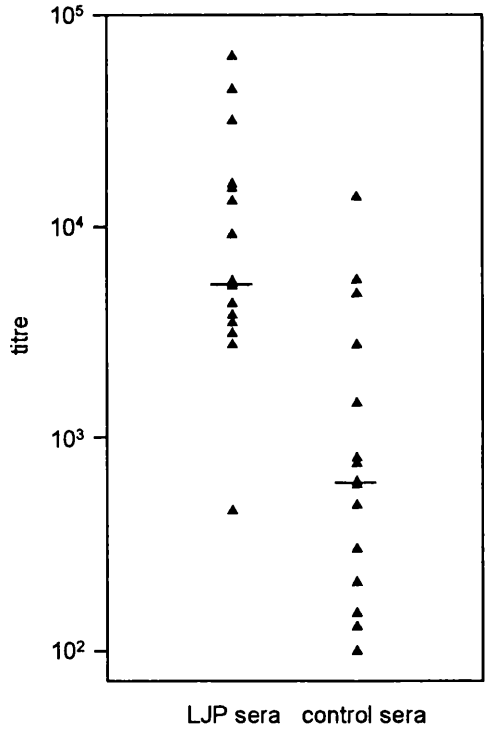


Figure 6.1. Range of IgG antibody titres to *A. actinomycetemcomitans* SAM in the sera of individuals with localized juvenile periodontitis (LJP) or in individuals with no evidence of periodontal disease. Horizontal bars indicate the median antibody titres. The difference in the median values was statistically significant, ($p<0.001$ using Wilcoxon's Rank sum test).

6.2.2. Inhibition of anti-proliferative activity by human sera

Neither patients sera nor sera from controls had any effect on the proliferation of MG63 cells when tested at a 1:50 dilution. Sera from 16 patients with LJP were added at a 1:50 or 1:500 dilution to MG63 cells incubated in the presence of 500ng/ml of SAM to determine if they could neutralize the anti-proliferative activity of the surface-associated protein/s (chapter 2, section 2.6.2.2.). At 1:50 dilutions, 9 of the 16 sera significantly blocked the anti-proliferative activity ($p < 0.0001$, using students T-test) and sera from patients 7 and 10 almost completely neutralized this activity (figure 6.2.).

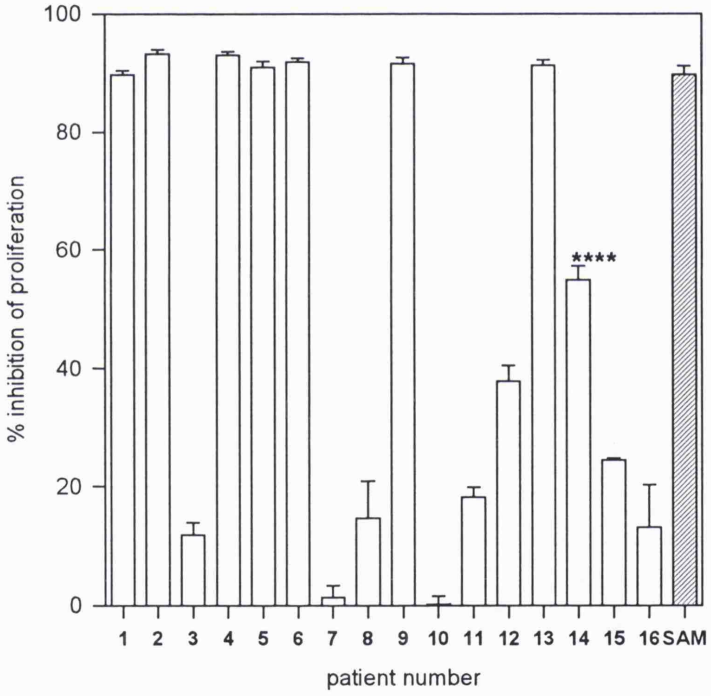


Figure 6.2. The influence of LJP sera, used at a 1:50 dilution, on the anti-proliferative activity of SAM from *A. actinomycetemcomitans* (incubated with cells at a concentration of 500ng/ml). Activity is measured as percentage inhibition of [^{3}H]-thymidine incorporation relative to control cultures to which SAM was not added. The final column on the right shows the effect of SAM when no serum has been added. The patient with the lowest titre of antibody against the whole SAM is shown on the left (patient 1) and highest titre patient on the right (patient 16). Results are expressed as the mean and SD of six replicate cultures. ****$p < 0.0001$.

At 1:500 dilutions, 5 patients (patients 3, 7, 8, 10 and 15) were still able to significantly block the anti-proliferative activity of the SAM ($p<0.001$) (figure 6.3.).

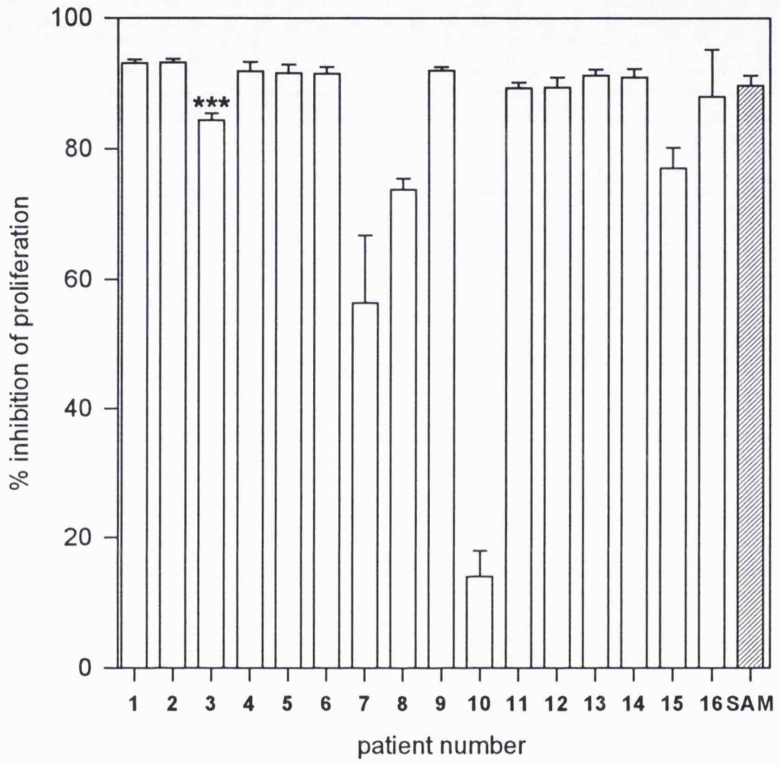


Figure 6.3. The influence of LJP sera, used at a 1:500 dilution, on the anti-proliferative activity of SAM from *A. actinomycetemcomitans* (incubated with cells at a concentration of 500ng/ml). Activity is measured as percentage inhibition of [^{3}H]-thymidine incorporation relative to control cultures to which SAM was not added. The final column on the right shows the effect of SAM when no serum has been added. The patient with the lowest titre of antibody against the whole SAM is shown on the left (patient 1) and highest titre patient on the right (patient 16). Results are expressed as the mean and SD of six replicate cultures. ***$p<0.001$.

High concentrations (1:50 dilutions) of sera from 15 individuals with no evidence of periodontal disease had no effect on the ability of the SAM to inhibit cell proliferation (figure 6.4.).

Serum from the patient which most potently blocked the anti-proliferative activity of SAM (patient 10) was tested at various concentrations to establish whether the effect was concentration-dependent (figure 6.5.). This graph shows that serum from a control, disease-free, volunteer was unable to block the anti-proliferative activity of 500ng/ml of SAM. In contrast patient 10, who had an antibody titre of 1:9,200, was capable of inhibiting this anti-proliferative activity in a concentration-dependent manner.

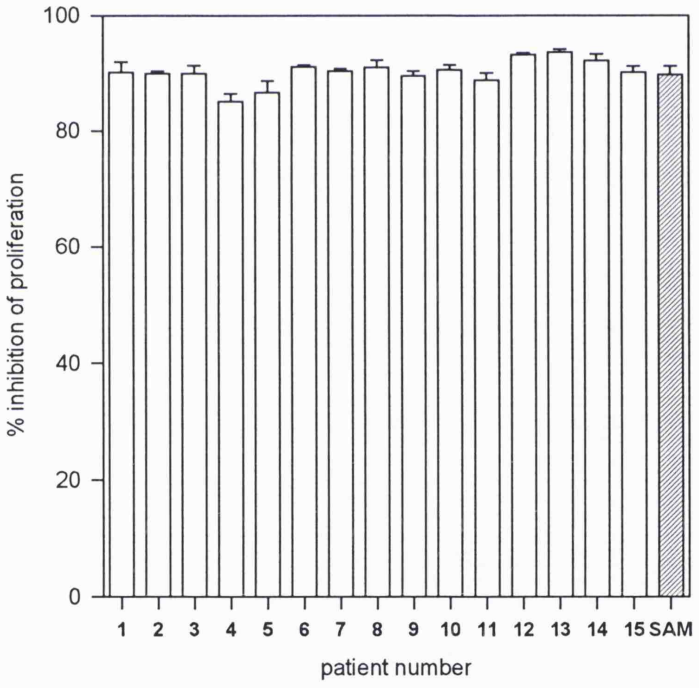


Figure 6.4. The influence of 15 control sera, used at a 1:50 dilution, on the anti-proliferative activity of SAM from *A. actinomycetemcomitans* (incubated with cells at a concentration of 500ng/ml). Activity is measured as percentage inhibition of [^{3}H]-thymidine incorporation against control cultures. The final column on the right shows the effect of SAM when no serum has been added. Results are expressed as the mean and SD of six replicate cultures.

From the previous chapter monoclonal antibody P3 was able to block the osteolytic activity of the SAM (chapter 5, section 5.2.2.). It was therefore tested for its ability to neutralize the anti-proliferative activity of the SAM, however mAb P3 demonstrated no neutralizing activity in this experiment.

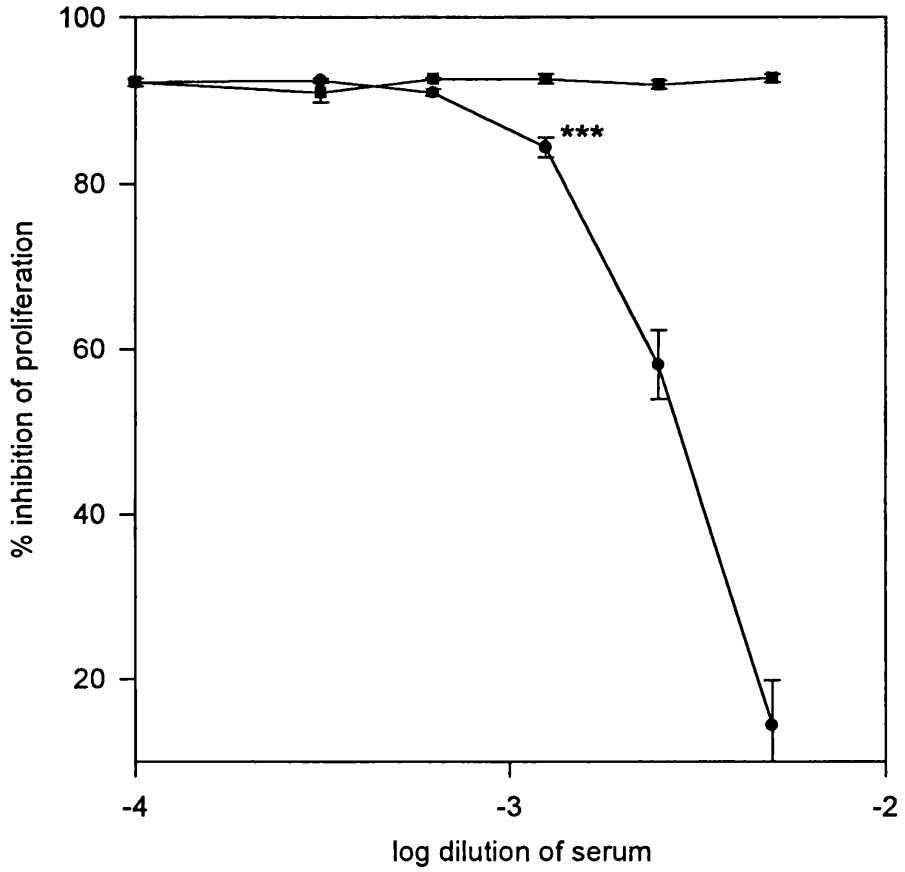


Figure 6.5. Titration curves showing the effect of serum from patient 10 on the anti-proliferative activity of 500ng/ml SAM from *A. actinomycetemcomitans* on MG63 cells. This graph shows that control serum from an individual with no evidence of periodontal disease was unable to block the anti-proliferative activity (square). In contrast serum from a patient with LJP (circle) was capable of inhibiting the anti-proliferative activity of the SAM in a concentration-dependent manner. Results are expressed as the mean and SD of three replicate cultures. ***$p<0.001$.

6.2.3. Depletion of serum antibody using protein A

Removal of antibodies from the serum of patient 10 using protein-A-Sepharose (chapter 2, section 2.10.4.) decreased the neutralizing ability after each treatment, reaching control levels (no neutralizing ability) after the third adsorption (figure 6.6.). Protein-A-Sepharose alone was not anti-proliferative, nor did it affect the anti-proliferative activity of the SAM.

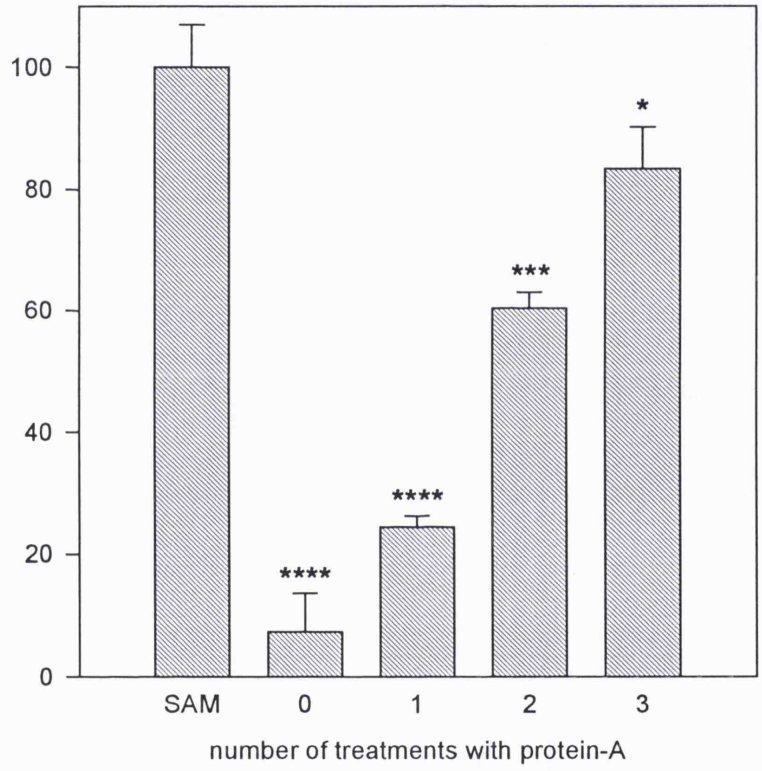


Figure 6.6. The effect of sequentially adsorbing serum from patient 10 with protein-A-Sepharose (to remove antibody) on the serum-mediated inhibition of the anti-proliferative activity of the SAM. Column 1 (SAM) is the normalized percentage inhibition of MG63 proliferation induced by SAM (500ng/ml). Column 0 is SAM plus unadsorbed serum. The remaining three columns show the inhibitory activity remaining after 1, 2 or 3 adsorptions of the serum with protein-A. Results are expressed as the mean and SD of three replicate cultures. *$p<0.05$, ***$p<0.001$, ****$p<0.0001$.

6.2.4. Western immunoblotting of sera

Western immunoblot analysis of SDS-PAGE-separated SAM from *A. actinomycetemcomitans* was carried out using the sera from all 16 LJP patients (chapter 2, section 2.10.3.). Antibodies bound to a large number of the proteins ranging in molecular mass from >66kDa to <14kDa. A representative immunoblot from patient 7 (figure 6.7.) shows the pattern of antibody binding. Both neutralizing and non-neutralizing sera showed a similar pattern of IgG binding antibodies with dominant bands at 16, 24 and 29kDa, and other prominent bands at approximately 34 and 39kDa.

Figure 6.7. 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 weights of various markers are displayed on the left-hand side.

6.3. Discussion

The present study confirms earlier findings (Meghji *et al.*, 1993, 1995) that the SAM from *A. actinomycetemcomitans* is strongly immunogenic and high titres of antibodies to this extract are present in the blood of patients with LJP. Of the sixteen patients with LJP tested in this study, the mean titre against *A. actinomycetemcomitans* SAM was nearly ten fold higher than that of control patients, 1:5,350 compared to 1:620. The role and effectiveness in overcoming disease, of antibodies is not fully established. Generally, the outcome of the antibody response against infecting bacteria results in erradication of the bacteria and protection against future infection. Historically those people who survived infectious diseases were seldom re-infected. In periodontal diseases the bacteria seem able to persist and survive the humoral response, why this occurs remains obscure. There is some evidence in periodontal diseases that antibodies help in reducing the severity of the infection. It has been shown that antibodies against *A. actinomycetemcomitans* are necessary for polymorphonuclear leucocytes (PMN) to most efficiently phagocytose and kill the organisms (Baker and Wilson, 1989). Whilst Zambon (1985) hypothesised that increased antibodies against *A. actinomycetemcomitans* in LJP tended to keep the disease localized. Studies by other workers have shown a relationship between reduced severity of the disease with an increase in antibody titre (Gunsolley *et al.*, 1987; Mouton *et al.*, 1987; Kinane *et al.*, 1993). Recently investigators have tried to show the protective nature of anti-pathogen antibodies by using animal models of periodontal diseases (reviewed by McArthur, 1993; Ebersole and Taubman, 1994). Evidence from these experiments suggests that antibodies to the suspected pathogens may inhibit bacterial colonization, tissue invasion, bacterial proliferation, as well as alveolar bone loss.

The anti-proliferative activity found in the SAM from *A. actinomycetemcomitans* may play a role in the pathology of LJP by preventing the replication of cells in the alveolar bone and periodontal ligament, thus causing a decrease in the rate of replacement of these labile tissues (Page and Ammons, 1974). The consequence of such inhibition of cellular proliferation could be the loss of connective tissue matrices and would therefore be equivalent to a loss of matrices by destructive processes, as is generally assumed to occur. Neutralization of the anti-proliferative activity by sera from patients with LJP could therefore be of benefit to the patient, resulting in a less destructive form of the disease. The ability of serum to neutralize the anti-proliferative activity of SAM was tested using sera from 16 patients suffering from LJP. At a dilution of 1:50, nine of the 16 sera could significantly block the anti-proliferative action of SAM ($p<0.0001$) and, at 1:500 dilution, 5 sera were still able to significantly block activity ($p<0.001$). The remaining sera, and sera from 15 individuals with no evidence of periodontal disease, failed to inhibit the anti-proliferative activity. There was no relationship between the levels of antibody to SAM and the neutralizing ability of the sera. This shows that although a patient may have an excellent antibody response against the SAM of infecting bacteria, he/she is not necessarily better protected against individual virulence proteins. A key question was whether the neutralizing activity of the sera was due to antibodies. This was shown to be so by the fact that 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 attributable to antibodies.

Western blot analysis showed that IgG antibodies in patients' sera reacted with many of the components in the SAM, the approximate molecular masses of the most prominent bands were 16, 24, 29, 34 and 39kDa. These results were similar with other studies, for instance, immunoblotting studies by Bolstad *et al.* (1990) revealed three major outer membrane antigens of *A. actinomycetemcomitans* with molecular masses of 16.5, 29 and 34kDa.

Furthermore, studies by Muller *et al.* (1990) identified four outer membrane proteins from *A. actinomycetemcomitans* with molecular masses of 30, 34, 36 and 39kDa. No differences in the binding pattern could be detected when neutralizing sera were compared with non-neutralizing sera. This polyclonal response to the many components in the SAM is presumably why there was no relationship between antibody titre to SAM and neutralizing capacity of the sera.

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. Work by my colleagues at the Eastman, has shown that 4 out of 6 high titre sera, to the SAM of *A. actinomycetemcomitans* from patients with LJP, could block the bone resorbing activity of the SAM from this organism (Meghji *et al.*, 1993). Tsai *et al.*, (1981) showed that 90% of sera from patients with juvenile periodontitis neutralized the leucotoxic activity of sonicated extracts of *A. actinomycetemcomitans*, whereas most sera from periodontally-healthy individuals and patients with adult periodontitis had no such activity. This could explain why an increase in antibodies to *A. actinomycetemcomitans* helps in the PMN-mediated killing of this organism (Baker and Wilson, 1989), as the antibodies may protect the PMNs from the leucotoxin produced by *A. actinomycetemcomitans*. Taichman *et al.*, (1984) reported that a bacterial sonicate of *A. actinomycetemcomitans* was capable of inhibiting endothelial cell growth at a concentration of 10μg/ml. They found that monoclonal antibodies which inhibited the leucotoxin from *A. actinomycetemcomitans* could not neutralize the endothelial cell inhibitor, but sera from patients with juvenile periodontitis could. Antibody neutralization of cell growth inhibition has also been reported by McAnally and Levine (1993), who found that the inhibition of HL60 cell growth by plaque was blocked by monoclonal antibodies raised against plaque extracts. The monoclonal antibodies were recognized by a range of bacteria, however, *A. actinomycetemcomitans* was not included in this group.

Neutralization of bacterial toxins by sera has also been looked at outside the context of periodontal disease. *Helicobacter pylori* infection is now recognized as the predominant cause of chronic gastritis in humans, and is strongly linked to peptic ulcer disease (Petersen, 1991). Cover *et al.* (1992) investigated the neutralizing effect of sera on the activity of an 87kDa vacuolating cytotoxin produced by *H. pylori.* They showed that sera from patients infected with *H. pylori* had a significantly higher neutralizing effect on the activity of the purified cytotoxin than uninfected patients sera.

These studies point to the fact that, whether beneficial or otherwise, bacterial proteins which affect mammalian cell growth can be neutralized by sera from patients who have the opportunity to raise antibodies against them. Certainly, common sense would suggest that neutralizing antibodies are likely to be more useful than harmful, as the host fight against infection continues against the infecting bacteria.

Chapter 7
Cloning of surface antigens from *A. actinomycetemcomitans*

7.1. Introduction

Surface structures of *A. actinomycetemcomitans* are likely to be involved in the colonization of the gingival pocket and in confering resistance to phagocytosis. Furthermore, the surface-associated material contains virulence factors which are anti-proliferative (Kamin *et al.*, 1986; Meghji *et al.*, 1992b), osteolytic (Wilson *et al.*, 1985 and Meghji *et al.*, 1994) and induce cytokine gene expression (Henderson and Wilson, 1995; Reddi *et al.*, 1995c)

A major hindrance in defining these factors is the inability to obtain them in sufficient quantities for analysis. Recombinant DNA technology not only offers a means of overcoming this problem but can also provide useful information about the cloned protein for example: (i) the boundaries of the gene can be defined; (ii) the DNA base sequence and subsequently the protein's amino acid sequence can be determined and; (iii) the cloned gene can be used as a probe and the gene can be modified.

From the previous chapter, and from other studies, it is well established that individuals with LJP have elevated serum antibodies to *A. actinomycetemcomitans* and its surface-associated material (SAM) (Ebersole *et al.*, 1991; Sim *et al.*, 1991; Saito *et al.*, 1993; Meghji *et al.*, 1995). The finding that the anti-proliferative activity can be blocked using sera from a proportion of patients with LJP led to the idea that serum from patients with LJP could be used to screen recombinant colonies.

The aim of this study was therefore to utilise the serum from a patient with severe LJP to screen a genomic library of *A. actinomycetemcomitans* with a view to cloning and expressing genes encoding immunogenic proteins and, in particular the anti-proliferative protein from *A. actinomycetemcomitans*.

7.2. Materials and Methods

Essentially, the methods used in this study are described in detail in the methods section. Figures 7.1. and 7.2. show schematic representations of the *A. actinomycetemcomitans* genomic library construction and the screening procedure developed for this study.

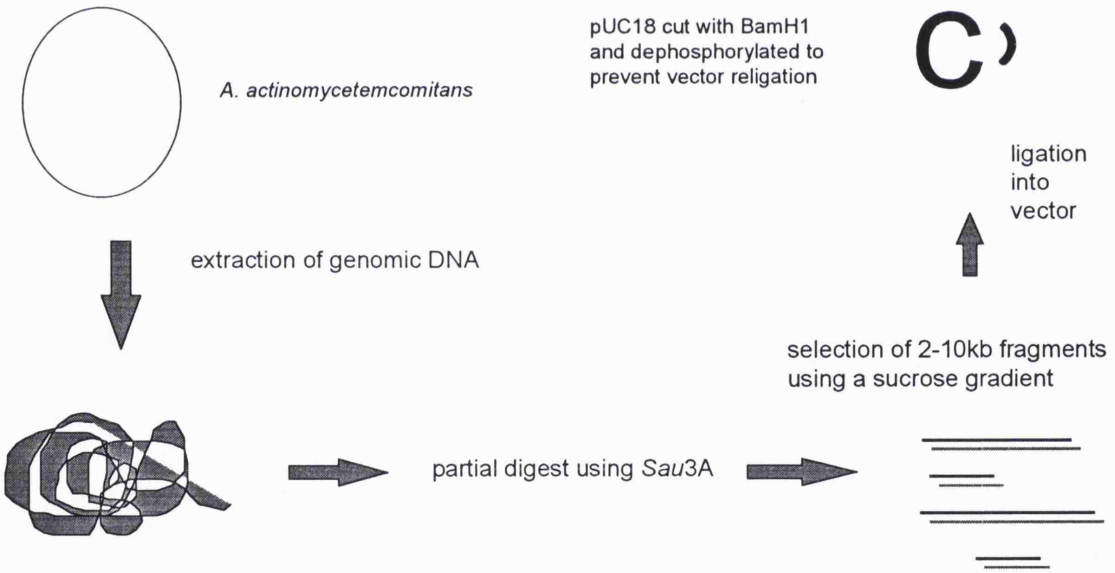


Figure 7.1. This diagram shows the construction of the genomic library. DNA was extracted from *A. actinomycetemcomitans* using phenol:chloroform, the DNA was partially digested using the restriction enzyme *Sau*3A and size fractionated using a 0-40% sucrose gradient. DNA in the size range 2-10 kb was ligated into the cloning vector pUC18, which had previously been cut with *Bam*H1 and dephosphorylated to prevent vector-to-vector ligation. For details see chapter 2, sections 2.12.1 - 2.12.3.

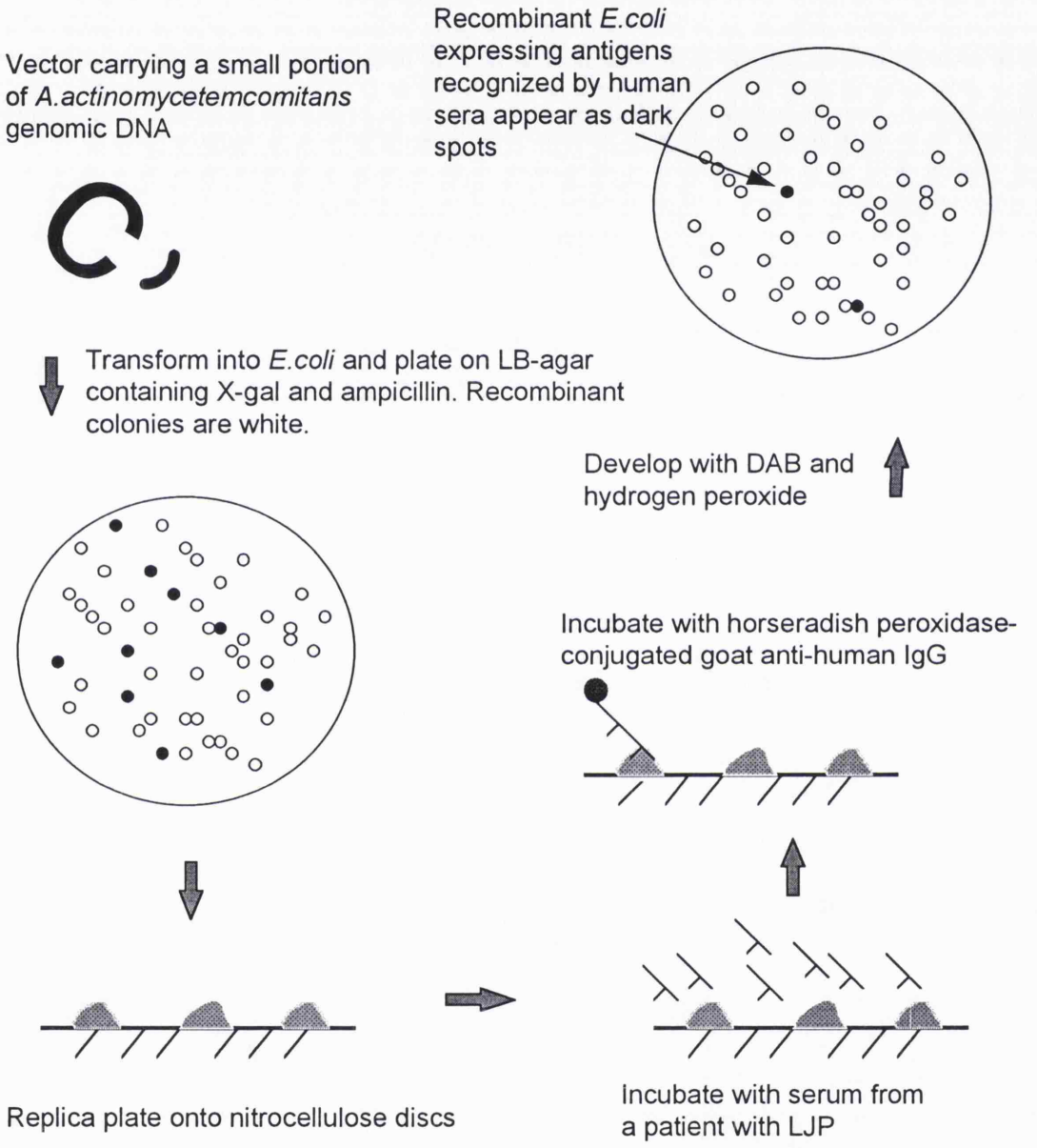


Figure 7.2. This diagram shows the screening procedure developed to identify clones expressing antigens from *A. actinomycetemcomitans*. The ligation reaction was transformed into *E. coli* and the bacteria plated on LB agar containing X-gal and ampicillin. White colonies contain recombinant plasmids. Colony lifts transfered the bacteria to membranes where they were washed, blocked using milk powder and incubated with serum from a patient with LJP. An enzyme labelled secondary antibody was used to identify colonies expressing antigenic proteins. For details see chapter 2, section 2.12.4.

7.3. Results

7.3.1. Immunoscreening of genomic library

Approximately 1000 *E. coli* transformants were screened for expression of *A. actinomycetemcomitans* antigens. 32 colonies reacted positively to the serum from a LJP patient (patient 7, see chapter 6) with on the initial screen. All 32 colonies which reacted positively were subcultured and screened for a second time, rescreening identified 3 colonies which reacted positively; clones **T**, **B** and **L** (figure 7.3.). All three clones remained positive when screened in the absence of IPTG.

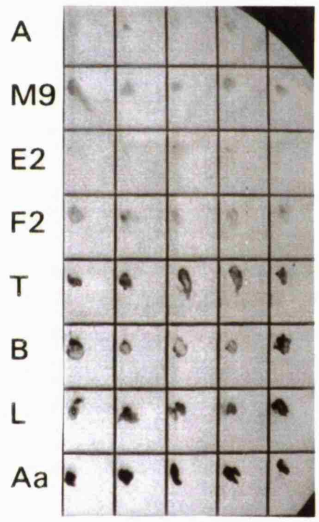


Figure 7.3. Rescreening for expression of *A. actinomycetemcomitans* antigens using a colony immunoblotting method. Colonies were dotted 5 times and screened using serum from a patient with localized juvenile periodontitis (LJP). Lane **A** and **M9** are controls-*E. coli* containing pUC18; lane **E2** and **F2** are recombinant colonies which when rescreened, show a negative response to the serum; lanes **T**, **B**, and **L** are recombinant colonies, which maintain a positive reaction on the second screen; lane **Aa** shows a positive response from *A. actinomycetemcomitans*

7.3.2. SDS-PAGE analysis of recombinant clones

Whole cell lysates of the three recombinant bacteria were prepared, separated by SDS-PAGE on 12% gels and stained with Coomassie blue (figure 7.4.) (chapter 2, section 2.12.7.1.). The gel shows the surface-associated material from *A. actinomycetemcomitans,* lysates of the three recombinant *E. coli* and a control (*E. coli* with pUC18). There was no clear indication of a recombinant protein in any of the clones.

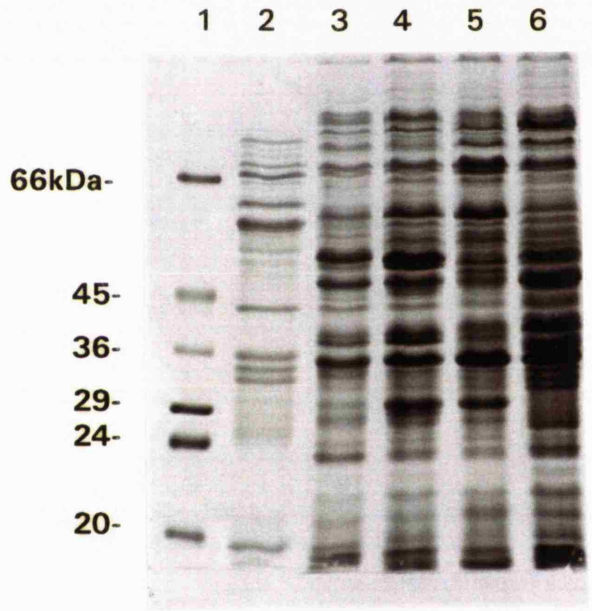


Figure 7.4. SDS-PAGE analysis of lysates of recombinant *E. coli*. Whole-cell lysates of reactive clones were electrophoresed on 12% gels and stained with Coomassie blue. Lane 1, molecular mass markers; lane 2, SAM from *A. actinomycetemcomitans*; lane 3, *E. coli* clone **L**; lane 4, *E. coli* clone **T**; lane 5, *E. coli* clone **B**; lane 6, *E. coli* control with pUC18.

7.3.3. Western blot analysis of recombinant clones

Initial analysis of the three clones by Western blotting (chapter 2, section 2.10.3) revealed that clone **L**, (which harboured plasmid designated pAAL91) contained a recombinant protein with a molecular weight of approximately

34kDa, which was not present in the *E. coli* control lysate. The protein could be detected using serum from a patient with LJP, however the presence of *E. coli* proteins which cross reacted with the patients serum caused a high background (figure 7.5.). The two other recombinant *E. coli,* **B** and **T**, showed no reactive protein bands which differed from the control lysate. In order to remove the cross reactivity to increase the sensitivity of the Western blot, the serum was pre-absorbed for 1h with whole cell *E. coli* lysate. This reduced the cross reactivity, hence increasing the intensity of the 34kDa recombinant protein band (figure 7.6.).

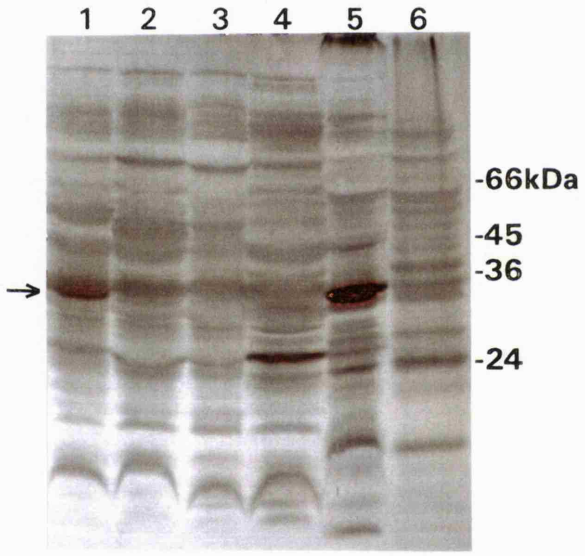


Figure 7.5. Western transfer analysis of lysates of recombinant *E. coli*. Immunoblots were probed with serum from a patient with LJP. Lane 1, *E. coli* clone **L**; lane 2, *E. coli* clone **T**; lane 3 *E. coli* clone **B**; lane 4, *E. coli* control with pUC18; lane 5, *A. actinomycetemcomitans* whole cell lysate; lane 6, *A. actinomycetemcomitans* SAM. Molecular mass markers are displayed on the right-hand side. The recombinant protein is indicated by the arrow.

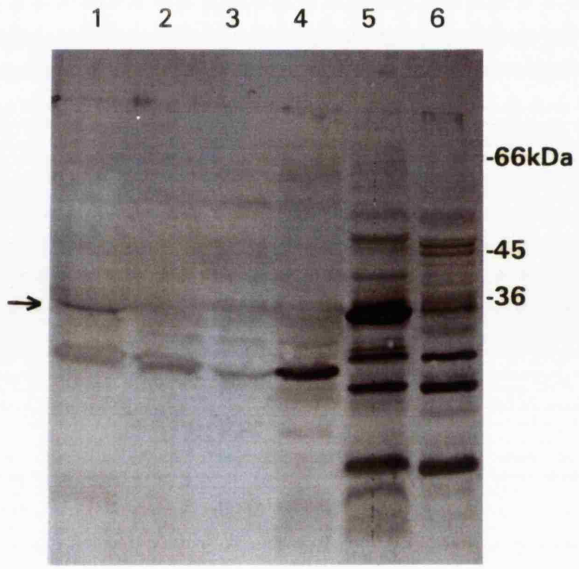


Figure 7.6. Western transfer analysis of lysates of recombinant *E. coli* following extensive pre-absorption of serum with the *E. coli* lysate to remove cross-reactivity. Immunoblots were probed with serum from a patient with LJP. Lane 1, *E. coli* clone **L**; lane 2, *E. coli* clone **T**; lane 3 *E. coli* clone **B**; lane 4, *E. coli* control with pUC18; lane 5, *A. actinomycetemcomitans* whole cell lysate; lane 6, *A. actinomycetemcomitans* SAM. Molecular mass markers are displayed on the right-hand side. The recombinant protein is indicated by the arrow.

Clones **B** and **T** did not reveal any recombinant proteins even after extensive pre-absorbtion with the *E. coli* lysate. Interestingly, from both Western blots there is a distinctive band with a molecular weight of approximately 34 kDa present in the *A. actinomycetemcomitans* whole cell lysate but absent in the SAM.

7.3.4. Restriction analysis of recombinant clones

All plasmids were cut with *Hind* III (figure 7.7.) to assess the size of the insert (chapter 2, section 2.12.5-2.12.6.). Clones **B,T** and **L** harboured plasmids with inserts of 370bp, 2650bp and 3700bp respectively. Plasmid pAAL91 was digested with a range of restriction endonucleases to produce a restriction map. Figure 7.8. shows the DNA fragments separated by agarose gel electrophoresis (chapter 2, section 2.12.7.2.). The fragment contained restriction sites for the enzymes *Eco*R1 and *Sac*1. Figure 7.9. shows the restriction map.

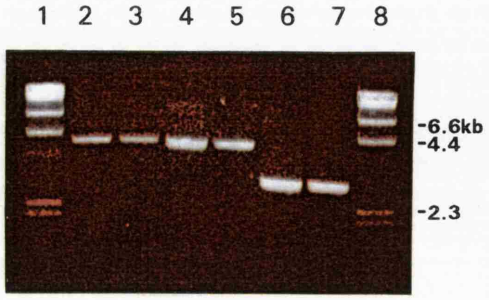


Figure 7.7. Restriction endonuclease analysis of plasmids isolated from recombinant *E. coli* . Lanes 1 and 8 lambda cut with *Hind* III; lanes 2 and 3, plasmid from clone **L** cut with *Hind* III; lane 4 and 5, plasmid from clone **T** cut with *Hind* III; lanes 6 and 7, plasmid from clone **B** cut with *Hind* III.

7.3.5. Anti-proliferative activity of recombinant *E. coli*

The recombinant bacteria were grown on solid media, harvested using saline and subjected to a saline wash to extract the surface-associated material (SAM) (chapter 2, section 2.2.1.). The SAM was dialysed, lyophilized and tested for anti-proliferative activity. All three recombinants and a normal *E. coli* control showed no anti-proliferative activity (chapter 2, section 2.6.2.1.).

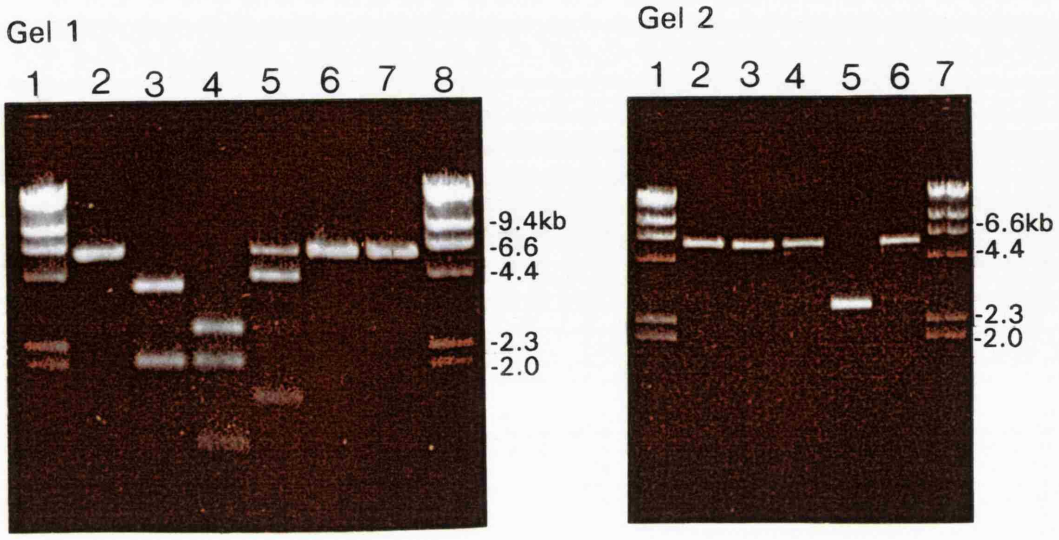


Figure 7.8. Restriction map analysis of plasmid pAAL91 from clone **L**.
Gel 1: Lane 1 lambda cut with *Hind*III; lane 2 pAAL91 cut with *Pst*I; lane 3 pAAL91 cut with *Eco*RI; lane 4 pAAL91 cut with *Pst*I and *Eco*RI; lane 5 pAAL91 cut with *Sac*I; lane 6 pAAL91 cut with *Bam*HI; lane 7 pAAL91 cut with *Hind*III; lane 8 lambda cut with *Hind*III.
Gel 2: Lane 1 lambda cut with *Hind*III; lane 2 pAAL91 cut with *Bam*HI; lane 3 pAAL91 cut with *Hind*III and *Bam*HI; lane 4 pAAL91 cut with *Hind*III; lane 5 pUC18 cut with *Bam*HI; lane 6 pAAL91 cut with *Sma*I; lane 7 lambda cut with *Hind*III.

7.4. Discussion

In order to facilitate the study of surface-associated proteins from *A. actinomycetemcomitans*, we constructed a library of the chromosomal DNA from this organism in *E. coli* JM109 using the plasmid vector pUC18. Three clones were isolated which expressed reactive proteins when immuno-screened with serum from a patient with LJP. ITPG was not needed to induce expression indicating the use of the indigenous *A. actinomycetemcomitans* promoter rather than the vector's lac promoter. All three clones produced a strong positive signal when immuno-screened, even without lysing the *E. coli*, demonstrating that recombinant proteins were probably expressed on the *E. coli* cell surface.

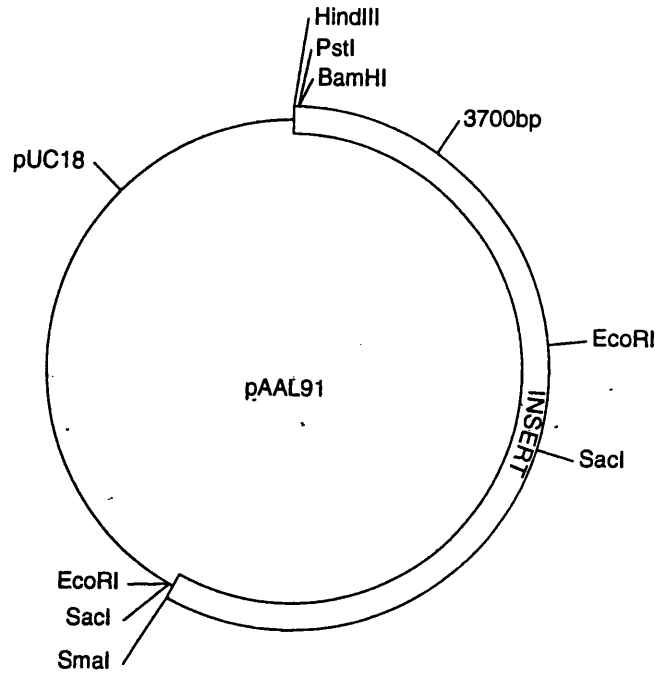


Figure 7.9. Plasmid map of clone pAAL91 showing restriction sites.

This could indicate that leader sequences used by *A. actinomycetemcomitans* to export proteins are recognized by *E. coli*. SDS-PAGE analysis of cell lysates from *E. coli* clones showed that recombinant proteins were not over-expressed. Subsequently, Western blot analysis of the three clones, revealed that clone **L** produced a reactive protein, which had an approximate molecular weight of 34kDa. Pre-absorbing the human serum with *E. coli* cell lysate reduced cross reactivity during immuno-screening, this strategy was adopted during Western blotting and resulted in clearer bands. Although clones **T** and **B** gave strong signals when immuno-screened, no recombinant protein could be detected upon Western blotting. Recognition of proteins by antibodies could be impaired due to the destruction of tertiary protein structures when lysates were boiled before separation by SDS-PAGE. A saline extract of each clone was prepared and assayed for anti-proliferative activity. The extract from all three clones failed to show any anti-proliferative activity at a concentration of 10μg/ml. These results show that the anti-proliferative protein (or at least in an active form) was not cloned into *E. coli* in this study.

Arakawa *et al.*, (1990) performed similar studies to those described in this chapter. An *A. actinomycetemcomitans* Y4 genomic library was constructed in λL47 and screened with serum from a patient with advanced destructive periodontitis. Six clones from 1000 screened were found to react positively. Two clones were found to harbour identical sequences, Southern analysis showed that these sequences were able to hybridize to other strains of *A. actinomycetemcomitans* but not to six other periodontopathogenic bacteria examined. Sunday *et al.*, (1990) also constructed a genomic library of *A. actinomycetemcomitans* Y4, this time in pUC13. Over 1000 recombinant colonies were screened with rabbit serum raised against the Y4 strain, 9 positive colonies were isolated and 3 reacted with the rabbit serum on a Western transfer. Other bacteria implicated in periodontal disease have been studied in this way. Hayakawa *et al.* (1992) cloned two proteins, 160 and 200kDa, from *Por. gingivalis* 381 using serum from a patient with severe adult periodontitis. Few cloning studies on *A. actinomycetemcomitans* have been reported. However, a considerable amount of work has involved the leucotoxin gene from *A. actinomycetemcomitans*, which has been cloned and sequenced by two groups (Lally *et al.*, 1989; Kraig *et al.*, 1990).

A study of the literature concerned with the use of sera to screen genomic libraries, reveals some interesting information. When bacterial lysates are subjected to Western transfer analysis with sera from patients suffering from an infection by the particular organism, only a few proteins react strongly. The literature shows that these immunodominant proteins are generally the ones identified when genomic libraries are screened with the same sera. For example, Brooks and Burnie (1994) identified three immunodominant bands from *Streptococcus sobrinus* following Western blot analysis. Six clones were identified from a genomic library of this organism when screened with sera from a patient with endocarditis caused by a bacterium from the mutans group. All six clones were found to express the same protein. Hendrix *et al.* (1990)

made a phage gene bank of DNA from *Coxiella burnetii* and screened the library with rabbit sera raised against whole bacteria. 3000 plaques were screened and 23 immunoreactive plaques were identified. Of the 23 plaques 22 produced a 60-65kDa protein. A final example perhaps most clearly demonstrates that only the most immunodominant proteins are cloned when screened with sera raised against the infecting bacteria. Won and Griffith (1993) identified 31,40, and 78kDa proteins from *Haemophilus somnus* using bovine sera raised against this bacterium. A genomic library constructed in pUC19 was screened and 45 reactive clones identified and 10 of the 45 recombinants expressed the 31 kDa protein.

Studies by a number of groups have identified a heat modifiable outer membrane protein (OMP) with a molecular weight of 29kDa which increases to 34kDa after incubation at 100°C (Bolstad *et al.*, 1990; Muller *et al.*, 1990; Wilson, 1991). Immunoblotting by Bolstad *et al.* (1990) and Wilson (1991) have shown that the humoral response of subjects with periodontitis to *A. actinomycetemcomitans* includes the production of antibodies which recognize this OMP. The 34kDa protein identified in these studies is by definition an immunodominant antigen of *A. actinomycetemcomitans*. As the literature indicates, generally only a minor number of immunodominant proteins can be cloned by screening libraries with sera, thus the 34kDa protein encoded from the 3.3kb fragment in pAAL91 could conceivably be this OMP. This protein could not only be an important surface antigen with regard to host immunity, but could also have potential as a marker for *A. actinomycetemcomitans* in LJP. Sequencing of plasmid pAAL91 could confirm whether the cloned protein is indeed the 34kDa OMP of *A. actinomycetemcomitans* as the N-terminal sequence is published (Wilson, 1991), a possibility that will have to be explored in future work.

This study demonstrates that *A. actinomycetemcomitans* genes can be cloned and successfully expressed in *E. coli*. This is not all that surprising, as *A. actinomycetemcomitans* is the most closely related subgingival plaque bacterium to *E. coli*, as assessed by 16S rRNA analysis (Shah and Gharbia, 1995). Thus recombinant DNA technology offers an excellent tool for studying the role of genes in colonization and virulence and could eventually help in the development of a vaccine against this pathogenic organism.

Chapter 8

Purification of the anti-proliferative protein

8.1. Introduction

By gently stirring *A. actinomycetemcomitans* in saline it is possible to release a proteinaceous fraction which is loosely associated with the bacterial surface. Two-dimensional SDS-PAGE of this surface-associated material (SAM) followed by Coomassie blue staining reveals that it contains 50 or more proteins or protein subunits. Surface-associated material exhibits potent anti-proliferative activity as assessed by measuring the incorporation of [^{3}H]-thymidine into MG63 cells. The growth of other cell types including fibroblasts is also inhibited by this material and anti-proliferative activity is present in different strains of *A. actinomycetemcomitans*. The aim of this study was to isolate the component from the SAM responsible for this activity. This will provide structural information for the cloning and expression of this molecule. Previous studies (Chapter 4) demonstrated that the active component was heat- and trypsin-sensitive, indicating that it is a protein. Extensive use has been made of a wide range of protein purification techniques, which will be described.

8.2. Results

8.2.1. Amicon Filtration

To quickly determine the molecular mass of the anti-proliferative component an Amicon ultrafiltration cell which separates molecules according to size was used (chapter 2, section 2.11.1). Initially 78mg of SAM (50mg of protein) was separated using a 30kDa cut-off membrane, which resulted in two fractions, one consisting of material less than 30kDa (<30kDa) and the other of greater than 30kDa (>30kDa). Analysis of the <30kDa material by SDS-PAGE and silver staining, revealed eight proteins ranging from a molecular mass of approximately 42kDa to 15kDa. The fractions were lyophilized and tested at known protein concentrations for anti-proliferative activity as assessed by measuring [^{3}H]-thymidine incorporation into MG63 cells. The activity was found

to reside entirely in the >30kDa fraction with no activity in the <30kDa fraction even at concentrations as high as 10μg/ml (table 8.1). The <30kDa material accounted for approximately 2% of the total material. This first experiment suggested that the anti-proliferative component had a molecular mass greater than 30kDa. Therefore 100kDa cut-off membranes were employed to generate two fractions of <100kDa and >100kDa material. Table 8.2. shows that of the 76% of activity recovered, 41.5% remained in the >100kDa fraction and 34% was able to pass through the membrane. The specific activities of the SAM and the two fractions were practically identical.The use of membrane ultrafiltration did not result in an increase in the specific activity of the fractions after separation, demonstrating the ineffectiveness of this technique for isolating the anti-proliferative protein.

Table 8.1
Purification using an Amicon 30kDa cut-off membrane

Purification step	Amount of protein (μg)	IC_{50} [a] (μg/ml)	Spec. act. [b] (IC_{50} units/μg^{-1})	Purification (-fold)[c]	IC_{50} units of activity [d]	% Recovery[e]
SAM	50,000	0.1	10	1	500,000	100
>30kDa	38,000	0.1	10	1	380,000	76
<30kDa	1,200	0.0	0	0	0	0

Table 8.2
Purification using an Amicon 100kDa cut-off membrane

Purification step	Amount of protein (μg)	IC_{50} [a] (μg/ml)	Spec. act. [b] (IC_{50} units/μg^{-1})	Purification (-fold)[c]	IC_{50} units of activity [d]	% Recovery[e]
SAM	200,000	0.1	10	1	2,000,000	100
>100kDa	83,000	0.1	10	1	830,000	42
<100kDa	68,000	0.1	10	1	680,000	34

[a] IC_{50} = μg of protein per ml required for 50% inhibition of proliferation.
[b] Specific activity = IC_{50} units per μg of protein.
[c] Purification = specific activity of fraction / specific activity of starting material.
[d] IC_{50} units = μg of protein × specific activity.
[e] (IC_{50} units in fraction / IC_{50} units in starting material) × 100

8.2.2. High performance liquid chromatography

High performance liquid chromatography (HPLC) was initially used to separate 40mg of SAM (26.4mg of protein). The crude SAM extract was separated by anion-exchange HPLC (chapter 2, section 2.11.3.1.). The elution profile showed that the majority of the anti-proliferative activity appeared in one well-defined peak which was collected in fraction 12 (figure 8.1.). Active fractions were subsequently retested at known protein concentrations to establish their IC_{50} values (concentration at which [^{3}H]-thymidine incorporation into MG63 cells was inhibited by 50%), fraction 12 was found to be 10 times more active than the crude material with an IC_{50} value of 10ng/ml (table 8.3.).

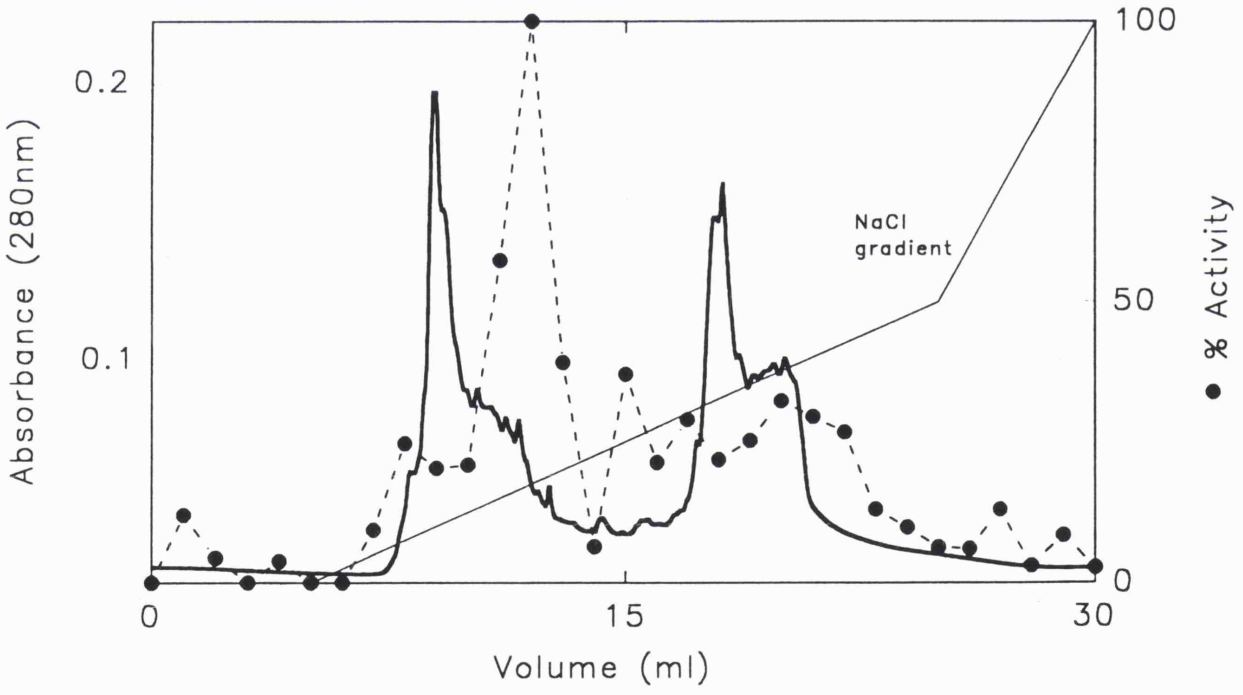


Figure 8.1. Anion exchange HPLC of SAM from *A. actinomycetemcomitans*. Protein elution profile is shown as absorbance at 280nm (solid line). Components were eluted with a 0-2M NaCl gradient. Each 1ml fraction was assayed for inhibition of [^{3}H]-thymidine incorporation into MG63 cells (dotted line) and the percentage activity was compared to the most active fraction (number 12) which was deemed 100% active relative to control cultures.

Fraction 12 was separated by gel-filtration HPLC on a Protein Pak 125 column (Waters) and the anti-proliferative activity assessed by measuring [^{3}H]-thymidine incorporation into MG63 cells (figure 8.2.) (chapter 2, section 2.11.4.1.). Bioactivity eluted as a broad peak, and based upon the retention time of standard markers, had a mean molecular mass of 8kDa (figure 8.3.). Bioactive fractions were pooled, concentrated and retested over a protein concentration range of 1 to 100 ng/ml. This semi-purified material was 25 times more active than the crude material with an IC_{50} value of 4ng/ml. (Table 8.3.) The pooled fraction, when analyzed by SDS-PAGE on a 15% gel, stained with colloidal Coomassie blue (chapter 2, section 2.5.), contained two proteins with molecular masses of less than 15kDa and two other minor proteins of approximately 26kDa (figure 8.4.).

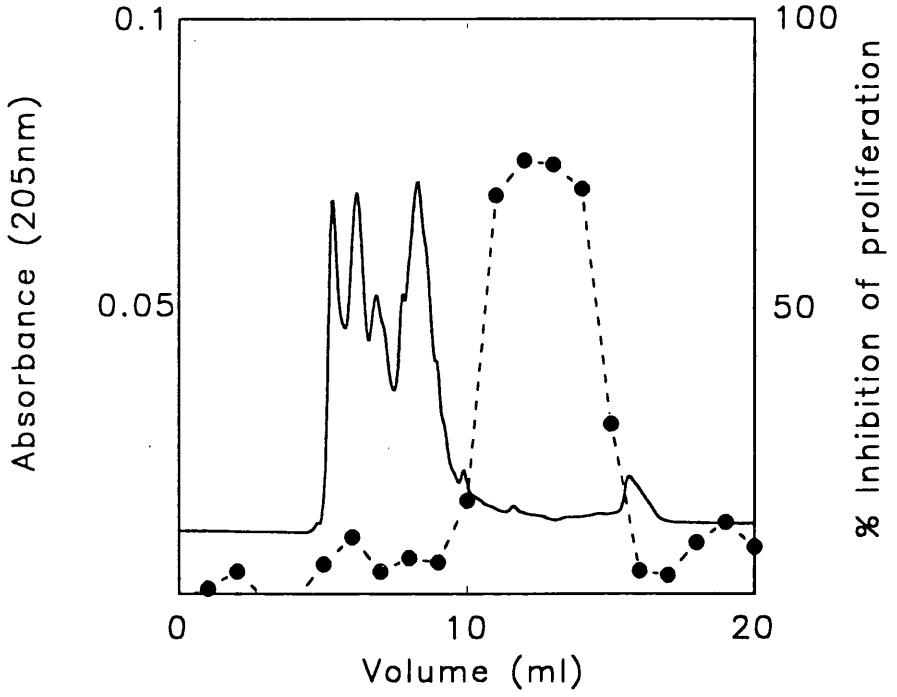


Figure 8.2. Size exclusion HPLC of the most active fraction (number 12) from the HPLC anion exchange column. The protein elution profile is shown as absorbance at 205nm (solid line). Each fraction was assayed for inhibition of [^{3}H]-thymidine incorporation into MG63 and compared to control cultures (dotted line).

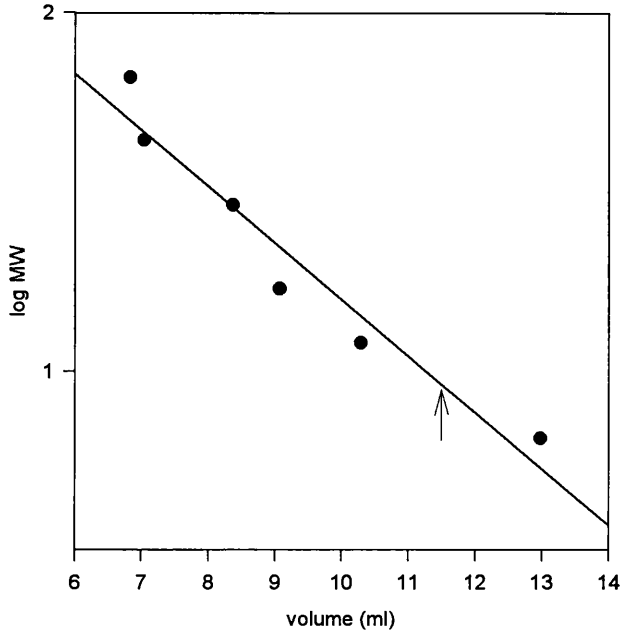


Figure 8.3. HPLC size exclusion of protein standards on a Protein Pak 125 column. The column was equilibrated with 0.1M phosphate buffer pH 6.7 containing 0.15M NaCl. A 10μl volume of the protein sample was injected on the column and the absorbance monitored at 205nm. Protein standards: aprotinin (6.5kDa), myoglobin (17kDa), cytochrome C (12kDa) carbonic anhydrase (29kDa), ovalbumin (44kDa) and bovine serum albumin (66kDa). The plot shows molecular mass vs. elution volume in minutes. The arrow denotes the elution time of the anti-proliferative activity, which corresponds to a molecular mass of approximately 8kDa based on the statistically-calculated line of best fit.

Table 8.3
Initial purification of anti-proliferative protein using HPLC.

Purification step	Amount of protein (µg)	IC_{50} [a] (µg/ml)	Spec. act. [b] (IC_{50} units/μg^{-1})	Purification (-fold)[c]	IC_{50} units of activity [d]	% Recovery[e]
SAM	26,000	0.10	10	1	260,000	100
Anion exchange	1,370	0.01	100	10	137,000	53
Size exclusion	62	0.004	250	25	15,500	6

[a] IC_{50} = µg of protein per ml required for 50% inhibition of proliferation.
[b] Specific activity = IC_{50} units per µg of protein.
[c] Purification = specific activity of fraction / specific activity of starting material.
[d] IC_{50} units = µg of protein × specific activity.
[e] (IC_{50} units in fraction / IC_{50} units in starting material) × 100

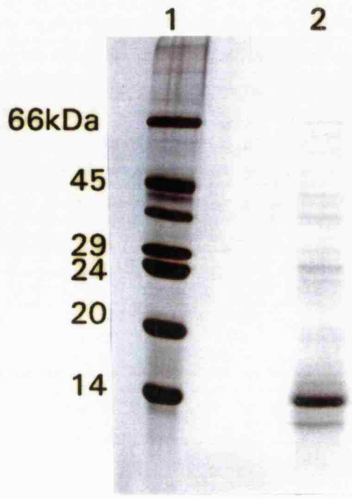


Figure 8.4. SDS-PAGE of the biologically active fraction eluted from the size exclusion column, activity being found in fractions 11-14. Molecular weight markers are displayed in lane 1. Fractions 11-14 were pooled and concentrated and the proteins present are shown in lane 2. The gel used contained 15% polyacrylamide and was stained with colloidal Coomassie blue to disclose protein bands.

8.2.3. Large scale anion exchange and gel-filtration chromatography

This initial work suggested that the anti-proliferative component accounted for only a very small proportion of the SAM. It was therefore deemed necessary to scale up the procedure in order to purify the active component to homogeneity.

400mg of SAM produced from bacteria grown on solid media were fractionated using a Q-Sepharose anion exchange column (chapter 2, section 2.11.3.2.). 10ml fractions were collected and assayed for anti-proliferative activity by measuring [^{3}H]-thymidine incorporation into MG63 cells. Fractions were initially tested at a 1:1000 dilution, however no activity was recovered. These were subsequently retested at a 1:100 dilution, figure 8.5. shows the activity and protein concentrations of each fraction.

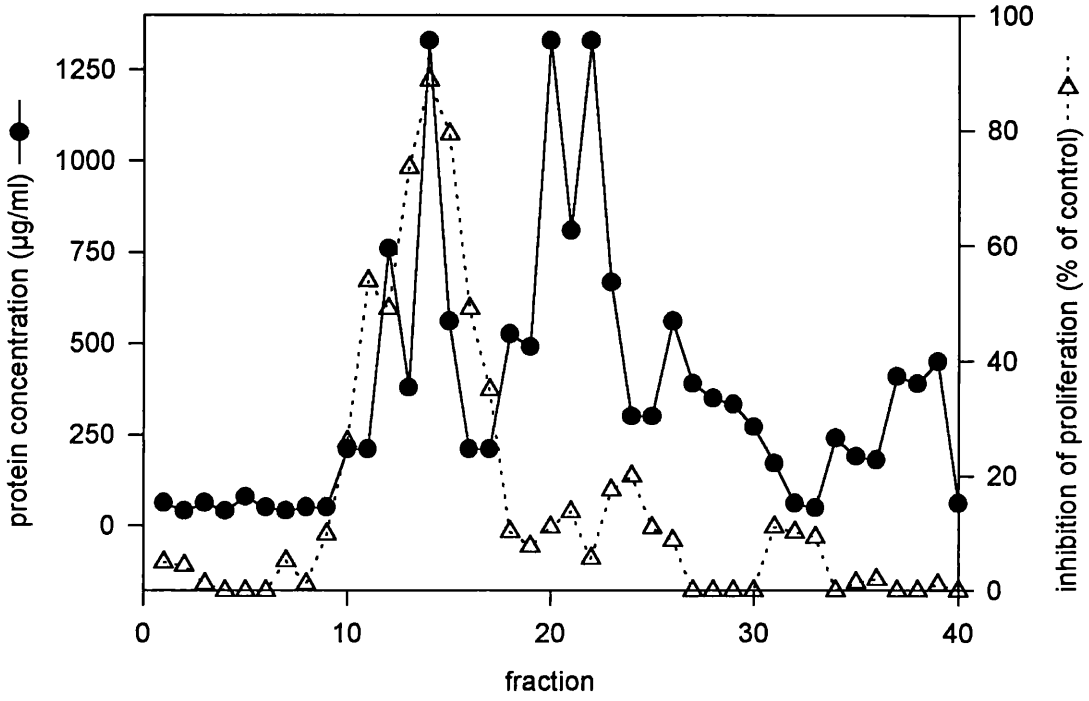


Figure 8.5 Elution profile of the SAM separated by Q-Sepharose anion exchange chromatography. Each fraction was assessed for protein content (solid circle) using the Bio-Rad Dc protein assay and for anti-proliferative activity (open triangle) by measuring [^{3}H]-thymidine incorporation into MG63 cells.

Table 8.4
Purification using large scale anion exchange chromatography.

Purification step	Amount of protein (µg)	IC_{50} [a] (µg/ml)	Spec. act. [b] (IC_{50} units/µg^{-1})	Purification (-fold)[c]	IC_{50} units of activity [d]	% Recovery[e]
SAM	256,000	0.10	10	1	2,560,000	100
Anion exchange	13,300	0.50	2	0.2	26,600	1

[a] IC_{50} = µg of protein per ml required for 50% inhibition of proliferation.
[b] Specific activity = IC_{50} units per µg of protein.
[c] Purification = specific activity of fraction / specific activity of starting material.
[d] IC_{50} units = µg of protein × specific activity.
[e] (IC_{50} units in fraction / IC_{50} units in starting material) × 100

Fraction 14, being the most active fraction, was retested at known protein concentrations to establish its IC_{50} value. Unfortunately, of the 2.56×10^6 units of activity loaded on to the column, only 26,600 (1%) were recovered (table 8.4.). Fraction 14 was analyzed by SDS-PAGE on a 4-20% gradient gel and silver stained (chapter 2, section 2.5.) (figure 8.6.). There was a 5-fold decrease in the specific activity of the most active fraction when compared to the crude material, demonstrating an unacceptable loss of activity. The remainder of the activity was separated by Sephadex G-100 gel-filtration (chapter 2, section 2.11.4.2.) to establish whether the active component was similar to the low molecular mass moiety previously identified using HPLC. Figure 8.7. shows the elution profile of the activity compared to the elution times of various molecular mass standards. The activity eluted as a single peak with an apparent molecular mass of less than 12kDa. The crude SAM was also passed through this column and fractions assayed for anti-proliferative activity by measuring [^{3}H]-thymidine incorporation into MG63 cells. Activity eluted immediately after the void volume with a gradual decline of activity in subsequent fractions.

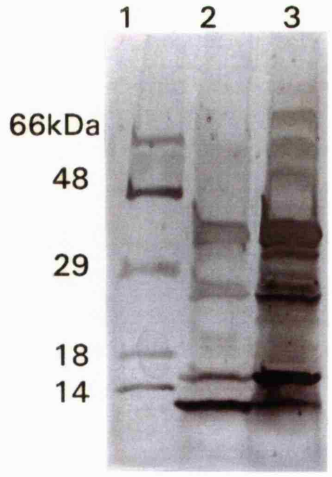


Figure 8.6. SDS-PAGE of the most active fraction eluted from the anion exchange column. Lane 1 shows the molecular weight markers. Lane 2 shows 5μl of fraction 14 and lane 3 shows 20μl of fraction 14. Protein samples were separated on a 4-20% gradient gel and silver stained to disclose proteins.

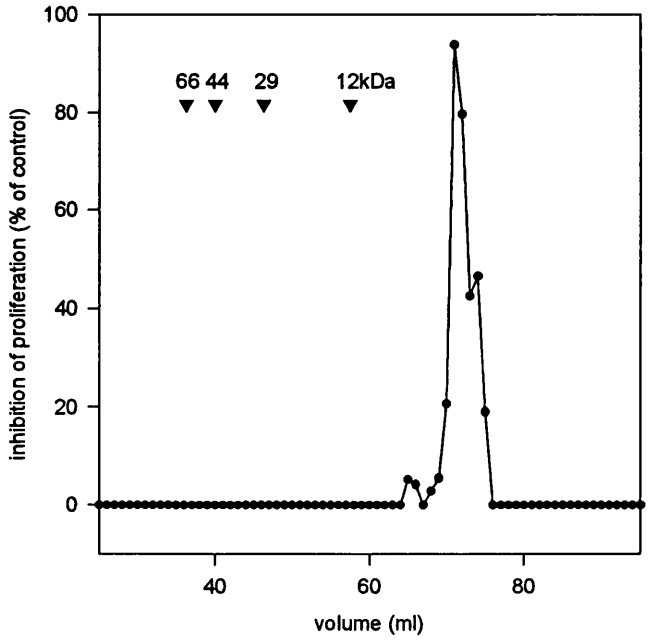


Figure 8.7. Sephadex G-100 gel-filtration of fraction 14. Activity of each 1ml fraction was assessed for anti-proliferative activity (solid circle) by measuring [^{3}H]-thymidine incorporation into MG63 cells. Protein standards (inverted triangle) were run separately with absorbance monitored at 205nm. Protein standards: cytochrome C (12kDa), carbonic anhydrase (29kDa), ovalbumin (44kDa) and bovine serum albumin (66kDa).

8.2.4. Purification of the anti-proliferative component of SAM extracted from liquid-cultured bacteria

8.2.4.1. Anion exchange

Liquid culture has the potential of allowing greater numbers of bacteria to be cultured due to the ease of growing and harvesting bacteria in this way (as opposed to solid media), thus providing a larger amount of starting material for the purification of the anti-proliferative component. Therefore, 421mg of SAM extracted from liquid cultured bacteria (84.2mg of protein) were separated by Q-Sepharose anion exchange chromatography (chapter 2, section 2.11.3.2.). Fractions were diluted 100 and 1000 times and tested for anti-proliferative activity. No anti-proliferative activity was recovered when fractions were assayed at a 1:100 dilution, demonstrating similar results to the previous separation. The loss of activity was probably due to one of three reasons: i)

The active component was inactivated during the separation. ii) A combination of two components are needed for activity and could have been separated during the procedure. iii) The active component was retained by the column and did not elute using 2 molar salt. To determine which of these explanations was correct the fractions were recombined, however, no activity was recovered. One possible explanation for retention of the activity on the Q-Sepharose column was that it had lectin-like activity and thus binds to carbohydrates. Methyl-mannoside is used for the removal of lectins from affinity columns. To determine if it would remove activity from the anion exchange column, a 20mM tris buffer, pH 7.0, containing 2M NaCl and 0.3M methyl mannoside was passed through the column. This resulted in approximately 100μg of protein being eluted, which demonstrated anti-proliferative activity with an IC_{50} of 10ng/ml - a 1.2% recovery of activity. Figure 8.8. shows this material separated on a 4-20% gradient gel and silver stained (chapter 2, section 2.5.).

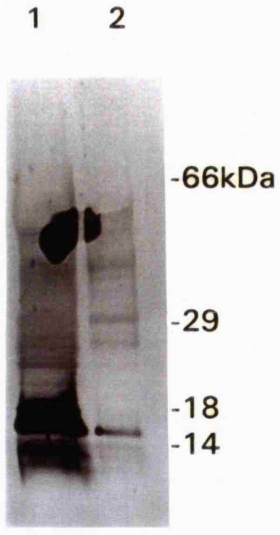


Figure 8.8. SDS-PAGE of material eluted from the Q-Sepharose anion exchange column using a 20mM tris buffer, pH 7.0, containing 2M NaCl and 0.3M methyl mannoside. Lane 1 shows 20μl of this fraction and lane 2 shows 5μl of this fraction. Protein samples were separated on a 4-20% gradient gel and silver stained to disclose proteins. Molecular mass markers are displayed on the right-hand side.

8.2.4.2. Dye binding chromatography

A growing trend in protein purification is to apply affinity interactions as early as possible in the separation process. The affinity ligands used are often low molecular weight triazine compounds, originally produced for textile dyeing such as Cibacron blue. In this procedure a selection of columns in which various dyes are bound covalently to agarose are used as affinity matrices. These columns selectively bind proteins which are able to interact with the immobilized dye. Unbound proteins are washed from the column, generally leaving a relatively small number of bound proteins. Bound material is subsequently washed off by adjusting the composition of the eluent.

Nine dyes were used in this study:

1) Reactive green 19
2) Reactive brown 10
3) Reactive yellow 86
4) Reactive green 5
5) Cibacron blue 3GA
6) Reactive blue 72
7) Reactive yellow 3
8) Reactive red 120
9) Reactive blue 4

1 mg of SAM extracted from bacteria gown in BHI-broth was passed through each column and eluted with 15ml of buffer A (10mM Tris pH 8.0, 5mM $MgCl_2$) (fraction 1). Columns were subsequently eluted with 15ml of buffer A plus 2M NaCl (fraction 2) (chapter 2, section 2.11.6.). Figure 8.9. shows the activity of each fraction recovered from the nine columns. Activity eluted in the unbound fraction from both the reactive yellow dyes, columns 3 and 7, demonstrating 79% and 70% inhibition of proliferation respectively. The specific activity of the material eluted from columns 3 and 7 was, however, not

higher than that of the crude SAM. Some activity was recovered from the bound fraction of all other columns, with the Cibacron blue 3GA column (column 6) yielding the most activity, at 33% inhibition of proliferation. Thus, there was a general failure to recover all of the activity from 7 of the 9 columns. When bound and unbound fractions were recombined, this still did not result in any further recovery of activity. Various buffers were passed through separate columns in order to elute retained activity. These included 6M urea, 0.1%, 2% and 10% ethylene glycol, and 10mM tris buffer pH 8.0 containing 0.3M methyl mannoside.

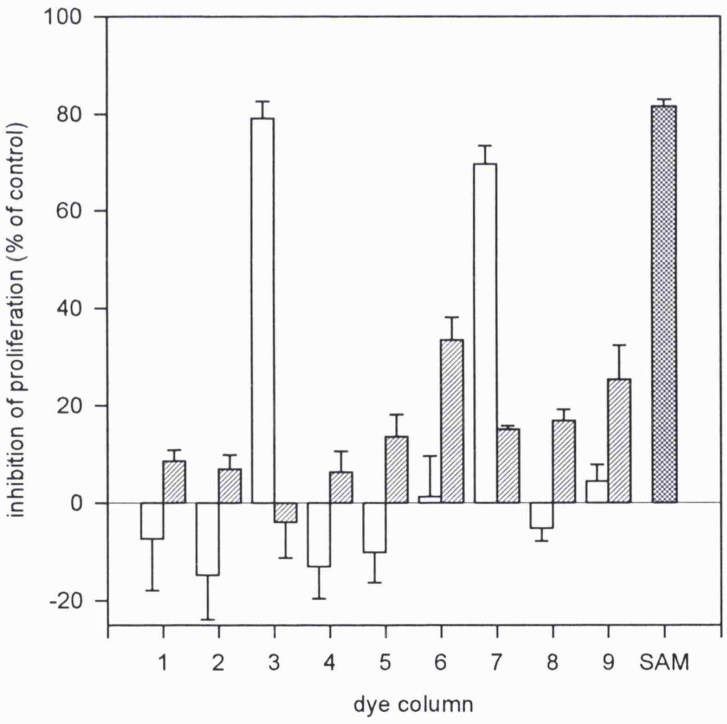


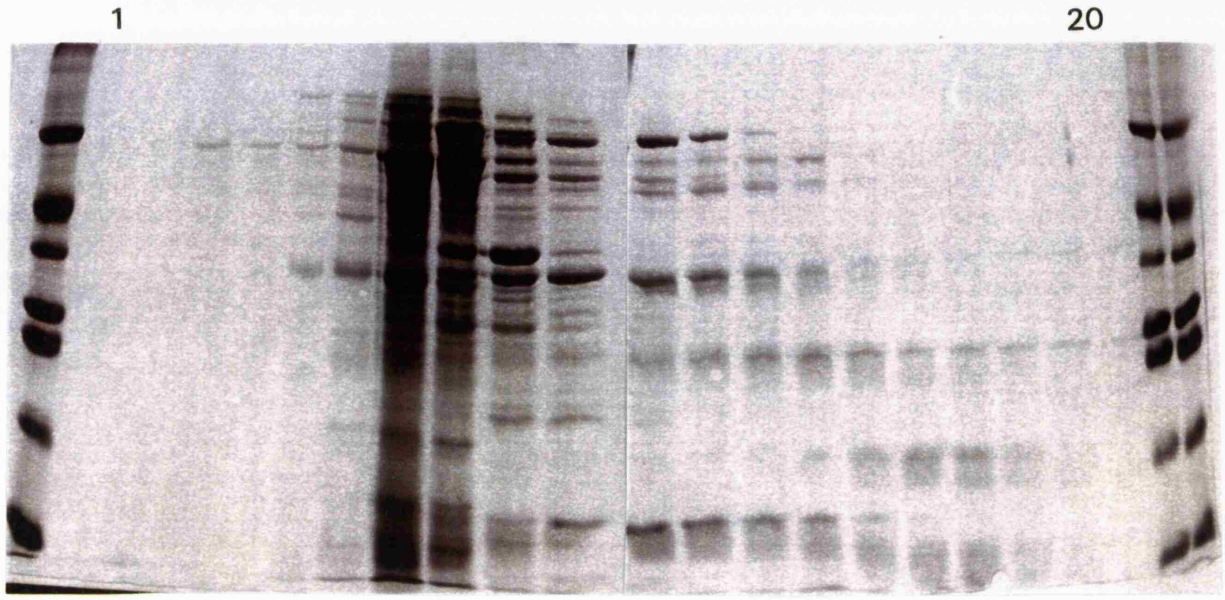
Figure 8.9. Separation of 1mg of SAM using a selection of dye-binding columns. Inhibition of proliferation was assessed by measuring [^{3}H]-thymidine incorporation into MG63 cells. Unbound material was eluted using 10mM Tris pH 8.0, 5mM $MgCl_2$ buffer (open bars) and bound material eluted with 10mM Tris pH 8.0, 5mM $MgCl_2$ containing 2M NaCl (hatched bars). The positive control is displayed on the right; the activity of 1mg of SAM diluted 1500 times in the same buffer.

Controls ensured that the buffers were not responsible for any reduction of activity. The use of 6M urea led to complete loss of activity and was therefore omitted from the experiment. Even with the use of these buffers no further activity was recovered.

8.2.5. Rotophor preparative isoelectric focusing

Surface-associated material from *A. actinomycetemcomitans* was separated using a Rotophor preparative isoelectrofocusing cell (chapter 2, section 2.11.7.). 40mg of SAM from bacteria grown on liquid media, and 50mg of SAM from bacteria grown on solid media, were fractionated. Figure 8.10 (panel A) shows the fractionation of SAM, produced from solid media, analyzed by SDS-PAGE on 12% gels and stained with Coomassie blue. Figure 8.10 (panel B) shows the pH of each fraction and clearly shows that most of the bacterial proteins are acidic and focus in the pH range of 4.5 - 6. Fractions from both separations were tested for anti-proliferative activity at a dilution of 1:100. Activity eluted in a similar pattern in both separations, with little activity in the initial fractions followed by a distinct peak of activity which dropped to form a secondary larger peak of activity (figure 8.11). Fractions were subsequently retested at a 1:1000 dilution, however at this dilution the most active fraction only produced 26% inhibition of proliferation. Fractions 4-8 from the fractionation of the SAM, produced from liquid cultured bacteria, were combined and separated a second time using the Rotophor. This purification was unsuccessful, when fractions were re-assayed all fractions contained anti-proliferative activity (approximately 70% inhibition of proliferation). Control fractions from a blank run in which SAM was omitted exhibited no anti-proliferative activity.

A



B

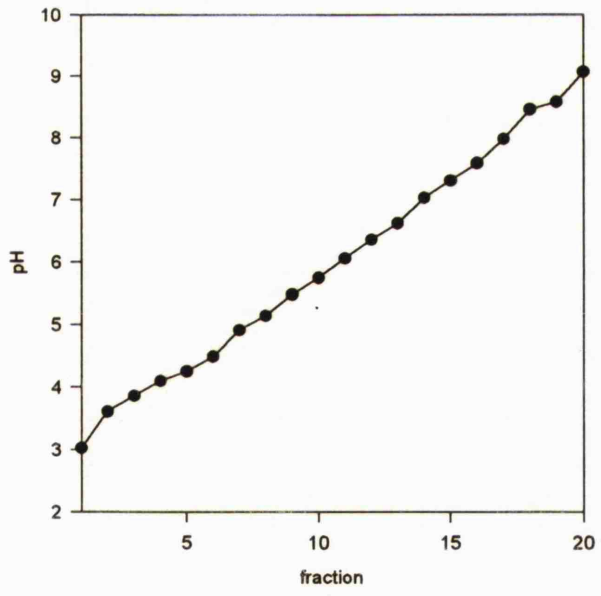


Figure 8.10. Separation of SAM using a Rotophor preparative isoelectric focusing cell. Panel A shows a 12% polyacrylamide gel of fractions 1-20 after staining with colloidal Coomassie blue. Panel B shows the pH gradient of each fraction

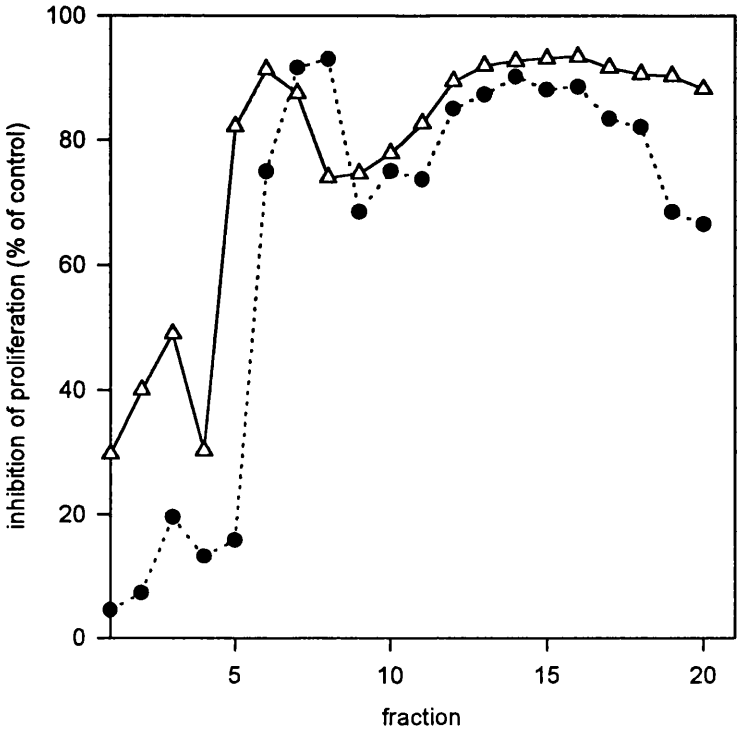


Figure 8.11. Activity profile of fractions from SAM separated using a Rotophor preparative isoelectric focusing cell. Fractions were diluted 1:100, inhibition of proliferation was assessed by measuring [^{3}H]-thymidine incorporation into MG63 cells. Two types of SAM were separated; 40mg of SAM from bacteria grown on liquid media (open triangle) and 50mg of SAM from bacteria grown on solid media (solid circle).

8.2.6. Purification of the anti-proliferative component

Following the initial attempts to purify the anti-proliferative component, it appeared that the small scale HPLC fractionation resulted in the highest purification and recovery of activity. However, the anion exchange column was only capable of separating 4mg of protein per run, making a large scale purification difficult. Therefore an additional purification step was needed to reduce the amount of starting material whilst maintaining activity. The use of an ammonium sulphate precipitation was therefore tested to see if it would aid purification.

8.2.6.1. Ammonium sulphate precipitation

In a pilot study, 25mg of SAM, extracted from bacteria cultured on BHI-agar supplemented with 5% horse blood, were precipitated using 30% (w/v) ammonium sulphate (chapter 2, section 2.11.2.1.). Residual protein in the supernatant was subsequently precipitated by increasing the amount of ammonium sulphate to 45%, 60% and 80% respectively. Each pellet and the unprecipitated material in the supernatant was tested for anti-proliferative activity at a protein concentration of 1μg/ml. Figure 8.12 (panel A), shows the activity of 1μg/ml of each fraction compared to 1μg/ml of SAM and a SAM-free control. The most bioactive material precipitated in the 30% ammonium sulphate cut, which exhibited an IC_{50} value of 20ng/ml. Figure 8.12 (panel B) shows the SDS-PAGE separation of each fraction compared to the starting material. The 45% ammonium sulphate cut precipitated the most protein, in subsequent fractions the amount of protein precipitated decreased sequentially.

This procedure was scaled up for 200mg of SAM. The 30% ammonium sulphate cut recovered 58.9% of the activity with a 5-fold increase in specific activity (Table 8.5.).

8.2.6.2. HPLC anion exchange

The material which precipitated using 30% ammonium sulphate was further fractionated by HPLC anion exchange chromatography (chapter 2, section 2.11.3.1.). Material was loaded on to the column and eluted over 30min using 1M NaCl. The activity eluted as a single well-defined peak (figure 8.13). 20μl of fractions 5-12 were analyzed by SDS-PAGE on a 4-20% gradient gel and silver stained (figure 8.14.). The most active fraction (fraction 9) was further separated by HPLC gel filtration.

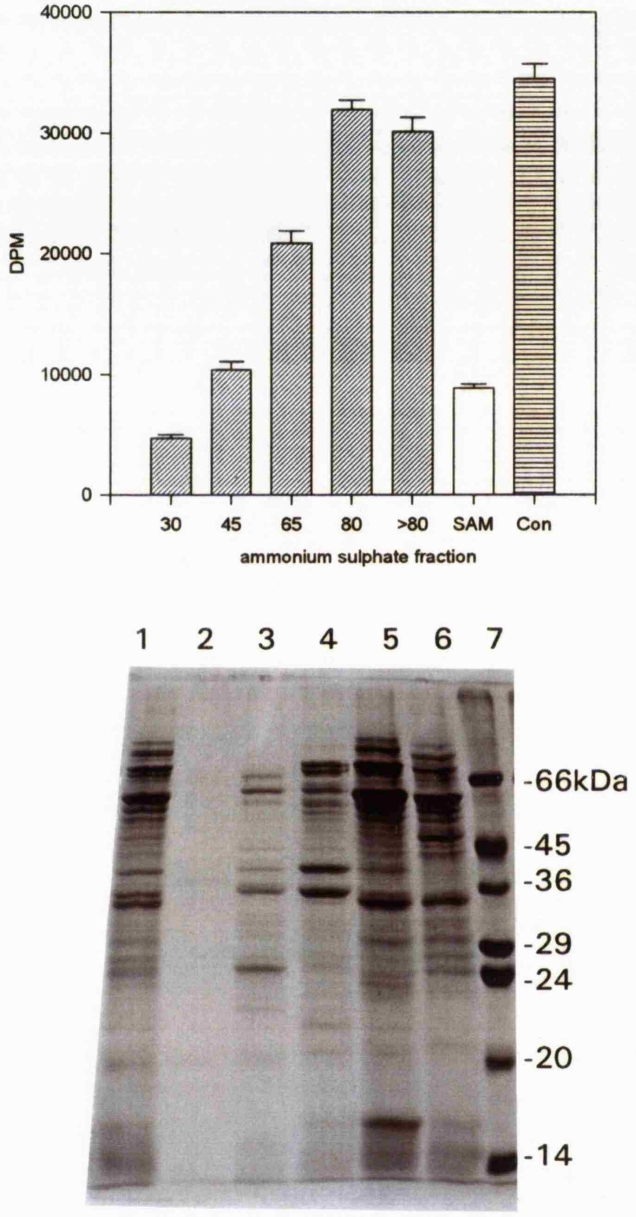


Figure 8.12. Ammonium sulphate precipitation of SAM. Panel A shows the activity of 1μg of protein of each fraction compared to the crude SAM and a SAM-free control. Anti-proliferative activity was assessed by measuring the incorporation of [^{3}H]-thymidine into MG63 cells. Panel B shows SDS-PAGE analysis of each fraction. Lane 1 shows the crude SAM; lane 2 shows the protein still in solution after the addition of 80% ammonium sulphate; lane 3 shows material precipitated using 80% ammonium sulphate; lane 4 shows material precipitated using 65% ammonium sulphate; lane 5 shows material precipitated using 45% ammonium sulphate; lane 6 shows material precipitated using 30% ammonium sulphate and lane 7 shows the molecular mass markers. The gel contained 12% polyacrylamide, proteins were stained with colloidal Coomassie blue.

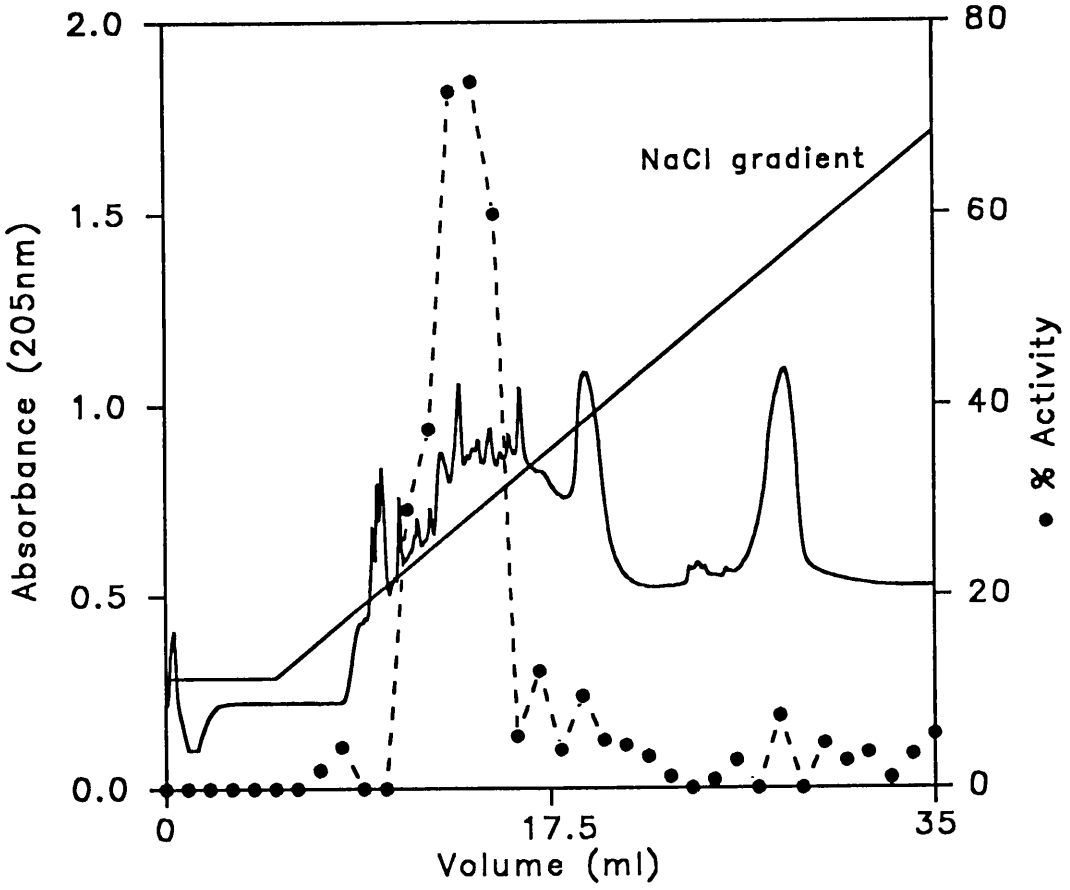


Figure 8.13. Anion exchange HPLC of the active fraction following ammonium sulphate precipitation. Protein elution profile is shown as absorbance at 205nm (solid line). Charged components were eluted with a 0-1M NaCl gradient. Each fraction was assayed for inhibition of [^{3}H]-thymidine incorporation into MG63 cells (dotted line). Activity is represented as the % inhibition of incorporation compared to control cultures.

Table 8.5.
Purification of anti-proliferative protein.

Purification step	Amount of protein (µg)	IC_{50} [a] (µg/ml)	Spec. act. [b] (IC_{50} units/μg^{-1})	Purification (-fold)[c]	IC_{50} units of activity [d]	% Recovery [e]
Saline extract	128,000.00	0.10	10	1.0	1,280,000	100.0
Ammonium sulphate	15,078.00	0.02	50	5.0	753,900	58.9
Anion exchange	243.00	0.005	500	50.0	121 500	9.5
Size exclusion	0.39	0.00042	2381	238.1	929	0.76

[a] IC_{50} = µg of protein per ml required for 50% inhibition of proliferation.
[b] Specific activity = IC_{50} units per µg of protein.
[c] Purification = specific activity of fraction / specific activity of starting material.
[d] IC_{50} units = µg of protein × specific activity.
[e] (IC_{50} units in fraction / IC_{50} units in starting material) × 100

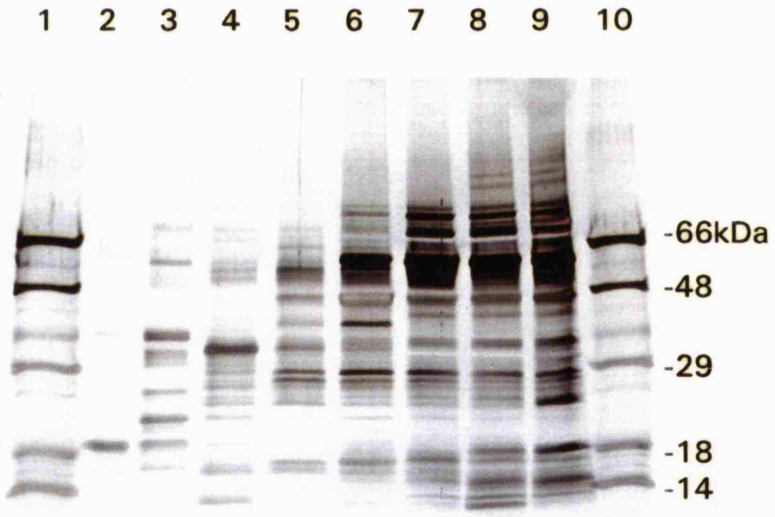


Figure 8.14. SDS-PAGE analysis of active fractions 5 to 12 separated on a 4-20% gradient gel. Proteins were silver stained to disclose proteins. Lanes 1 and 10 show the molecular mass markers; lane 2 shows fraction 5; lane 3 shows fraction 6; lane 4 shows fraction 7; lane 5 shows fraction 8; lane 6 shows fraction 9 (most active fraction); lane 7 shows fraction 10; lane 8 shows fraction 11; lane 9 shows fraction12.

8.2.6.3. Reverse phase chromatography

9.8μg of fraction 9 from anion exchange HPLC was separated by reverse phase on a Resource 3ml column (chapter 2, section 2.11.5.). A 0-100% linear gradient of acetonitrile containing 0.8% TFA was used to elute the proteins with absorbance measured at 220nm. Fractions were dried using a Speed-vac and tested for anti-proliferative activity. No activity was recovered in any fraction. Figure 8.15. shows the elution profile with two major peaks eluting immediately.

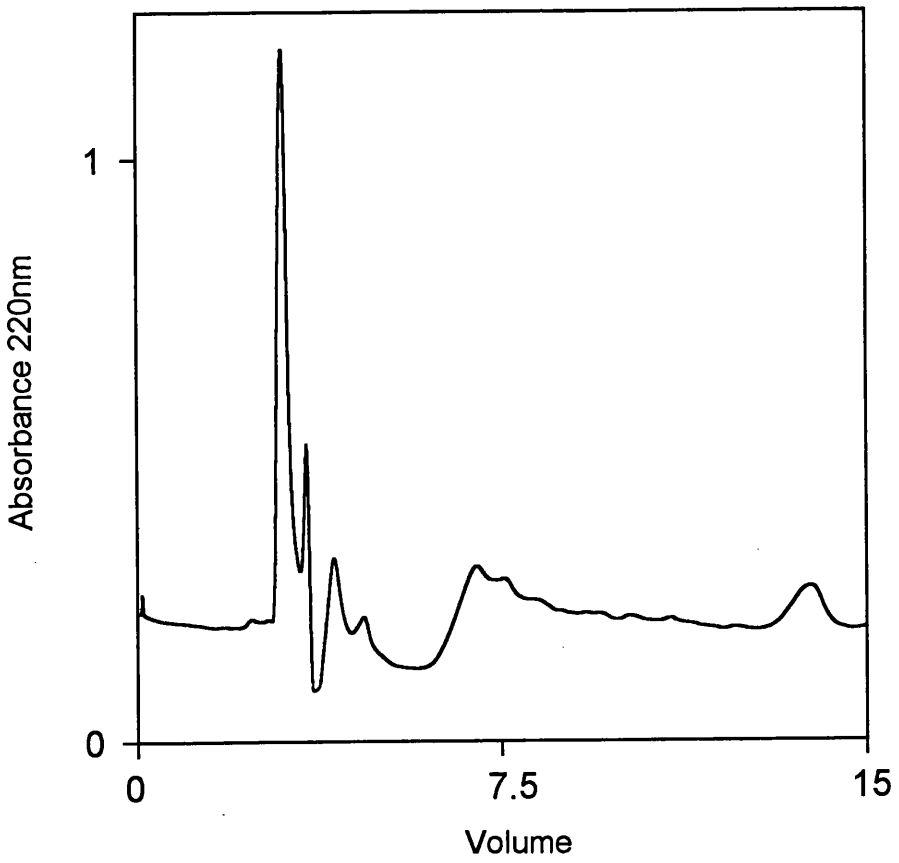


Figure 8.15. HPLC reverse phase using a Resource column. Proteins were eluted using a 0-100% linear gradient of acetonitrile containing 0.8% TFA, the protein elution profile is shown as absorbance at 220nm.

8.2.6.4. Size-exclusion HPLC

Previous studies using a Protein-Pak 125 HPLC column showed that the anti-proliferative activity eluted as a low molecular mass moiety of approximately 8kDa. To confirm the mass of the anti-proliferative protein this procedure was therefore repeated. (chapter 2, section 2.11.4.1.). 9.8μg of protein from the anion exchange HPLC was separated on the Protein Pak column, fractions were diluted 1:20 and assayed for anti-proliferative activity. Activity eluted as a broad peak with the most active fraction eluting with an apparent molecular mass of 8kDa when compared to molecular mass standards.

In this study another HPLC size exclusion column was utilized, namely a TSK 250 column (chapter 2, section 2.11.4.1.). Initially samples were separated using a low ionic strength mobile phase buffer, 20mM phosphate buffer pH 6.7. 9.8μg of protein of the most active fraction (fraction 9) recovered from the previous anion exchange HPLC was separated in this way. When fractions 1-20 were assayed for anti-proliferative activity no activity was recovered. To minimize the retention of proteins by the column the ionic strength of the mobile phase was increased by using 10mM phosphate buffer pH 6.7 containing 0.5M NaCl. Activity eluted as a very broad peak in fractions 17-25. There was clear evidence that the anti-proliferative protein interacted with the matrix as it eluted after the total column volume (14.3ml). Finally, a 0.1M phosphate buffer pH 6.7 containing 0.15M NaCl was used, activity eluted as a tailing peak in fractions 14-20 (figure 8.16.).

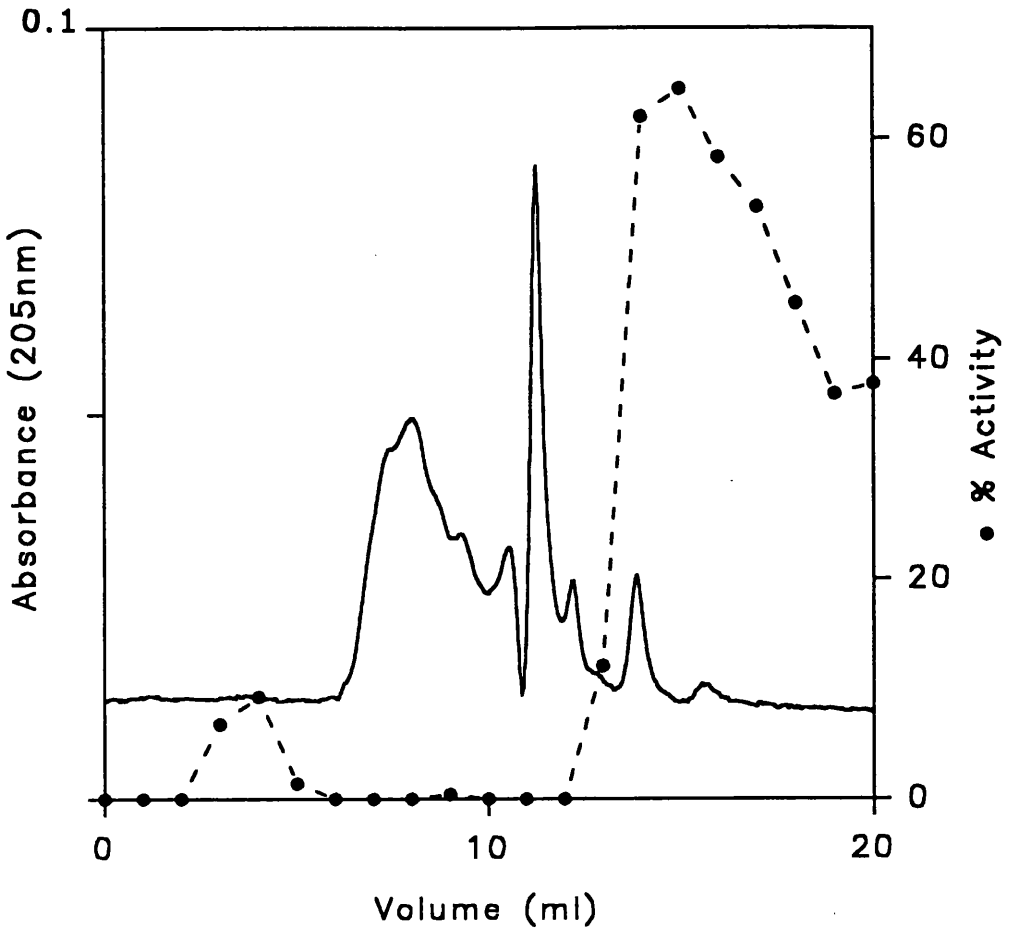


Figure 8.16. TSK-250 size exclusion HPLC of the most active fraction (number 9) from the previous HPLC anion exchange separation. The column was equilibrated with 0.1M phosphate buffer pH 6.7 containing 0.15M NaCl and 9.8μg of protein injected. The protein elution profile is shown as absorbance at 205nm (solid line). Each fraction was assayed for inhibition of [^{3}H]-thymidine incorporation into MG63 cells (dotted line).

The 0.1M phosphate buffer pH 6.7 containing 0.15M NaCl appeared the most adept in suppressing interactions with the column matrix. This interaction was fortuitous as it allowed the isolation of a single protein, as assessed by silver stained SDS-PAGE gel (figure 8.17.). The purified protein, potently inhibited the incorporation of tritiated thymidine with an IC_{50} value of 0.42 ng/ml, showing a 238-fold increase in specific activity. Unfortunately we were only able to isolate 390ng of this protein and, in this diluted form, it proved to be very unstable; activity was lost after storage at 4°C for 4 days or after dialysis.

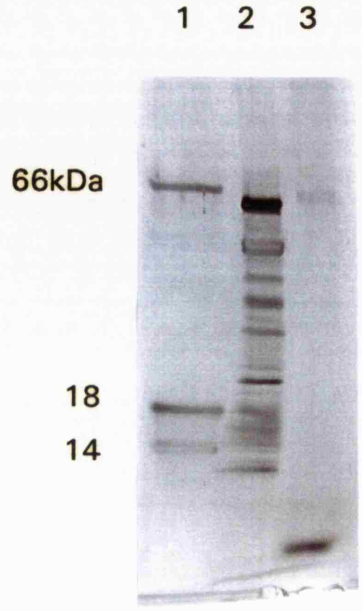


Figure 8.17. SDS-PAGE analysis of active fractions obtained upon purification of the anti-proliferative protein. Lane 1 shows the molecular weight markers; lane 2 shows 1μg of protein from the most anti-proliferative fraction following HPLC anion exchange; lane 3 shows approximately 50ng of the purified 8kDa anti-proliferative protein following a separation by HPLC gel filtration of 10μg of protein from lane 2. The 4-20% gradient gel was silver stained to reveal proteins.

8.3. Discussion

A. actinomycetemcomitans surface-associated material contains potent anti-proliferative activity, with an IC_{50} value of approximately 100ng/ml, as assessed by measuring the inhibition of [^{3}H]-thymidine incorporation into MG63 cells. This chapter describes the attempts to isolate the component responsible for this activity.

Virtually nothing concerning the nature of the active molecule was known at the beginning of this study. On the basis of its lability to heat and trypsin, it was considered more likely that the anti-proliferative component was a protein. The surface-associated material of *A. actinomycetemcomitans* consists of approximately 75 proteins or protein subunits as assessed by two dimensional SDS-PAGE and silver staining (chapter 3, section 3.2.3.2.). Thus, the task of isolating and characterizing the anti-proliferative component was not likely to be a simple procedure.

The first fractionation attempts to define the nature of the anti-proliferative activity were aimed at establishing its molecular mass range and involved the use of Amicon ultrafiltration. Separation of the SAM using a 30kDa cut-off membrane generated two fractions, one with material >30kDa and the other <42kDa (as assessed by SDS-PAGE). Three of the eight proteins in the <30kDa fraction had a molecular mass exceeding 30kDa, demonstrating that the cut-off of the membrane is not exactly precise. Activity resided in the >30kDa fraction indicating that the active component had a molecular mass exceeding 30kDa. Subsequently, a 100kDa cut-off membrane was utilized and this revealed that activity fractionated approximately equally between the >100kDa and <100kDa fractions. Each fraction demonstrated similar specific activities, which were no higher than the specific activity of the starting material. At first sight, these results suggested that the molecular mass of the active protein was approximately 100kDa, as a protein of this mass would generate activity in both fractions with similar specific activities. However, as shall be explained these initial experiments were misleading.

Having found that the anti-proliferative activity was due to a molecule of high molecular mass (an incorrect assumption as it turned out), the isolation of this molecule was pursued. This was initially done using high-performance liquid chromatography (HPLC). Use was made of a computer-controlled Bio-Rad HRLC system for these studies. Purification of 40mg of SAM by anion exchange HPLC, which separates molecules on the basis of their charge characteristics, revealed one peak of activity. Further purification by size-exclusion HPLC also showed a single peak of activity and demonstrated that the active component had a molecular mass of approximately 8kDa. These results therefore indicated that only one molecule within the SAM was responsible for the anti-proliferative activity. This purification procedure proved to be successful, with a 25-fold increase in the specific activity of the active fractions generated by the size exclusion HPLC. However, a major limitation was the small amount of material which could be separated by anion exchange HPLC. This procedure demonstrated that the anti-proliferative protein was only a minor constituent of the SAM, the final protein sample (fractions 11-14 from size exclusion) contained only 62μg of protein and contained at least 4 proteins as assessed by colloidal Coomassie blue staining. These results suggested that increased amounts of starting material would be necessary to purify the anti-proliferative protein.

These results are paradoxical. The Amicon membrane filtration showed that the anti-proliferative activity was exclusively present in the >30kDa fraction and approximately half of the activity failed to pass through an Amicon 100kDa cut-off membrane. Conversely, the HPLC size-exclusion separation demonstrated that the activity eluted as a low molecular mass moiety of approximately 8kDa. Two possible explanations for these anomalous findings are: i) the active molecule is self-associating under particular circumstances used in these studies and/or ii) the active protein binds to a carrier protein or proteins.

From the HPLC fractionation it was clear that the anti-proliferative component was a minor constituent of the SAM. Purification of the anti-proliferative protein could only be achieved if the amount of starting material was dramatically increased. Therefore over 750 plates of bacteria were grown and the SAM extracted. This produced 400mg of SAM which was separated using a Q-Sepharose anion exchange column. When fractions were assayed for their ability to inhibit cell proliferation, activity eluted in a single peak. However, 99% of the anti-proliferative activity was lost. Furthermore, the most active fraction recovered had a 5-fold lower specific activity than the starting material. The little activity which was recovered was further analyzed using Sephadex G-100 gel filtration. The activity eluted as a low molecular mass component of less than 12kDa. These findings confirmed earlier results that the active component had a low molecular weight. The crude SAM was also passed through the G-100 column and the fractions assayed. Activity eluted as a high molecular weight component. It was therefore apparent that when the crude SAM is separated, the activity elutes as a high molecular weight component, as shown by Amicon ultrafiltration and Sephadex G-100 gel filtration. However, after anion exchange chromatography the active component eluted as a low molecular weight component as demonstrated by Sephadex G-100 gel filtration and HPLC Protein Pak 125 gel filtration. These results support the hypothesis that the active protein binds to a carrier protein, eluting as a much larger molecule until separated from its carrier molecule by, for example, anion exchange chromatography.

In a second fractionation, 421mg of SAM produced from bacteria grown in liquid culture were separated by Q-Sepharose anion exchange. When fractions were tested at 1:100 dilution no activity was recovered. The exact reasons for the loss of activity when using Q-Sepharose were unclear, but three main possibilities existed; i) the anti-proliferative protein was inactivated during the separation, ii) a combination of two components are needed for activity and could have been separated during the purification procedure, iii) the protein was

retained on the column and did not elute with 2M NaCl (the highest concentration of salt passed through the column). Recombination of fractions did not lead to a recovery in activity. Anion exchange is generally considered to be a mild technique with excellent recovery of biological activity. It was unclear, therefore, why the HPLC anion exchange resulted in a good recovery of activity, whereas the Q-Sepharose anion exchange resulted in very poor recovery of anti-proliferative activity. The mobile phase buffers and the functional group (quaternary amine) were identical. However, the main difference between the two columns was the stationary phase matrices. The HPLC MA7Q anion exchange column's matrix consisted of a nonporous methacrylate polymer, whereas the Q-Sepharose column contained porous cross-linked agarose. Lectins are a family of carbohydrate binding proteins that are produced by slime moulds, plants and animals. If the anti-proliferative component had lectin-like properties it may therefore be able to bind to carbohydrate, and could be retained by an agarose matrix. Methyl-mannoside is used for the removal of glycoproteins from lectin-affinity columns. Therefore to determine if the anti-proliferative moiety had lectin-like activity a methyl-mannoside buffer was passed through the column. In this case methyl mannoside would compete with the column to bind the anti-proliferative protein, resulting in the elution of the active molecule. Passing a methyl mannoside buffer through this column resulted in approximately 100μg of protein being eluted. The specific activity of this material was 10-fold higher than the crude material, demonstrating a degree of purification. SDS-PAGE analysis of this material showed the presence of numerous protein bands, with two dominant bands of low molecular mass. The exact nature of the protein binding was inconclusive as only 1% of the activity was recovered in this manner. What was more conclusive was that this method of purifying the anti-proliferative protein was unsatisfactory.

A trend in protein purification is to apply affinity interactions as early as possible in the separation process. The affinity ligands used are often low molecular weight triazine compounds, originally produced for textile dyeing such as Cibacron blue. Generally, only a small number of proteins in a crude protein mixture are liable to interact with such dyes and these can then be removed by increasing the ionic strength of the mobile phase. The end result can often mean an extremely good purification with excellent recovery of activity. However, in this study dye affinity chromatography resulted in a poor recovery of activity with 7 of the 9 columns. Ethylene glycol or methyl-mannoside buffer did not result in any subsequent recovery of activity. These results provide further evidence of the strong binding potential of this protein. Where activity was recovered in columns 3 and 7, the activity eluted in the unbound fraction along with the majority of the other SAM proteins and exhibited no increase in specific activity. Interestingly, both these columns contained yellow dyes, this would indicate that binding was mediated by the binding mechanism of the dye rather than the agarose to which the dye was covalently bound.

Isoelectric focusing is a simple and practical method of purifying proteins. Modern instruments such as the Rotophor preparative electrofocusing cell (Bio-Rad) are now recognised as being effective for purifying large amounts of starting material. Two types of SAM were separated in this way; SAM extracted from bacteria grown on BHI agar supplemented with 5% horse blood and SAM extracted from bacteria grown in BHI broth. Isoelectric focusing in the Rotophor cell was carried out at 12 W of constant power at 4°C for 4 h. Focusing continued for 30 min after the voltage had stabilized. Fractions 1-20 were assayed for anti-proliferative activity and activity was present in all fractions except the first 5 fractions. The activity profile revealed the presence of two peaks, the fact that both separations demonstrated this phenomenon indicated this was not due to experimental error. The initial peak of activity eluted in fractions 5-8, where the bulk of the protein eluted. If one considers

the earlier hypothesis of a carrier molecule, the finding of two peaks of activity could be explained if the anti-proliferative protein eluted at the pI of the carrier protein and at the actual pI of the anti-proliferative protein. This would happen when separation of the anti-proliferative protein from the carrier protein occurred. This technique did not separate the anti-proliferative activity. However there was no loss in activity as seen during other purification procedures, indicating that this method may have proved useful if used later on in the purification procedure.

Numerous techniques and procedures as described above, were used to isolate the anti-proliferative protein with little success. At this stage it was thought wise to return to a method which had previously worked rather than to try any further purification techniques. Anion exchange HPLC had proved to be a reliable technique with an excellent recovery of activity. However, there were limitations with this technique, as only 4mg of protein could be separated per run. Another step was therefore introduced to reduce the amount of starting material, whilst maintaining the activity. A purification strategy was eventually developed which involved an initial precipitation of protein by ammonium sulphate, followed by HPLC anion exchange chromatography and HPLC gel filtration chromatography. Using a Protein Pak 125 column, activity eluted as a broad peak with a molecular mass approximately 8kDa. A second gel-filtration column was subsequently used. Initially, the mobile phase used was a low ionic strength buffer, 20mM phosphate buffer pH 6.7. Fractions 1-20 were assayed for anti-proliferative activity. Surprisingly, no activity was recovered. Increasing the salt concentration by using a 10mM phosphate buffer pH 6.7, containing 0.5M NaCl resulted in activity eluting in fractions 17-25, after the total volume of the column (14.3ml). Finally, a 0.1M phosphate pH 6.7 containing 0.15M NaCl was used, this resulted in activity eluting slightly earlier in fractions 14-20, but still after the total column volume. This proved to be a fortuitous finding, the active protein interacted anomalously with the TSK 250 silica gel-filtration column enabling it to be cleanly separated from

contaminating proteins. This phenomenon is not uncommon. Silica supports containing surface charge, due to free silanols, or producing hydrophobic interactions, have been shown to retain peptides by ionic or hydrophobic binding and in extreme cases the peptides have been shown to elute after the total permeation volume of the column, this is probably what was happening during the TSK 250 gel-filtration HPLC (Ahmed and Modrek, 1992). Fractions 16-20 were pooled, dialysed, and concentrated. The concentrate was analysed by SDS-PAGE and silver stained to reveal a single protein band with a molecular mass of approximately 8kDa. The purified protein potently inhibited the incorporation of [^{3}H]-thymidine, demonstrating a 238-fold increase in specific activity when compared to the crude SAM.

Attempts to isolate sufficient amounts of the anti-proliferative protein for N-terminal sequence analysis are continuing and will unfortunately have to extend beyond the time span available for my study. Information about the N-terminal sequence could led to subsequent cloning and expression of the molecule, thus providing sufficient material for detailed studies of the mechanism of action of this molecule. The anti-proliferative protein isolated from the SAM of *A. actinomycetemcomitans* has now been termed gapstatin.

Chapter 9

Characterization and mechanism of action of the anti-proliferative protein from the surface associated-material of *A. actinomycetemcomitans*

9.1. Introduction

A number of studies have established that *A. actinomycetemcomitans* contains anti-proliferative activity (Shenker *et al.*, 1982; Kamen, 1983; Stevens *et al.*, 1983; Taichman *et al.*, 1984; Kamin *et al.*, 1986; Stevens *et al.*, 1988; Meghji *et al.*, 1992a; Meghji *et al.*, 1992b). The work in this thesis has shown that such activity is concentrated on the surface of this organism. No group has yet succeeded in isolating this anti-proliferative activity. In the last chapter the isolation of an 8kDa protein, which accounts for all the anti-proliferative activity of the SAM, was reported. In this chapter the mechanism of action of this activity is explored in relation to its effect on cell cycle progression

9.2. Results

9.2.1. Cytotoxic effect of SAM on human gingival fibroblasts and MG63 cells.

9.2.1.1. Acridine orange staining

One simple explanation for the anti-proliferative activity of SAM from *A. actinomycetemcomitans* is that it was cytotoxic. To test this possibility, cytotoxicity was monitored by incubating MG63 cells with 100μg/ml of SAM for 24 h and measuring acridine orange uptake (chapter 2, section 2.8.2.). The proportion of acridine orange-stained cells in control cultures, and in those exposed to SAM, were determined and compared with the incorporation of [^{3}H]-thymidine into cells in parallel control cultures or cultures exposed to SAM. Acridine orange uptake demonstrated that the SAM was not cytotoxic, as the number of non-viable cells in cultures incubated with SAM was not significantly different to that in the controls. However, at the concentration of SAM used, there was almost complete inhibition of [^{3}H]-thymidine incorporation (figure 9.1.).

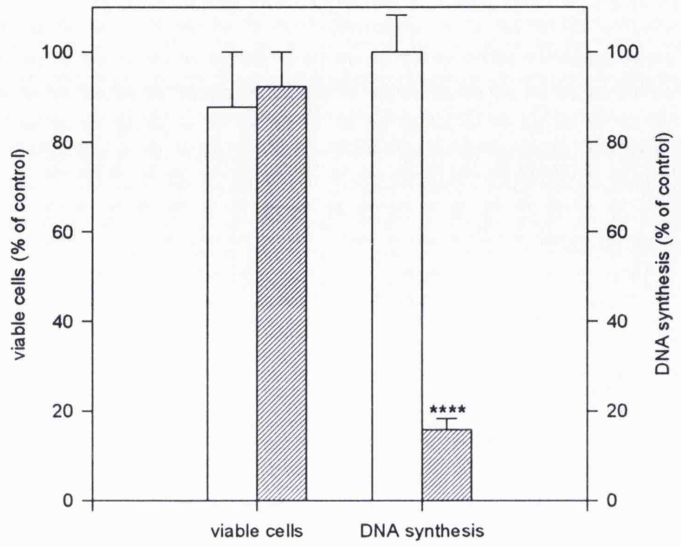


Figure 9.1. Comparison of the effect of SAM on cell viability and on cellular replication, as measured by [^{3}H]-thymidine incorporation into DNA. MG63 osteosarcoma cells were exposed to 100μg/ml of SAM for 24h. The control, to which SAM was not added, (open bars) and SAM-treated cultures (hatched bars) 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 the mean and SD of 3 replicate cultures. ****$p<0.0001$.

9.2.1.2. Cytotoxicity determined by lactate dehydrogenase release

The cytotoxicity of the SAM was also determined by measuring lactate dehydrogenase (LDH) release, using the CytoTox 96 non-radioactive cytotoxicity assay (Promega) (chapter 2, section 2.8.2.). MG63 cells were cultured for 24 h and human gingival fibroblasts were cultured for both 24 and 48 h in the presence of various concentrations of SAM ranging from 0.1 to 100μg/ml. LDH levels in culture supernatants were measured with a 30min coupled enzymatic assay which results in the conversion of a tetrazolium salt (INT) into a red formazan product. Absorbance at 592nm was measured and the percentage cell lysis was calculated. The SAM was not cytotoxic to either

type of cell over a period of 24h even at concentrations as high as 100μg/ml. However, when human gingival fibroblasts were exposed to SAM for 48h, concentrations greater than 1μg/ml were slightly cytotoxic (figure 9.2.).

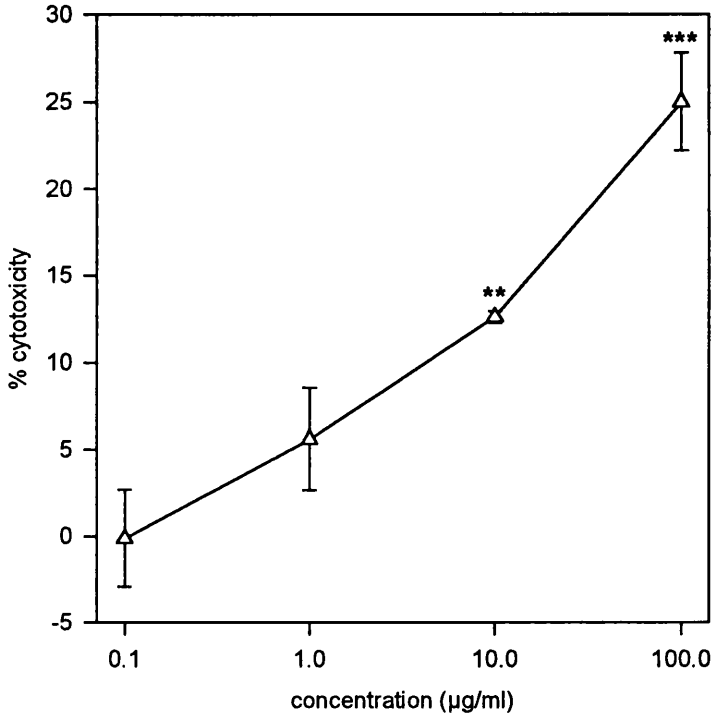


Figure 9.2. The dose-dependent cytotoxicity of SAM to human gingival fibroblasts after 48h of culture. Cytotoxicity was assessed by measuring LDH release using a commercial kit. The results are expressed as mean and SD of six replicate cultures. $^{**}p<0.01$, $^{***}p<0.001$.

9.2.2. The effect of inhibitors of intracellular signal transduction on the anti-proliferative activity of SAM.

9.2.2.1. Inhibition of protein kinase A

H-89 [N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide] is a potent and selective inhibitor of cAMP-dependent protein kinase A (PK-A). This kinase exists as a tetramer in its holoenzyme form and is composed of two

types of subunit, one which possesses catalytic activity and one which regulates the activity of the enzyme. Two catalytic subunits complex with two regulatory subunits to form the active enzyme. PK-A phosphorylates cellular proteins and is involved in cell signalling.

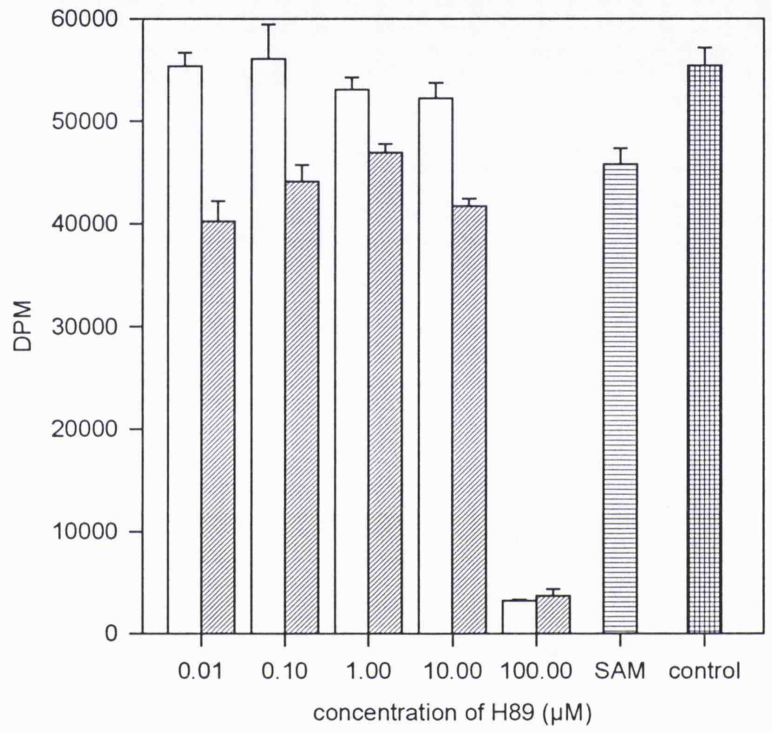


Figure 9.3. The effect of various concentrations of H89 on the anti-proliferative activity of 50ng/ml of SAM. Cells were incubated with various concentrations of H89 in the presence of SAM (diagonal hatched bars) and in the absence of SAM (open bars). Anti-proliferative activity was assessed by measuring the incorporation of [^{3}H]-thymidine into MG63 cells. The right hand column show the anti-proliferative activity of 50ng/ml of SAM (horizontal hatched bars) and an H89-free and SAM-free control (cross-hatched bars). The results are expressed as mean and SD of six replicate cultures.

To establish whether the anti-proliferative effect of the SAM was working through cAMP-dependent PK-A, 50ng/ml of SAM was incubated with MG63 cells in the presence of H-89 over the concentration range 0.01μM to 100μM. The activity of the SAM was then assessed by measuring [^{3}H]-thymidine incorporation into MG63 cells. Figure 9.3. shows the effect of H-89 in the presence and absence of SAM. At concentrations of 100μM the compound inhibited the incorporation of the radiolabel, whilst at lower concentrations H-89 was not anti-proliferative. SAM at 50ng/ml inhibited [^{3}H]-thymidine incorporation by 17.3% when compared to the control. Concentrations of H-89 up to and including 10μM appeared not to affect the anti-proliferative activity of the SAM.

9.2.2.2. Inhibition of protein kinase C

Two compounds obtained from Roche, 7549 and 8220, are potent and selective inhibitors protein kinase C (PK-C). It is generally accepted that P-KC plays a role in physiological cellular responses to external signals such as those related to growth promotion and cell differentiation (Nishizuka, 1984). PK-C is therefore a key enzyme for signal transduction and the SAM could be acting to inhibit cell proliferation through a PK-C mediated pathway.

1μg/ml of SAM was incubated with MG63 cells in the presence of compounds 7549 and 8220 over the concentration range 0.005μM to 5μM. The activity of the SAM was then assessed by measuring [^{3}H]-thymidine incorporation into MG63 cells. The results for both compounds were very similar (figure 9.4. and 9.5.). At 5μM, both compounds inhibited [^{3}H]-thymidine incorporation by approximately 80% in the absence of SAM. At lower concentrations the inhibitors of PK-C were unable to affect the anti-proliferative activity of the SAM.

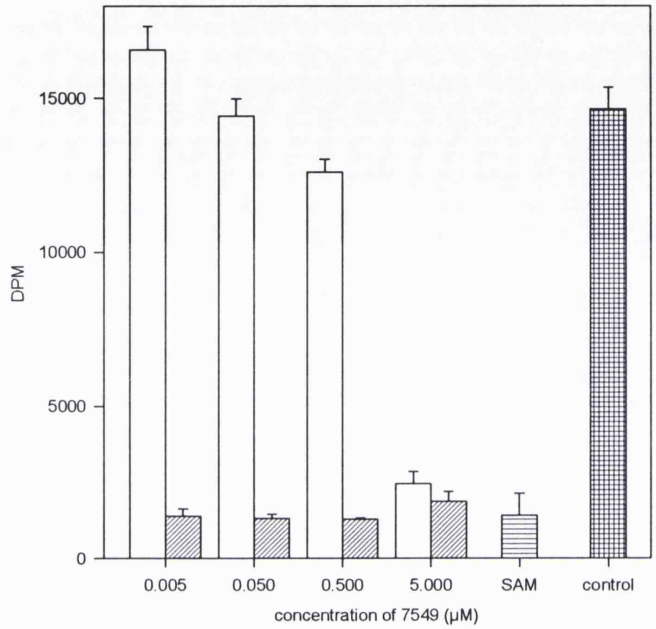


Figure 9.4. The effect of various concentrations of compound 7549 on the anti-proliferative activity of 1μg/ml of SAM. Cells were incubated with various concentrations of compound 7549 in the presence of SAM (diagonal hatched bars) and in the absence of SAM (open bars). Anti-proliferative activity was assessed by measuring the incorporation of [^{3}H]-thymidine into MG63 cells. The right hand column shows the anti-proliferative activity of 1μg/ml of SAM (horizontal hatched bars) and a 7549-free and SAM-free control (cross-hatched bars). The results are expressed as mean and SD of six replicate cultures.

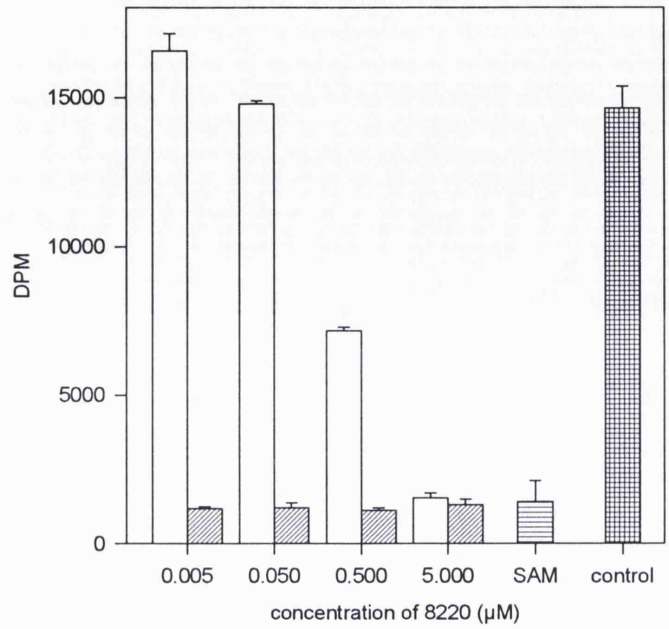


Figure 9.5. The effect of various concentrations of compound 8220 on the anti-proliferative activity of 1μg/ml of SAM. Cells were incubated with various concentrations of compound 8220 in the presence of SAM (diagonal hatched bars) and in the absence of SAM (open bars). Anti-proliferative activity was assessed by measuring the incorporation of [^{3}H]-thymidine into MG63 cells. The right hand column show the anti-proliferative activity of 1μg/ml of SAM (horizontal hatched bars) and a 8220-free and SAM-free control (cross-hatched bars).

9.2.3. Kinetics of inhibition of isotope incorporation

To further investigate the anti-proliferative effect of SAM, a more detailed analysis of the kinetics of incorporation of thymidine, uridine and amino acids, into DNA, RNA and protein respectively was performed. Each precursor was added separately to human gingival fibroblast cultures at 0h and incorporation was measured at various times from 1.5 to 24h (chapter 2, section 2.6.3.). As can clearly be seen (figure 9.6.) SAM (used at a concentration of 1μg/ml) had no inhibitory effect on DNA, RNA or protein synthesis during the first 6h of culture. However, at 21 and 24h there was clear evidence of inhibition of DNA and protein synthesis although there was less of an effect on RNA synthesis.

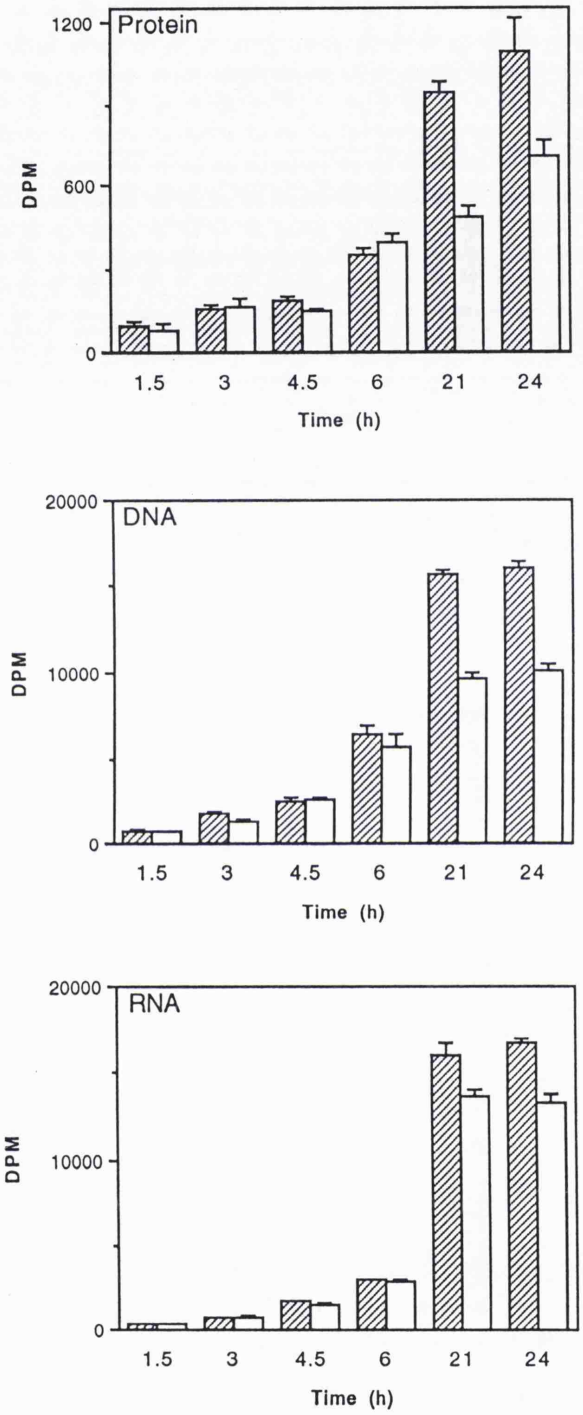


Figure 9.6. Effect of 1μg/ml of SAM from *A. actinomycetemcomitans* on DNA, RNA and protein synthesis (open bars), measured as incorporation of radiolabelled thymidine, uridine or amino acid mixture, by human gingival fibroblasts over a 24h period. Control cultures are depicted by hatched bars. The precursors were added separately to the cultures at 0 h and incorporation measured at various periods from 1.5 to 24h. The results are expressed as mean and standard deviation (SD) of six replicate cultures.

8.2.4. Comparison of the anti-proliferative protein with known inhibitors

The kinetics of inhibition of [^{3}H]-thymidine incorporation by MG63 cells exposed to 10μg/ml of SAM or 10ng/ml of the purified protein was compared to MG63 cells cultured in the presence of various inhibitors of DNA synthesis (cytosine arabinoside, hydroxyurea), or with an inhibitor of cell cycle progression (colcemid) (chapter 2, section 2.9.1). As shown in figure 9.7., SAM exhibited the same kinetics of inhibition of [^{3}H]-thymidine incorporation as caused by colcemid, which inhibits cell cycle progression by blocking cells in mitosis (M). In marked contrast, cytosine arabinoside and hydroxyurea had an immediate effect on DNA synthesis, demonstrating their known direct inhibition of S phase.

9.2.5. Flow cytometry (FCM)

Asynchronous cultures of human gingival fibroblasts were exposed to hydroxyurea, colcemid or SAM for 24h then stained with propidium iodide and the population DNA content measured by FCM (chapter 2, section 2.9.2.). Untreated cells showed a distribution of DNA content typical of proliferating mammalian cell cultures (figure 9.8.), with the majority of cells in the G_1/S phase of the cell cycle and many fewer in G_2/M. Cells treated with hydroxyurea had few cells in the G_2 or M phases of the cell cycle with most cells containing the 2n DNA, as expected of an inhibitor of DNA synthesis. In contrast, cells treated with colcemid showed a preponderance of 4n cells. Cells exposed to SAM also showed a large percentage of cells with 4n DNA (figure 9.8.). Thus, colcemid and SAM had apparently similar effects on the cell cycle, suggesting that the bacterial SAM could be an inhibitor of M phase.

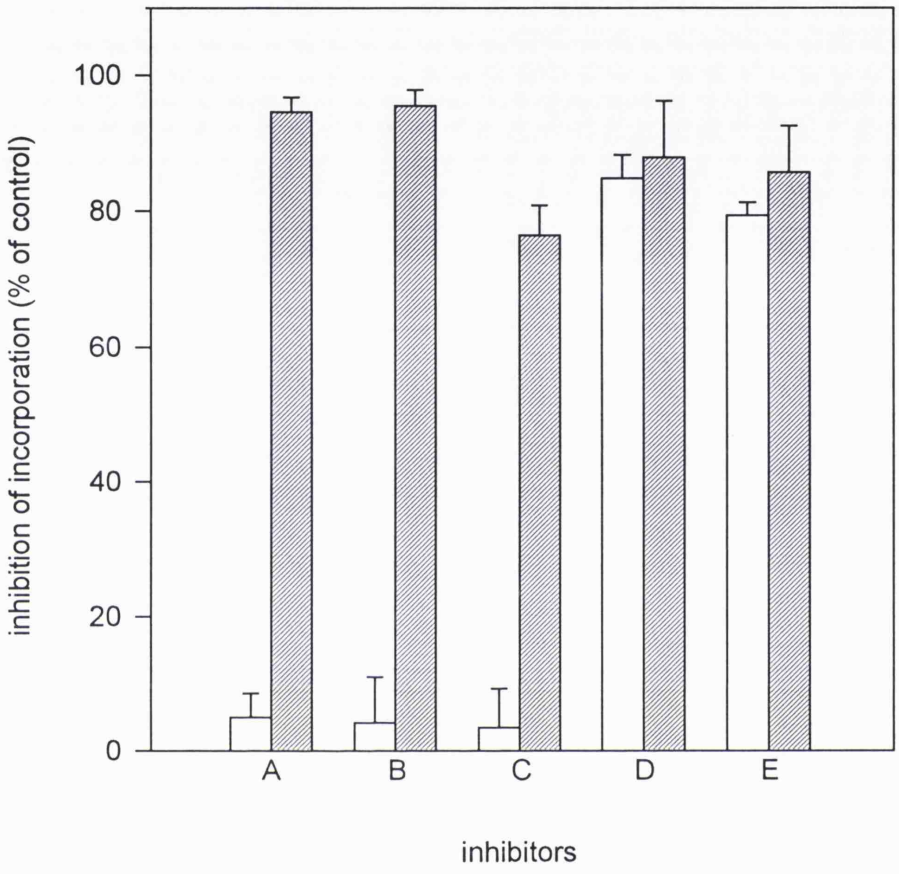


Figure 9.7. Delayed inhibition of DNA synthesis by the anti-proliferative protein from *A. actinomycetemcomitans*. MG63 cells were incubated with (A) SAM, 10μg/ml, (B) purified anti-proliferative protein, 10ng/ml, (C) colcemid, 0.1μM, (D) β-D-arabinofuranoside, (50μM) (E) hydroxyurea, 1mM. [^{3}H]-thymidine was added immediately to one-half of the wells containing each inhibitor, for 3h (open bars) and to the other half after 21h of culture with the inhibitors, also for a period of 3h (hatched bars). The results are expressed as mean and SD of six replicate cultures.

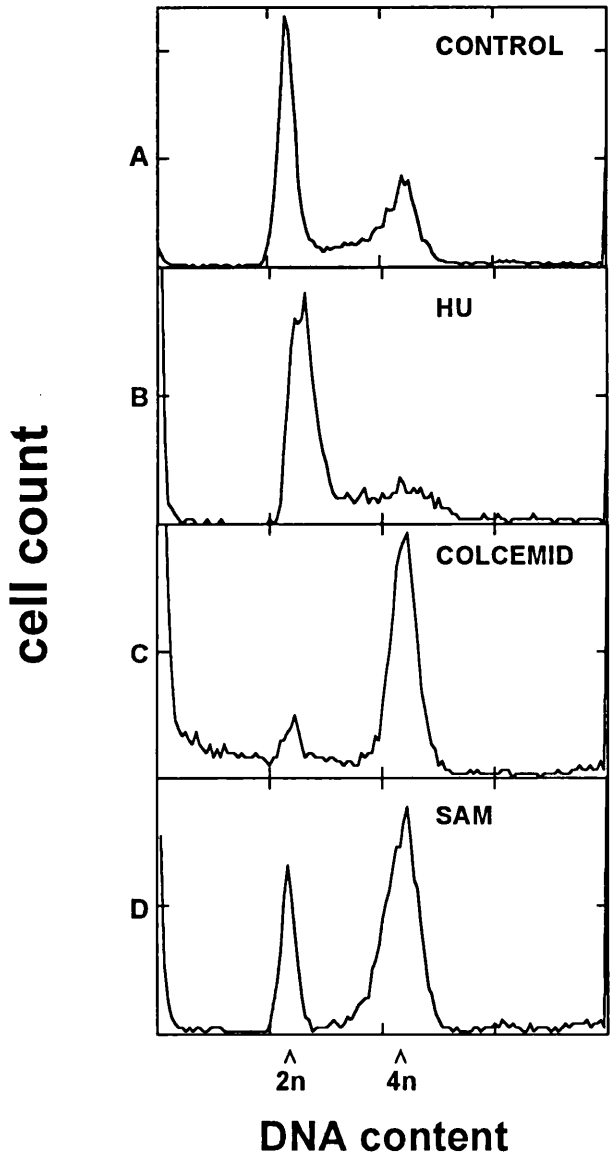


Figure 9.8. FCM analysis of the effects of the bacterial SAM on the DNA content of mammalian cells. Asynchronous cultures of cells were exposed to hydroxyurea, colcemid or SAM from *A. actinomycetemcomitans* for 24h then stained with propidium iodide and the population DNA content measured by FCM. Untreated cultures show a normal distribution of DNA content (A), with the majority of cells containing 2n DNA and some with 4n DNA. Cultures treated with hydroxyurea had very few cells containing 4n DNA (B). In contrast, cultures treated with colcemid showed a preponderance of 4n cells (C), the same as cultures exposed to the bacterial surface-associated material (D).

9.2.6. Cell synchronization

The cell cycles of MG63 cultures were synchronized by incubating cells in the presence of 1mM hydroxyurea for 24h to arrest the cells at the G_1/S boundary. Removal of hydroxyurea by washing then allowed cells to enter the S-phase of the cell cycle (chapter 2, section 2.9.3.).

9.2.6.1. Use of synchronized MG63 cells to monitor number of mitotic cells after treatment with the anti-proliferative protein

The proportion of mitotic cells was determined every 2h after release from hydroxyurea-induced G_1/S phase arrest (chapter 2, section 2.9.3.). As shown in figure 9.9, the highest proportion of cells in control cultures were in mitosis within 10h, the number declining thereafter. In the case of the colcemid-treated cultures, 56% had reached mitosis within 10h, and by 12h 83% were arrested in mitosis. In contrast, cultures treated with either 500ng/ml of the semi-purified anti-proliferative protein (active material, following ammonium sulphate precipitation and anion exchange HPLC of SAM), or 10μg/ml of the SAM showed virtually no cells in mitosis throughout the 12h period. Figure 9.10. shows the appearance of cells after a 10h exposure to colcemid, SAM or the semi-purified protein.

9.2.6.2. Time-lapse photography of cells treated with the semi-purified anti-proliferative protein

Cells treated with semi-purified protein were released from hydroxyurea-induced arrest, and filmed and compared to control cultures (chapter 2, section 2.9.4.). Over a 48h period none of the cells (approximately 100 analysed) treated with the anti-proliferative protein were seen to divide, whilst the control cells were clearly observed passing through mitosis (results not shown).

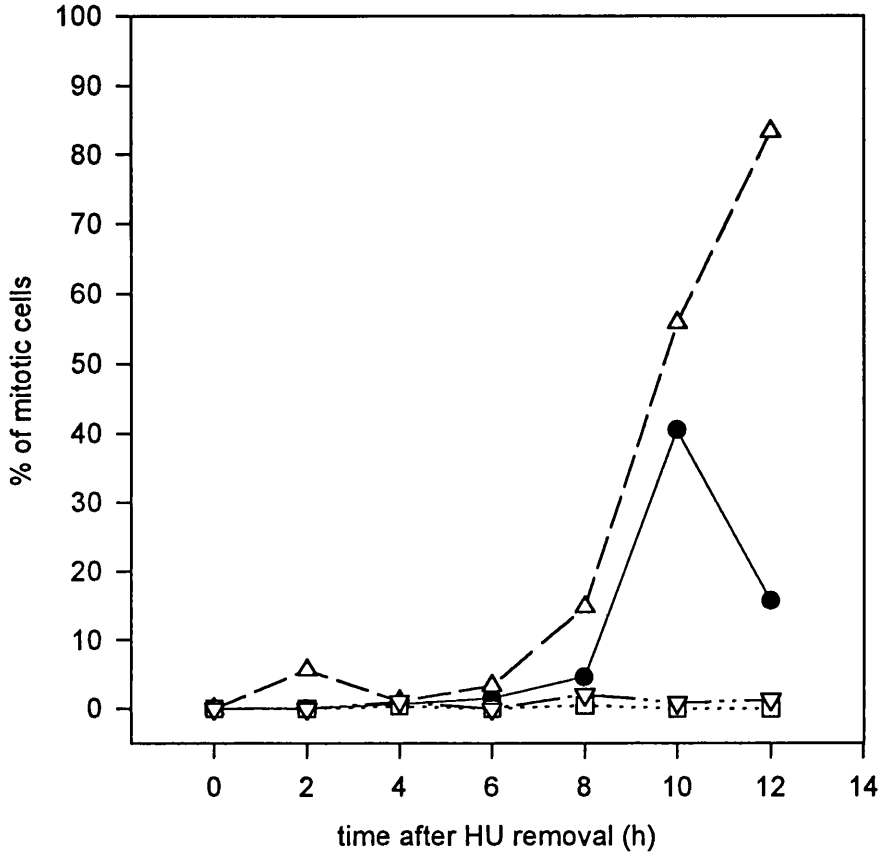


Figure 9.9. Appearance of mitotic cells after release from hydroxyurea-induced G_1/S arrest. MG63 cultures were synchronized by incubating cells in the presence of hydroxyurea for 24h to block cells at the G_1/S boundary, as described. The proportion of mitotic cells was measured every two hours after removal of the hydroxyurea. In control cultures (circle) the highest number of cells were in mitosis within 10 h, the proportion declining by 12 h. In the case of colcemid-treated cultures (triangle) 83% had reached and been trapped in mitosis after 12 hours. In contrast cultures treated with either 500ng/ml of the semi-purified anti-proliferative protein, gapstatin (inverted triangle) or 10μg/ml of SAM (square) showed virtually no cells in mitosis throughout the 12 hour period. Approximately 250 cells were counted for each data point.

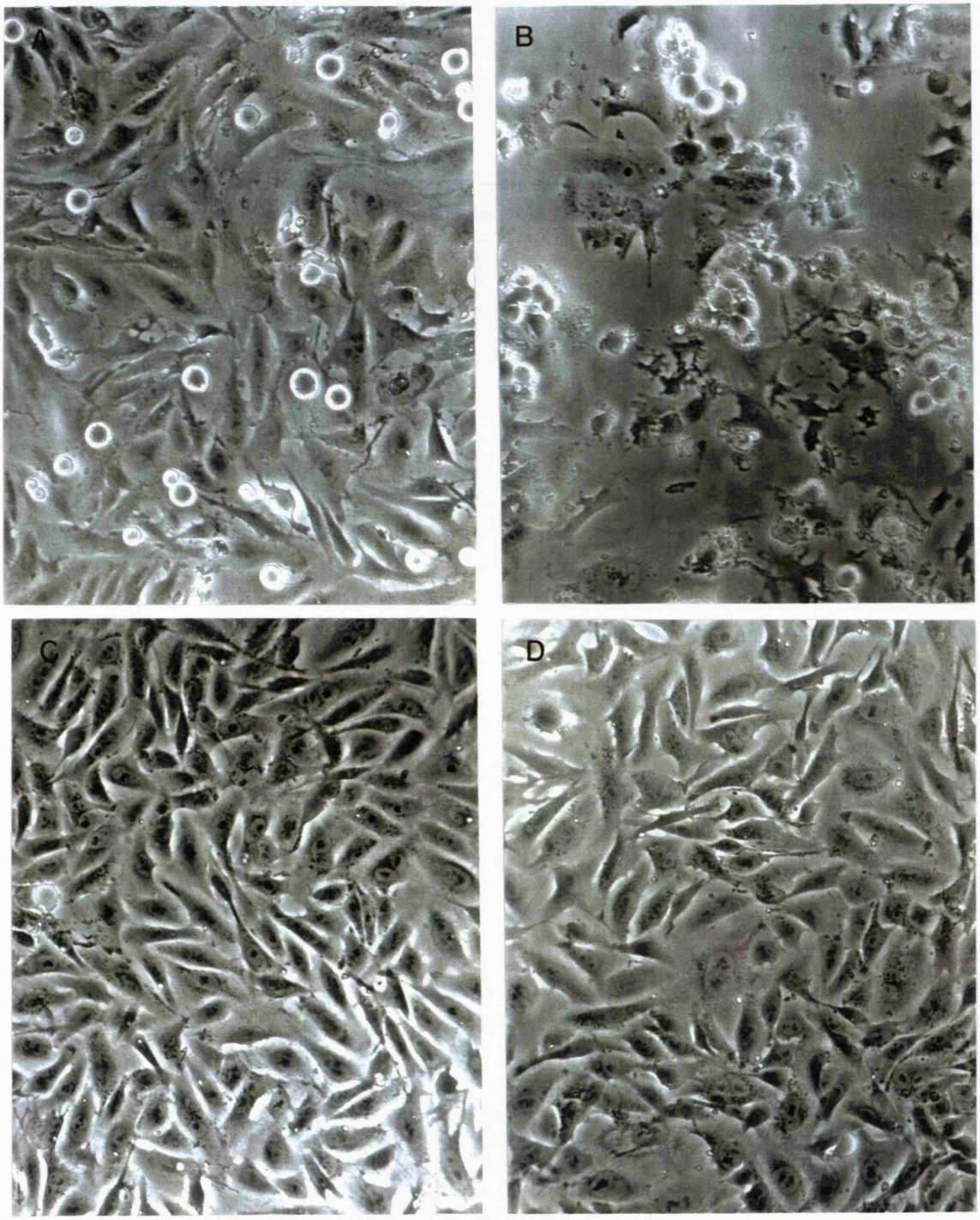


Figure 9.10. The cell cycles of MG63 cultures were synchronized by incubating cells in the presence of hydroxyurea for 24h to block cells at the G_1/S boundary. Removal of hydroxyurea by washing then allowed cells to enter the S-phase of the cell cycle. The proportion of mitotic cells (rounded cells) was photographed 10 hours after release from synchrony. Control cultures showed numerous cells in mitosis (A). In the case of the colcemid treated cells the majority of the cells were trapped in mitosis after 10 hours (B). In contrast cells treated with either 10μg/ml of the crude surface-associated fraction (C) or 500ng/ml of the semi-purified anti-proliferative protein (D) showed virtually no cells in mitosis. x400

9.2.7. Is activity due to cell-surface receptor binding

MG63 cells were cooled to 4°C, and these cells, and cells maintained at 37°C, were incubated with SAM for various times from 5min to 18h. Cells were then washed to remove any free bacterial proteins and then re-cultured for a total of 24h at 37°C, being pulsed with [^{3}H]-thymidine over the last 6h (chapter 2, section 2.9.5.). Cells which had been exposed to the SAM for only 5min showed significant inhibition of thymidine incorporation (figure. 9.11.).

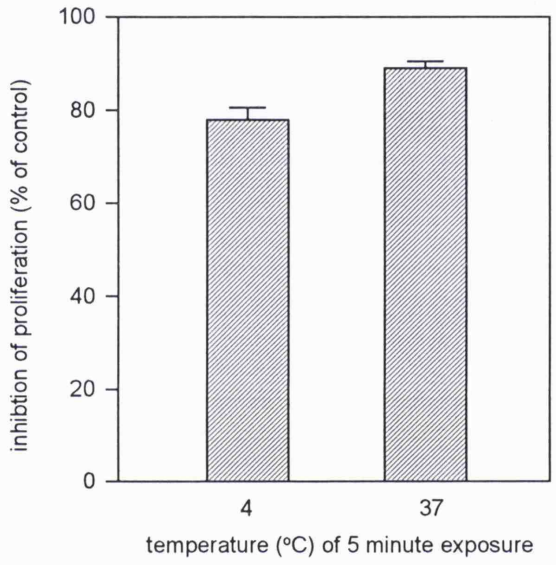


Figure 9.11. The effect of a 5 min exposure of the surface-associated material on the proliferation of MG63 cells. Cultures were treated at either 4°C or 37°C and incubated for 24 h, being pulsed with [^{3}H]-thymidine over the last 6 h. The results are expressed as mean and SD of six replicate cultures.

Further evidence for the presence of a cellular receptor was obtained from experiments in which 1μg/ml of the surface-associated material was serially incubated with monolayers of MG63 cells (chapter 2, section 2.9.3.). The surface-associated material was incubated with an MG63 cell monolayer for

2h, then the medium was removed and incubated with a fresh monolayer, this process being repeated a third time. Cells were cultured for 24h, being pulsed with [^{3}H]-thymidine over the last 6h, and the inhibition of incorporation assessed relative to control cells. At concentrations of 1μg/ml there was obvious depletion of the anti-proliferative activity after one exposure to cells and there was almost complete removal of activity by the third exposure to cells, showing that cells were able to bind a substantial fraction of the active molecule in the 2h period of contact (figure 9.12.).

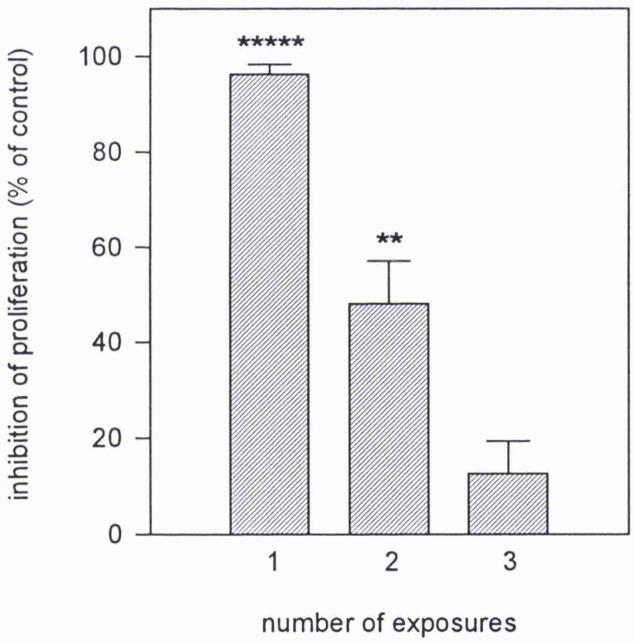


Figure 9.12. Depletion of the anti-proliferative protein by binding to MG63 cells. The surface-associated material (1μg/ml) was serially incubated with a monolayer of MG63 cells. After a 2 h exposure the medium was removed and re-incubated with a fresh monolayer, this process being repeated a third time. Cells were cultured for 24 h, being pulsed with [^{3}H]-thymidine over the last 6 h. The results are expressed as mean and SD of six replicate cultures. **$p<0.01$, *****$p<0.00001$.

9.3. Discussion

There have been a number of reports during the past two decades that macromolecular fractions from *A. actinomycetemcomitans* can inhibit the proliferation of mammalian cells (Shenker *et al.*, 1982; Kamen, 1983; Stevens *et al.*, 1983; Taichman *et al.*, 1984; Kamin *et al.*, 1986; Stevens *et al.*, 1988; Meghji *et al.*, 1992a; Meghji *et al.*, 1992b). However, the active component/s have not been studied in detail and remain poorly defined. Moreover, the mechanism by which proliferation is inhibited has not been elucidated and may in some cases be attributable to their cytotoxicity. Previous studies at the Eastman showed that surface-associated material was able to inhibit the incorporation of radiolabelled thymidine by osteoblasts, myelomonocytic cells and epithelial cells (Meghji *et al.*, 1992). Furthermore, treatment of human gingival fibroblasts with SAM resulted in the development of enlarged nuclei (Kamin *et al.*, 1986).

To establish whether or not SAM-induced inhibition of proliferation was attributable to cytotoxicity, experiments involving measurement of LDH release and acridine orange staining were performed. SAM was shown to be non-cytotoxic to human gingival fibroblasts or MG63 cells at 100μg/ml after a 24 h incubation, the time period used for these studies. Thus, inhibition of proliferation could not be attributable to a cytotoxic effect.

The surface-associated material from *A. actinomycetemcomitans* did not inhibit DNA, RNA and protein synthesis during early times of exposure, it was only after 21h in culture that there was significant inhibition of radiolabel incorporation. Following the purification of the active protein, the activity of this molecule was compared with known inhibitors of DNA synthesis such as hydroxyurea and cytosine arabinoside. These low molecular mass direct inhibitors produced an immediate inhibition of DNA synthesis. However, the crude SAM and the anti-proliferative protein, like colcemid, only showed activity after being in contact with the cells for significant periods of time, for

example 21h. This suggested that the mechanism of action of the cell cycle inhibitor might be a delayed action, similar to colcemid. Colcemid causes an inhibition of DNA synthesis due to interference with microtubule assembly resulting in cells being trapped in mitosis, thus inhibiting further replication. Analysis of the human gingival fibroblast population DNA content by FCM showed that hydroxyurea-treated cells were blocked at the S phase of the cell cycle and had 2n levels of DNA. In contrast, both the colcemid- and SAM-treated cultures showed a preponderance of cells with 4n levels of DNA. The effects of the anti-proliferative protein on synchronized MG63 cells, following release from hydroxyurea-induced arrest at G_1/S, was examined. In these experiments the proportion of mitotic cells in the presence of SAM, or the semi-purified anti-proliferative protein, was counted. Virtually no cells were able to enter mitosis during the 12h incubation, whilst 83% of cells entered mitosis in the control cultures. Thus, it appeared that the anti-proliferative protein was able to inhibit DNA synthesis by blocking mammalian cell cycle progression from G_2 to M. Further evidence for a G_2 blockade was demonstrated when cells were synchronized, treated with the semi-purified anti-proliferative protein and filmed for 48h. In this period no cells were seen to enter mitosis as assessed by rounding up of the cell followed by division into two daughter cells. In contrast numerous control cells were clearly seen passing through mitosis.

These studies demonstrated similar results to that of Helgeland and Nordby (1993), who described the presence of a cell cycle-specific inhibitory activity in culture media supporting *A. actinomycetemcomitans.* In this study the authors also found that cells were blocked in the G_2 phase of the cell cycle, however the activity was associated with an uncharacterized component of approximate molecular mass 50kDa.

We have few clues to the mechanism by which cells are blocked in G_2. In studies in which cells were exposed to the SAM for very short periods of time at 4°C it was clear that there was marked inhibition of DNA synthesis, possibly indicating binding of the active protein to the cell surface. In other experiments it was found that the anti-proliferative activity of the SAM was depleted by repeated exposure to MG63 cells. Taken together these results suggest that there is a high affinity receptor for this anti-proliferative protein on mammalian cells. Whether this triggers some intracellular signalling process has not been clearly established. Inhibitors of protein kinases A and C had no effect on the inhibition of DNA synthesis by the anti-proliferative protein, suggesting that intracellular signalling may not involved in the anti-proliferative activity of the SAM. At high concentrations these compounds caused an inhibition of DNA synthesis in the absence of SAM.

The mechanism of action of the anti-proliferative protein is unclear. It blocks cells in G_2 and may be having an effect on the generation of the cyclinB-cyclin kinase mitosis-promoting factor (MPF) complex which is required to drive cells into the mitotic cycle (Sherr, 1993). The anti-proliferative protein is one of the most potent inhibitors of cell proliferation, producing 50% inhibition of cell proliferation (IC_{50}) at a concentration of 50 picomolar. This shows that *A. actinomycetemcomitans* produces a protein which functions to modulate the eukaryotic cell cycle. This may represent a novel type of virulence factor.

Chapter 10
General Discussion

10.1. Introduction

During the past two decades an increasing number of studies have implicated *A. actinomycetemcomitans* as the aetiological agent of localized juvenile periodontitis (Slots *et al.*, 1980; Hammond and Stevens, 1982; Slots *et al.*, 1982; Slots and Genco, 1984; Zambon, 1985; Moore, 1987; Zambon, 1988). LJP is characterized by rapid loss of alveolar bone resulting in tooth mobility and loss. This could be due to the stimulation of bone breakdown processes or the inhibition of bone formation or a combination of both. In this thesis I have tested the hypothesis that proteins loosely-associated with the cell wall of *A. actinomycetemcomitans* could be responsible for the bone loss seen in LJP. The aims of this thesis were therefore to isolate, characterize and clone these putative bone virulence proteins.

The thesis has concentrated on two potential bone-modulating activities detected in the proteinaceous surface-associated material of *A. actinomycetemcomitans*. The SAM, isolated by washing the bacteria gently in saline, accounts for a large proportion of the dry weight of the bacteria and contains approximately 75 proteins or protein subunits when it is separated by two-dimensional SDS-PAGE and silver stained. The two bone-modulating activities examined include a direct osteolytic activity, as assessed using the mouse calvarial bone resorption assay, and an anti-proliferative activity, which is particularly active against osteoblasts, assayed by measuring the incorporation of [^{3}H]-thymidine into DNA. The osteolytic component was isolated and has been shown to be a member of the chaperonin 60 family of heat shock proteins. The anti-proliferative component was shown to be non-cytotoxic, heat- and trypsin-sensitive and could be neutralized by the sera from a proportion of patients with localized juvenile periodontitis. In an attempt to clone surface antigens, and in particular the anti-proliferative protein, from *A.*

actinomycetemcomitans these sera were used to screen a genomic library of this organism in *E. coli*. Although three recombinant clones were isolated, none possessed anti-proliferative activity. The anti-proliferative component, extracted from the SAM, was purified to homogeneity and was shown to have an apparent molecular mass of 8kDa. Studies to elucidate the mechanism of action of the anti-proliferative protein revealed that it caused cell cycle arrest by blocking progression from G_2 to M. The anti-proliferative protein from *A. actinomycetemcomitans* has been termed gapstatin.

10.2. Chaperonin 60

Molecular chaperones are a ubiquitous family of cellular proteins which mediate the correct folding of other polypeptides. Therefore, the finding that a molecular chaperone, chaperonin (cpn) 60, was able to cause bone destruction is a surprising and fascinating discovery. Cpn 60 is an oligomeric complex of 14 approximately 60kDa identical subunits with a total molecular mass of 850-900kDa. Molecular chaperones are evolutionarily-conserved proteins, the human cpn 60 known as P1 being 48% homologous to the *A. actinomycetemcomitans* protein.

As well as *E. coli* groEL, other members of the mammalian molecular chaperone families have recently been shown to be osteolytic using the mouse calvarial bone resorption assay (Kirby *et al.*, 1995; Nair *et al.*, 1996). These results could indicate that a conserved region of all molecular chaperones, responsible for the correct folding of peptides, is also responsible for osteolytic activity. Whether these results are coincidental, or the osteolytic activity is indeed intrinsically linked to the ability of the molecules to fold proteins, is as yet uncertain. Cpn 60 also has ATP-ase activity associated with the N-terminus of the molecule. It is not known whether the osteolytic activity is dependent on this enzymatic activity, however it would be interesting to test this hypothesis. Although the exact mechanisms by which cpn 60 stimulates bone resorption are unclear, recent work by my colleague Dr. Kris Reddi has suggested that this

molecule acts directly on the osteoclast as it was able to directly stimulate rat osteoclasts to form resorption pits on dentine slices (Reddi *et al.*, 1996). These studies imply a much larger role for the cpn 60 protein in the pathogenesis of other bacterial infections than has hitherto been considered.

10.3. The cell cycle

The anti-proliferative activity of gapstatin could represent a unique mechanism of growth inhibition, which raises the question; how is this protein able to prevent cell cycle progression from the G_2 to M phase? The following section outlines the cell cycle and the key events during G_2 to M phase transition.

10.3.1. Cell cycle progression

Mitosis is the only visible part of the cell cycle, during which the chromosomes condense, pair, split and the cell divides in the process called cytokinesis. Stefan Pelc (1956) was responsible for the cell cycle nomenclature of the G_1,G_2 and S phases. G stands for gap as Pelc could see no signs of cell activity between mitosis and the start of DNA synthesis and, the end of DNA synthesis and mitosis. The cell cycle thus consists of the M (mitotic) phase where cell division occurs. Following M phase there is a gap termed the G_1 phase (first gap) which precedes the S (synthetic) phase, where DNA synthesis occurs. The final G_2 phase (second gap) follows the S phase prior to mitosis (figure 10.1.). Many non-dividing cells in tissues (for example, all resting fibroblasts) suspend the cycle after mitosis and just prior to DNA synthesis, these cells are said to be in G_0 state.

Progression of eukaryotic cells through the major cell cycle transitions is mediated by complexes of proteins known as cyclins, and a family of related protein kinases, the cyclin-dependent kinases (CDKs) (Matthias and Herskowitz, 1994). Cell cycle regulation is controlled at discrete points called check points which ensure correct cell cycle transitions by monitoring the execution of specific events (such as DNA replication or spindle assembly) and coupling

them to further progression. Perhaps the most significant development recently has been the isolation of a family of small cyclin-CDK inhibitor proteins (CDIs) that bind and inactivate the CDKs. Individual inhibitor proteins are often absent from transformed cells, marking them out as potential products of tumour suppressor genes (Hunter and Pines, 1994). For example, the CDI p53 mediates arrest of the cell cycle in G_1 in response to DNA damage. Figure 10.1. shows a schematic view of points of action of mammalian cyclin-CDK complexes in the cell cycle.

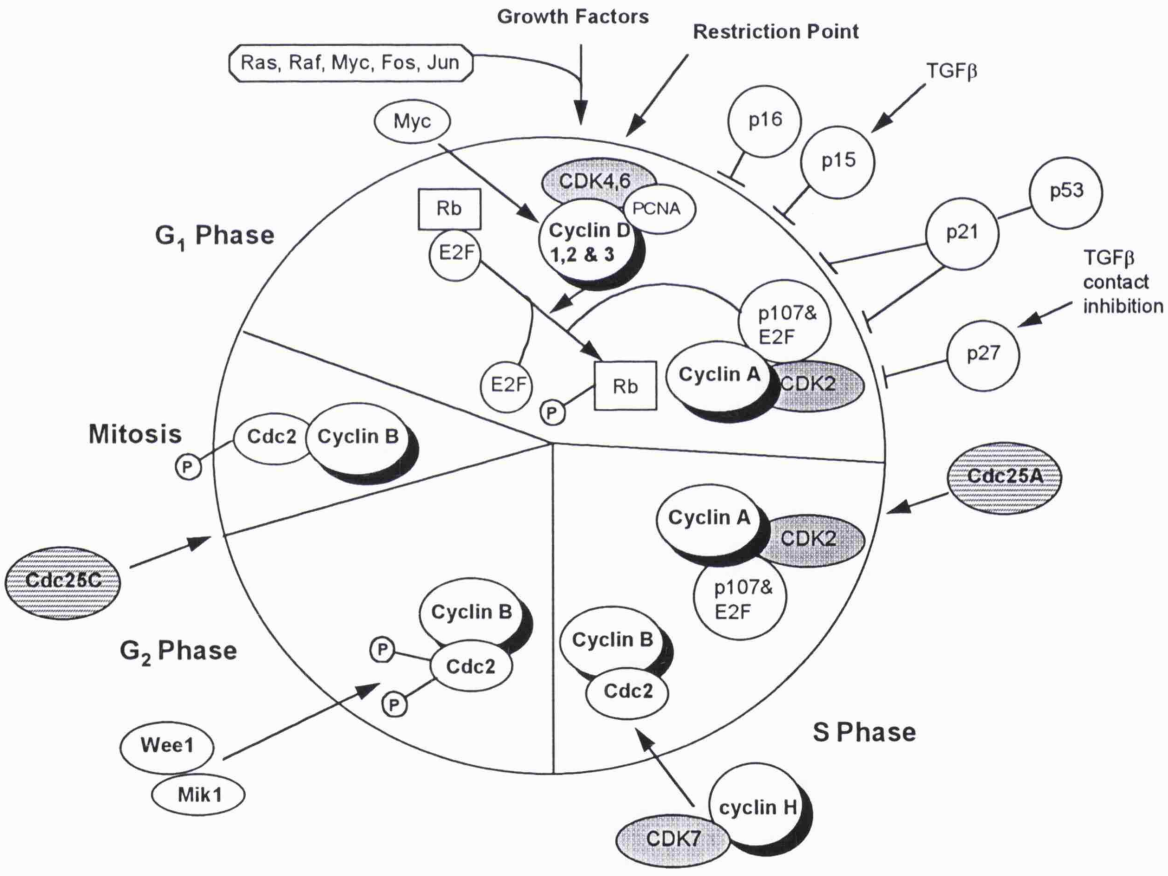


Figure 10.1. A schematic view of the points of action of mammalian cyclin-CDK complexes in the cell cycle. Cyclins are depicted by shadows.

In mammalian cells, the cyclin-CDK complexes most closely linked to the regulation of the start of the cell cycle (G_1 phase) are the D-type cyclins and their partner CDKs (Sherr, 1993), they are also thought to act as growth factor sensors. The S phase check point is controlled by the E-type cyclins which act at the G_1/S transition itself and are important in the initiation of DNA replication. A-type cyclins are required continuously for progression through S phase and again for the G_2/M transition.

Of particular interest to this thesis are the control mechanisms involved in the transition from the G_2 to the M phase of the cell cycle. The key component of this pathway is a mitotic regulator composed of the protein subunit Cdc2 and its positive regulatory subunit, cyclin B. Together, these form mitosis-promoting factor (MPF). Since Cdc2 is constitutively present, it is the accumulation and periodic degradation of cyclin B that determines the oscillation of MPF activity. The mitotic cell cycle can be defined by three transitions that involve cyclin B (K ing *et al.*, 1994). In the first, cyclin B activates MPF and initiates prophase. In the second, MPF activates a ubiquitin-dependent proteolytic system, causing both cyclin B destruction and the initiation of anaphase. In the third, the degradation machinery for cyclin B is turned off and the cell cycle reset.

10.3.2. Gapstatin and G_2/M transition

In this discussion I will concentrate on the transition from the G_2 to M phase as this is the key event which appears blocked by the anti-proliferative protein, gapstatin. In all cells cyclin B must accumulate above a threshold level to enter mitosis. Therefore by blocking the expression of cyclin B, cells would arrest at G_2, this is an obvious mechanism by which gapstatin could exert it effect. To generate active MPF, Cdc2 must also bind to cyclin B. Gapstatin could also block this binding event by binding to either Cdc2 or MPF. As well as the binding of Cdc2 to cyclin B, active MPF relies on Cdc2 phosphorylation at Thr-161. The kinase that phosphorylates this site is the Cdc2-activating kinase (CAK), and its catalytic subunit called Cdk7 (Poon *et al.*, 1993). The story gets

somewhat complicated here as CAK itself also requires phosphorylation and a positive regulatory subunit called cyclin H for its activation. It is unclear whether the absence of CAK activity would inhibit the progression of the cell cycle, however this remains a possible area for the activity of gapstatin, thus arrest could occur due to; (i) inactivation of CAK (ii) inhibition of the phosphorylation of CAK, or (iii) inactivation of cyclin H.

Negative regulators of MPF could also be targets for gapstatin. In the mammalian cell, in late S phase and G_2, Cdc2 is phosphorylated on Try-15 and Thr-14 by Wee1/Mik1-related protein kinases, which maintains Cdc2 in a catalytically inactive state (Draetta *et al.*, 1988). Connecting Wee1 to other cell processes is another protein kinase, Nim1, which phosphorylates and inhibits Wee1 to activate mitosis. Dephosphorylation of Cdc2 is correlated with its activation. At the end of G_2, Cdc25 phosphatase is stimulated to dephosphorylate T14/Y15 and activate Cdc2 as part of a positive feedback loop. In normal cells, DNA damaged by radiation or alkylating reagents prevents T14/Y15 dephosphorylation, so that cells arrest in G_2 (Hunter and Pines, 1994). Inactivation of Cdc25 could be the most likely target for the mechanism of action of gapstatin, by inactivating this protein or preventing its expression gapstatin could cause G_2 arrest. Phosphorylation of Cdc25 is related to its ability to promote mitosis as demonstrated by the activity of protein phosphatases PP1 and PP2A to inhibit Cdc25 through its dephosphorylation (Izumi *et al.*, 1992; Maller, 1994). Due to the small mass of gapstatin it is unlikely that it could cause G_2 arrest by inhibiting Cdc25 through dephosphorylation as phosphatase activity is normally associated with proteins of a larger molecular mass. Figure 10.2 shows a simplified diagram of the complicated events involved in MPF activation which allows G_2 transition to M phase and the possible points at which gapstatin could interfere.

Finally going back to cyclin-CDK inhibitor proteins (CDIs) that bind and inactivate the CDKs. Low molecular weight stoichiometric inhibitors of Cdk2 such as p21 (Sherr, 1994) have been shown to weakly inhibit Cdc2-cyclin B *in vitro* (Xiong *et al.*, 1993). So far, no direct stoichiometric inhibitor specific to Cdc2 has been identified, it is possible that gapstatin is acting like a CDI to inhibit Cdc2. I have speculated on a number of possible key events in the transition of G_2 to M phase where gapstatin could be acting, although there remains a vast number of other possibilities to explain exactly how gapstatin exerts its effect.

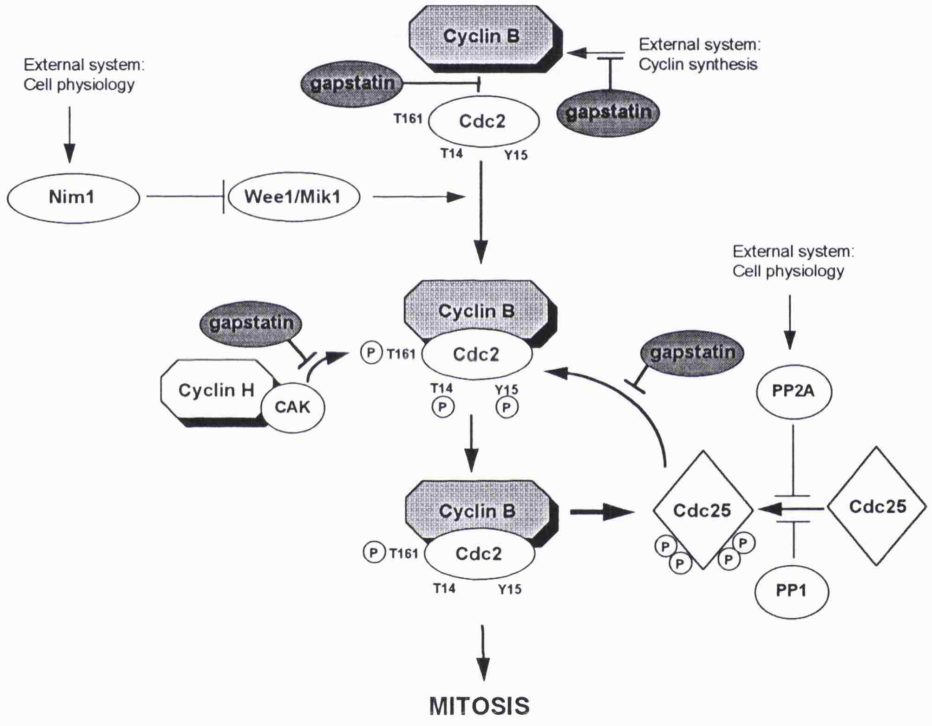


Figure 10.2. Mitotic phosphorylation and the activation of MPF. Following translation, cyclin B associates with unphosphorylated Cdc2; Cdc2 is then activated by phosphorylation on Thr-161 (T161). In interphase, the activation of Cdc2 is restrained through tyrosine phosphorylation by the active Wee1 tyrosine kinase or related Mik1. The tyrosine phosphatase Cdc25 will dephosphorylate the inactive, tyrosine-phosphorylated Cdc2-cyclin B complex and activate the complex. This active complex constitutes MPF. Cdc25 is activated when phosphorylated during mitosis and possibly by Cdc2 directly. Active Cdc25 promotes Cdc2 dephosphorylation on tyrosine and MPF activation. These regulations comprise the Cdc25 autoactivation loop. External control include cyclin synthesis and the control of some regulators like the kinase Nim1 or the phosphatase PP2A. The four main key control points where gapstatin may be acting are shown (see text).

10.4. Bacterial proteins which affect cell division

There are few studies of bacterial proteins which can inhibit mammalian cell proliferation without being cytotoxic. Inhibiting the division of cells rather than killing them could be of advantage to bacteria, cells which are not killed but are unable to proliferate could prove more of an impedance to the host in the eradication of the infecting bacteria, than dead cells, which would be destroyed and replaced. This is analogous to the wounded soldier strategy used in warfare, whereby the enemy is deliberately wounded rather than killed outright to impose a burden on the rival forces.

Of the few bacterial proteins identified which affect cell division, an interesting finding is that they are all from bacteria associated with diseases involving bone loss. *Por. gingivalis*, is an oral bacterium implicated in the aetiology and pathogenesis of adult periodontitis (White and Maynard, 1981; Loesche *et al.*, 1985). Mihara and Holt (1993) reported the isolation of a 24kDa outer membrane protein termed fibroblast activating factor (FAF) from this organism, which significantly stimulated proliferation. Concentrations of 150ng/ml resulted in a rate of [^{3}H]-thymidine incorporation into HGF four to five times higher than control cultures. Although quite an opposite effect is exerted by FAF when compared to gapstatin, it is interesting to note that purified gapstatin is two log orders more active than this protein. The authors hypothesised that FAF was acting in a similar manner to epidermal growth factor (EGF) or fibroblast growth factor (FGF). The purified protein was tested for its bone-resorptive capability and was shown to promote *in vitro* bone resorption and also to stimulate the formation of multinucleated, tartrate-resistant acid phosphatase-positive monocytes; indicators of osteoclast cell formation (Mihara *et al.*, 1993). It is unclear exactly how FAF caused bone destruction or indeed whether its ability to stimulate proliferation was linked to its osteolytic activity, yet here is clear evidence that a protein which affects cell division can also cause bone destruction.

One of the most potent bacterial bone resorbing factors described to date is *Pasteurella multocida* toxin (PMT) (Felix *et al.*, 1992). The 146kDa toxin has been established as the primary aetiologic factor in the pathogenesis of progressive atrophic rhinitis in pigs, which results in the destruction of the pig's snout (Foged, 1992). PMT is extremely potent at stimulating the proliferation of fibroblasts and several other cell types (Rozengurt *et al.*, 1990). PMT has recently been shown to down-regulate the expression of several markers for osteoblast differentiation and to inhibit the *in vitro* deposition of mineral in cultures of primary osteoblasts and osteoblast-like osteosarcoma cells (Mullan and Lax, 1996). The exact mechanisms of bone resorption by PMT are unclear; it may cause the inhibition of osteoblast development, promoting a pre-osteoblast/fibroblast population incapable of forming a normal bone matrix (Mullan and Lax, 1996). This, in effect, could be similar to the action of gapstatin whereby the differentiation of pre-osteoblasts into osteoblasts is impeded through inhibition of proliferation.

Bordetella bronchiseptica produces an 145kDa dermonecrotic toxin (DNT) which has also been implicated in the pathogenesis of atrophic rhinitis (Horiguchi *et al.*, 1993). When added to the murine osteoblastic cell line MC3T3-E1 it caused changes in cellular architecture and potently inhibited the osteoblast's capacity to produce alkaline phosphatase and collagen *in vitro* (IC_{50} - 100pg/ml) (Horiguchi *et al.*, 1993). DNT, like PMT, is a potent stimulator of [^{3}H]-thymidine incorporation into MC3T3-E1 cells with activity at concentrations as low as 0.3ng/ml. In culture, the number of DNT-treated cells does not increase, although they become multinucleated. It therefore appears that DNT inhibits cytokinesis, but it is not clear how this is achieved or how it relates to the inhibition of osteoblast function, such as collagen synthesis. Although the ability of SAM to inhibit collagen synthesis has not been directly linked to gapstatin, this is likely to be the case. Thus it could be hypothesised that although DNT and gapstatin affect cell division in different ways, they may help to contribute to bone loss through the modulation of osteoblast function.

Fusobacterium nucleatum has also been implicated in destructive periodontal disease (Slots *et al.*, 1983). Of particular interest is a recent paper by Shenker and Datar (1995) which describes the isolation of a 90-100kDa protein, termed *F. nucleatum* immunosuppressive protein (FIP), which was capable of inhibiting mitogen-induced proliferation of human T-cells in a dose-dependent manner. FIP, like gapstatin was shown to inhibit proliferation by preventing cell cycle progression. Cell cycle analysis indicated that although FIP-treated T-cells were activated and had entered the G_1 phase of the cell cycle, they were prevented from exiting the G_0/G_1 phase of the cell cycle. Analysis of the expression of cyclins indicated that the phase of the cell cycle that is FIP-sensitive resides somewhere beyond the restriction point of cyclin D (early to mid-G_1) but prior to cyclins D3 and E (mid to late-G_1). Analysis of the expression of the proliferating cell nuclear antigen (PCNA) indicated that this was the earliest detectable defect in T-cells exposed to FIP.

A recent paper (Kurita-Ochiai and Ochiai, 1996) reported the isolation of a 14kDa protein from *A. actinomycetemcomitans* with the ability of suppressing mitogen-stimulated proliferation of, and cytokine production by, C3H/HeN mouse spleenic T-cells. Unfortunately the affect of the purified protein was not tested on the proliferation of fibroblasts or osteoblasts, although it is unlikely that this protein is gapstatin as it was sensitive to heat, where gapstatin is heat labile.

The concept is emerging that bacteria produce a range of proteins which modulate eukaryotic cell cycle progression, rather than inhibiting DNA synthesis. Thus, these combined studies may herald the discovery of a new class of virulence factor implicated in destructive bone diseases.

10.5. Implications of bone-modulating proteins in LJP

Alveolar bone supports the teeth and its loss is the most serious problem in periodontal disease. Like the rest of the skeleton, alveolar bone continues to be remodelled throughout life and, in the absence of disease, resorption of bone by osteoclasts is equilibrated by "coupled" deposition of new bone by osteoblasts. Bone resorption occurs when there is an imbalance between bone resorption and formation. Thus the loss of bone in periodontal disease could be due to: (i) increased resorption by osteoclasts; or (ii) a decrease in bone formation by osteoblasts.

It is now widely accepted that the loss of alveolar bone in periodontal disease is associated with the release of certain bacterial factors within the periodontal pocket. Both bacterial virulence factors themselves, or host factors released via virulence factor stimulation, may contribute to bone loss. Although it is likely that there is a role for host factors in mediating bone resorption, this thesis has favoured the hypothesis that bone loss in LJP is caused directly by the bacterial factors. Support for this in LJP is provided by the fact that the diseased gingiva are only mildly inflamed, thus indicating a limited release of pro-inflammatory mediators (Schluger *et al.*, 1990.). Work in this thesis has concentrated on two putative bone-modulating proteins isolated from the surface-associated material; the first being cpn 60 of *A. actinomycetemcomitans* which demonstrates potent osteolytic activity; and the second being the anti-proliferative protein, gapstatin.

Several bacterial components can stimulate bone resorption, the most widely studied being LPS. This study, and other studies, have shown that the SAM from *A. actinomycetemcomitans* is extremely active at promoting bone resorption with significant destruction being observed at concentrations as low as 10ng/ml in some experiments. In contrast the LPS from this organism is a weak osteolytic agent only showing activity at concentrations of 1-10μg/ml, thus it is approximately 100-1000 times less active (Wilson *et al.*, 1985;

Meghji *et al.*, 1994). Indeed, further evidence for the bone-modulating activity of SAM is demonstrated in this thesis by its ability to inhibit osteoblast-like protein synthesis, and in previous studies (Meghji *et al.*, 1992a; Wilson *et al.*, 1988) where SAM was shown to be a potent inhibitor of collagen synthesis. This activity could adversely effect the ability of the osteoblast to deposit new bone. Other possible osteolytic factors associated with *A. actinomycetemcomitans* include a family of proteins known as the Lipid A-associated proteins (LAPs). The LAP from *A. actinomycetemcomitans* which can be co-extracted with LPS, has been shown to be more potent than LPS, exhibiting activity at a concentration of just 10ng/ml (Reddi *et al.*, 1995a).

In this thesis I have termed gapstatin a bone-modulating protein. Where is the evidence for this connection? Although not directly osteolytic, gapstatin could prove to be a very damaging molecule within the periodontal pocket, especially in conjunction with the osteolytic cpn 60. When one considers the rapid rate of tissue turnover within the periodontal pocket, it is likely that a protein with the ability to inhibit cell division would prove extremely detrimental to these labile tissues. Gapstatin could cause bone resorption by a number of different mechanisms. Following osteoclastic bone resorption, possibly stimulated by cpn 60, growth factors are released from within the bone matrix. This stimulates the division of pre-osteoblasts in preparation for a wave of bone deposition. If upon stimulation, pre-osteoblasts cannot replicate and subsequently differentiate into osteoblasts, this would lead to a deficit in the amount of bone laid down and hence could result in bone loss. The purified protein, gapstatin, demonstrated potent anti-proliferative activity against MG63 cells with an IC_{50} value of 0.4ng/ml. Purification studies demonstrated that gapstatin is solely responsible for the anti-proliferative activity found in SAM. Taking this into consideration, of importance to the hypothesis for the role of gapstatin is the observation that the SAM of *A. actinomycetemcomitans* is a far more potent inhibitor of osteoblast-like cell division as opposed to other cell types. This suggests that the activity of gapstatin is directed against

osteoblasts rather than other cell types. In contrast, the SAM from *Eik. corrodens*, although shown to be a potent inhibitor of HGF proliferation, was only weakly active against the MG63 osteoblast-like cell line.

One interesting finding in this thesis is the fact that both bone-modulating proteins can be neutralized by antibodies. Sera from patients with LJP have been shown to block both the anti-proliferative activity of SAM in this thesis, and the osteolytic activity of SAM (Meghji *et al.*, 1993). Whether this inhibition is repeated in the *in vivo* situation is a key point for future study. These data certainly indicate that a vaccine developed against both proteins could be of enormous therapeutic potential in helping to combat LJP. Numerous studies have linked the production of antibodies against periodontopathogenic bacteria with host protection (reviewed by Ebersole and Taubman, 1994). It is likely that cpn 60 and gapstatin are acting synergistically, whereby direct destruction by cpn 60 is coupled with inhibition of osteoblast proliferation following the release of local growth factors from the matrix.

What advantage would *A. actinomycetemcomitans* accrue by inhibiting cell proliferation? As well as bone cells, the proliferation of other cell types could be inhibited by gapstatin *in vivo*. Evidence for this is shown in this thesis when crude SAM was shown to inhibit the proliferation of numerous cell types and lines, including fibroblasts which are the main cell type found in the periodontium. The periodontal tissues divide and regenerate rapidly, this could lead to the organism being continually shed from within the periodontal pocket. By inhibiting cell division, the organism may survive for longer periods, giving it an obvious evolutionary advantage over competing organisms. Inhibiting the proliferation of other cells could also prove advantageous to *A. actinomycetemcomitans*, for instance, decreased proliferation of T-cells could adversely effect the immune response within the periodontal pocket.

10.6. Future Studies

A key objective would be to obtain an N-terminal sequence for gapstatin. For this to be achieved increased amounts of gapstatin would need to be purified, using the procedure developed in this study. This could provide the vital information needed for the construction of a probe with which to isolate the gene expressing gapstatin from a genomic library. The subsequent over-expression and purification of gapstatin, could help reveal much more about this interesting molecule.

The mechanisms of action of bacterial toxins have provided novel insights into the control of cellular regulatory processes, including protein synthesis, ion channel activity and signal transduction (Staddon *et al.*, 1990). It is therefore possible that gapstatin may prove a useful tool to help elucidate the remaining mysteries of cell cycle control mechanisms. Future experiments could therefore include a detailed analysis of the expression, and translation of key proteins involved in G_2 to M phase progression following treatment with gapstatin. Indeed, the role of gapstatin as an anti-cancer agent cannot be ruled out. In contrast to non-dividing cells in G_0, the cells that are constantly in cell cycle constitute the "growth fraction" of a tumour. Tumours with a high-growth fraction are therefore targets for phase-specific agents. Taxol is one example of a molecule which has been used as an anti-tumour drug in this way. Taxol, like gapstatin, inhibits cell division by blocking cells in the late G_2 to M phase of the cell cycle, by promoting the formation of discrete bundles of stable microtubules that result from the reorganization of the microtubule cytoskeleton (Brand-Horwitz, 1994).

This thesis has concentrated on the bone-modulating activities of surface-associated proteins of *A. actinomycetemcomitans* with particular emphasis on the anti-proliferative activity of this organism. The major type of periodontal disease caused by *A. actinomycetemcomitans* is juvenile periodontitis, a disease characterized by minimal gingival inflammation and rapid loss of bone.

This study has established that the SAM of this bacterium contains a potent bone resorbing protein subsequently identified as a cpn 60. It is envisaged that a combination of this active osteolytic protein, with gapstatin acting to inhibit the proliferation of osteoblasts and thus slowing the rate of normal bone replacement, could account for the rapidity with which alveolar bone is lost in juvenile periodontitis.

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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_{592} 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

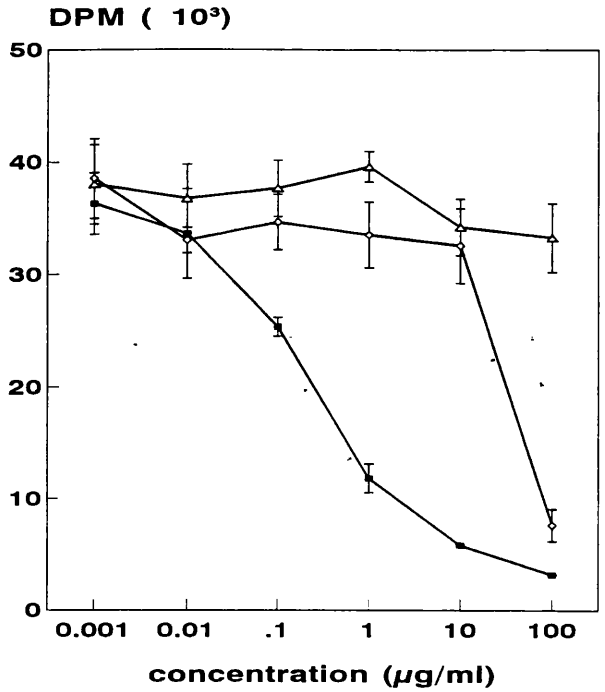

FIG. 1. Inhibitory effect of increasing concentrations of SAMs from *A. actinomycetemcomitans* (square), *P. gingivalis* (diamond), and *E. corrodens* (triangle) on DNA synthesis, measured as incorporation of [^{3}H]thymidine into DNA, by MG63 cells. The results are expressed as means and standard deviations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

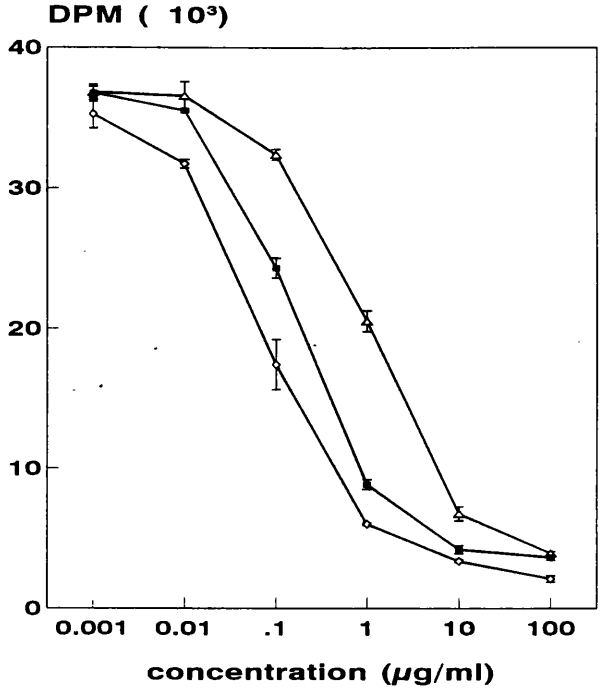

FIG. 2. Inhibitory effect of increasing concentrations of SAMs from *A. actinomycetemcomitans* serotypes a (triangle), b (diamond), and c (NCTC 9710) (square) on DNA synthesis, measured as incorporation of [^{3}H]thymidine into DNA, by MG63 cells. The results are expressed as means and standard deviations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

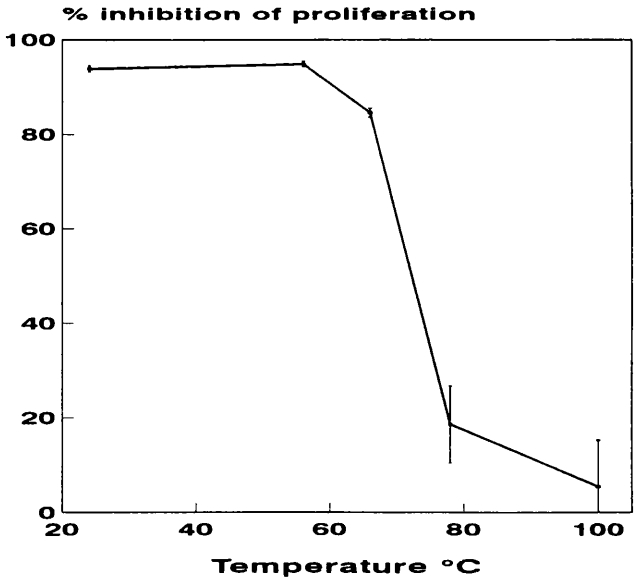

FIG. 3. Effect of temperature on the inhibitory activity of SAM from *A. actinomycetemcomitans* on DNA synthesis, measured as incorporation of [^{3}H]thymidine into DNA, by MG63 cells. Solutions of SAM were heated at various temperatures for 1 h and tested at a concentration of 10 μg/ml. The results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

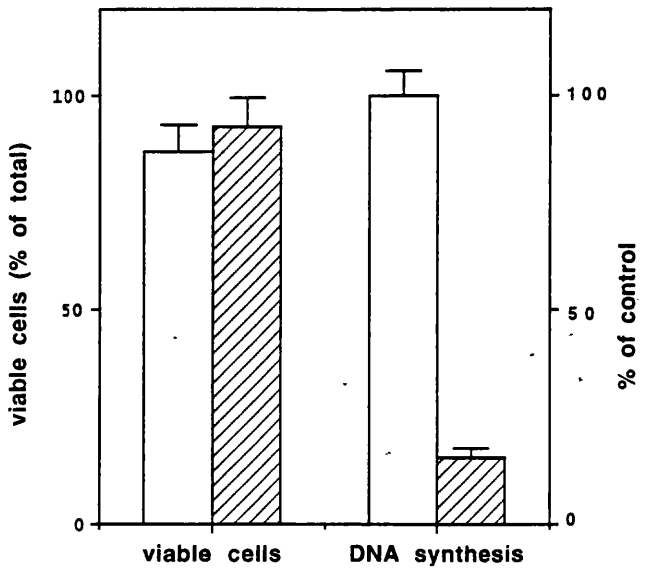

FIG. 4. Comparison of the effect of SAM on cell viability and on cellular replication, as measured by [^{3}H]thymidine incorporation into DNA. MG63 osteosarcoma cells were exposed to 100 μg of SAM per ml for 24 h. The control cultures, to which SAM was not added (open bars), and SAM-treated cultures (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

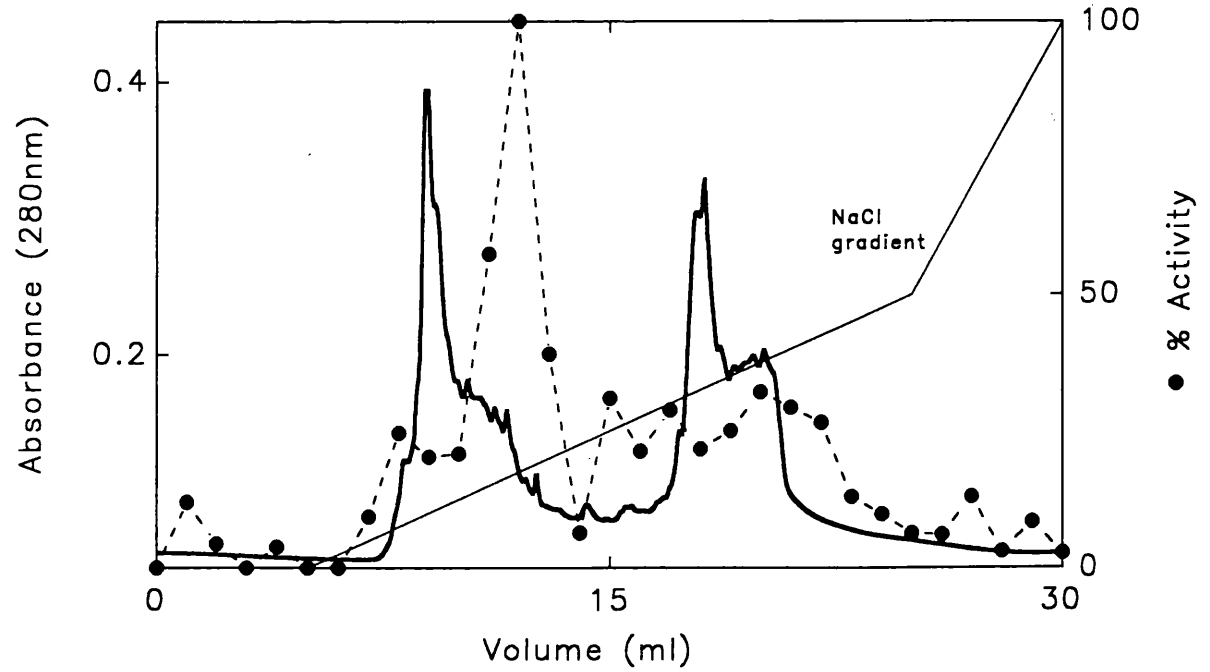

FIG. 5. Anion-exchange HPLC of SAM from *A. actinomycetemcomitans*. The protein elution profile is shown as A_{280} (solid line). Charged components were eluted with a 0 to 2 M NaCl gradient. Each fraction was assayed for inhibition of [^{3}H]thymidine incorporation into MG63 cells (dotted line), and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

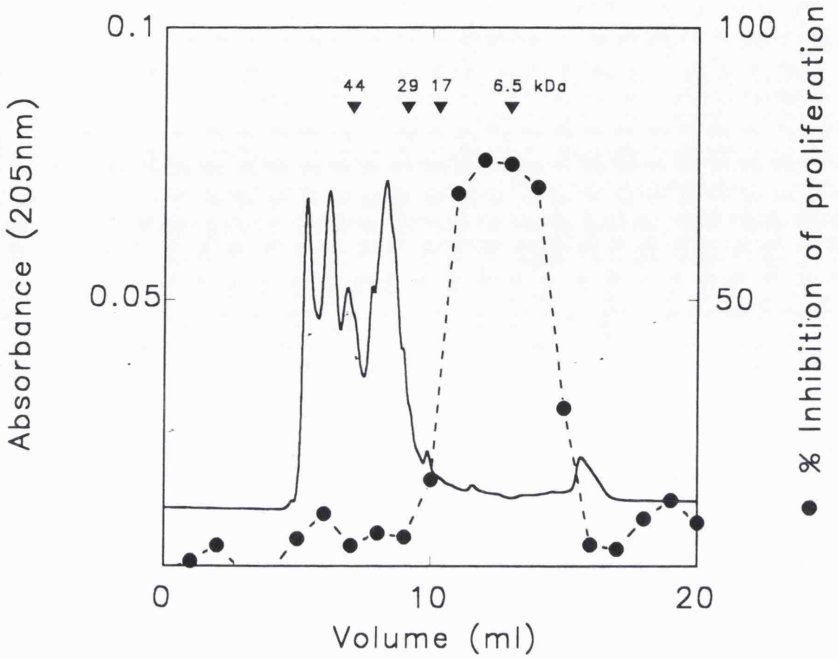

FIG. 6. Size exclusion HPLC of the most active fraction (fraction 12) from the HPLC anion-exchange column. The protein elution profile is shown as A_{205} (solid line). Each fraction was assayed for inhibition of [^{3}H]thymidine incorporation into MG63 cells (dotted line). Protein standards: aprotinin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

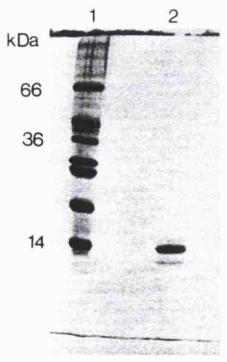

FIG. 7. SDS-PAGE of the biologically active fraction eluted from the size exclusion column, activity being found in fractions 10 to 14. Molecular weight markers are displayed in lane 1. Fractions 10 to 14 were pooled and concentrated, and the proteins present are shown in lane 2. The gel used contained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

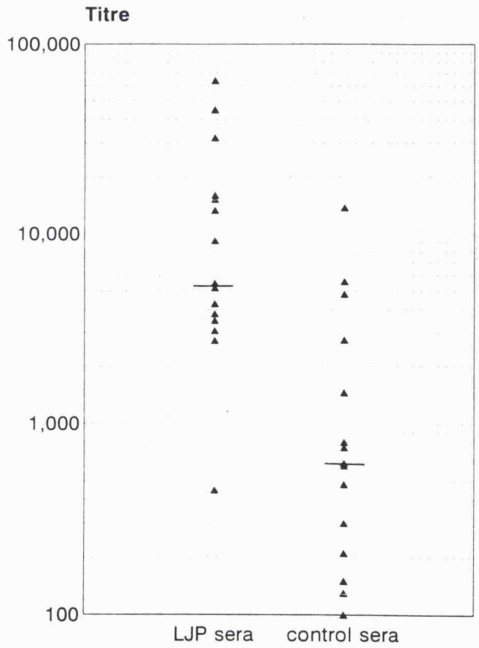

FIG. 8. Range of IgG antibody titers to *A. actinomycetemcomitans* SAM in the sera of individuals with LJP and individuals with no evidence of periodontal disease. Horizontal lines, median antibody titers. $P < 0.001$ (Wilcoxon's rank sum test).

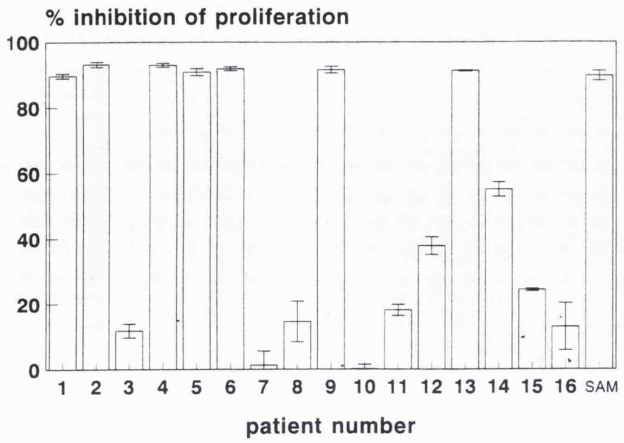

FIG. 9. Influence of LJP sera, used at a 1:50 dilution, on the antiproliferative activity of SAM from *A. actinomycetemcomitans* (incubated with cells at a concentration of 500 ng/ml). Activity is measured as percent inhibition of [^{3}H]thymidine incorporation relative to control cultures. The effect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

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

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

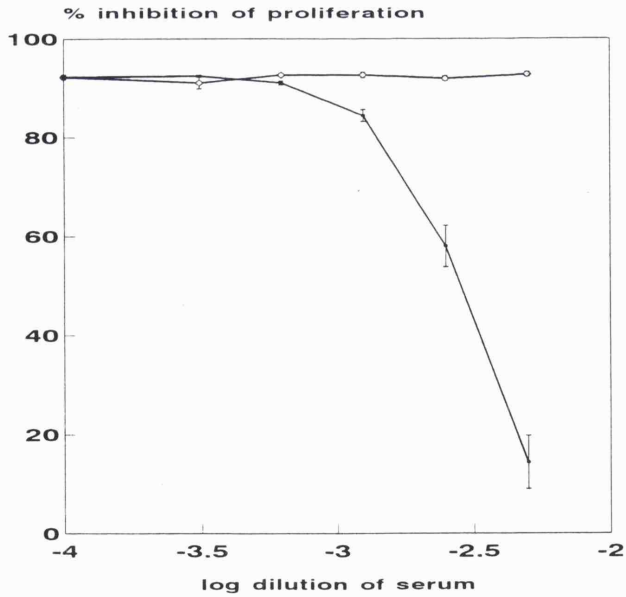

FIG. 10. Titration curves showing the effect of serum from patient 10 on the antiproliferative activity of 500 ng of SAM from *A. actinomycetemcomitans* per ml on MG63 cells. The graph shows that control serum from an individual with no evidence of periodontal disease was unable to block the antiproliferative 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.

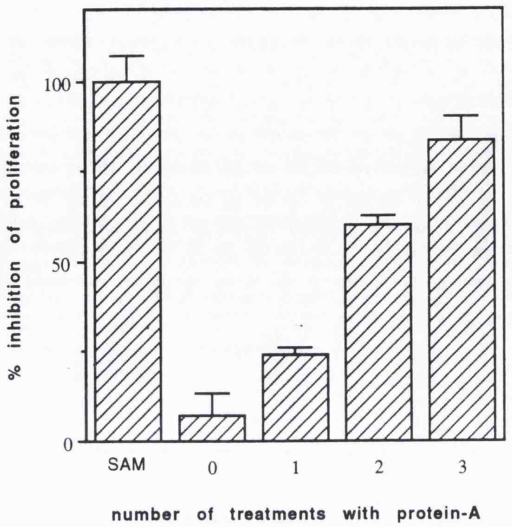

FIG. 11. Effect of sequentially adsorbing serum from patient 10 with protein A-Sepharose (to remove antibody) on the serum-mediated inhibition of the antiproliferative activity of SAM. The normalized percent inhibition of MG63 proliferation induced by SAM (500 ng/ml) is shown on the far left (SAM). 0, 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-

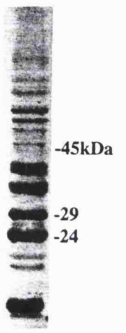

FIG. 12. Representative Western blot of SDS-PAGE-separated components of *A. actinomycetemcomitans* SAM. The blot was stained to show IgG binding of serum from a patient with LJP (patient 7). The molecular sizes of markers are indicated on the right.

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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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

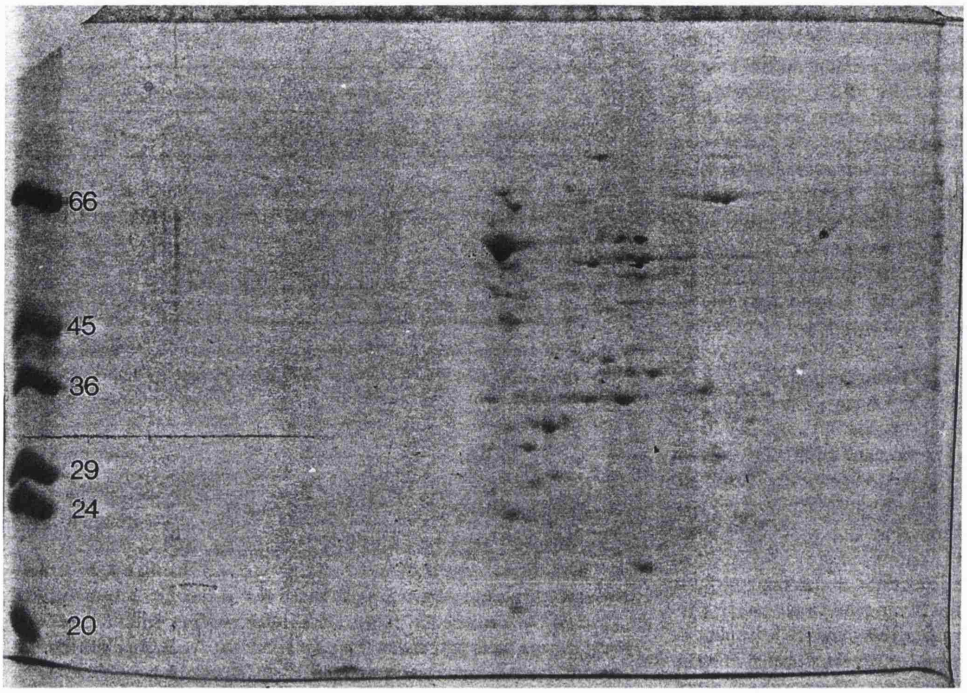

Figure 1. Determination of the protein composition of the SAM of *A. actinomycetemcomitans* by two-dimensional SDS-PAGE. Proteins have been separated in one dimension on the basis of their isoelectric point and in the other dimension on the basis of their molecular mass. The gel was stained with Coomassie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

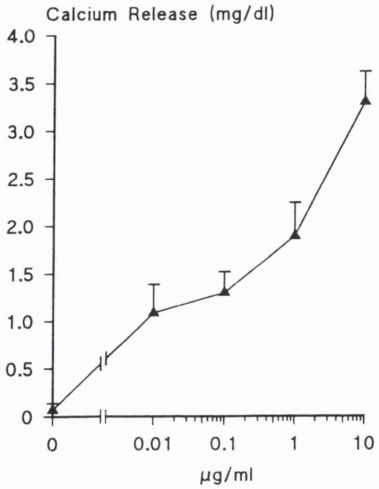

Figure 2. Dose response of the stimulation of calvarial bone resorption induced by the SAM from *A. actinomycetemcomitans*. Bone breakdown is measured as the release of calcium from the bone. Each point represents the mean and standard deviation of five separate cultures. The concentrations of SAM used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

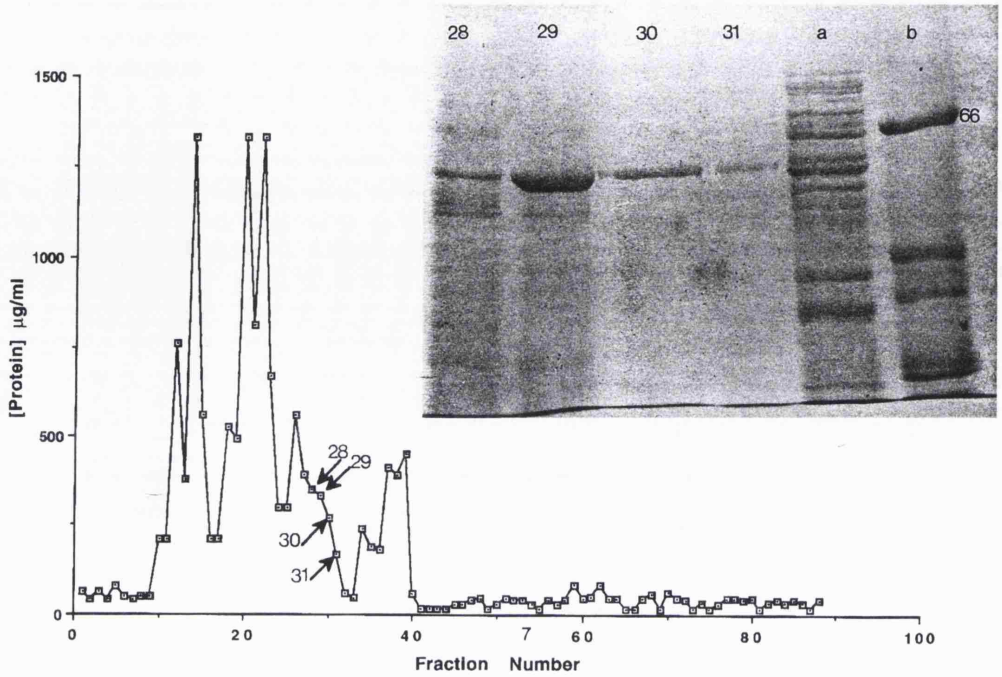

Figure 3. Elution profile of the SAM on Q-Sepharose anion exchange chromatography. Each fraction was assessed for protein content and for bone-resorbing activity in the murine calvarial bone resorption assay. The protein content of each fraction is shown and the fractions with osteolytic activity are 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).

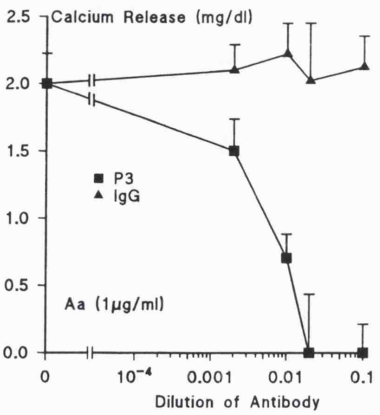

Figure 4. Dose-dependent inhibition of the bone resorption (measured as calcium release) induced by 1 µg/ml SAM from *A. actinomycetemcomitans* by addition of mAb P3 to the bone explants. The lack of effect of equivalent concentrations of mouse IgG is also seen. Results are expressed as the mean and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

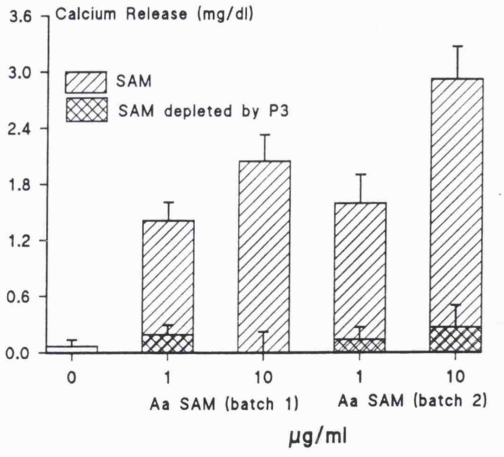

Figure 5. Effect of depletion of the SAM, by immunoabsorption with mAb P3, on the stimulation of murine calvarial bone resorption. Cultures were exposed to 1 or 10 µg/ml SAM which had either been depleted with mAb P3 or sham-depleted by incubation with heat-killed *S. aureus* (Cowan strain) in the absence 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.

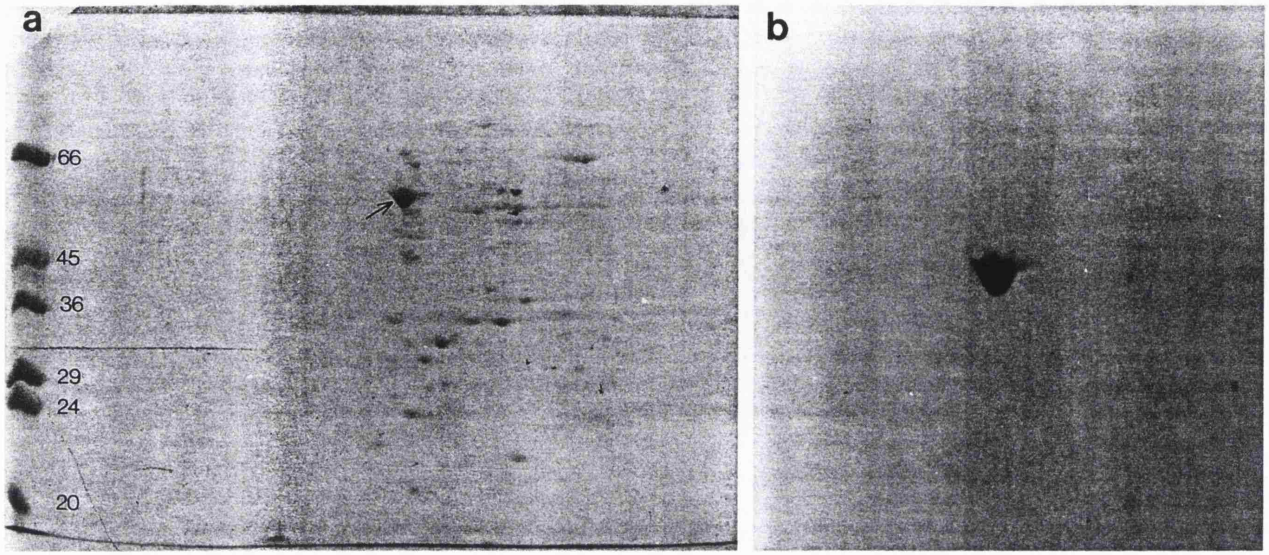

Figure 6. Western immunoblotting of *A. actinomycetemcomitans* SAM separated by two-dimensional SDS-PAGE with mAb P3. (*a*) Coomassie blue–stained two-dimensional gel with a prominent protein spot of 62 kD arrowed; (*b*) gel immunoblotted with antibody P3 to demonstrate that this antibody only binds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

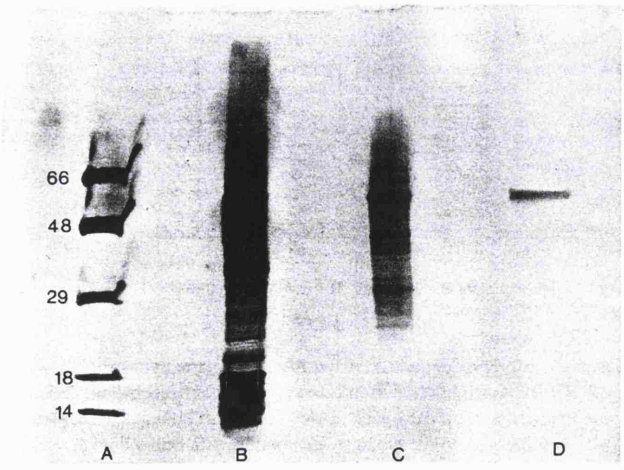

Figure 7. Affinity purification of the 62-kD osteolytic protein on ATP-Sepharose. (*A*) Molecular weight standards; (*B*) starting material; (*C*) protein eluted from Q-Sepharose column; (*D*) material eluted from ATP-Sepharose column. The SDS-PAGE gel was stained with silver.

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.

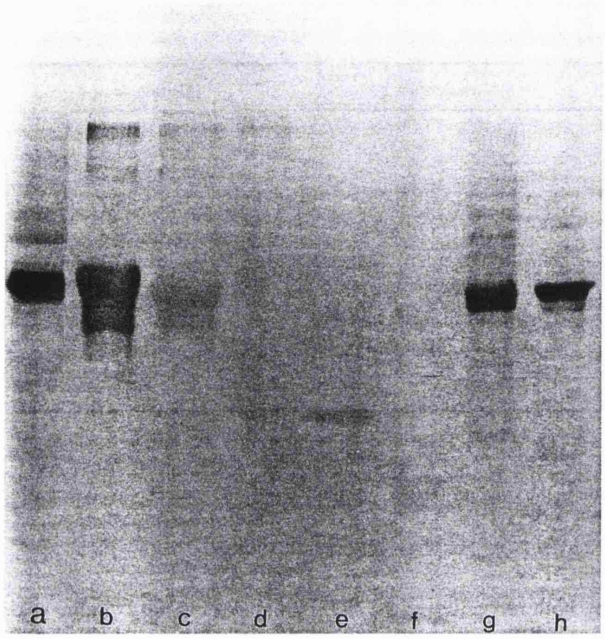

Figure 8. Western immunoblotting of bacterial components with antibody P3: (*a*) a crude lysate of *E. coli;* (*b*) *M. tuberculosis* hsp65; (*c*) *M. leprae* hsp65; (*d*) SAM from *S. aureus;* (*e*) SAM from *P. gingivalis;* (*f*) SAM from *E. corrodens;* (*g*) > 30-kD fraction of the SAM from *A. actinomycetemcomitans;*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

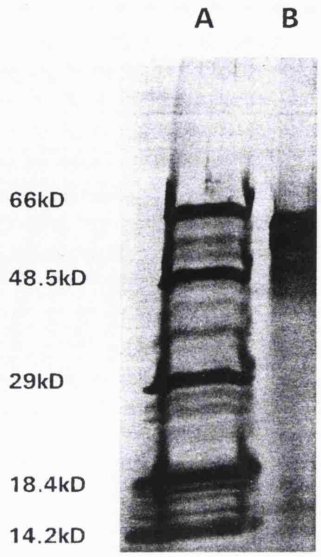

Figure 10. Silver-stained SDS-PAGE gel overloaded with the purified GroEL homologue (*B*) showing lack of a ladder pattern indicative of LPS contamination. The molecular mass markers are shown (*A*).

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

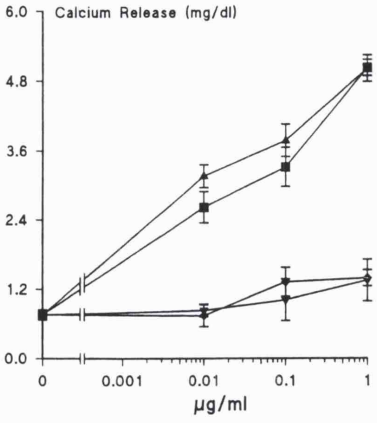

Figure 9. Stimulation of calvarial bone resorption by increasing concentrations of SAM from *A. actinomycetemcomitans* (■), purified *E. coli* GroEL (▲), or the GroEL-like proteins from *M. tuberculosis* (♦) or *M. leprae* (▼). Results are expressed as the mean and standard deviation of five replicate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

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
GroEI 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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Surface-associated material from the bacterium *Actinobacillus actinomycetemcomitans* contains a peptide which, in contrast to lipopolysaccharide, directly stimulates fibroblast interleukin-6 gene transcription

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The oral commensal Gram-negative bacterium *Actinobacillus actinomycetemcomitans* is believed to be the causative organism of localized juvenile periodontitis, a disease in which there is rapid loss of alveolar bone supporting the teeth. Previously, we have reported that gentle saline extraction of this bacterium removed a loosely adherent proteinaceous fraction from the cell surface of the bacterium, which we have termed surface-associated material. This material contained potent bone-resorbing activity. We now report that surface-associated material is also a potent stimulator of cytokines and, in particular, interleukin-6 (IL-6) synthesis, while the lipopolysaccharide from this bacterium is only a weak stimulator of IL-6 synthesis by fibroblasts and monocytes. In contrast to enteric lipopolysaccharide (LPS), which induces fibroblast IL-1, IL-6 and tumour necrosis factor (TNF) α synthesis, surface-associated material stimulated gingival fibroblasts to synthesize only IL-6, with no induction of IL-1 or TNF (the normal inducers of IL-6 synthesis). Reverse transcriptase PCR also failed to detect mRNA for IL-1 or TNF in surface-associated-material-stimulated fibroblasts, although both mRNAs were present in *Escherichia coli* LPS-stimulated cells. Neutralizing antibodies to IL-1 and/or TNF or the natural IL-1 receptor antagonist (IL-1ra) inhibited enteric LPS-induced IL-6 synthesis, but did not inhibit surface-associated-material-induced synthesis. In addition, dexamethasone, which completely suppressed LPS-induced IL-6 synthesis, only inhibited surface-associated-material-induced IL-6 synthesis by 50%. This suggests that the active constituent in the surface-associated material stimulates IL-6 gene transcription by a transcriptional control mechanism distinct to that of *E. coli* LPS.

The IL-6-stimulating activity of the surface-associated material is inhibited by both heat and trypsin, suggesting that it is proteinaceous. The activity has been isolated using anion-exchange, reverse-phase and size-exclusion HPLC. The active moiety is a peptide of molecular mass 2 kDa which may be the product of a bacterial short open reading frame.

Keywords: lipopolysaccharide; bacteria; cytokine; interleukin-6; bacterial surface proteins.

The mouth contains a diverse population of commensal Gram-negative bacteria. Normally, these bacteria are present in low numbers. In periodontal diseases, the most prevalent chronic inflammatory diseases of *Homo sapiens* [1], anaerobic Gram-negative bacteria accumulate between the tooth and the gum. This results in inflammation of the gums and the loss of the alveolar bone supporting the teeth. Eventually, bone loss can become so severe that the teeth are lost. The bacteria implicated in the pathology of these diseases do not penetrate the periodontal tissues, i.e. the tissues surrounding the tooth, and tissue pathology is believed to be largely driven by soluble factors released by the bacteria [2, 3]. Localized juvenile periodontitis is a relatively rare form of periodontal disease which, unlike other forms of this disease, demonstrates minimal inflammation. One organism in particular, *Actinobacillus actinomycetemcomitans*, has been implicated in the pathogenesis of this disease [4]. Over the past decade, we have established that gentle extraction of this bacterium in 0.15 M NaCl releases a proteinaceous fraction, which we have termed surface-associated material, that contains a number of putative virulence factors.

Initial studies established that the surface-associated material contained a potent osteolytic activity [5, 6] which we have recently established is the chaperonin 60 of this bacterium [7]. The surface-associated material also contains a protein, that we have called *gapstatin*, which blocks cell-cycle progression in G_2 by a unique mechanism of action [8]. We have recently established that the surface-associated material also has a potent pro-inflammatory cytokine-stimulating activity, which is significantly greater than that of the lipopolysaccharide (LPS) from this bacterium [9]. Of particular interest is the finding that the

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Abbreviations. DMEM, Dulbecco's modified Eagles medium; FCS, foetal calf serum; HBSS, Hank's balanced salt solution; IL, interleukin; LPS, lipopolysaccharide; PDGF, platelet-derived growth factor; RT-PCR, reverse transcriptase PCR; TNF, tumour necrosis factor.

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surface-associated material is an extremely potent inducer of interleukin-6 (IL-6) synthesis by monocytes and fibroblasts. We now report on the mechanism by which the surface-associated material induces IL-6 production by human gingival fibroblasts and on the physicochemical characterization of the active constituent.

MATERIALS AND METHODS

Bacterial growth. *A. actinomycetemcomitans* NCTC 9710 was cultured on brain heart infusion agar (Oxoid) supplemented with 5% (by vol.) 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% (by vol.) saline prior to freeze drying and extraction.

Extraction of bacterial components. The surface-associated material was removed by stirring suspensions of the lyophilized bacteria very slowly in 0.15 M NaCl at 4°C for 1 h, the bacteria then being sedimented by centrifugation. Extraction was repeated and the combined supernatants were dialysed against distilled water using benzoylated dialysis tubing (Sigma; molecular mass cut-off of 2 kDa) and lyophilized.

LPS was prepared from the surface-associated-material-extracted bacteria by the butanol extraction method of Morrison and Leive [10]. Briefly, cells were suspended in 0.15 M NaCl at 4°C and an equal volume of butanol added, the suspension mixed thoroughly for 10 min and centrifuged for 20 min at 35000 *g*. The aqueous phase was removed and the butanol, together with the insoluble residue, was re-extracted twice with 50% the initial volume of saline. The combined aqueous extracts were centrifuged at 35000 g for 20 min to remove particulate matter and dialysed against distilled water for 48 h. This crude extract was ultracentrifuged at 100000 *g* at 4°C for 1 h and lyophilized. This material was then subjected to hot phenol/water extraction [11] and purified by ultracentrifugation. The aqueous phase was digested by addition of RNase and DNase (Sigma; 20 μg/ml in 0.05 M Tris/HCl, pH 7.3) overnight at 37°C, then with 1 mg/ml pronase (Sigma) for 6 h. The suspension was then ultracentrifuged for 1 h at 100000 *g* and the LPS, which appeared as a clear gel at the base of the tube, was removed and lyophilized. The endotoxin content of this preparation was measured by use of a colorimetric *Limulus* amoebocyte lysate assay. Highly purified international standard *Escherichia coli* LPS (84/650; NIBSC) which contains 7000 IU/μg was also used in these studies.

The protein content of the isolated components was estimated by the method of Lowry [12] or by use of a commercial Lowry reagent kit (Bio-Rad). The carbohydrate content was assessed by the method of Dubois et al. [13]. The nucleic acid contamination was estimated by the Warburg Christian method by measuring the ratio of absorption at 260/280 nm. The lipid content was crudely estimated by extraction of the surface-associated material with methanol:chloroform (2:1) and determining the mass of the lyophilized extract. The content of LPS was measured using a commercial chromogenic *Limulus* amoebocyte lysate assay (Pyrogent, Byk-Mallinckrodt).

The sensitivity of the isolated components to heat was tested by heating solutions of the components at either 80°C or 100°C for various times. To determine sensitivity to proteolytic digestion, solutions were incubated in 0.25% trypsin at room temperature for various times with 0.75% soya bean trypsin inhibitor being added to stop proteolysis. The effects of this inhibitor alone or of inhibitor-trypsin complexes on cytokine production were also determined (as controls).

Cell culture and cytokine induction. Human gingival fibroblasts were obtained from explants of normal gingivae obtained during minor oral surgical procedures. Cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS; Sigma), 100 U/ml penicillin/streptomycin (Gibco) and 2 mM glutamine (Sigma). Cells were subcultured at twice-weekly intervals using 0.25% trypsin (Sigma) with viability being routinely checked; cells were used for experimental purposes at passages 6–12. To determine fibroblast cytokine synthesis, 30000 cells were added to each well in 24-well plates (ICN Flow) in 0.5 ml DMEM supplemented with 2% FCS and cells were incubated overnight at 37°C in 5% CO_2/air. Plates were then washed with Hank's balanced salt solution (HBSS, Sigma) and further incubated with serial dilutions of surface-associated material, fractionated surface-associated material or LPS in groups of three wells/concentration for 24 h under the same culture conditions. Control cultures were incubated with DMEM containing 2% FCS. At the end of the culture period, the media were removed and immediately frozen at −70°C for assay of cytokines. In studies of the intracellular levels of cytokines, cells were lysed by removing media, adding distilled water and freeze thawing three times. The lysed cells were then centrifuged at 1000 *g* for 30 min to pellet cell debris and the supernatant assayed for IL-1*β*.

Cytokine assays. The media from stimulated cells were assayed for the presence of IL-1*β*, IL-6 and TNF*α* using two-site ELISAs. These assays have recently been described in detail in [15].

Cytokine-neutralizing agents. Neutralizing antibodies to human IL-1*α*, IL-1*β* and to human TNF*α* were raised in sheep. Recombinant IL-1 receptor antagonist (IL-1ra) was a kind gift from Dr Bob Thompson (Synergen). A neutralizing antiserum to human platelet-derived growth factor (PDGF) was obtained from R&D Systems.

Inhibitor studies. Dexamethasone (Sigma) was added to cells at concentrations of 1–1000 nM. Indomethacin (Sigma) was used at a concentration of 1 μM. The selective 5-lipoxygenase inhibitor BW70C (Wellcome Foundation) and the 5-lipoxygenase activating protein (FLAP) inhibitor, MK886 (Merck Frosst) were dissolved in dimethyl sulphoxide and added to cell cultures at concentrations of 1–1000 nM with appropriate solvent controls.

Reverse transcription polymerase chain reaction (RT-PCR) for cytokine mRNA. Human gingival fibroblasts were grown to confluence in 3.5-cm culture dishes and incubated for 24 h in DMEM supplemented with 2% FCS at 37°C in 5% CO_2/air. After washing with ■■ M NaCl, ■■ M potassium phosphate, pH ■■, cells were incubated in media containing *E. coli* LPS or surface-associated material at 10 ng/ml and 1000 ng/ml, respectively. At various times, total RNA was extracted from cells as described in [16]. To improve the yield of cellular RNA, 10 μg 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 (100 U; Gibco/BRL) in a 20-μl reaction mixture containing reaction buffer (50 mM Tris/HCl, pH8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol), 0.5 mg each of dNTP mix (dATP, dCTP, dGTP, dTTP), 0.5 μg oligo$(dT)_{12-18}$, 10 U rRNasin (Promega) and 10% of each pooled total RNA. The reaction was carried out for 1 h at 42°C. Amplification of cDNA by PCR was performed using oligonucleotide primer pairs for IL-1*β*, IL-6 or TNF as described in [17]. The reactions were performed in a thermal cycler (Eppendorf) in a 30-μl reaction volume containing reaction buffer (20 mM Tris/HCl, pH8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM each dNTP mix (dATP, dCTP, dGTP, dTTP), 1.5 U *Taq* polymerase (Gibco/BRL) and 2 μl first-strand

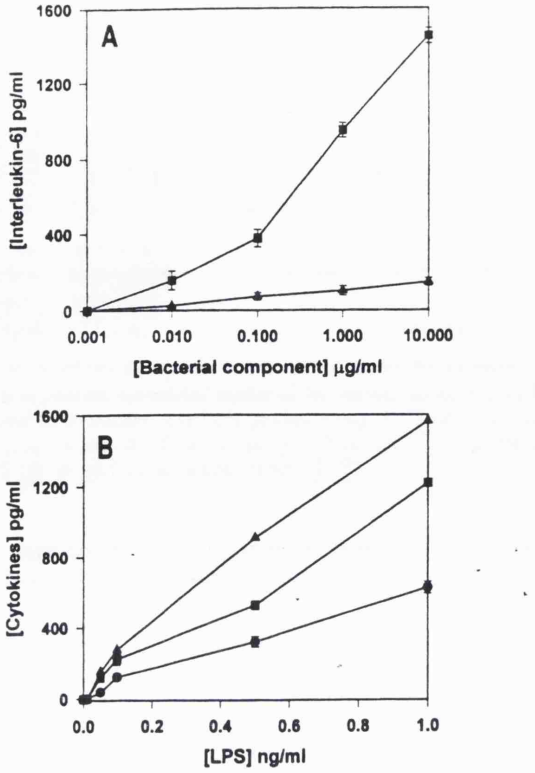

Fig. 1. Cytokine synthesis by human gingival fibroblasts exposed to the surface-associated material and LPS of *A. actinomycetemcomitans* and the LPS from *E. coli*. Results are expressed as the mean and SD of six replicate cultures. (A) Dose-dependent stimulation of IL-6 synthesis by surface-associated material (■) or to the LPS (▲) of *A. actinomycetemcomitans*. (B) Dose-dependent stimulation of IL-1β (■), IL-6 (●) or TNFα (▲) synthesis in response to LPS from *E. coli*.

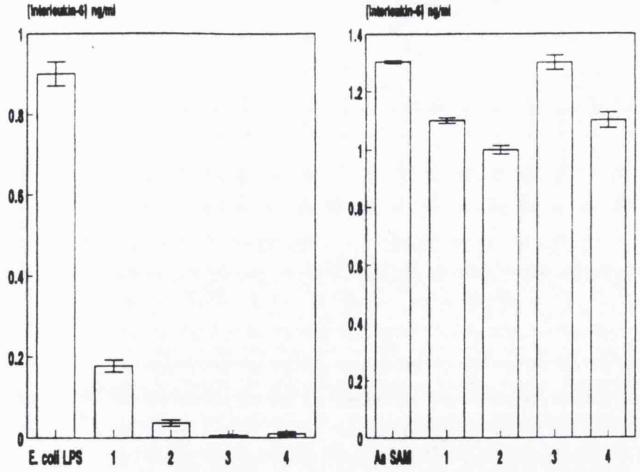

Fig. 2. The effect of excess concentrations of neutralizing antibodies to: (1) IL-1 (α and β), (2) TNFα, (3) a combination of IL-1 (α and β) and TNFα or (4) the natural IL-1 receptor antagonist (IL-1ra) on the synthesis of IL-6 by HGFs stimulated by *E. coli* LPS (10 ng/ml, left-hand graph) or *A. actinomycetemcomitans* surface-associated material (10 μg/ml, right-hand graph). Results are expressed as the mean and standard deviation of six replicate cultures.

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°C and annealing/extension at 60°C for 2 min and 30 s. The PCR products were separated on 1% agarose gels containing 50 μg/ml ethidium bromide, visualised and photographed under ultraviolet light.

Protein purification and analysis. The surface-associated material was fractionated by a combination of anion-exchange, size-exclusion and reverse-phase chromatography using a Bio-Rad HRLC computer-controlled HPLC system. Protein purification was monitored by SDS/PAGE using the method of Laemmli [18] and by assaying the bio-activity of fractions.

The surface-associated material was initially fractionated on a MA7Q (Bio-Rad) HPLC anion exchange column (5 cm ×0.78 cm), equilibrated with 20 mM Tris/Hcl, pH 8.5 (buffer A). 1 ml 4 mg/ml solution of surface-associated material in buffer A was injected onto the column and eluted at a flow rate of 1 ml/min, with 5 ml buffer A, followed by linear gradients of 0−50% buffer B (buffer A + 2 M NaCl) in 20 ml and 50−100% buffer B in 5 ml. The protein absorbance was monitored at 280 nm and 1-ml fractions were collected, dialysed repeatedly against distilled water, lyophilized and the IL-6-stimulating activity of each fraction measured. Active fractions were re-fractionated by reverse-phase HPLC on C_4 (Vydac; Sigma) and C_8 columns (Phenomenex) using 0.1% trifluoroacetic acid /acetonitrile gradients (0−100%) to elute proteins. Fractions were dried on a Gyrovap (Savant) and tested for IL-6-stimulating activity. Bioactive fractions were further fractionated by size-exclusion chromatography. The bio-active peak from the C_4 column was run through a calibrated Protein-Pak 125 (Waters) gel-filtration column (30 cm×0.75 cm). The major peak from this column

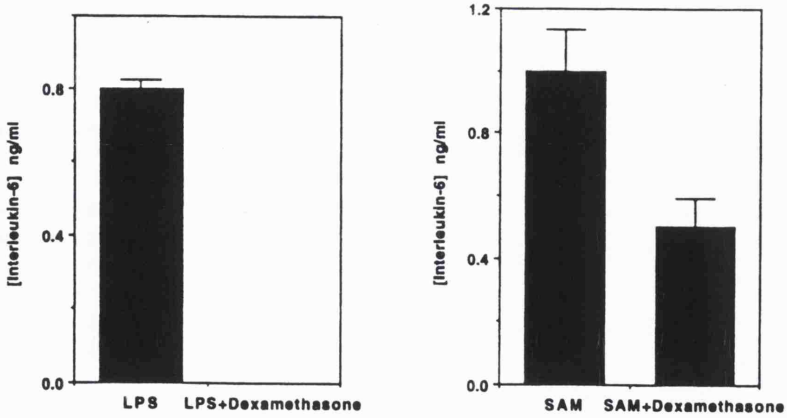

Fig. 3. The effect of 1 nM dexamethasone on the induction of IL-6 by HGFs exposed to 10 ng/ml *E. coli* LPS (left-hand graph) or 10 μg/ml surface-associated material (right-hand graph). Results are expressed as the mean and SD of six replicate cultures.

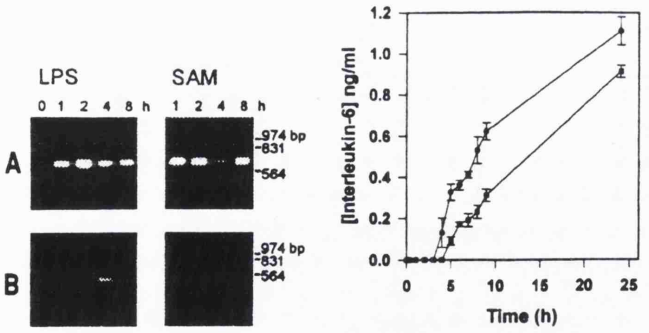

Fig. 4. RT-PCR of the IL-1β and IL-6 mRNA in HGFs exposed to *E. coli* LPS or surface-associated material for various times. (A) PCR product using IL-6 primers; (B) PCR product using IL-1β primers. The associated graph shows the time course of IL-6 released in response to *E. coli* LPS (■) or surface-associated material (●).

was then refractionated on a C_8 reverse-phase column (Phenomenex, 25 cm×0.46 cm) using a trifluoroacetic/acetonitrile gradient.

RESULTS

Characterization of the surface-associated material. The average value of the protein content of the surface-associated material was 60−70%, with the remainder being carbohydrate and lipid. The LPS content of the surface-associated material was low, generally in the order 0.0001−0.001 IU/ng. On SDS/PAGE, the surface-associated material showed a relatively large number of protein bands. On a silver-stained SDS/PAGE, the LPS from this organism showed a classic ladder pattern.

Induction of cytokine synthesis. As little as 10 ng/ml (mass/vol.) of the surface-associated material could stimulate human gingival fibroblasts to release immunoreactive IL-6. In contrast, the highly purified LPS from this organism was virtually inactive (Fig. 1 A). IL-6-stimulating activity was significantly inhibited by heat and exposure to trypsin (results not shown). When fibroblast culture media were assayed for the presence of the other two major pro-inflammatory cytokines, IL-1β and TNFα, none was detected. In contrast, very low concentrations of LPS from the enteric bacterium *E. coli* was a potent inducer of all three cytokines (Fig. 1 B).

Mechanism of HGF IL-6 synthesis. The release of IL-6 by HGFs stimulated with *E. coli* LPS was significantly inhibited by neutralizing monospecific polyclonal antibodies to IL-1α/β or to TNFα, but not by a neutralizing antibody to PDGF. The natural antagonist IL-1ra also significantly inhibited *E. coli* LPS-induced IL-6 synthesis. In contrast, the neutralization of these cytokines by antibodies or antagonists consistently failed (in four separate experiments) to have any significant effect on the synthesis of IL-6 induced by the surface-associated material (Fig. 2). Addition of 1 nM dexamethasone to *E. coli* LPS-stimulated fibroblasts completely inhibited IL-6 synthesis by HGFs. At this, and at higher concentrations (1mM), dexamethasone only blocked IL-6 synthesis by 40−50% (Fig. 3).

Indomethacin, the 5-lipoxygenase inhibitor BW70C (Wellcome) or the 5-lipoxygenase-activating peptide (FLAP) inhibitor MK886 (Merck Frosst) all failed to inhibit IL-6 synthesis induced by the *E. coli* LPS or the surface-associated material.

Measurement of intracellular IL-1β in cell lysates demonstrated the presence of the immunoreactive protein in *E. coli* LPS-stimulated HGFs but the levels in surface-associated mate-

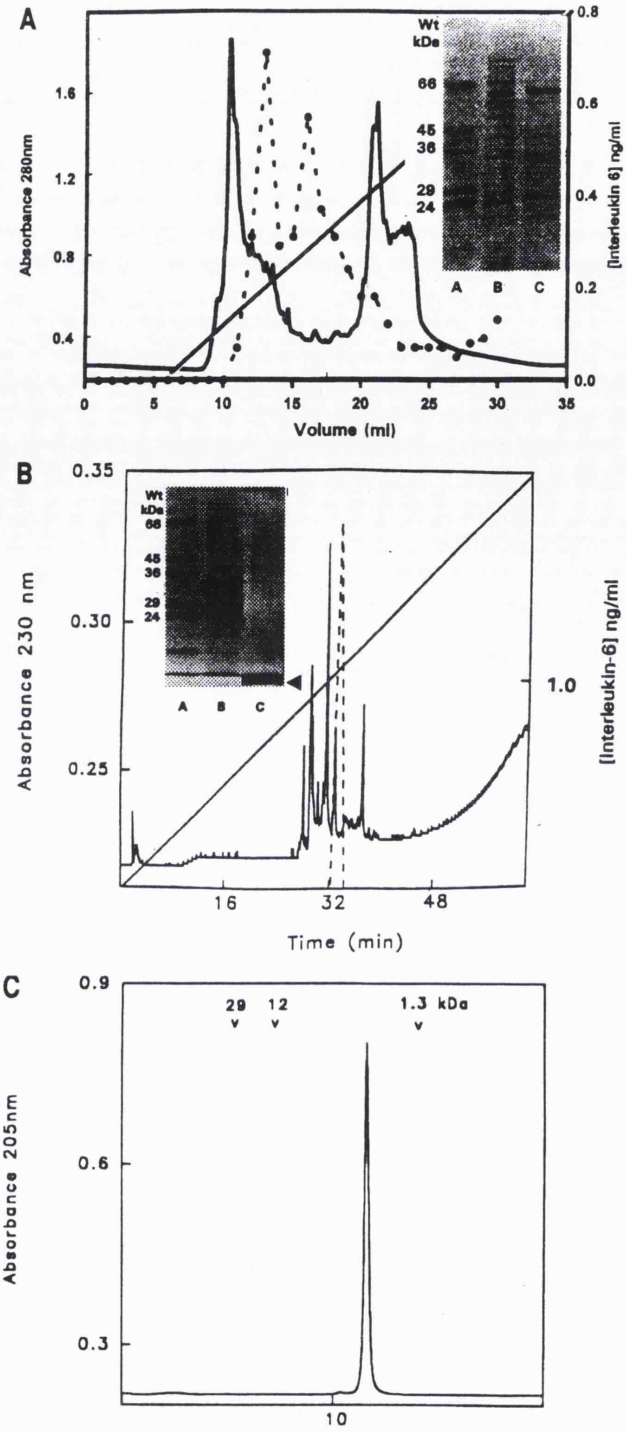

Fig. 5. Elution profile of surface-associated material (A_{280}). (A) MA7Q anion exchange column with a gradient of NaCl from 0−2 M. (– – –) The IL-6-stimulating activity of each fraction is shown (A). The inset is the SDS/PAGE of molecular-mass markers, the starting material and the most biologically active fraction. (B) Fractionation of the most biologically active anion-exchange peak on a Vydac C_4 reverse-phase column eluted with an trifluoroacetic acid/acetonitrile gradient (0−100%). (- - -) IL-6-stimulating activity of the various fractions. Only one peak showed biological activity. The inset is the SDS/PAGE of molecular-mass markers, the starting material and the silver stain of the fraction which showed biological activity (protein band arrowed). (C) Size-exclusion chromatography, on a Protein-Pak 125 column, of the active IL-6-stimulating peak from the C_4 reverse-phase column. Only one peak with an apparent molecular mass of 2 kDa eluted from this column.

rial-stimulated cells was below the detection limit of the assay (4 pg/ml).

Using RT-PCR, it was possible to indirectly amplify mRNA for IL-6 in HGFs within 1 h of adding the *E. coli* LPS or surface-associated material. IL-1 cDNA was also amplified from the mRNA of *E. coli* LPS-stimulated HGFs. In contrast, the surface-associated material reproducibly failed to produce sufficient IL-1 or TNF mRNA to allow amplification by RT-PCR (Fig. 4).

Protein purification. Initialfractionation of the surface-associated material using a YM30 Amicon membrane revealed that all the IL-6-stimulating activity passed through this membrane, suggesting that the molecular mass of the active components was <30 kDa. There was no binding of the surface-associated material to a cation-exchange column. On anion-exchange HPLC, two major protein peaks were separated. The trailing edge of the first peak contained all the bioactivity which eluted as two closely opposed peaks of activity (Fig. 5A). The first peak was further fractionated on a Vydac C_4 reverse-phase column, which appeared to resolve all the proteins present in this peak with only one peak demonstrating IL-6-stimulating activity (Fig. 5B). On silver-stained SDS/PAGE, this bioactive peak contained only one band of low molecular mass. This IL-6-stimulating peak was further fractionated on a calibrated gel-filtration column and the activity eluted with an apparent molecular mass of 2 kDa (Fig. 5C). This was then refractionated on a C_8 reverse-phase column which again revealed the presence of only one protein peak.

DISCUSSION

There is a growing realization that LPS is not the only bacterial component capable of stimulating cytokine synthesis and that other bacterial constituents are also able to induce cytokine gene transcription. Some of the most active cytokine-inducing components and products are toxins such as pneumolysin from *Streptococcus pneumoniae* and toxin B from *Clostridium difficile*. Both proteins are active in the pico-molar to femto-molar concentration range (reviewed in [19]). However, little is known about the mechanisms by which such non-LPS components induce the transcription of cytokine genes.

We have previously demonstrated that the surface-associated material from *A. actinomycetemcomitans*, in contrast to the LPS from this bacterium, is a potent inducer of cytokine synthesis by human peripheral blood mononuclear cells, the myelomonocytic cell line Mono-Mac-6 and human gingival fibroblasts [9]. While the surface-associated material could stimulate human peripheral blood mononuclear cells to produce IL-1, IL-6 and TNF, it only appeared to stimulate IL-6 release from fibroblasts. This contrasted with the capacity of very low concentrations of highly purified LPS from *E. coli* to induce HGFs to produce all three pro-inflammatory cytokines.

To examine the mechanism by which the surface-associated material induced fibroblast IL-6 synthesis, use was made of neutralizing antibodies to IL-1, TNF or PDGF or the natural IL-1 receptor antagonist protein, IL-1ra. It is now recognized that IL-6 synthesis is, in a range of cell types, dependent upon the prior synthesis and autocrine feedback of cytokines such as IL-1, TNFα and PDGF [20–23]. As with most pro-inflammatory cytokines, LPS-induced synthesis of IL-6 is blocked by glucocorticoids [24]. When HGFs were stimulated with *E. coli* LPS, the release of IL-6 was significantly inhibited by neutralizing antibodies to IL-1 (α and β), by IL-1ra or by a neutralizing monospecific antiserum to TNFα. In contrast, the induction of IL-6 synthesis in response to surface-associated material was unaffected by the neutralization of these cytokines. Neutralization of PDGF had no inhibitory effect on *E. coli* LPS-associated or surface-associated-material-induced cytokine synthesis.

Dexamethasone was also tested for its ability to inhibit IL-6 synthesis. As expected, concentrations of this steroid as low as 1 nM completely blocked the synthesis of IL-6 by fibroblasts stimulated with *E. coli* LPS. This was not the case with the surface-associated material-stimulated synthesis of IL-6, which could only be inhibited by approximately 50%, even with concentrations of dexamethasone as high a 1 μM.

Lipid mediators such as prostaglandin E_1 and leukotrienes [25], have been reported to stimulate cellular IL-6 synthesis. However, neither the cyclo-oxygenase inhibitor indomethacin nor selective inhibitors of 5-lipoxygenase or FLAP (a protein essential for the activation of 5-lipoxygenase) had any inhibitory effect on surface-associated material-induced or *E. coli* LPS-induced IL-6 synthesis. This shows that lipid metabolites of arachidonic acid are not involved in the upregulation of the IL-6 gene in cells exposed to this bacterial peptide.

To demonstrate that the effects seen were due to differences in cytokine gene transcription by the two bacterial agonists, use was made of RT-PCR to amplify cytokine mRNA in stimulated fibroblasts. In cells exposed to *E. coli* LPS or surface-associated material, there was the rapid appearance of amplifiable IL-6 mRNA within 1 h of cell activation. In contrast, IL-1 mRNA was found only in *E. coli* LPS-stimulated cells. This confirms the finding that only *E. coli* LPS-stimulated cells contained intracellular IL-1β. Measuring the time course of IL-6 release from surface-associated material-treated or *E. coli* LPS-treated HGFs revealed the earlier appearance of IL-6 in surface-associated-material-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 *E. coli* LPS rapidly induces the transcription of the gene for IL-1β, which then acts in a feedback manner to induce IL-6 gene transcription via nerve factor κB or nerve factor IL-6 [26]. It has recently been reported that dexamethasone induces the transcription of the gene for the inhibitory protein IκBα, an inhibitor of the translocation of NF-κB, one of the transcriptional control elements of the IL-6 gene, into the nucleus [27, 28]. This is now believed to be the mechanism by which dexamethasone blocks LPS-induced cytokine synthesis. The finding that surface-associated-material-induced cytokine synthesis is only partially blocked by this steroid suggests that NF-κB is not the only transcriptional element activated by this stimulus and that, in terms of transcriptional activation, it differs from both LPS [27, 28] and lipoarabinomannan [26].

Initial studies of the molecular nature of the IL-6-stimulating activity revealed that it was sensitive both to heat and trypsin, indicating that it contained protein and was not a lipid or carbohydrate. The activity was not due to LPS contamination, as purified LPS from *A. actinomycetemcomitans* was only a very weak cytokine-inducing agonist. On the assumption that activity was due to a protein-containing molecule, a purification strategy was devised in an attempt to isolate the active constituent. After anion-exchange HPLC, reverse-phase HPLC and gel-filtration HPLC of the surface-associated material, only one bio-active peak with an apparent molecular mass of 2 kDa was resolved. To confirm that only one peptide was responsible for the biological activity, the peak from gel filtration was refractionated on a C_8 reverse-phase column with only one protein peak being resolved. Thus, we conclude that the IL-6-stimulating activity of the surface-associated material from *A. actinomycetemcomitans* is a peptide with an apparent molecular mass of 2 kDa. We are currently attempting to prepare sufficient quantities of this material to sequence. However, this IL-6-stimulating peptide is present in very low concentrations and this will be a major under-

taking. It is surprising that activity is due to such a small peptide and we initally thought that, in spite of our precautions, there had been proteolysis during isolation. However, the protease activity in the surface-associated material is extremely low and we have never found any evidence for protein breakdown during protein isolation. Thus, this peptide may be the product of a bacterial short open reading frame of which a number, encoding for molecules of the size of this peptide, have been reported [29].

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