Periapical Infection and the Inflammatory Radicular Cyst

Submitted in fulfilment of the degree of Doctor of Philosophy,

University of London

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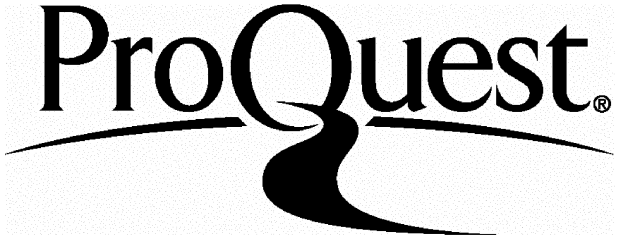

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Abstract

Much attention has focused on the activity of lipopolysaccharides (LPS), in periapical lesions; however, the surface associated material (SAM) from oral bacteria was found to be 100-1000 times more potent in inhibiting mammalian cell division. SAM also significantly inhibited DNA and collagen synthesis by murine calvaria and isolated osteoblasts and had potent bone resorbing activity.

However, where the radicular microbial component is small and contained by the local immune response the outcome appears to be a chronic granuloma or a cyst. To test this hypothesis, cyst fluids were investigated for the presence of endotoxin, microorganisms and cytokines. The results showed significantly high concentrations of endotoxin in radicular cyst fluids. All were sterile. Immunoassay showed IL-1α and β in the inflammatory radicular cysts, whereas the developmental keratocysts and follicular cysts only showed IL-1α. All the cyst fluids contained IL-6 and none contained any TNF.

The influence of endotoxin and these cytokines on the epithelial cell rests was explored. Endotoxins, IL-1, IL-6 and TNF significantly stimulated keratinocyte proliferation. PGE_2 had no effect except at 10μM where it induced cytotoxicity. As epithelial cell rests are undoubtedly influenced by the adjacent connective tissue, the role of fibroblasts was also explored. Fibroblast culture media were shown to have a dose related proliferative effect on keratinocytes.

Antibodies against the cytokines IL-1, TNF, IL-6, were used to localise these molecules histochemically in cysts. All radicular cyst epithelia showed positive staining for IL-1α and β and IL-6. Keratocysts revealed the presence of IL-1α and IL-6.

It is postulated that growth of radicular cysts involves endotoxin stimulation of the epithelium, the autocrine stimulation of cyst epithelial cell division by IL-1 and IL-6 and the paracrine activity of these cytokines on the fibroblast component of the cyst wall. The consequence of this is osteolysis by fibroblast/inflammatory cell factors such as the eicosanoids.

Table of Contents

List of Tables

Table of Figures

Abbreviations

A. actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*
Con A	concanavalin A
CPC	cresolphthalein complexone
dpm	disintegrations per minute
EGF	epidermal growth factor
EP	endogenous pyrogen
E. coli	*Escherichia coli*
E. corrodens	*Eikenella corrodens*
FCS	foetal calf serum
FGF	fibroblast growth factor
HBSS	Hank's buffered salt solution
HPLC	high performance liquid chromatography
8HQ	8-hydroxy-quinolone
IFN	interferon
IL-	interleukin-
IL-1ra	Interleukin-1 receptor antagonist protein
KC	keratocyst
KDO	keto deoxyoctanoic acid
KGF	Keratinocyte Growth Factor
LAF	lymphocyte activating factor
LAL	Limulus amoebocyte lysate
LPS	lipoploysaccharide
MCF	mononuclear cell factor
MEM	Minimum essential medium
OAF	osteoclast activating factor
PBS	phosphate buffered saline
PHA	phytohaemaglutinin
PGs	prostaglandins
PGE	prostaglandins of the E series
PTH	parathyroid hormone
P. gingivalis	*Porphyromonas gingivalis*
TGF	transforming growth factor
TNF	tumour necrosis factor
S. aureus	*Staphylococcus aureus*

SECTION I

The Outcome of Inflammation and Necrosis of the Dental Pulp

Chapter 1

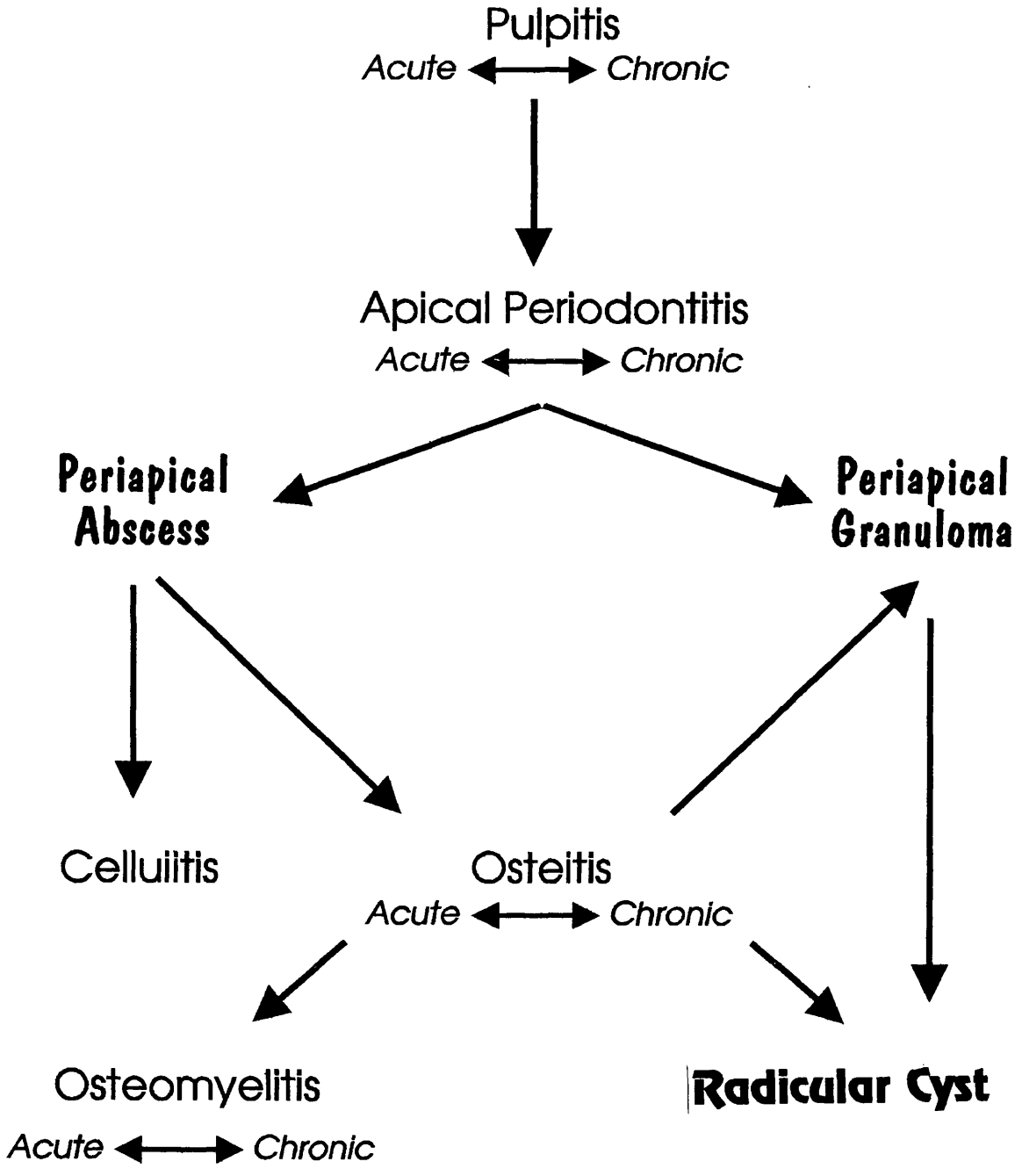
1. Introduction

The sequence of events which lead to pulpal necrosis, periapical inflammation and radicular cyst formation are well recognised (Figure 1); however many details of these stages in pathogenesis remain unexplained. This work is intended to provide a better understanding of this process by the addition of new information derived from studies of several important stages.

1.1 Pulpitis

The dental pulp is a connective tissue system enclosed within the calcified walls of a tooth. It is this enclosure, that makes this tissue different, in the way it reacts to tissue damage or infection. Most cases of pulpitis are primarily a result of dental caries where bacterial invasion of the dentine and pulp tissue occurs. Occasionally there is bacterial invasion in the absence of caries, as in cases of tooth fracture that expose the dental pulp to the oral fluids and microorganisms or as a result of a bacteraemia. This has been shown by Kakehashi et al, (1965) who demonstrated the relationship between bacterial infection and periapical lesion formation. In these studies, exposure of the pulp from the oral cavity resulted in the development of periapical lesions in rats maintained in a conventional environment, whereas germ-free animals with pulp exposures failed to develop lesions.

Figure 1. Interrelations of Periapical Inflammation



The inflammatory process in the pulp is basically the same as in any other connective tissue. However because of the unique environment the response is altered. For example the excessive swelling that occurs in the hyperaemic and edematous phase of inflammation cannot occur. The pulp chamber is filled with nerves, vascular tissue, fibres, ground substance, interstitial fluid, odontoblasts, fibroblasts and other minor cellular components. Since each of these constituents is relatively incompressible, the total volume of blood within the pulp chamber cannot be increased. Thus, the tissue regulation of blood flow is of critical importance. However many pulps have a collateral circulation which can be seen by examination of serial sections of extracted teeth. Here the pulp is supplied with blood not only by vessels that enter the apical foramina but also through some vessels that enter along the lateral aspects of the roots and the interradicular regions. Consequently, inflammatory pulp changes do not always cause self-strangulation of the pulp as had been believed. Thus resolution of pulp inflammation can occur (Seltzer and Bender; 1990).

In acute inflammation, chemical mediators such as histamine, bradykinin, serotonin, prostaglandins, released from injured cells, excite sensory nerve fibres. These then act on the muscular elements of the blood vessels and cause dilation of vessels. The permeability of the capillaries which do not have muscle cells, is increased by action of these substances (for example; histamine) on the ground substances of the capillary walls. The increased permeability of the vessels permits the escape of plasma proteins and

leukocytes from the capillaries into the inflamed area in an attempt to carry out neutralisation, dilution, and phagocytosis of the irritant.

During chronic inflammation, pulp tissue pressure is also thought to be elevated, although less than from the high pressures resulting from acute inflammation (Van Hassel; 1973, Tónder and Kvinnsland; 1983). However, capillary permeability is gradually decreased as repair occurs.

In severe inflammation, the lymphatic vessels are closed by persistently increased fluid and pulp pressure. The end result may be pulp necrosis. Thus untreated pulpitis, whether acute or chronic ultimately results in complete necrosis of the pulp tissue.

1.2 Periapical Abscess

The periapical abscess is an acute or chronic suppurative process of the dental periapical region. It usually arises as a result of infection following carious involvement of the tooth and pulp infection. It can also occur after traumatic injury to the teeth, resulting in necrosis of the pulp. It may also develop directly as an acute apical periodontitis following an acute pulpitis. The abscess contains a central area of microorganisms and disintegrating polymorphonuclear leucocytes surrounded by viable leucocytes and some lymphocytes. It may remain limited to the osseous structures or, through bone resorption, follow the path of least resistance, breaking through the periosteum and invading the soft tissues.

Both aerobic and facultative anaerobic bacteria have long been considered to be responsible for most of these abscesses (Alin and Aagren; 1954) but recent studies have emphasised a major role for anaerobes (Moore and

Russell; 1972, Megran, Scheifele and Chow 1984, Brook, Frazier and Gher; 1991). The predominant anaerobes recovered were *Bacteroides* spp. (Brook, Frazier and Gher 1991).

The significant role of oral bacteria in the induction of periapical inflammation has been confirmed experimentally. Möller et al (1981) exposed pulpal tissue of monkey teeth to the oral environment for 7 days. The teeth were than sealed and examined 6 months later. All of the exposed teeth became infected with microorganisms such as alpha-haemolytic streptococci, enterococci, coliforms and anaerobes such as bacteroides, eubacterial, propionibacteria and peptostreptococci. Ninety percent of the teeth developed periapical lesions that could be seen radiographically. In a subsequent study, Fabric ius et al., (1982) found that the ratio of anaerobes to aerobes increased with the passage of time following the closure of the 7-day-exposed pulps. The ratio increased from 3.9 at 90 days to 11.3 at 1,060 days.

1.3 The Periapical Granuloma

The periapical granuloma is one of the most common of all sequelae of pulpal death. It is essentially a localised mass of granulation tissue formed in response to low grade chronic infection. This chronic inflammatory tissue contains plasma cells, macrophages, lymphocytes, polymorphonuclear leukocytes and mast cells, in addition to fibroblasts, strands of epithelial cells and blood vessels.

The pathogenesis of the chronic periapical granuloma is not well understood. Although the presence of oral bacteria could account for

much of the pathosis seen, several studies have shown these lesions to be sterile (Grossman, 1959; Shindell, 1961 and Melville and Birch, 1967). A more recent study has shown low ($10^{2.2}$CFU/ml) levels of facultative anaerobes and strict anaerobes (Iwu et al; 1990).

A possible explanation for the pathogenesis of the chronic periapical granuloma is that bacterial products originating in septic root canals diffuse into the apical tissue. Indeed Schonfield et al., (1982) found that 75% of inflamed granuloma tissue contained endotoxin.

1.4 Radicular Cyst

Radicular cyst formation may arise from the periapical granuloma. Radicular cysts are by far the most common cystic lesions in the jaws. The pathogenesis of the radicular cysts can be divided into three phases; (a) initiation, (b) formation, and (c) enlargement. The precise mechanisms involved in these phases are not known. It is generally accepted that this epithelial lining is derived from the epithelial cells rests of Malassez in the periodontal ligament. These is also no doubt that these cells may proliferate, and when they do, either in vivo or in tissue culture experiments, that there are consistent morphological and histochemical changes (Ten Cate, 1972). The proliferating cells have a decreased nuclear-cytoplasmic ratio, they utilise glycogen, synthesise neutral lipids and ribonucleic acid and show an increased glucose-6-phosphate dehydrogenase activity, but depressed succinic dehydrogenase activity. The latter two chemical changes indicated that the activated epithelial cells preferentially use the hexose monophosphate shunt (Ten Cate, 1972).

The next phase in the pathogenesis of a radicular cyst is the process by which a cavity is formed and lined with epithelium. Two possibilities are generally recognised. One theory proposes that the epithelium proliferates and covers the bare connective tissue surface of an abscess cavity or a cavity which may occur as a result of connective tissue breakdown by proteolytic enzyme activity (Summers, 1974). The other hypothesis is that a cavity forms within the proliferating mass of epithelial cells by degeneration and death of cells in the centre.

The third phase, the enlargement of the cyst is also controversial. The enlargement of a cyst is dependent on continued epithelial proliferation. However the osmotic theory (reviewed by Main, 1970) is still popular. This proposes that bone destruction is caused by mechanical stress exerted by the cyst wall on the surrounding bone, resulting from the gradually increasing volume of the cyst fluid. The theory is that hyperosmolar cyst fluid draws in fluid from the surrounding tissue, giving rise to a positive intra-cystic pressure which is transmitted to the bone. The osmotic theory was proposed by Toller's (1948) experiments. He measured the intra-cystic hydrostatic pressure by inserting a fine bone needle attached to a manometer and found values above atmospheric pressure.

However, the concept that epithelial cell breakdown produces a hyperosmolar cyst fluid which draws in fluid from the surrounding tissue is difficult to sustain. The presence of large intra-cystic molecules such as globulin, fibrinogen and fibrin degradation products make it impossible to consider that the complex cyst wall acts simply as a semi-permeable

membrane. In fact, the composition of radicular cyst fluid suggest that transduction, exudation and haemorrhage all take place through the mural vessels to contribute to the contents. Also the amount of pressure exerted on the bone is unknown, and the pressure measured by Toller (1948) is more likely a function of a cyst wall contractility and does not necessarily signify any outwardly acting mechanical force. These biomechanical theories ignore both the cellular aspects of cyst growth and the biochemistry of bone destruction. The production of prostaglandins (Harris et al., 1973), leukotrienes (Matejka et al., 1985), collagenase (Donoff et al., 1972), interleukin-1 (Meghji et al., 1989) by the cyst wall, have contributed to the clarification of this problem.

1.5 Specific Considerations of Connective Tissue Destruction by the Periapical Abscess.

1.5.1 Bacterial Mediators of Connective Tissue Destruction

Extracts from several species of bacteria have been shown to inhibit the growth of mammalian cells in vitro. Sonic extracts from *Actinobacillus actinomycetemcomitans* were found to be cytotoxic to fibroblasts (Shenker et al, 1982, Stevens et al., 1983), epithelial cells (Kamen, 1983), and endothelial cells (Taichmen et al., 1984). Other oral organisms which inhibit fibroblast growth and proliferation include *S. intermedius* and *A viscosus* (Higerd et al., 1978) and *C. sputigena* (Stevens et al. 1980). Besides inhibiting DNA synthesis, sonicates from *A. actinomycetemcomi tans* also inhibit fibroblast protein synthesis in vitro (Shenker et al., 1982).

There is also evidence that bacteria in culture release soluble factors with biological activity. For example filtrates of culture media from *Porphyromonas gingivalis* and *B. asaccharolyticus* were shown to be cytotoxic to fibroblast (van Steenbergen et al., 1982) and inhibit collagen synthesis by chondrocytes (Touw et al., 1982).

Several bacterial factors (Figures 2,3,4), lipopolysaccharide, lipoteichoic and teichoic acids, peptidoglycans, muramyl dipeptide and capsular material have be shown to have a destructive effect on connective tissue.

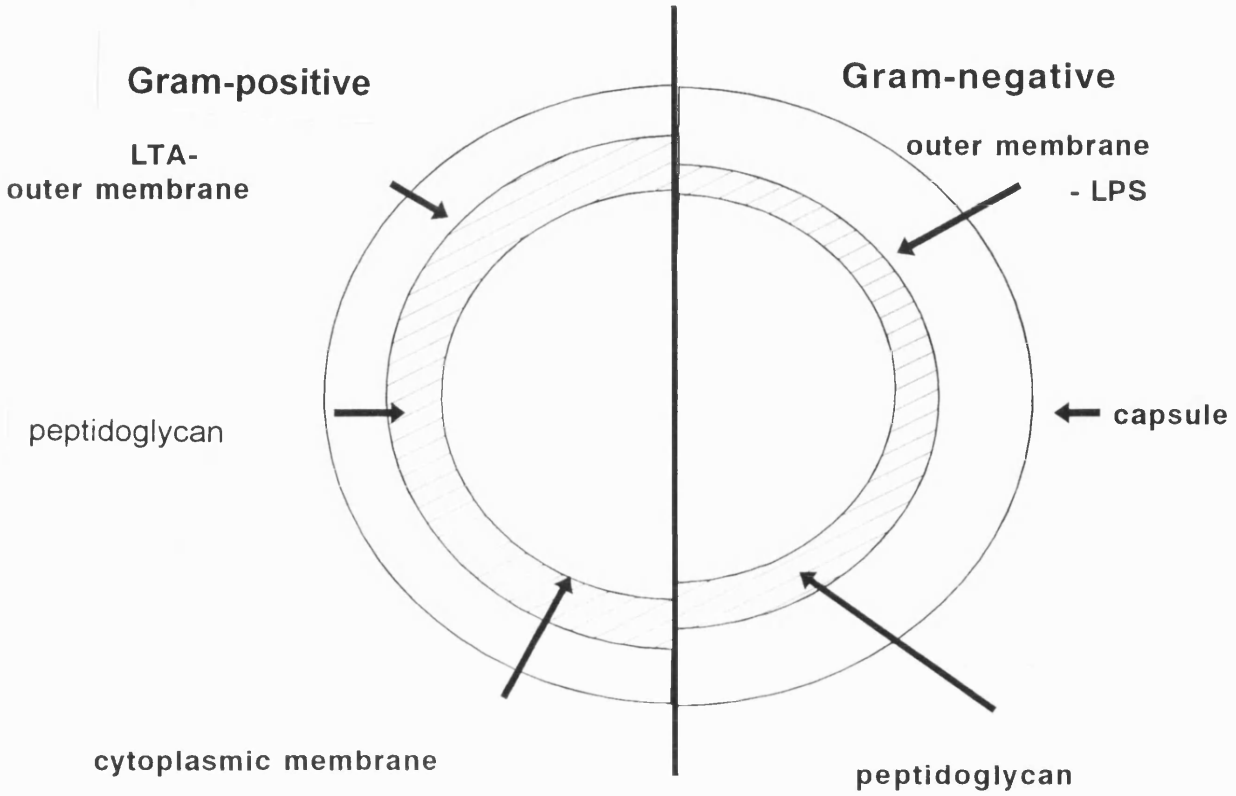


Figure 2. Cross-Section of Bacteria

1.5.2 Lipopolysaccharide

Lipopolysaccharide (LPS) or endotoxin is the most well known bacterial component. It is fundamental to the membrane structure of Gram-negative bacteria and is thought to be responsible for their biological properties (Morrison and Ulevitch, 1978). Endotoxins are released from the cell during its active growth and also during cell lysis (Crutchley, Marsh and Cameron, 1967).

The presence of endotoxin in necrotic pulps has been documented by Schein and Schilder (1975) and Dahlén and Bergenholtz (1980). Experimentally, endotoxin has been demonstrated to induce periapical inflammatory lesions and bone resorption in experimental animals (Dwyer and Torabinejad, 1981; Pitts et al., 1982). Dwyer and Torabinejad found that when endotoxin was inoculated into pulp chambers of cats' canines, larger apical radiolu cent areas were induced after 2 weeks. Root canals inoculated with either saline or detoxified endotoxin failed to elicit this reaction. Pitts et al., (1982) obtained similar results with *Salmonella* endotoxin in the root canals of dog molars and premolars. The induced periapical inflammatory lesions were heavily populated with neutrophils, and the periapical bone was resorbed.

1.5.2.1 Biological Activities of LPS

LPS is also known to be a potent toxin. Its injection into animals induces effects which include fever, bone marrow necrosis, changes in the levels of circulating leukocytes and metabolic changes (for example blood glucose levels, shock and death (Nowotny, 1969) . It is thought that the lipid A

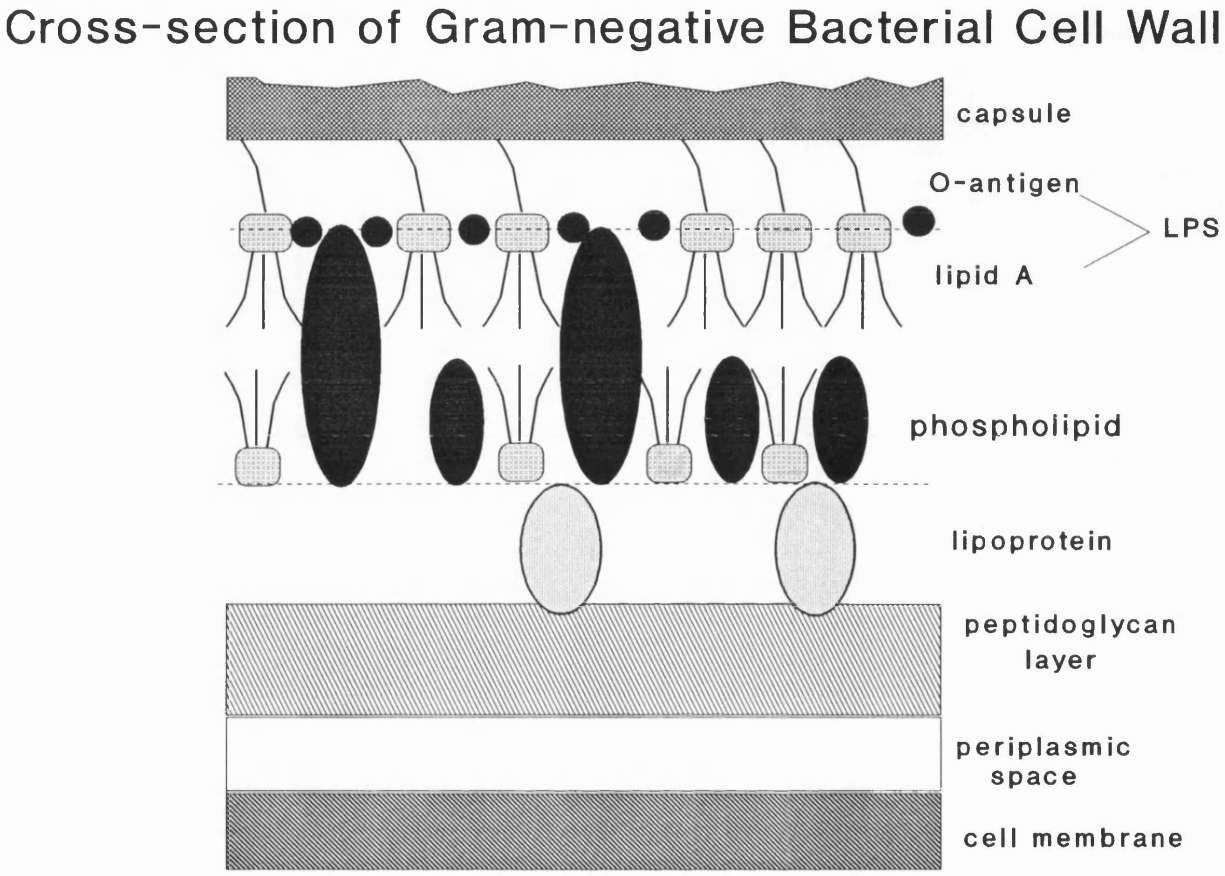


Figure 3. Cross-Section of Gram-negative bacteria

part of the molecule carries most of the biological activity (Galanos et al., 1972; Kotani et al., 1984; Rietschel et al., 1982). The effects of LPS are complicated by the multiplicity of its interactions with cells and molecular systems of inflammation, immunity and haemostasis. Indeed, it is difficult to distinguish primary effects from secondary changes.

1.5.2.2 The Effect of LPS on Fibroblasts

LPS has been shown to affect the ability of fibroblasts to adhere to surfaces and to proliferate. Neiders and Weiss (1973) demonstrated that LPS from *E. coli* caused detachment of mouse fibroblasts from glass and Aleo (1980) has shown LPS to inhibit the attachment of human gingival fibroblasts to root surfaces of teeth.

The cytotoxic effects of LPS on fibroblasts were first reported by Bergman and Nilsen (1963) who showed that that endotoxin from *Proteus mirabilis* inhibited the growth of chick fibroblasts. In an ultrastructural study of fibroblasts, endotoxin appeared to affect most cellular organelles i.e. disruption of mitochondria and Golgi apparatus, disorientation of cytofilaments, formation of autophagic vacuoles, lipid droplets, increased pinocytosis, disruption of plasma membrane (De Reniz and Chem, 1981). However, another study by Lucas et al. (1985), using an avidin-biotin complex method, demonstrated that LPS was concentrated only in the nucleus and there was no localisation in other cell organelles. This could indicate that the changes observed by De Reniz and Chen were due to late effect on the cell which was initiated at the nucleus rather than a

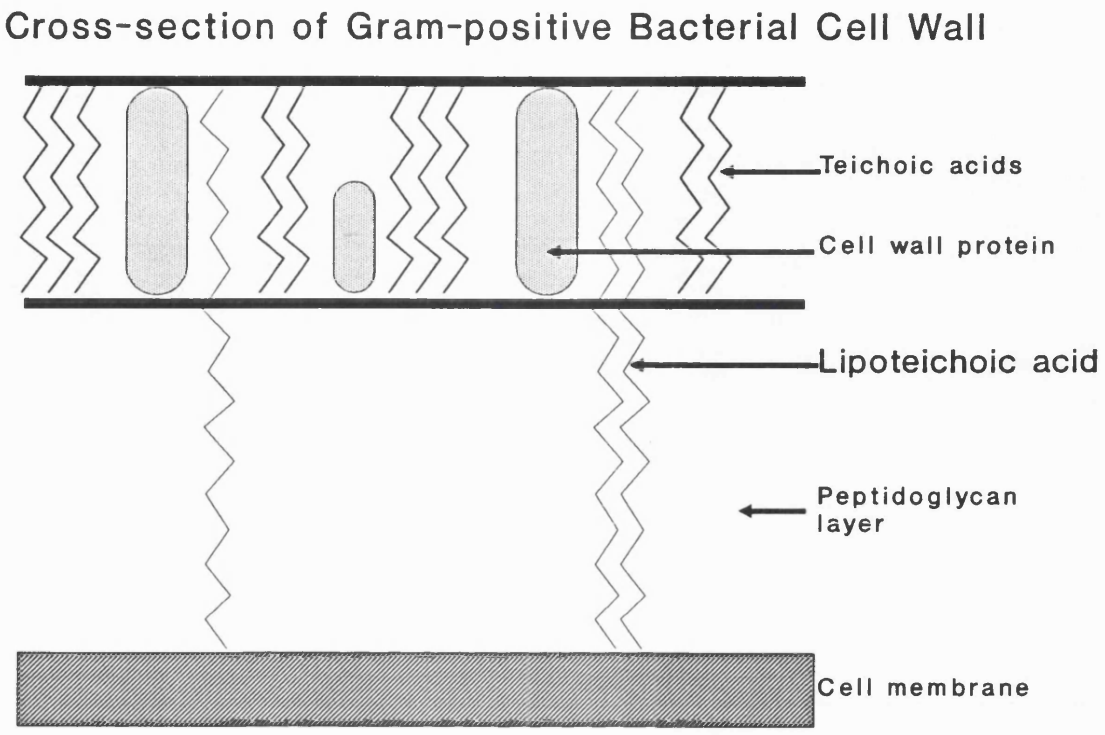


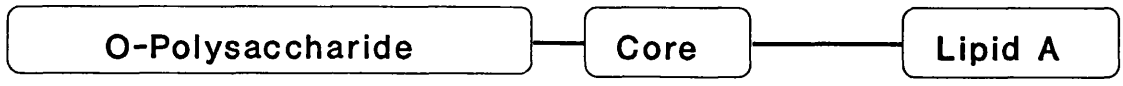
Figure 4. Cross-Section of Gram-positive bacteria

direct effect on the organelles. Alternatively, they could be due to the occurrence of cell death arising from the use of such large concentrations of LPS (300-500μg/ml).

At concentrations of 100μg/ml and above LPS has been shown to reduce proliferation of mouse and gingival fibroblasts (Aleo et al., 1974 ; Singer and Dutton, 1979 ; Olsen, Adams and Layman, 1985 ; Layman and Dietrich 1986). At these concentrations, LPS has also been shown to inhibit protein and collagen synthesis (Singer and Dutton, 1979; Aleo, 1980). However, at nanogram concentrations, LPS stimulates fibroblast proliferation (Vaheri et al., 1973; Hughes and Smales, 1988). The dose response of fibroblasts to LPS appears to be biphasic.

1.5.2.3 Bone Resorbing Activity of LPS

A large number of studies have assessed the bone resorbing potential of LPS. These studies have been conducted mainly in vitro, and have examined hydroxyproline, lactate and calcium release as means of assessing bone resorption. The bone resorbing activity of LPS was first described by Hausmann et al., (1970). LPS from a number of oral and non-oral species have been investigated. Most studies show that LPS at concentrations between 1 and 10μg/ml in vitro causes an increase in bone resorption. Sveen and Skaug (1980) showed that LPS from *Bacteroides fragilis* to be more potent than that from *Fusobacterium nucleatum* or *Veillonella parvula*. Iino and Hopps (1984) found that *B. gingivalis* LPS to be significantly more active than LPS from *A. actinomycetemcomitans* or



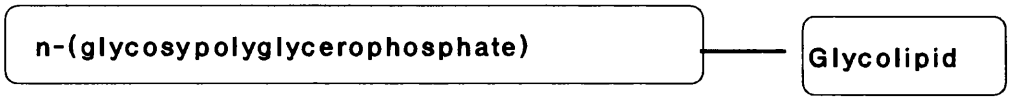
Endotoxin (Lipopolysaccharide)

Figure 5. Diagram of structure of Endotoxin

Capnocytophaga ochracea.

Little information is available on that portion of the LPS molecule responsible for the bone resorbing activity (Figure 5). Although detailed structural analyses of the LPS from all oral species is lacking, it is known that *B. gingivalis* and *B. fragilis*, which lack KDO and heptose, are able to stimulate bone resorption (Hausmann et al., 1975). Thus, the lack of these sugars does not seem to affect their ability to resorb bone. In order to determine whether the lipid or polysaccharide regions are essential for activity, Hausmann et al. (1975) have examined the bone resorbing activities of LPS from various forms of *Salmonella minnesota*. They included the smooth form, which consisted of an O-specific polysaccharide and another which contained KDO plus lipid A. Isolated lipid A was also studied. The results from this study suggest that the lipid A portion of the LPS molecule is responsible for the bone resorbing acitivy. However, another study (Sveen and Skaug, 1980) attributed the bone resorbing ability of fusobacterium LPS to both lipid A and polysaccharide portions. The two components were separated by heating in 1% acetic acid. Both portions stimulated bone resorption with the polysaccharide portion showing the greater activity. In contrast, studies performed by Harvey, Wilson and Meghji (1986) show that polymyxin B, a molecule which binds to the lipid A region inhibited LPS-induced bone resorption.

In addition to being able to stimulate bone resorption, low concentrations of LPS have been shown to interact with other bone resorbing agents. For example Raisz et al., (1981) have shown that LPS acts synergistically with



Lipoteichoic acid

Figure 6. Diagram of Lipoteichoic Acid

certain host factors, parathyroid hormone (PTH), prostaglandin E_2 (PGE_2) and osteoclast activating factor now known to be IL-1 (Dewhirst, 1985) to give increased levels of bone resorption in vitro. These effects were not due to a stimulation of endogenous prostaglandin synthesis, as a concentration as high as 10μM of indomethacin did not affect the synergism. This could suggest the LPS may have a different receptor and/or mechanism of action from these other bone resorbing agents.

The dependence of LPS-induced bone resorption on endogenous prostaglandin synthesis is unclear. The inhibition of LPS-induced resorption by indomethacin has been taken as evidence for the role of PG in mediating the effect of LPS on bone (Meryon and Perris, 1981), but other investigators have found no such inhibition (Hausman et al., 1975). There is evidence that the dependence of endogenous prostaglandin synthesis for bone resorption varies with the source of the LPS. Iino and Hopps (1984) found that indomethacin significantly inhibited resorption induced by LPS from *A. actinomycetemcomitans* but not from *B. gingivalis* and *C. ochracea*.

1.5.3. Gram-Positive Cell-Wall Components

The cell walls of Gram-positive bacterial, such as streptococci, staphlyococci and actinomyces, are also capable of influencing inflammatory reactions.

1.5.3.1 Peptidoglycans

Peptidoglycan is made up of glycan chains (N-acetyl glucosamine and N-acetyl muramic acid) with peptide cross bridges. Together they form a large, net-like structure which lends rigidity to the bacterial cell (Shockman and Barrett, 1983). Peptidoglycans are also found in smaller amounts in Gram-negative bacteria. Purified cell wall preparation and isolated peptidoglycan from streptococcus Gram-positive organisms have also been shown to stimulate bone resorption in organ culture (Lesgraf, Greenblatt and Bawden, 1979; Meikle, Gowen and Reynolds (1982).

1.5.3.2 Muramyl dipeptide

Muramyl dipeptide (MDP; N-acetylmuramy-L-alanyl-D-glutamine) is a degradation product of peptidoglycans from both Gram-negative and Gram-positive bacteria. It is the smallest fraction of the peptidoglycan which shows biological activity. It can act as an adjuvant (Holton and Schwab, 1966; Adam et al, 1974), activates complement (Greenblatt et al., 1978) and can also supress lymphocyte proliferation (Dziarski et al, 1978). It is now genenerally accepted that the adjuvant activity of MDP is mediated by an action on macrophages, which releases IL-1 (Fevrier et al., 1978; Guenounou, Vacheron and Nauciel, 1985). This increases the number of T-helper lymphocytes, which in turn stimulate B-lymphocytes to produce antibodies. MDP is a potent bone resorbing agent (Alander et al., 1980), active at concentrations as low as 10^{-7}M (Douglas et al., 1985). The bone resorbing activity is independent of endogenous PG synthesis (Raisz et al.,

1982) in the long bone system. However, Douglas et al., (1985) found that when mouse calvaria were used, indomethacin caused up to 50% decrease in bone resorbing activity. In addition, MDP is also capable of stimulating the secretion of PG and collagenase by macrophages (Wahl et al., 1979 ; Dewhirst et al., 1981; Whithead et al., 1981).

Thus, peptidoglycan has the potential to play an active role in cell and tissue destruction in periapical lesions. Bacterial death will yield peptidoglycan fragments, which because of their small size could easily penetrate the tissue. Gram-positive bacterial peptidoglycan can form 50-90% of the dry weight, whereas in Gram-negative bacteria it forms 5-20% of the dry weight (McGhee et al., 1982).

1.5.3.3 Lipoteichoic Acids

Lipoteichoic acids (LTA) are covalently linked to peptidoglycan. They are composed of polymers of glycerol phosphate with a glycolipid moiety at one end. LTA has many biological activities including the stimulation of bone resorption (Hausmann et al., 1975), antigenicity (Wicken and Knox 1980) and stimulation of macrophage lysosomal enzyme release (Wicken and Knox; 1980).

Experimentally Gram-positive bacteria have been shown to induce pulpal and periapical inflammation. Whole cells or sonicates of *Streptococcus mutans* induced pulpal necrosis and periapical bone resorption 6 months after injection into pulps of cats (Stabholz and Sela, 1983).

1.5.4 Bacterial Capsules

Not all investigators agree that LPS constitutes a major factor in the initiation or maintenance of chronic inflammation and the sequelae of such inflammation.

Repeated application of endotoxin can induce immunological non-specific tolerance, as measured by passive haemagglutination and lymphoblast assays (Beutler et al., 1986). Some strains of mice become markedly resistant to endotoxin. In these strains, macrophages do not produce tumour necrosis factor, interleukin-1, interferon or colony stimulating factor in response to endotoxin (Beutler et al., 1986). In these endotoxin-resistant rats, alveolar bone resorption is significantly reduced (Nowotny and Sanavi, 1983).

Many of the oral bacteria, *B. gingivalis*, *A. actinomycetemcomitans, C. sputigens, Eikenella corrodens* possess an outer membrane around their cell walls (Woo, Holt and Leadbetter, 1979) yet the role of the capsule in the pathogenesis of periapical lesions has been neglected. Capsules enable bacteria to evade phagocytosis by PMNL by virtue of their hydrophilic nature (Dudman, 1977; Smith 1977) and protect the cells against antibody-dependent complement-independent cytotoxic killing (Finegold, 1969) by masking the surface antigen to which antibodies are directed (Orskow et al., 1963; Jann et al., 1970). Capsules are also important for selective adherence to cells and other surfaces (Troy, 1979). The thickness of the capsule may contribute to bacterial virulence (Kasper et al., 1977; Troy, 1979). Kasper et al., (1977) found that encapsulated

strains of *B. fragilis* caused abscess formation rats wheras non-encapsulated strains did not.

Early studies carried out using outer membrane complex of *P. gingivalis* show it to have significant bone resorbing activity in vitro (Woo et al., 1979). Nowotny et al., (1982) demonstrated the presence of a proteolysis-sensitive bone resorbing factor from microvesicles of *A. actinomycetemcomitans*. Isolation of these microvesicles by sucrose gradient centrifugation of the culture medium followed by phenol-water extraction of LPS yielded 10% LPS (of the dry weight) and a proteolysis-sensitive, heat-resistant non-endotoxin bone resorbing factor. They concluded therefore that this factor was not endotoxin or leukotoxin. Since the microvesicles were derived from the cell wall, it is probable that it contained capsule exopolymers. In 1985 Wilson et al., extracted a material from *A. actinomycetemcomitans* with saline and purified it by gel filtration and ion exchange chromatography which had potent bone resorbing properties, with activities that were a thousand-fold lower than LPS from the same organism. Apart from stimulating bone resorption it also inhibited bone collagen and DNA synthesis (Wilson, Meghji and Harvey, 1988) and inhibited fibroblast cell division (Kamin et al, 1986). The high solubilty of this material would facilitate penetration of tissues, whereas it s potent cytotoxic, bone resorbing activity could contribute to tissue destruction not only in the periapical granuloma, but also in the abscess.

1.5 OBJECTIVES OF THE STUDY - 1

The purpose of the experiments described in this section was to investigate the potential role of bacterial outer capsule or surface associated material (SAM; capsular material) in the tissue destruction which characterises the periapical abscess. This will be presented in three interrelated investigations. They are (i) the antiproliferative and cytotoxic activity of surface associated material from oral bacteria (Chapter 3), (ii) inhibition of bone DNA and collagen production by bacterial surface associated material (Chapter 4) and (iii) the effect of this material on bone resorption (Chapter 5). In addition, the activities of the bacterial capsular preparations has been compared with those of LPS, which, to date, has been the bacterial component most widely implicated in the pathogenesis of these lesions.

CHAPTER 2

METHODS AND MATERIALS

This chapter describes the preparation of SAM and LPS and the tissue culture methods used throughout this study.

2.1 Preparation of Surface Associated Material (SAM) and Lipopolysaccharide (LPS)

2.1.1 Culture of Bacteria

Actinobacillus actinomycetemcomitans Y4 was grown at 37°C on Brain Heart Infusion agar (Oxoid, Basingstoke, England) under anaerobic conditions (Oxoid Gas Pak System, Oxoid).

Prophyromonas gingivalis W50 was grown at 37°C under anaerobic conditions on a medium consisting of 5g trypticase, 5g proteose peptone, 2.5g yeast extract (Oxoid, Basingstoke, England), 2.5g glucose, 2.5g sodium chloride, 0.375g cysteine HCl, 0.25g haemin and 0.05g menadione per litre (BDH, England).

Eikenella corrodens NCTC 10596 was grown at 37°C under anaerobic conditions on a medium consisting of Brain Heart Infusion agar (Oxoid) supplemented with 0.375g/L cysteine HCl, 0.25g/L haemin and 0.05g/L menadione (BDH).

Staphylococcus aureus NCTC6571 was cultured aerobically at 37°C for 24 hours on either Wilkins-Chalgrens agar (Oxoid) containing 10% horse blood (Oxoid) or on nutrient agar (Oxoid).

All bacteria were grown on solid media to enhance capsule production.

2.1.2 Purity Checks

In order to ensure that the cultures were free from contamination, purity checks were carried out at the time of inoculation of the cultures on to fresh agar plates. After 3-4 days all cultures were inspected visually for contamination, Gram-stained, removed from the plate with sterile saline and then centrifuged at 30,000xg for 30 min at 4°C. The cells were resuspended in saline, recentrifuged and then lyophilized.

2.1.2.1 Gram's Stain

Bacterial smears were heated in a flame and then stained as follows:

i) 1 minute in 0.5% aqueous crystal violet

ii) 1 minute in Lugol's iodine (1% solution of iodine in 2% aqueous potassium iodine)

iii) decolorised for 5 seconds in acetone

iv) 1 minute in 1% carbol fuchsin

v) washed in tap water

vi) air dried

2.1.3 Extraction of SAM from the Bacteria

An extract containing surface-associated components from the cells was obtained by washing in saline as previously described by Wilson et al. (1985). Lyophilized cells were suspended in 0.15M sodium chloride at a concentration of 1% (weight per volume) and stirred gently for 1 hour at 4°C. The bacteria were then sedimented by centrifugation at 10,000g for 30 minutes at 4°C. This was repeated twice more. The combined aqueous phases were pooled and the SAM was precipitated by addition of 1.5

volumes acetone, centrifuged at 30,000 G for 30 minutes, dialysed and then lyophilized. The protein content of the precipitated SAM was determined by the Lowry method using bovine serum albumin as a standard and the endotoxin content was determined using the chromogenic *Limulus* amoebocyte lysate assay (Pyrogent, Byk-Mallinckrodt, England) according to the manufacturers instructions.

2.1.3.1 The Limulus Amoebocyte Lysate Assay

Preparation of reagents: Aseptic techniques were employed throughout this assay to avoid exogenous endotoxin contamination.

Substrate 5-1423: 9mg of lyophilised chromogenic substrate (AC-I1e-Glu-Gly-Arg-pNA HCL) was reconstituted with 6.00ml of sterile pyrogen-free water to a concentration of 2.2 mM/l and stored at 4°C. For the assay, this stock was diluted with equal volumes of 50nM Tris buffer pH 9.00 and pre-warmed at 37°C.

Limulus amoebocyte lysate (LAL): The lyophilised LAL was reconstituted with 1.4 ml of sterile, pyrogen-free water and mixed gently. This was incubated at 20-25°C for 10 minutes to ensure it had dissolved, then kept on ice until used.

Endotoxin Standard E. Coli 0111:B4: 2.0ng of the lyophilised endotoxin was reconstituted with 1 ml of sterile pyrogen-free water. From this 2ng/ml (24 endotoxin units /ml; EU/ml) stock solution 1, 0.8, 0.5 0.2 EU/ml were prepared using sterile, pyrogen-free water.

SAM: Lyophilised SAM from *A. actinomycetemcomitans*,*P. gingivalis* and *E. corrodens* was dissolved in sterile, pyrogen free water to give a

concentration of 0.1ng/ml.

The Assay Procedure: 100μl triplicate samples of pyrogen-free water (blank), standard or test SAM were placed into 5ml, pyrogen free, glass test tubes and warmed to 37°C for 3-5 minutes. 100μl of LAL was added to each tube which was then mixed thoroughly and incubated at 37°C for a further 10 minutes. 200μl pre-warmed substrate buffer solution was then added, mixed and incubated for a further 3 minutes to allow cleavage of the substrate to take place. The reaction was stopped by adding 200μl of 50% acetic acid.

Duplicate 200μl aliquots of the resulting solution were then placed in a microtitre plate (Flow). The optical densities were measured in a microtitre plate reader (Titretek) at 405nm wavelength. The mean optical density for each standard was calculated and a standard curve was drawn. From this, the concentration of endotoxin was obtained for the corresponding absorbance of the SAM test sample.

2.1.4 Electron Microscopy

To check on the efficacy of extraction and the integrity of the resultant cells, bacteria were examined by transmission electron microscopy before and after saline extraction. A portion of the bacterial suspension was fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer for 1h at room temperature (control cells). A second portion was fixed in the same manner except that the fixative also contained 0.15% ruthenium red. Bacteria were then pelleted by centrifugation at low speed, the fixative decanted and the pellet washed in cacodylate buffer. Control cells were

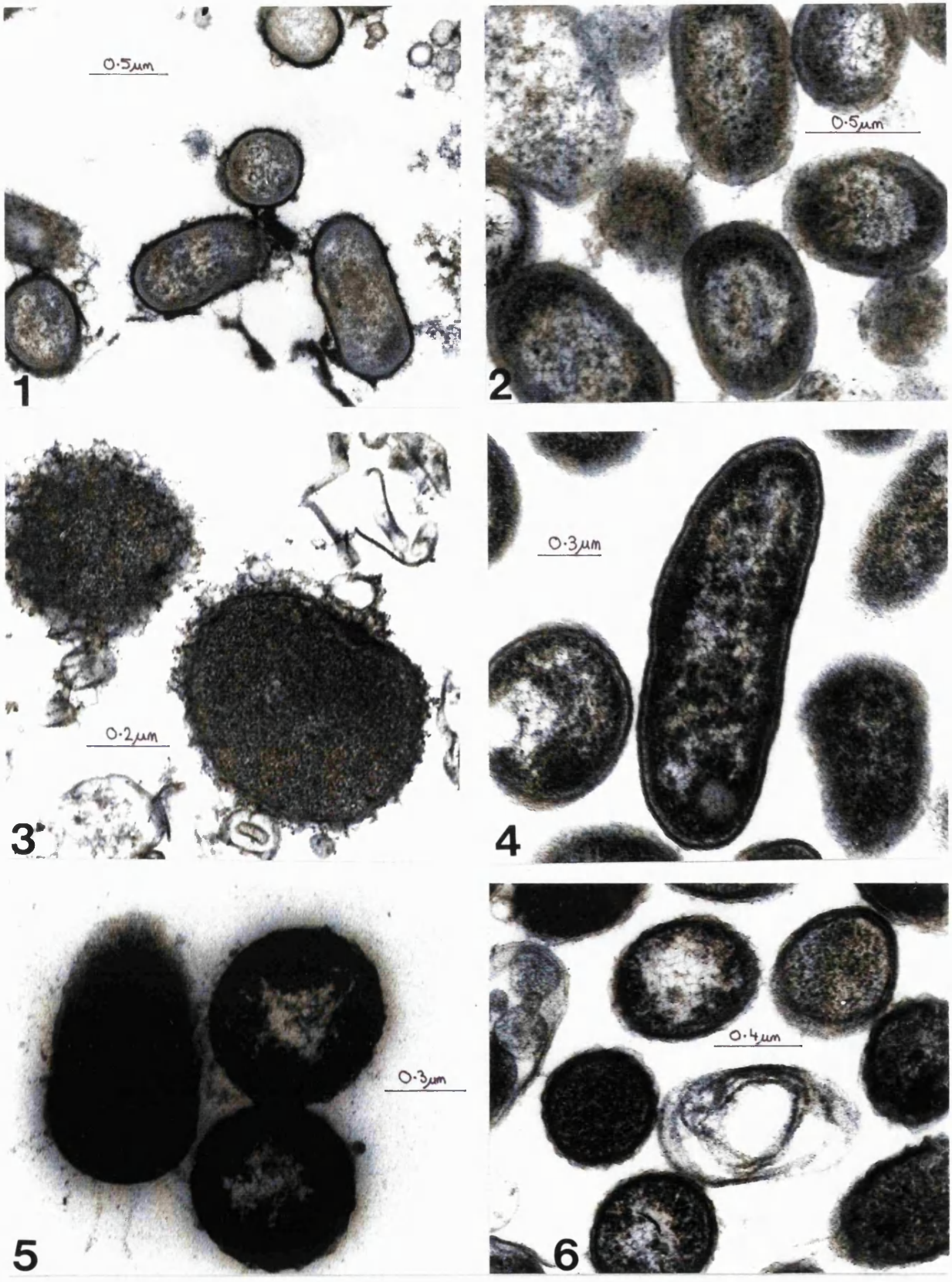


Figure 7. Electron micrographs demonstrating the staining of the surface associated material of the three bacteria with Ruthenium red prior to and after extraction of the bacteria with sterile normal saline. **(1)** and **(2)** show *A. actinomycetemcomitans* prior to and following saline extraction respectively. Likewise **(3)** and **(4)** show *P. gingivalis* before extraction and after extraction respectively. In the same manner **(5)** and **(6)** demonstrate the effect of extraction on the *E. corrodens*.

then fixed in 1% osmium tetroxide in cacodylate buffer for 2h at room temperature. The test cells were treated identically except that the post-fix also contained 0.15% ruthenium red. The fixative was decanted after centrifugation and the pellets washed in cacodylate buffer. All specimens were then dehydrated in a graded series of concentrations of ethanol and embedded in Araldite.. Sections were cut on an Reichert Ultracut E ultramicrotome using a diamond knife and examined (unstained) in a JEOL 100CXII electron microscope. (Figure 7).

In the case of *A. actinomycetemcomitans* and *P. gingivalis*, SAM was fractionated by gel filtration and ion-exchange chromatography as described previously (Wilson et al., 1985). In each case, the major component obtained was a protein- and carbohydrate-containing material which was used in experiments with PMNs.

2.1.5 Preparation of LPS

After extraction of the SAM from *A. actinomycetemcomitans, P. gingivalis and E. corrodens* LPS was extracted from the resulting cells as described by Kiley and Holt (1983). Briefly, cells were extracted three times with phenol/water at 68°C and the resulting aqueous phases were lyophilized after dialysis against distilled water. The crude LPS obtained was purified by treatment with RNase, DNase and pronase and then ultracentrifuged. The purity of the LPS was assessed by SDS-PAGE.

2.2 TISSUE CULTURE

All tissue culture manipulations were carried out in a sterile laminar flow

cabinet (Microflow).

2.2.1 Tissue Culture Material

Tissue culture materials were obtained from Gibco Ltd. (Paisley, Scotland) and the disposable plastic tissue culture items were obtained from Sterilin (Feltham, England) unless otherwise stated.

1. *Dulbecco's Minimal Essential Medium (MEM).*

   This was a 10 x concentrate and was diluted to single strength with double-distilled deionised water, and supplemented with the following:

   a) Foetal calf serum (FCS) (virus and mycoplasma screened)

   b) Sodium bicarbonate 2.25 g/l

   c) L-glutamine 2.0 mM

   d) Penicillin and streptomycin 100 U/ml each

All other media were made up and stored in the same way. Powdered media solutions were sterilised using Sartorius minisart filters, pore size 0.2 μM. Serum was heat-inactivated in a water bath at 56°C for 30 mins.

2. *Hank's Balanced Salt Solution (HBSS) without sodium bicarbonate.*

This was a 10 x concentrate and was diluted to single strength with double-distilled deionised water, supplemented with the following:

   a) Sodium bicarbonate 0.45 g/l

   b) Penicillin and streptomycin 100 U/ml each

HBSS was used as a transport medium for the tissues studied and as a

rinsing medium.

3. Biggers, Gwatkin and Hyner Medium (BGJ)

(Flow Laboratories, Irvine, Scotland)

This was obtained in powder form; it was dissolved in double distilled deionised water and filter sterilised. It was supplemented with the following:

a) Heat inactivated rabbit serum (5%), (Flow)

b) Sodium Bicarbonate 3 g/l

c) L-glutamine 2.0mM

d) Penicillin and streptomycin 100u/l each

4. RPMI 1640 (Rosewell Park Memorial Institute)

This was obtained in powder form, and was dissolved in double distilled, deionised water, filter sterilised and supplemented by antibiotics, sodium bicarbonate, and 1-glutamine as described for BGJ medium above.

5. Trypsin solution for subculturing.

Sterile trypsin was bought in the lyophilised form, and was reconstituted to 2.5% (w.v) and stored at -20°C. Prior to use, this was diluted to single strength in HBSS and stored at 4°C.

6. Phosphate Buffered Saline (PBS)

KCl 200 mg/l

KH2PO4 200 mg/l

Na2HPO4 1.15 g/l pH 7.6

NaCl 8 g/l

7. Borate Buffer

Boric acid 0.01M

Adjust pH to 8.5 with NaOH

2.3 Cyst and Normal Tissue Culture

2.3.1 Preparation of Normal tissue and Cyst Explant Media

The tissue was minced with two No.23 scalpel blades into fragments approximately 1mm^3 and approximately 50mg (wet weight) was placed on the culture surface of a 25^2 cm flask with 5 ml serum-free MEM. The flask was incubated 5% CO_2 incubator at 37°C for three days. At the end of the incubation period, the contents of the flask were placed in a sterile conical bottom test tube, and centrifuged for 20 min at 2000 rpm. The supernatant was removed and stored at -20°C, the pellet was blotted and weighed.

2.3.2 Normal Gingival Fibroblasts

Human gingival fibroblasts were grown from gingival tissue explants obtained during minor oral surgical procedures, usually the removal of impacted wisdom teeth. The tissue was minced with two No.23 scalpel blades into fragments approximately 1mm^3 and 10-12 of these placed on the culture surface of a 25^{2}cm flask with 1.5ml MEM with 10% FCS; this was enough to cover the tissue but allowed it to remain in contact with the culture surface. Again in the initial stage of establishing the cell culture, Fungizone (Squibb, N. Jersey, USA) (5 μg/ml) was incorporated in the culture medium to minimise the risk of yeast and

fungal contamination. As soon as cell growth was established, the medium was replaced with 5ml MEM with 10% FCS per flask, and the cells fed with this at twice-weekly intervals. The fibroblast cell lines were used for experimental purposes from passages 4 to 12.

2.3.3 Mouse Calvarial Cells

Osteoblast-like cells were grown from calvaria of 5 day old mice using a sequential enzyme digest method. The mice were killed by cervical dislocation, and the cranial bones removed and trimmed. The parietal and frontal bones of the calvaria were washed in HBSS. The periosteum and endosteum were stripped from the bones to minimise contamination of bone cells by fibroblasts. They were placed in a sterile test tube with 0.25% trypsin and 0.05% bacterial collagenase (Clostridiopeptidase; Sigma) in PBS (Flow, Scotland) and incubated at 37°C for 15 min. To facilitate digestion, the test tube was rotated at 20 rpm. The supernatant was removed and discarded. 0.05% Collagenase and 10% FCS in PBS were added to the bones and incubated for a further 20 min. At the end of the incubation period, it was removed, centrifuged at 900 rpm for 10 min. and saved. This was repeated twice more. The cells obtained were pooled, washed, counted and seeded at $3x10^6$ in $25cm^2$ flasks in MEM with 10% FCS. The yield was about $15x10^6$ cells per 40 mice. Cells were fed twice weekly and subcultured when confluent. The bone cells were used for experimental purposes at the first passage.

2.4 Subculturing

Cell suspensions for both subculturing and experimental cultures were

prepared by trypsinisation. The culture medium was removed and cell layer washed with 1.0ml of 0.25% trypsin in HBSS. Excess trypsin was removed, leaving only a thin film covering the cell layer, and the flask incubated for 20 min. at 37°C. The process was arrested by adding 1.0ml MEM containing 10% FCS into the flask and single cell suspensions were obtained by vigorously pipetting the cell clumps through a finely-drawn Pasteur pipette several times. The cells were counted, and either placed into two new flasks at 10^6 per flask or used for experimentation. Cell growth was routinely examined using a phase contrast microscope (Olympus, Japan).

2.5 Mycoplasma screening

All cell lines were routinely screened for mycoplasma using the method described by Kaplan et al (1984) which depends on the incorporation of 3H-thymidine. Spent culture media from cells were collected and centrifuged twice at 900 rpm for 10' in order to remove any cells. 100 µl aliquots of each supernatant were plated in triplicate, in wells of a 96-well flat-bottomed microtitre plate with an equal volume of fresh medium containing 10% filter-sterilised FCS. After 1, 3, 5 and 7 days of culture, 0.2 µCi of ^{3}H- thymidine (sp. act. 24 Ci.mMol-1, Amersham International, U.K.) in 10 µl MEM was added to each well using Hamilton syringe. The plates were further incubated for 6 hours and the contents of each well harvested with a semi-automated cell harvester (Skatron AS, Norway). Material insoluble in 5% TCA was retained on 10mm glass fibre filter discs (Skatron AS, Norway). The discs were

transferred to 5ml polypropylene scintillation vial inserts. 3ml of scintillation fluid (Unisolve 1, Koch Light) was added to each vial. Radioactivity was measured for 100 seconds on an LKB Wallac Rackbeta Liquid Scintillation Counter with external standardisation and radioactivity expressed as disintegrations per minute (dpm).

2.6 Mouse Calvarial Bone Resorption Assay

Bone resorption was measured by the release of calcium (measured colorimetrically; Zanelli et al., 1969) from explants of neonatal mouse calvaria in culture. This culture technique was basically the same as that described by Reynolds and Dingle (1970).

2.6.1 Dissection Technique

Mice were killed by cervical dislocation. The skin was removed to expose the cranial bones. The dissection was then continued using the lamboidal suture as a guide line, taking care not to damage the periosteum. The fronto-parietal bones were trimmed of any adhering connective tissue and interparietal bone.

Dissected calvaria were pooled in HBSS and washed free of blood and adherent brain tissue. The calvaria were divided along the sagittal suture and placed in a dish with HBSS.

2.6.2 Culture Procedure

Each half was cultured separately on stainless steel grids (1 cm^2 Minimesh FDP quality, Expanded Metal Co. W. Hartlepool) in a 30mm plastic petri dish with 1.5 of BGJ medium containing 5% heat inactivated rabbit serum. The bones were incubated for 24 hours at 37°C in

humidified atmosphere of 5% CO_2, 95% air.

The 24h pre-incubation period prior to addition of experimental factors was included for two reasons: firstly, it enabled the bones to adapt to their new environment and to permit calcium exchange to reach an equilibrium. Secondly it has been reported that the release of prostaglandins from freshly explanted bone cultures is relatively high (Katz et al., 1981). These authors recommended that the assay for bone-resorbing substances, which may themselves act by modifying PG synthesis in the bone, should be postponed until PG levels had normalised.

At the end of the 24h pre-incubation, the medium was removed and replaced with fresh medium containing test substances. In the case of conditioned media, this was diluted 1 in 10 in BGJ medium; PGE_2 (10^{-6}M) used as positive control to stimulate resorption, was dissolved first in ethanol, then diluted in BGJ medium. The maximum concentration of ethanol was 0.02%. The resorption in these cultures was compared with that in control cultures containing BGJ alone. The cultures were incubated for a further 48h and resorption measured as the release of calcium into the culture medium over this period. Each experimental group contained five separate cultures.

2.6.3 Measurement of calcium release

At the end of the incubation period, the culture medium was removed from each dish with a Pasteur pipette into autoanalyser cups (conical-bottomed, 2 ml, Chem Lab Instruments, Essex). Calcium concentrations

were measured colorimetrically on an autoanalyser (Chem Lab Instruments, Essex), by using the metal complexing dye cresolphthalein complexone (CPC) (Gitelman, 1967). Calcium was separated from proteins by continuous flow dialysis under acidic conditions. The concentration of dialysed calcium was then determined colorimetrically by complexing with CPC.

The sample cups containing the media or standard calcium solutions (8, 10, 12 and 14 mg/ml) were placed on the sample tray which held 40 samples at a time. One aliquot of 100 µl was removed from each sample through the stainless steel sampling probe followed by a 20 s wash with distilled water after each sample. The samples were mixed with 1M HCl containing 8-hydroxy-quinoline (8HQ) at 2.5 g/l (which eliminated interference by magnesium), and dialysed against a solution of similar composition containing CPC at 0.7 g/l). The dialysate was then mixed with 2-amino-2-methylpropano-l-ol (AMP) (90 g/l). The absorbance of the resultant purple-coloured solution was measured in a 15mm flow cell at 570nm, and plotted on a chart recorder at 0.5cm/min. Calcium concentrations were calculated from the absorbance peak heights measured against the standard curve.

2.7 Statistical Methods.

The unpaired Student's t test was used for comparison of experimental and control means. The 2 sample t-test was used.

CHAPTER 3

Anti-proliferative and Cytotoxic Activity of Surface-associated Material from Oral Bacteria

3.1 Introduction

That there is a close association between bacterial infection and the pathogenesis of the periapical diseases is now widely accepted (Kakehashi, Stanley and Fitzgerald; 1965). However, the exact nature of this association is still unclear. In attempting to ascertain the role of bacteria in these diseases, attention has focused on the biological activity of one component common to many of the disease-related bacteria, namely lipopolysaccharides (LPS). One particular activity which has been associated with LPS is the inhibition of mammalian cell division. Numerous studies have shown that LPS from various bacteria inhibits the growth of murine and human fibroblasts (Aleo et al, 1974; Singer and Dutton ; 1979, Layman and Diedrich, 1987). However, in these studies the concentrations needed of purified LPS to inhibit fibroblast cell division were 100μg/ml or over. In contrast, capsular material from *A. actinomycetemcomitans* obtained by gentle solubilization in saline at 4°C, was between two and three log orders more potent in inhibiting fibroblast cell division (Kamin et al, 1986). Furthermore, LPS stimulates fibroblast proliferation at nanogram concentrations (Vaheri et al., 1973, Hughes and Smales, 1988).

Fibroblasts are only one of the cell populations in the inflamed periapical tissue which are exposed to bacteria and their components. The response of epithelial cells, macrophages, polymorphonuclear leukocytes (PMNs) and

other cell populations, to LPS and/or other bacterial components has not been investigated in a systematic manner. In this chapter the response of cultured human fibroblasts, epithelial cells, the macrophage cell line - U937 and peripheral blood PMNs to surface-associated material (SAM) from three oral bacteria has been investigated.

3.2 Materials and Methods

3.2.1 Fibroblast Culture and DNA Synthesis

Human gingival fibroblasts were grown from explants obtained during minor oral surgical procedures. The cells were grown in Eagles' MEM containing 10% foetal calf serum (FCS), 2mM L-glutamine, 2.25g/l $NaHCO_3$, penicillin and streptomycin (each 100U/ml) and incubated at 37°C in 5% CO_2/air. Cells were subcultured at weekly intervals and used for experiments between passages 6 and 12.

Fibroblast suspensions were seeded into 96-well culture plates (Microtitre, Linbro) at concentrations of 15,000 cells/well in 100µl of MEM with 10% FCS and left overnight to attach. The culture medium was then replaced with MEM containing 2% FCS and various concentrations of either purified LPS or SAM, in groups of 6 wells per concentration. Control cultures received only unsupplemented medium. Cells were incubated for a further period of 24h and then 0.5uCi of 5-^{3}H-thymidine (^{3}H-TdR: specific activity 20Ci/mmol: Amersham) was added to each well 6h before termination of cultures. Trichloroacetic acid-insoluble material in each cell layer was dissolved in 0.4M NaOH and radioactivity measured in 3ml of scintillant by

scintillation spectrometry (Rackbeta: LKB) with external standardization.

3.2.2 Keratinocyte Culture and DNA Synthesis

Keratinocytes (GPK cell line, derived from guinea pig ear epidermis) were obtained from Dr. M. Hola, Department of Biochemical Pathology, University College School of Medicine, London. The cells were grown in Eagles' MEM containing 10% foetal calf serum (FCS), 2mM L-glutamine, 2.25g/1 $NaHCO_3$, penicillin and streptomycin (each 100U/ml) and incubated at 37°C in 5% CO_2/air.

Keratinocyte suspensions were seeded into 96-well culture plates (Microtitre, Linbro) at concentrations of 20,000 cells/well in 100ul of MEM with 10% FCS and left overnight to attach. The culture mediumwas then replaced with MEM containing 2% FCS and various concentrations of either purified LPS or SAM, in groups of 6 wells per concentration. Control cultures received only unsupplemented medium. Cells were incubated for a further period of 24h and then 0.5uCi of 5-^{3}H-thymidine (specific activity 20Ci/mmol: Amersham) was added to each well 6h before termination of cultures. The incorporation of tritiated thymidine was measured as described above.

3.2.3 Macrophage Cell Line Culture and DNA Synthesis

U937, a monocytic cell line derived from a human histiocytic lymphoma was used in these experiments. The cells were seeded into 96-well culture plates (Microtitre, Linbro) at a concentration of 500,000 cells per well in 100ul of RPMI (Gibco) with 5% heat-inactivated FCS containing various concentrations of either purified LPS or SAM, in groups of 6 wells per

concentration. Control cultures received only unsupplemented medium. Cells were incubated for 48h and then 0.5uCi of 5-^{3}H-thymidine (specific activity 20Ci/mmol; Amersham) was added to each well 6h before termination of cultures. The cells were collected using a cell harvester (Skatron) and radioactivity was measured in 3ml of scintillant by scintillation spectrometry (Rackbeta: LKB) with external standardization.

3.2.4 Preparation of PMNs and Cytotoxicity Assay

3.2.4a Preparation of PMN monolayers: Peripheral venous blood was collected in plastic tubes containing 20U/ml heparin, mixed 1:4 with Dulbecco's phosphate buffered saline (PBS) and separated on a cushion of Ficoll-Hypaque according to Boyum (1968). Pelleted red blood cells and PMNs were resuspended in fresh lysing buffer (0.155M NH_4C1, 10mM $KHCO_3$ and 0.1M EDTA) to disrupt red blood cells, washed twice with PBS and then in RPMI medium. Cells pelleted after the final wash were resuspended in RPMI 1640 containing 10% foetal calf serum (FCS), diluted to $2x10^5$ cells per ml and stored on ice until required. Glass coverslips 13mm in diameter were placed in sterile cluster trays and overlaid with one ml of leukocyte suspension.

3.2.4b Neutrophil cytotoxicity assay: After a 60 min incubation at 37°C to allow the PMN to adhere to coverslips, the monolayers were washed twice with PBS and then various concentrations of LPS or the protein/carbohydrate material purified from SAM were added. Dose and time response experiments were performed for each of the 4 microbial

extracts. After incubation the coverslips were washed with PBS and viability was tested by adding 0.1% trypan blue and counting, by phase contrast microscopy, the number of cells capable of excluding the dye. Assays were performed in triplicate and for each coverslip 200 PMN were counted. The number of cells taking up trypan blue gave the percentage of cells killed by the specific concentration of microbial extract after the appropriate incubation time.

3.3 Results

3.3.1 Inhibition of Fibroblast DNA Synthesis (Figure 8)

The SAM from the three pathogens inhibited the incorporation of ^{3}H-TdR into human gingival fibroblasts in a dose-dependent manner over the range 10ng/ml to 10µg/ml (Fig 8).

At 10 ng/ml the preparations exhibited very similar potencies, achieving a reduction in thymidine incorporation of between 17 and 18 %. At the highest concentration tested, 10 µg/ml, SAM from *P. gingivalis* was the most effective inhibitor of thymidine incorporation, achieving a 50 % reduction compared with control SAM-free cultures.

In contrast, LPS from *A. actinomycetemcomitans* had no significant effect on fibroblast proliferation even at concentrations as high as 10 µg/ml.

3.3.2 Inhibition of Keratinocyte DNA Synthesis (Figure 9)

Over the concentration range of 10ng/ml to 10ug/ml SAM from *A. actinomycetemcomitans, P. gingivalis* and *E. corrodens* inhibited thymidine uptake into cultured guinea pig keratinocytes in a dose-dependent manner. At 10

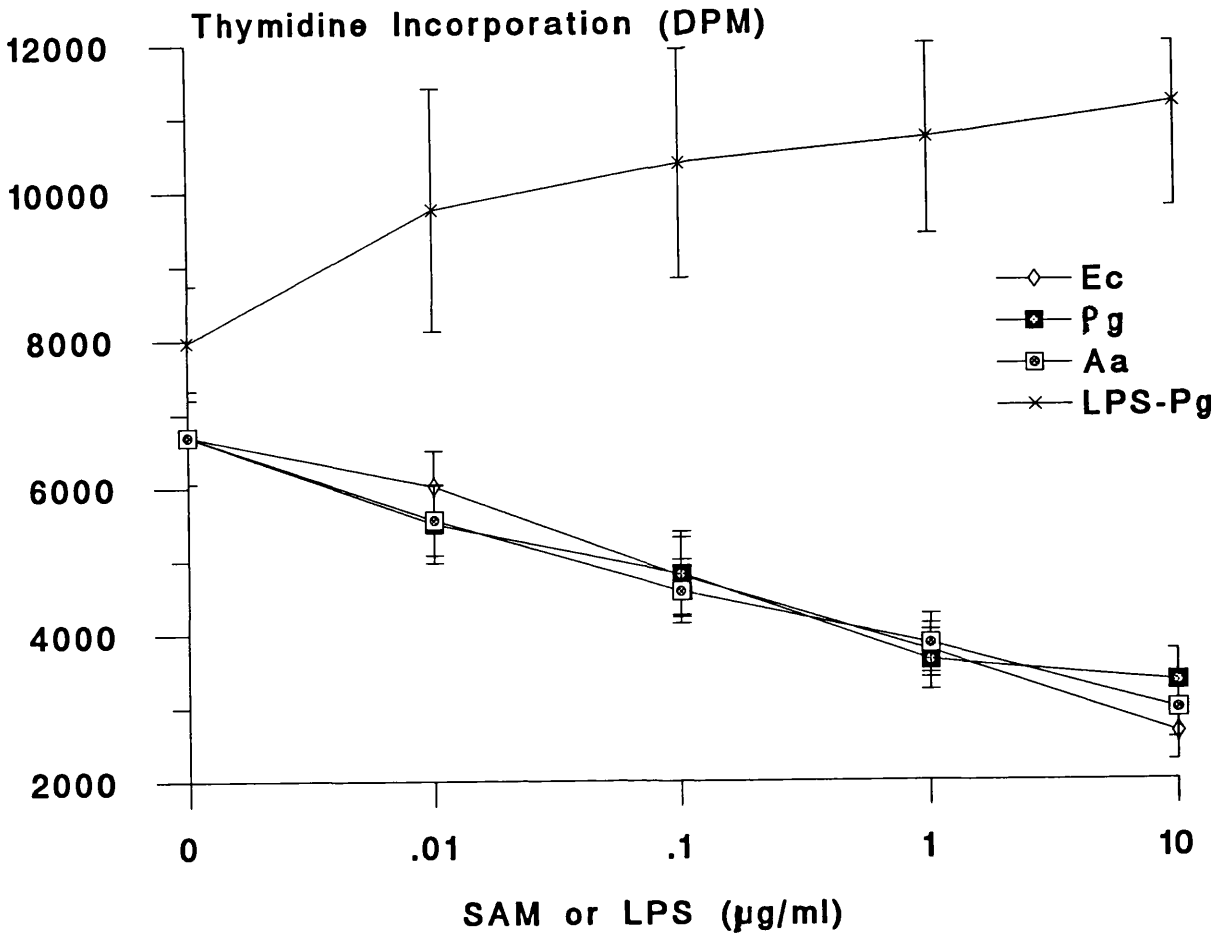


Figure 9: Inhibitory effect of increasing concentrations of SAM from *A. actinomycetemcomitans* (Aa; open square), *P. gingivalis* (Pg; closed square), *E. corrodens* (Ec; diamond) and LPS from *A. actinomycetemcomitans* (LPS-Aa; cross) on keratinocyte DNA synthesis, measured as incorporation of tritiated thymidine into DNA by keratinocytes. The results are expressed as mean and standard deviation of 6 cultures. The significance of the results has been calculated by Student's t-test. SAM from all three bacteria significantly inhibited thymidine incorporation at 10ng/ml ($p<0.01$), whereas LPS had slight stimulatory effect over the dose-range tested.

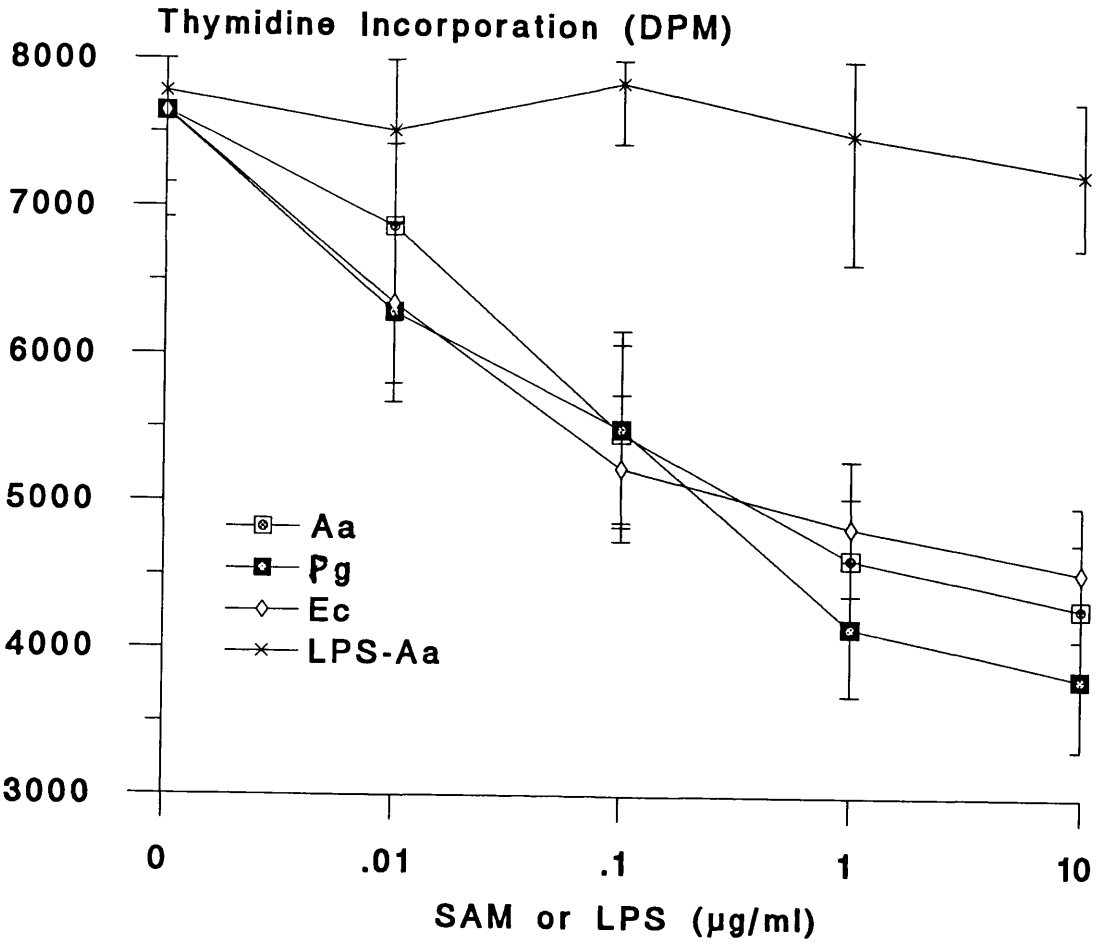


Figure 8: Inhibitory effect of increasing concentrations of SAM from *A. actinomycetemcomitans* (Aa; open square), *P. gingivalis* (Pg; closed square), *E. corrodens* (Ec; open diamond) and LPS from *P. gingivalis* (LPS-Pg; cross) on fibroblast DNA synthesis, measured as incorporation of tritiated thymidine into DNA by human gingival fibroblasts. The results are expressed as mean and standard deviation of 6 cultures. The significance of the results has been calculated by Student's t-test. SAM from all three bacteria significantly inhibited thymidine incorporation at 10ng/ml ($p<0.01$). Whereas LPS had no effect over the dose-range tested.

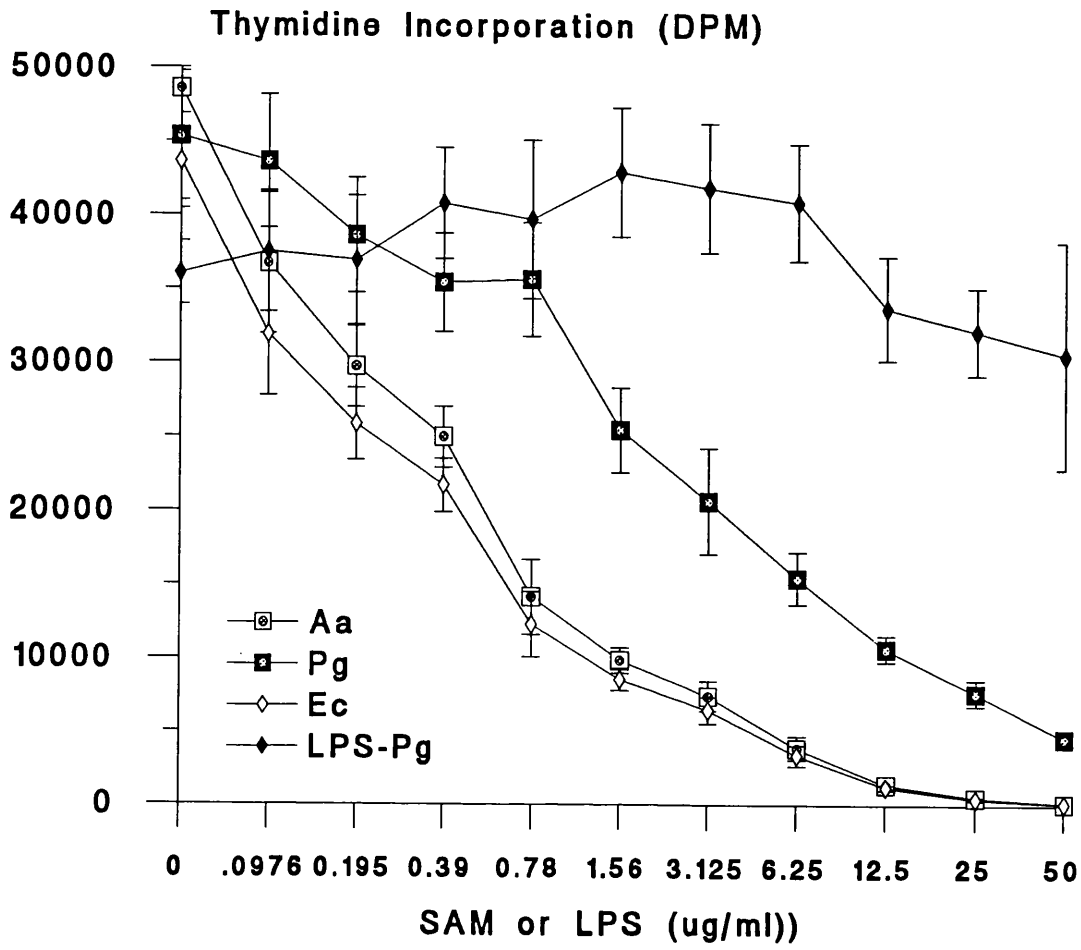


Figure 10: Inhibitory effect of increasing concentrations of SAM from *A. actinomycetemcomitans* (Aa; open square), *P. gingivalis* (Pg; closed square), *E. corrodens* (Ec; diamond) and LPS from *P. gingivalis* (LPS-Aa; cross) on macrophage DNA synthesis, measured as incorporation of tritiated thymidine into DNA by U937 cells. The results are expressed as mean and standard deviation of 6 cultures. The significance of the results has been calculated by Student's t-test. SAM from all three bacteria significantly inhibited thymidine incorporation at 10ng/ml ($p<0.01$). In contrast LPS form *P. gingivalis* failed to inhibit thymidine incorporation even as 50μg/ml.

ng/ml, SAM from both *A. actinomycetemcomitans* and *P. gingivalis* achieved a statistically significant degree of inhibition, amounting to 18 % in both cases. At higher concentrations, the inhibition achieved by all 3 preparations was significantly different from control cultures. In contrast, addition of LPS from *A. actinomycetemcomitans* or *P. gingivalis* to keratinocytes resulted in a dose-dependent stimulation of thymidine incorporation over the concentration range 0.01 - 10 µg/ml (Fig 9).

3.3.3 Inhibition of U937 DNA Synthesis (Figure 10)

SAM from the three pathogens was added to U937 cells over the dose range 100ng/ml to 50ug/ml. Again, all three SAM preparations had the capacity to inhibit thymidine incorporation by these cells (Fig. 10). The material from *A. actinomycetemcomitans* and *E. corrodens* was most active producing 50% inhibition at between 200-400ng/ml. The SAM from *P. gingivalis* was less active causing 50% inhibition at approximately 2-3ug/ml.

In contrast LPS from *P. gingivalis* failed to inhibit ^{3}H-TdR incorporation even at 50ug/ml (Fig 10).

3.3.4 PMN Cytotoxicity (Figures 12, 13)

Preliminary experiments with LPS indicated that maximum cytotoxicity could be achieved by 5.0 µg/ml and 50.0 µg/ml of LPS from *A. actinomycetemcomitans* and *P. gingivalis* respectively, whereas with the material isolated from the crude SAM preparations the respective concentrations were 0.6 ng/ml and 12.5 ng/ml. These concentrations were used to determine the time course of cytotoxicity for the four bacterial

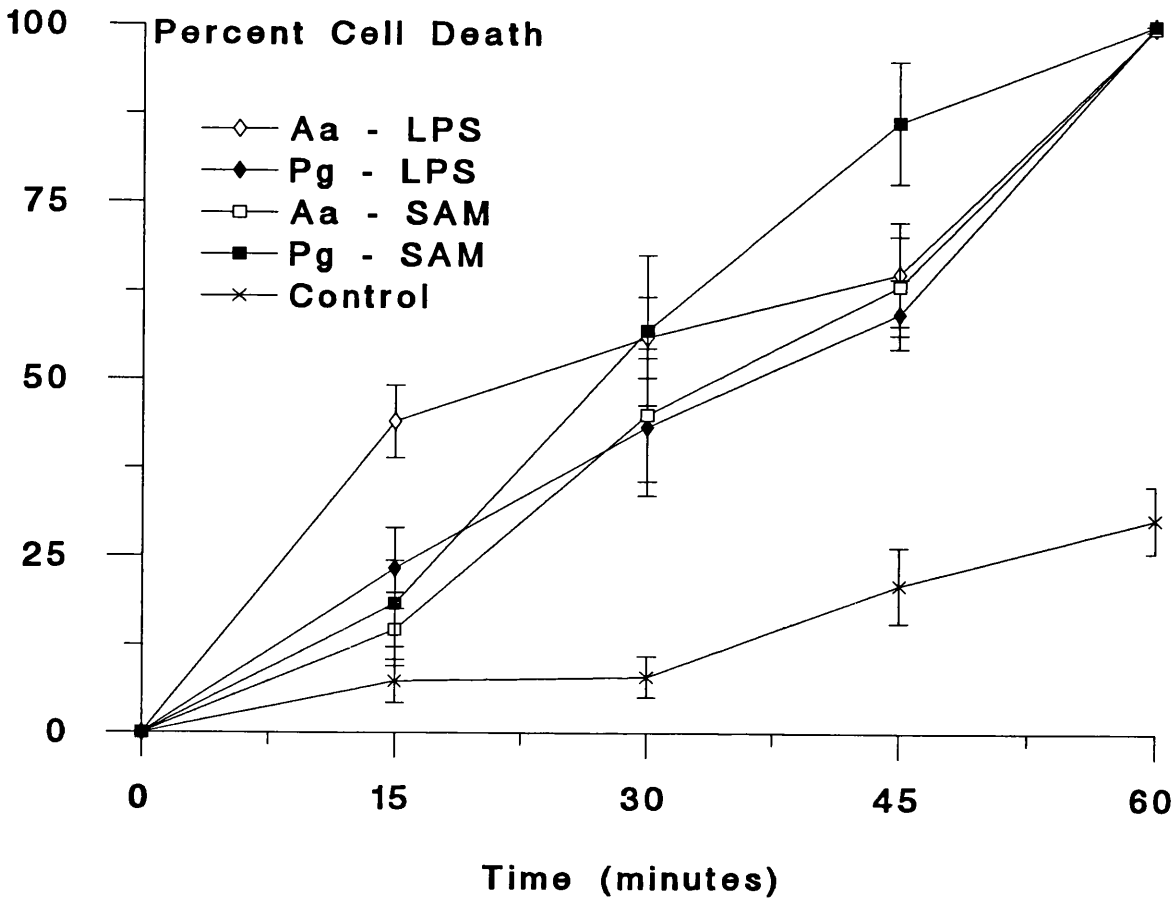


Figure 11: PMN cytotoxicity effects of SAM from *A. actinomycetemcomitans* (0.6ng/ml; Aa-SAM; open square) and *P. gingivalis* (12.5ng/ml; Pg-SAM; closed square) and LPS from *A. actinomycetemcomitans* (5μg/ml; Aa-LPS; open diamond) and *P. gingivalis* (50μg/ml; Pg-LPS; closed diamond) over 60 minutes.

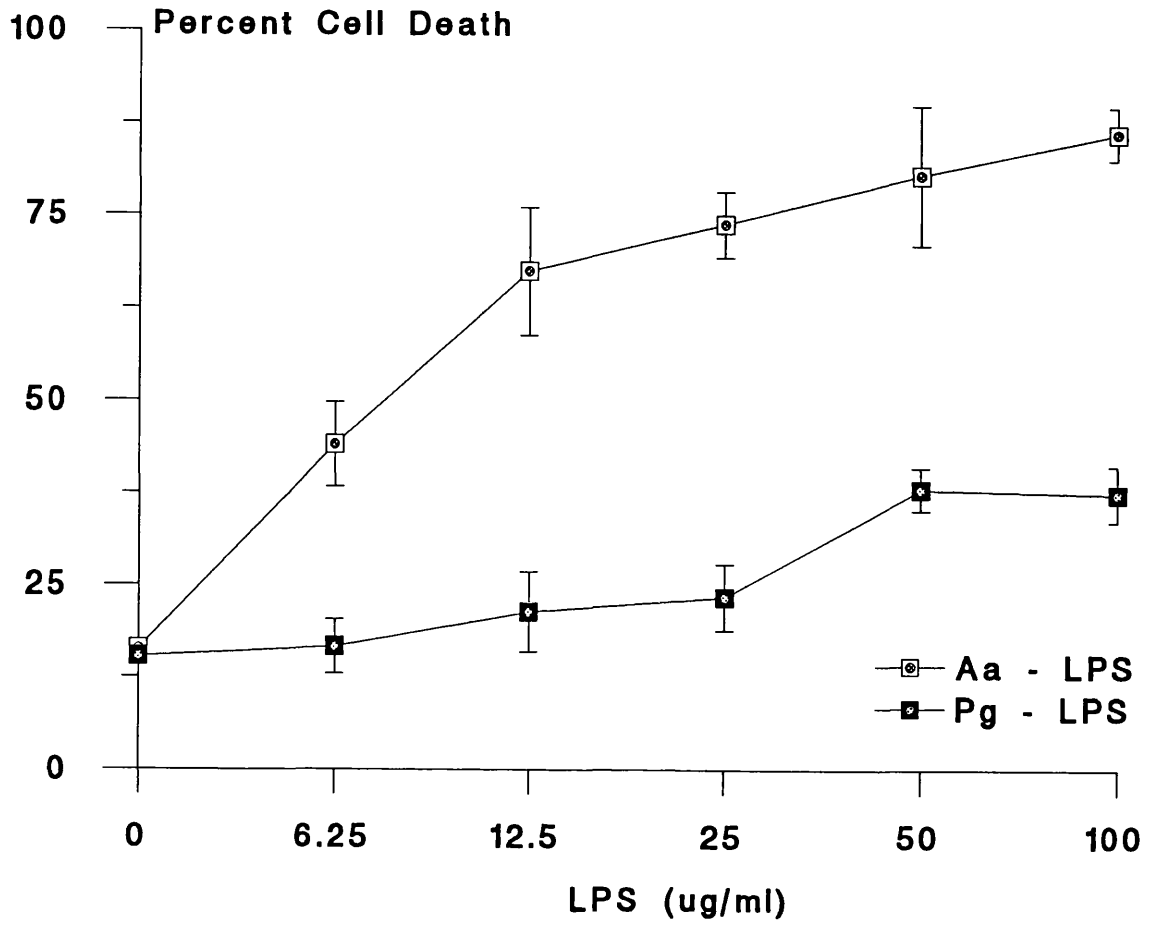


Figure 12: Increasing percent PMN cell death with increasing concentrations of LPS from *A. actinomycetemcomitans* (Aa-LPS; open square) and *P. gingivalis* (Pg-LPS; closed square) over 30 minutes. Results are expressed as the mean and standard deviation of 3 cultures.

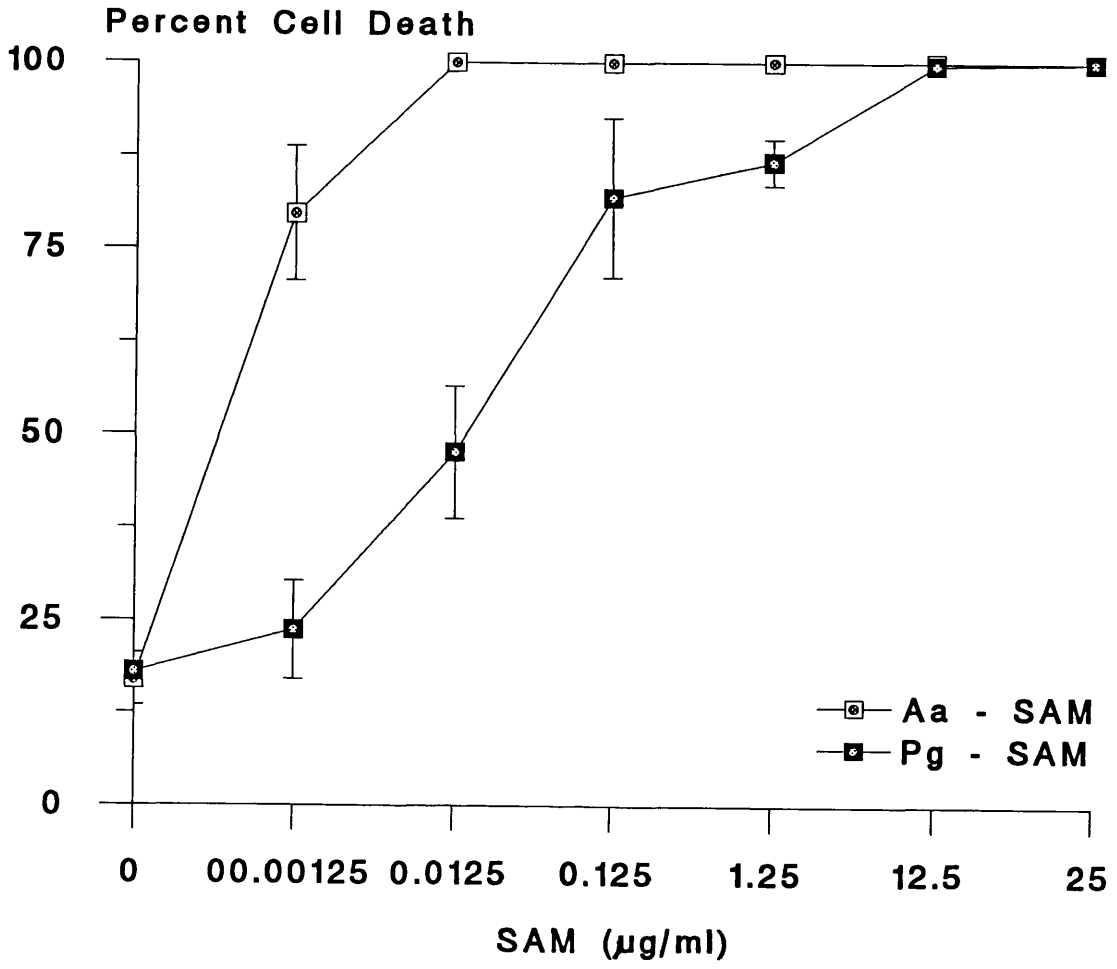


Figure 13: Increasing percent PMN cell death with increasing concentrations of SAM from *A. actinomycetemcomitans* (Aa-SAM; open square) and *P. gingivalis* (Pg-SAM; closed square) over 30 minutes. Results are expressed as the mean and standard deviation of 3 cultures. With maximum cytotoxicity at 0.6µg/ml for Aa-SAM and 12.5µg/ml for Pg-SAM.

extracts (Fig 11). At these concentrations all PMNs are killed in 60 min. The dose response for these four bacterial constituents was determined in assays lasting for 30 min. This showed that LPS from A. actinomycetemcomitans was much more active than that from P. gingivalis although even with 100μg/ml LPS from the former organism there was only 80% PMN cytotoxicity. In contrast, as little as 12.5ng/ml of the material isolated from the crude SAM from A. actinomycetemcomitans produced 100% cytotoxicity in 30 min with 50% killing at less than 1ng/ml. The material purified from the SAM of P. gingivalis produced 100% killing at 12.5ug/ml with 50% activity at 12.5ng/ml (Fig 12 and 13).

3.4 Discussion

Many of the bacterial species associated with periapical infection have been shown to be capable of exerting a cytotoxic effect on a number of mammalian cells (Boehringer, 1984; Stevens and Hammond, 1988; Fotos et al., 1990). The majority of such studies have been concerned with the effect of bacterial cell sonicates on human gingival fibroblasts. Hence, it has been shown that sonicates from *A. actinomycetemcomitans, Porphyromonas gingivalis, Fusobacterium nucleatum, Eikenella corrodens* (Stevens and Hammond, 1988) *Prevotella intermedia* (Fotos, 1990) and *Treponema denticola* (Boehringer, 1984) all exhibit some degree of toxicity towards fibroblasts. Comparatively few studies have been directed at evaluating the potential of oral pathogens to adversely affect growth of other mammalian cell types such as epithelial and endothelial cells, lymphocytes and monocytes. The exceptions to this generalisation are the many investigations concerning the leukotoxicity of *A. actinomycetemcomitans* (Zambon et al, 1988). Despite the demonstrable cytotoxicity of crude bacterial extracts reported in the above studies, few investigators have attempted to characterise the active constituents or utilised purified bacterial components in their assay systems. The few such studies that have been carried out have invariably involved the leukotoxin from *A. actinomycetemcomitans* or lipopolysaccharide from a number of species. Indeed, a review of the literature creates the impression that "cytotoxicity" is attributable solely to LPS of Gram-negative bacteria and, in the case of *A. actinomycetemcomitans*, a proteinaceous leukotoxin (Tsai

et al, 1984).

In this investigation saline extracts of three oral bacteria have been prepared; *A. actinomycetemcomitans, P. gingivalis* and *E. corrodens.* The bacteria have a number of surface-associated materials, including capsules and fibrils (Handley et al, 1991), and the procedure used was sufficient to remove such material. The extraction procedure was gentle, and electron microscopic examination of the bacteria revealed that, after extraction, the SAM had been almost totally removed but that the bacteria were intact (Figure 7). This was reinforced by the finding of very low levels of endotoxin in the SAM.

Previous studies have demonstrated that one of the components of SAM from *A. actinomycetemcomitans* was 1-2 log orders more active than the corresponding LPS in inhibiting human fibroblast thymidine incorporation (Kamin et al, 1986). Such inhibition of cell function may contribute to the pathology of periapical lesions by compromising repair processes. It was therefore of interest to determine if this anti-proliferative activity of SAM from *A. actinomycetemcomitans* was unique to this bacterium and to the fibroblast. It was also of interest to determine the relative activities of SAM and LPS from the same organism.

With regard to fibroblast cytotoxicity, the results of the present investigation have shown that SAM from *P. gingivalis, A. actinomycetemcomitans* and *E. corrodens* are potent inhibitors of fibroblast proliferation achieving statistically significant inhibition (approximately 17 % in each case) at concentrations as low as 10 ng/ml whereas LPS from

one of these organisms, *A. actinomycetemcomitans*, had little effect on fibroblast proliferation at concentrations as high as 10 μg/ml.

In the case of *A. actinomycetemcomitans*, several studies have indirectly implicated SAM as the component(s) responsible for fibroblast inhibition. For example, Shenker et al (1982) and Stevens et al (1983) demonstrated that the component responsible for fibroblast toxicity was neither endotoxin nor the leukotoxin. Furthermore, Stevens et al (1983) showed that the inhibitory activity was present in a phosphate buffer-extract of the organism implying that the factor responsible was a surface component.

P. gingivalis sonicates have been shown to be toxic to human gingival fibroblasts although the concentrations required to achieve a statistically significant effect were very high, amounting to between 10 and 50 ug/ml of protein (Larjava et al., 1987; Fotos et al. 1990). Once again, LPS was considered to be the active component responsible for fibroblast toxicity. However, the results of the present investigation imply that surface components other than LPS are the prime agents responsible for the fibroblast cytotoxicity of *P. gingivalis*.

There has been only one report of the toxicity of *Eikenella corrodens* towards fibroblasts (Stevens and Hammond, 1988). These investigators found that sonic extracts containing 50 μg/ml of protein inhibited thymidine incorporation by human gingival fibroblasts but only to the extent of approximately 10%. The component(s) responsible for the cytotoxic effect was not identified.

There are few reports concerning the *in vitro* effects of oral bacteria on

epithelial cells. Kamen (1981) found that sonic extracts of *P. gingivalis* inhibited thymidine uptake by human foreskin keratinocytes in a dose-related manner over the concentration range 0.25 - 25 µg/ml. However, a statistically significant degree of inhibition was obtained only at a concentration of 2.5 µg/ml of sonicate and this amounted to between 47 and 66 % depending on the time of exposure to the sonicate. Kamen (1983) also found that a sonic extract of *A. actinomycetemcomitans* inhibited keratinocyte proliferation. The keratinocyte-inhibitory factor present in these bacterial sonicates was not identified, but it was suggested that LPS may have been responsible (Kamen, 1983). The finding in the present study that SAM from *A. actinomycetemcomitans, E. corrodens* and *P. gingivalis* inhibited keratinocyte proliferation at concentrations as low as 10 ng/ml, while LPS from these organisms actually stimulated proliferation, would suggest that the inhibitory component of the bacterial sonicates may have been SAM rather than LPS.

With the exception of *A. actinomycetemcomitans*, few organisms have been investigated for their potential cytotoxicity towards monocytes. The toxic effects shown by *A. actinomycetemcomitans* towards these cells have been attributed to its leukotoxin (Tsai et al 1979) which Simpson et al. (1988) have shown to be effective against the monocytic cell line (U937) used in the present investigation at doses as low as 9 ng/ml. In the present study, although a leukotoxic strain (Y4) of *A. actinomycetemcomitans* was used, Gel electrophoresis of the SAM

showed that no protein-containing band with a molecular weight of the order of 115 KDa was present, implying the absence of the leukotoxin. SAM from both *P. gingivalis* and *E. corrodens* also exhibited appreciable toxicity towards the monocytic cell line used in the investigation, the SAM from *E. corrodens* being considerably more potent than that of *P. gingivalis* and very similar in potency to SAM from *A. actinomycetemcomitans*, achieving 27% inhibition at a concentration of approximately 100 ng/ml. The adverse effects of *A. actinomycetemcomitans* on PMN have been attributed to a 115 KDa outer membrane-associated leukotoxic protein (Tsai et al., 1984). In the present study, one of the constituents of SAM, a protein- and carbohydrate-containing material, from a "non-leukotoxic" strain of this organism (Ohta and Kato 1991) has been shown to be a potent leukocidal agent. At concentrations as low as 1.25 ng/ml this material killed 80 % of PMN in a suspension containing $2x10^5$ cells/ml. Gel electrophoresis of this material revealed that no protein-containing band with a molecular weight corresponding to the leukotoxin (115 KDa) was present (Kamin, 1986). Material purified from the SAM of *P. gingivalis*, although exhibiting a considerable degree of cytotoxicity, was far less potent, requiring a concentration 100-fold greater to achieve a comparable degree of killing. In the case of both organisms, the material purified from the SAM had a potency at least 3 log orders greater than LPS from the corresponding organism.

These studies have shown that the easily-solubilized surface components of three bacteria have the capacity to inhibit thymidine incorporation by a

number of mammalian cells and have potent cytotoxic activity against human PMN. The corresponding LPS from each bacterium was either inactive or several orders of magnitude less active. Thus anti-proliferative activity could contribute to the loss of tissue. The consequence of inhibiting monocyte cell division is unclear but it would be of interest to determine if the SAM can inhibit monocyte maturation into macrophages. It must be emphasized that the influence of the SAM on keratinocytes and monocytes has used continuous cell lines. While these are commonly used in biological research as convenient sources of relevant cells their response to stimuli may be qualitatively or quantitatively different from that of primary cultures. This has to be borne in mind when interpreting these results. However, it was clear that with both cell lines there was a definite response to the SAM but that LPS was relatively inactive. The lethal effect of SAM, particularly from *A. actinomycetemcomitans,* on PMN may have a profound role in disease pathology. The main function of these cells will be removal of living and dead bacteria through opsonisation and phagocytosis, therefore disruption of this activity could have profound inflammatory effects.

CHAPTER 4

Inhibition of Bone DNA and Collagen production by Surface-Associated Material.

4.1 Introduction

Bacterial infection of the dental pulp usually results in the formation of a dental periapical lesion such as an abscess and/or granuloma with the concomitant breakdown of bone (Griffee et al., 1980; Yoshida et al., 1987). Acute inflammation and the destruction of this bone can occasionally lead to osteomyelitis. At present there are no clinical therapies which will inhibit the destruction of the extracellular matrix of the alveolar bone other than the elimination of the infection.

The bacteria implicated in periapical disease include oral bacteria such as *Porphyromonas gingivalis, Eikenella corrodens* and *Wolinella recta* . With regard to the bone loss associated with these lesions, most attention has been focused on the mechanisms of bone destruction and the role played in this by lipopolysaccharide (LPS) (Schonfeld et al., 1982; Yamasaki et al., 1992). However, it is possible this bone loss is the result of the inhibition of bone formation or of a combination of increased breakdown and decreased synthesis.

Many oral bacteria have capsules and/or other surface components (Progulski and Holt, 1980; Yamamoto et al., 1982 ; Holt et al. 1980). It has been previously shown that solubilized capsular components from *Actinobacillus actinomycetemcomitans*, when added to cultured murine calvaria, inhibited both DNA and collagen synthesis (Wilson, Meghji and Harvey, 1988). The bone cell population responsive to the capsular

material is believed to be the osteoblast, but this has not been formally demonstrated. It is not known if this inhibitory activity is unique to the capsular components of this organism or is a general action of the outer, easily solubilized, components of such bacteria. Therefore, in this chapter solubilized surface-associated material (SAM) from three oral bacteria have been examined for their ability to inhibit DNA and collagen synthesis in cultured murine calvaria. Furthermore, the mechanism of inhibition has been explored by use of the cyclo-oxygenase inhibitor - indomethacin. In order to assess the effects of these bacterial components on their potential target cells, osteoblasts, these cells have been isolated from murine calvaria and exposed to graded concentrations of SAM.

4.2 Methods and Materials

4.2.1 Collagen Synthesis by Calvaria

Calvaria from 5 day-old mice were halved along the sagittal suture and incubated in groups of 6 for 18h in 50mm culture dishes containing BGJ medium (Gibco) supplemented with 50μg/ml ascorbic acid (Sigma), 2mM glutamine (Flow) 5% heat-inactivated rabbit serum (Wellcome Foundation) and 50μg/ml beta-aminopropionitrile fumarate (Sigma) to inhibit collagen cross-linking. ^{3}H-proline (specific activity 31.5 Ci/mmol: Amersham) was added at 2μCi/ml for the last 6h of a 24h incubation period with various concentrations of SAM.

The incorporation of tritiated proline into collagen was measured by a pepsin extraction method modified by Meghji et al., (1992b). Briefly,

collagen was extracted from each halved calvaria by limited pepsin digestion (0.5mg/ml pepsin in 0.5M acetic acid for 16h at 4°C). Insoluble debris was removed by centrifugation and the collagen was precipitated from solution by the addition of 5% (w/v) NaCl. The precipitate was centrifuged, redissolved in 0.5M acetic acid and then reprecipitated with 5% NaCl. The final collagen pellet was dissolved in 0.5M acetic acid, mixed with 3ml scintillant (Unisolve 1: Koch Light) and the radioactivity measured by scintillation spectrometry on an LKB Rackbeta scintillation counter with external standardization.

4.2.2. DNA Synthesis by Calvaria

DNA synthesis was measured by adding tritiated thymidine (specific activity 20Ci/mmol: Amersham) at 0.5μCi/ml to halved calvaria (in groups of 6 prepared as above) for the last 6h of a 24h culture period in the presence of SAM. The calvaria were individually extracted with 5% trichloroacetic acid (TCA) to remove free isotope, and then dissolved in 150μl hyamine hydrochloride for 2h at 60°C and acidified with 1M HCl prior to addition of scintillant. Radioactivity was measured as described above.

4.2.3 Histology of Calvaria

Calvaria from unstimulated or SAM-stimulated cultures were fixed in glutaraldehyde, embedded in Epon and sectioned on a Reichert Jung microtome at 1μm. Sections were stained with toluidine blue and

examined by light microscopy. With some specimens ultrathin specimens were prepared and examined by electron microscopy using a JEOL 100CXII microscope.

4.2.4 Actions of Indomethacin

To assess the role of prostanoids in the inhibition of collagen and DNA synthesis indomethacin was added at 1μM to control osteoblast cultures or to cultures exposed to various concentration of SAM.

4.2.5 Preparation of Murine Osteoblasts

Osteoblasts were released from calvaria of 5-day old mice using a sequential enzyme digestion method as described in Chapter 2. The purity of the final osteoblast preparation was ascertained by a number of criteria. Cell morphology was determined by phase contrast microscopy and alkaline phosphatase positivity was determined by a standard histochemical method using alpha-naphthyl phosphate. The ability of cells to accumulate cyclic AMP (cAMP) was also assessed. Primary cultures of osteoblasts at 10^5cells/30mm dish were exposed to $5x10^{-9}$M parathyroid hormone (PTH) for 5 min. cAMP was extracted by extraction in ethanol-HCl at -20°C overnight and measured by commercial radioimmunoassay (Amersham).

4.2.6 Collagen Synthesis by Isolated Osteoblasts

Trypsinised osteoblast suspensions were seeded into 96-well culture plates (Microtitre, Linbro) at concentrations of 15,000 cells/well in 100 μl of MEM

with 10% FCS and left overnight to attach. The culture media were then replaced with fresh MEM supplemented with 10% FCS and containing various concentrations of SAM, in groups of 6 wells per concentration. Control cultures received only unsupplemented medium. The cells were incubated with 0.5μCi of ^{3}H-proline for a further period of 24h.

The incorporation of tritiated proline into collagen was measured by the pepsin extraction method of Webster and Harvey (1979). Briefly, culture media were placed in LP3 tubes with 100μl of 1mg/ml pepsin in 1M acetic acid. The tubes were incubated for 16h at 4°C. At the end of the incubation period 100 μl of rat acid-soluble collagen (RASC) was added as a carrier and the volume in each tube was made up to 1ml with 0.5M acetic acid. Collagen was precipitated from solution by the addition of 5% (w/v) NaCl and incubation for 2h at 4°C. The precipitate was centrifuged, redissolved in 0.5M acetic acid and then reprecipitated with 5% NaCl. The final collagen pellet was dissolved in 0.5M acetic acid, mixed with 3ml scintillant (Unisolve 1: Koch Light) and the radioactivity measured by scintillation spectrometry on an LKB Rackbeta scintillation counter with external standardization.

4.2.7 DNA Synthesis by Isolated Osteoblasts

Osteoblast suspensions were seeded into 96-well culture plates (Microtitre, Linbro) at 15,000 cells per well in 100 μl of MEM with 10% FCS. These were incubated overnight to allow the cells to attach. The culture media were then replaced by MEM with 2% FCS containing various dilutions of

SAM in groups of 6 wells per dilution. Control cultures received only unsupplemented medium. The culture plates were incubated for 18h, and then 0.5μCi of 5-^{3}H-thymidine were added to each well and the incubation continued for a further 6 h. Trichloroacetic acid-insoluble material in each cell layer was dissolved in 0.5M NaOH and radioactivity measured in 3ml of scintillant (Unisolve 1: Koch Light) by scintillation spectrometry on an LKB Rackbeta scintillation counter with external standardization.

4.2.8 Cell Viability

The cytotoxic activity of bacterial SAM on isolated osteoblasts was assessed by measurement of lactate dehydrogenase release into the media by a commercial assay (Sigma).

4.2.9 Statistics

The significance of results was determined by the Student's t-test.

4.3 Results

4.3.1 Endotoxin Content of SAM

The endotoxin content of SAM preparations was measured by the chromogenic *Limulus* assay. Solutions of SAM at a concentration of 1mg/ml were assayed to determine the endotoxin content. The amount of endotoxin in these SAM preparations was in the range 1-10ng/ml.

4.3.2 Effect of SAM on Calvarial DNA and Collagen Synthesis (Fig. 14, 15)

Lyophilized SAM was dissolved in medium and added to calvarial cultures at concentrations ranging from 10ng/ml to 10μg/ml (w/v). The SAM from all three bacteria had a dose-dependent inhibitory effect both on DNA synthesis (Fig. 14) and collagen synthesis (Fig. 15). SAM from *P. gingivalis* and *E. corrodens* were most potent showing significant inhibition of both DNA and collagen synthesis at 10ng/ml. In the case of SAM from *P. gingivalis* both DNA and collagen synthesis were inhibited by 18 % at a concentration of 10 ng/ml. At a concentration of 100 ng/ml, SAM from all three bacteria caused significant inhibition of collagen and DNA synthesis.

4.3.3 Histology of Calvaria

Calvaria were examined by both light and electron microscopy. In control calvaria the bone was intact with a surrounding periosteal lining. In SAM-stimulated cultures there was clear evidence of osteolysis with the appearance of osteoclasts in areas of resorption. Detailed examination of

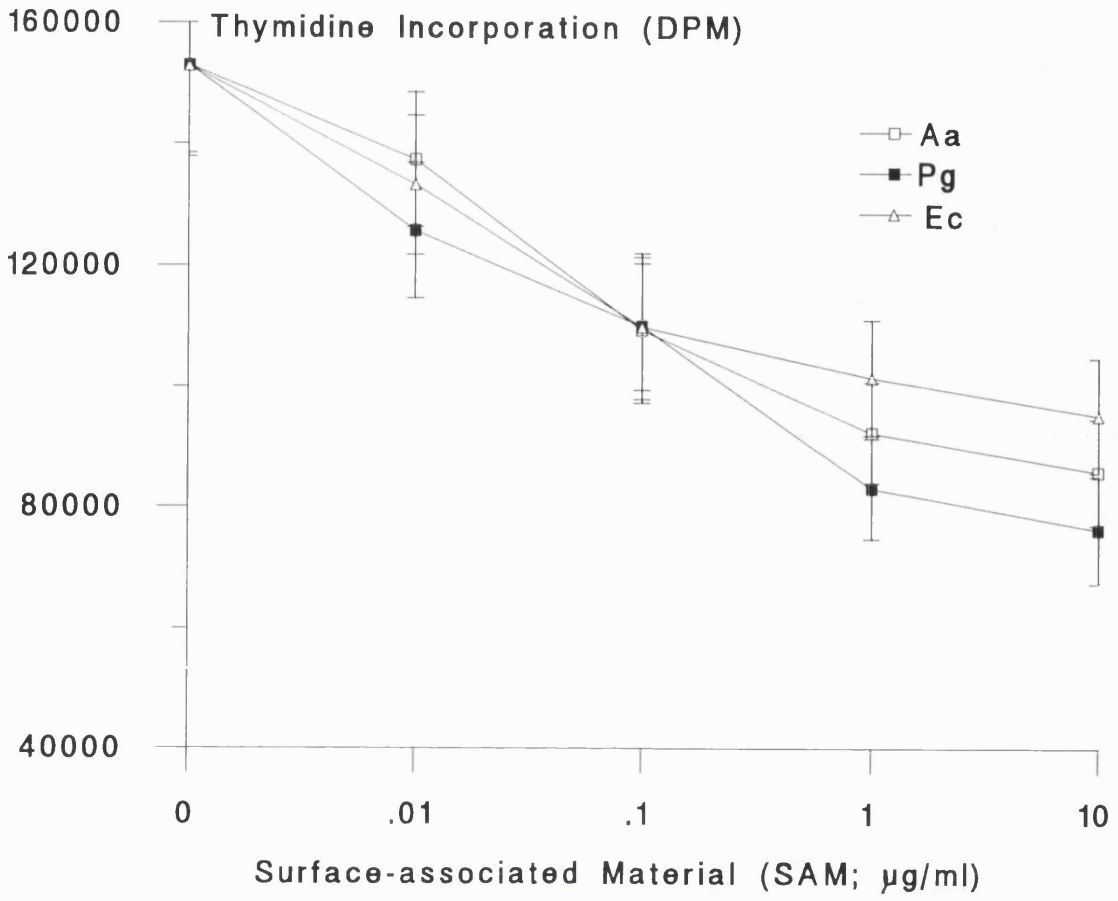


Figure 14. Inhibitory effect of increasing concentrations of SAM from *A. actinomycetemcomitans (Aa; open square), P. gingivalis (Pg; closed square) or E. corrodens (Ec; triangle)* on calvaria DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by murine calvaria. Results are expressed as the mean and standard deviation of 6 cultures. The significance of the results has been calculated by Student's t-test. Both SAM from *P. gingivalis* ($p<0.01$) and *E. corrodens* ($p<0.05$) significantly inhibited thymidine incorporation at 10ng/ml. At a concentration of 100ng/ml all SAM preparations significantly inhibited thymidine incorporation ($p<0.001$).

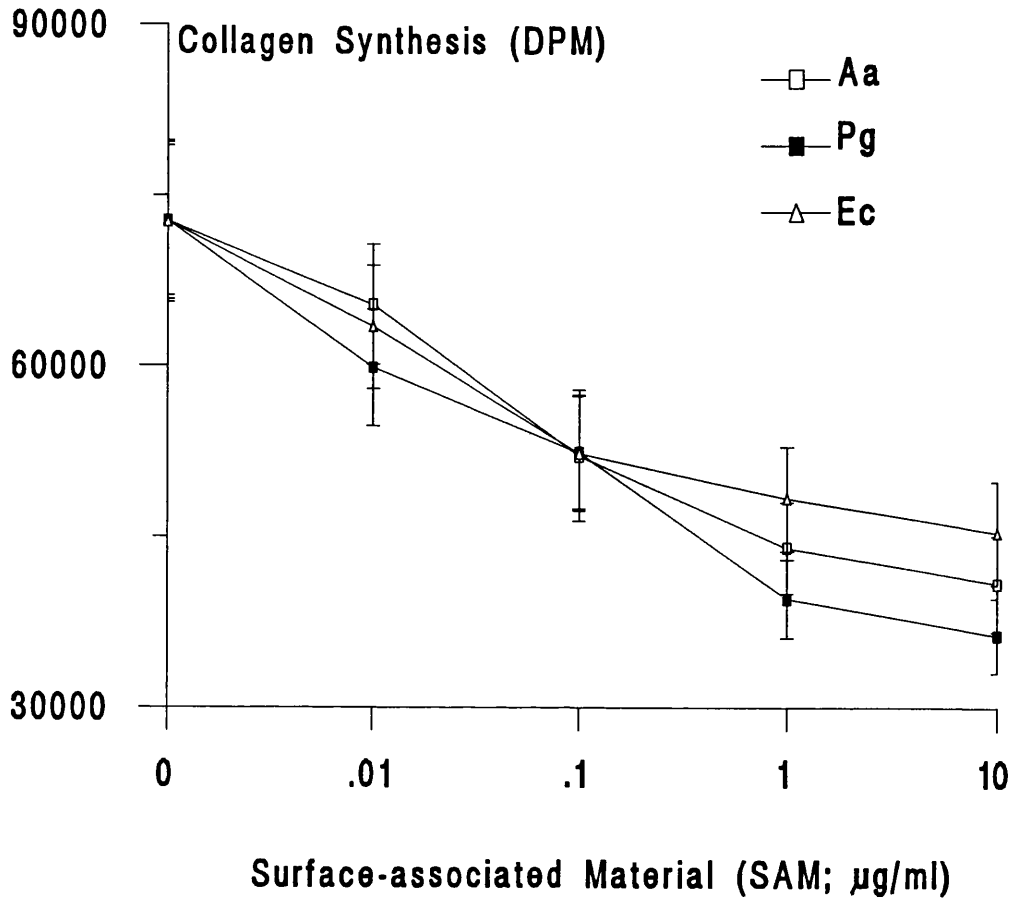


Figure 15. Inhibitory effect of increasing concentrations of SAM from *A. actinomycetemcomitans (Aa; open square), P. gingivalis (Pg; closed square) or E. corrodens (Ec; triangle)* on radiolabelled proline incorporation into collagen of cultured murine calvaria. Results are expressed as mean and standard deviation of 6 replicate cultures. Both SAM from *P. gingivalis* ($p<0.01$) and *E. corrodens* ($p<0.05$) significantly inhibited tritiated proline incorporation. At a concentration of 100ng/ml all SAM preparations significantly inhibited proline incorporation ($p<0.001$).

sections exposed to the various SAMs failed to detect evidence of cytotoxicity due to these bacterial constituents.

4.3.4. Role of Prostanoids in the Action of SAM (Table 1)

To ascertain whether products of cyclo-oxygenase activity were contributing to the inhibition of calvarial DNA and collagen synthesis induced by SAM, indomethacin (1μM) was added to cultures containing 100 ng/ml of each SAM. Indomethacin had no stimulatory effect on collagen synthesis in control calvaria but almost completely blocked the inhibitory effect on DNA and collagen synthesis of SAM from *P. gingivalis* (Table 1). In the case of SAM from *A. actinomycetemcomitans*, indomethacin restored DNA and collagen synthesis to levels that were not statistically significantly different from those in control cultures. The inhibitory effect of SAM from *E. corrodens* on DNA and collagen synthesis was only partially blocked by indomethacin.

4.3.5 Effect of SAM on DNA and Collagen Synthesis by Cultured Osteoblasts (Figures 16, 17)

Cells isolated by sequential enzyme digestion from murine calvaria and identified as osteoblasts on the basis of morphology, alkaline phosphatase staining and their response to PTH were used in these studies.

Isolated osteoblasts were cultured in the presence of graded concentrations of the three bacterial SAM preparations. All three SAM preparations inhibited DNA and collagen synthesis in a dose-dependent fashion (Figs 16

and 17 respectively). As with whole calvaria, the SAM from P. gingivalis significantly inhibited collagen and DNA synthesis at 10ng/ml to the extent of 31.5 % and 31.8 % respectively. SAM from E. corrodens significantly inhibited DNA synthesis but not collagen synthesis at 10ng/ml, whereas SAM from A. actinomycetemcomitans did not significantly inhibit either collagen or DNA synthesis at this concentration. At 100 ng/ml SAM from all three bacteria significantly inhibited DNA and collagen synthesis.

Measurement of lactate dehydrogenase release from osteoblasts exposed to various concentrations of SAMs failed to demonstrate any significant degree of cytotoxicity.

Table 1

Blocking Effects of 1μM Indomethacin on the Inhibition of DNA and Collagen
Synthesis by Murine Calvarial Explants

Treatment	p	DNA synthesis DPM X10^4	p	Collagen Synthesis DPM X10^4
Control		1.33+/-0.15		7.32+/-0.84
Aa		0.95+/-0.11		5.23+/-0.60
Aa+Indo	0.01	1.17+/-0.13	0.01	6.44+/-0.74
Pg		0.96+/-0.11		5.30+/-0.61
Pg+Indo	0.001	1.3+/-0.15	0.002	7.16+/-0.82
Ec		0.96+/-0.11		5.33+/-0.60
Ec+Indo	0.049	1.11+/-0.13	0.068	6.15+/-0.70

Aa - Actinobacillus actinomycetemcomitans
Pg - Porphyromonas gingivalis
Ec - Eikenella corrodens
Indo - indomethacin

The p value givesthe statistical significance between the activity in the presence of 100ng bacterial SAM and with the bacterial SAM plus 1uM indomethacin

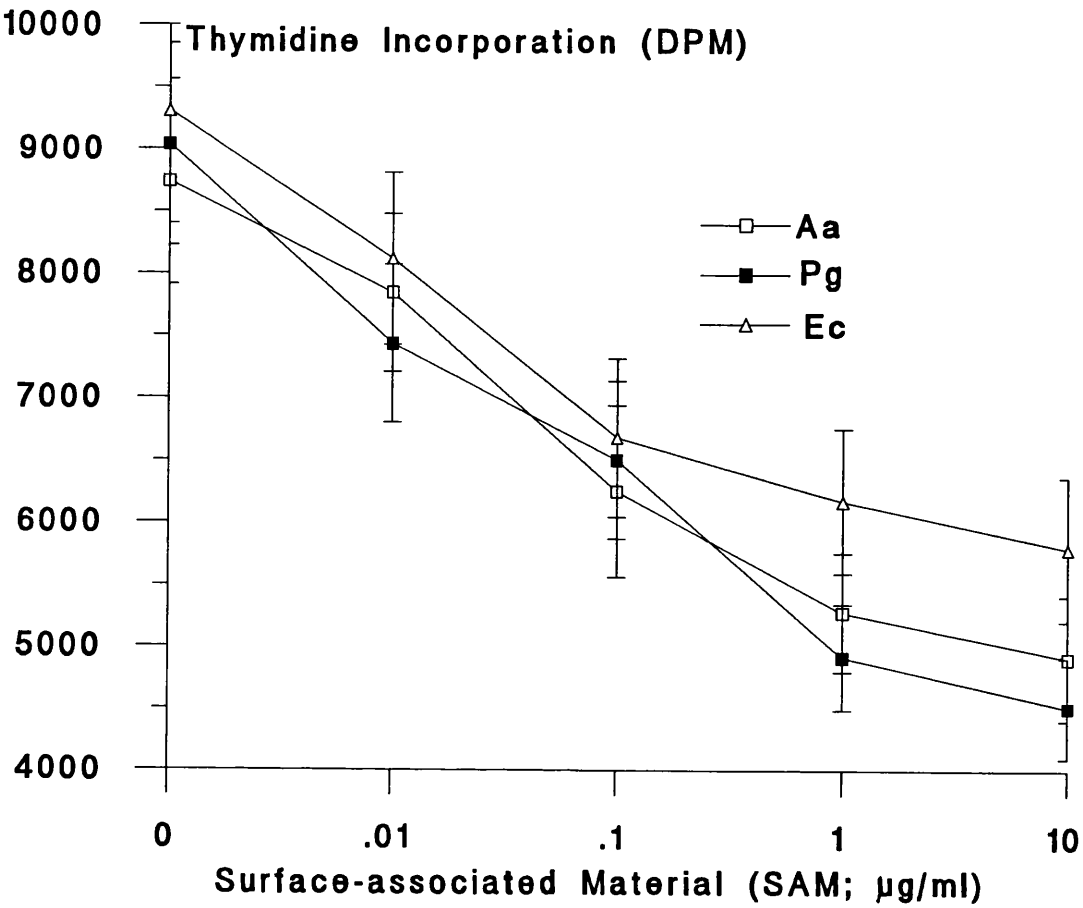


Figure 16: Inhibitory effect of increasing concentrations of SAM from *A. actinomycetemcomitans (Aa; open square), P. gingivalis (Pg; closed square) or E. corrodens (Ec; triangle)* on DNA synthesis (measured by tritiated thymidine incorporation) by isolated murine calvarial osteoblasts. Results are expressed as the mean and standard deviation of 6 replicate cultures. SAM from *P. gingivalis* and *E. corrodens* significantly ($p<0.05$) inhibited thymidine incorporation at 10ng/ml. At 100ng/ml all SAM preparations significantly inhibited thymidine incorporation ($p<0.001$).

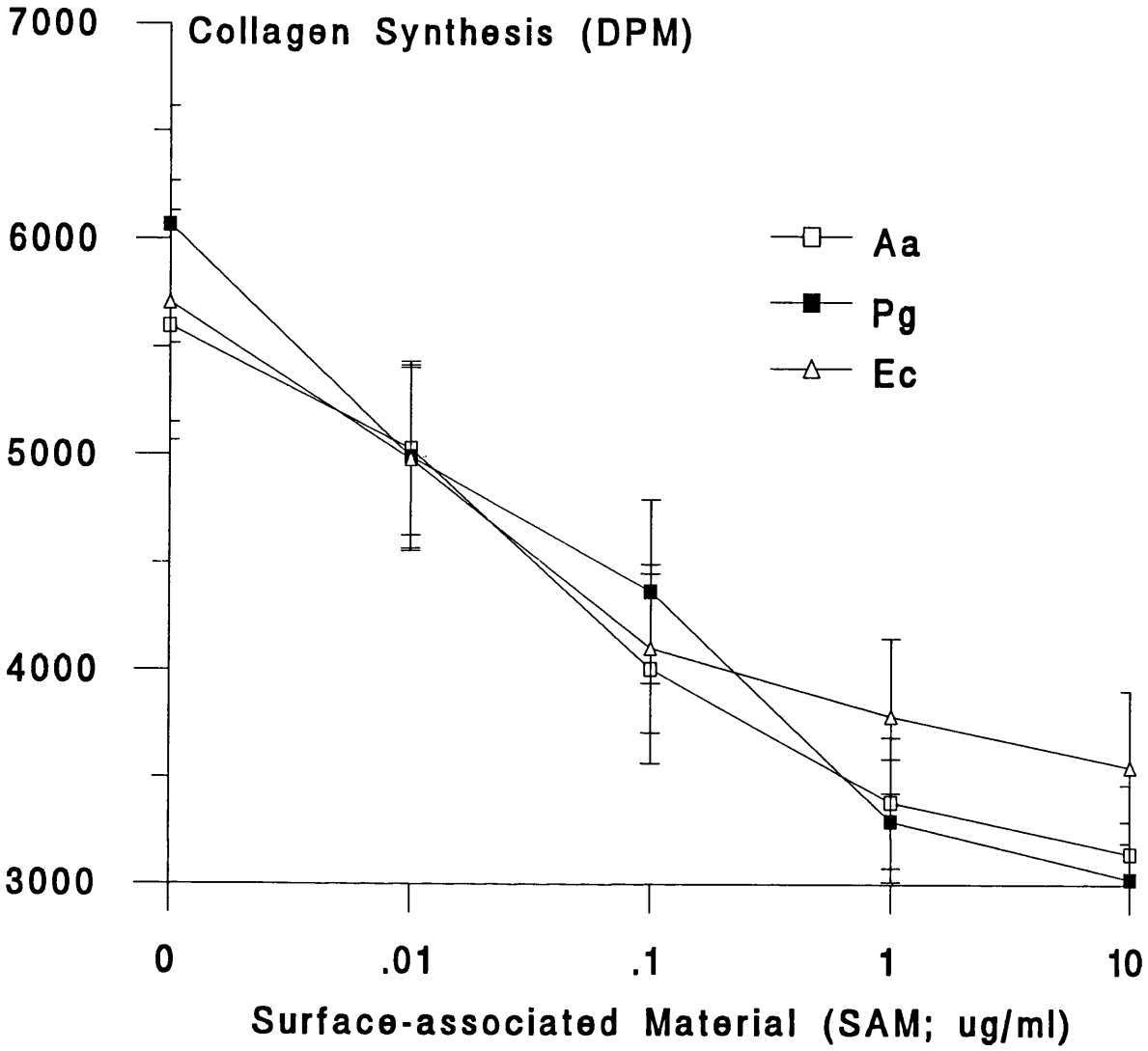


Figure 17. Inhibitory effect of SAM from *A. actinomycetemcomitans (Aa; open square), P. gingivalis (Pg; closed square) or E. corrodens (Ec; triangle)* on incorporation of tritiated proline into collagen by isolated murine calvarial osteoblasts. Results are expressed as mean and standard deviation of 6 replicate cultures. SAM from *P. gingivalis* significantly ($p<0.01$) inhibited proline incorporation at 10ng/ml. At 100ng/ml all SAM preparations significantly inhibited proline incorporation ($p<0.001$).

4.4 Discussion

A number of major human diseases demonstrate localized or systemic progressive loss of bone matrix. Amongst such diseases are: periodontal disease, periapical lesions, odontogenic cysts and rheumatoid arthritis. Research into the mechanisms of bone matrix loss has invariably concentrated on bone destruction. However, as bone homeostasis is a balance between formation and removal of matrix, a decrease in bone synthesis without any change in the rate of destruction would also result in a reduction in bone mass.

In both the slow growing granulomas to acute alveolar abscesses there is loss of the periapical and alveolar bone. Although bacteria are the etiological agents of periapical lesions (Kakehashi, Stanley and Fitzgerald; 1965), the mechanism of this bone loss has not been studied in detail and the intermediate pathway(s) linking infection and mineralised tissue destruction are poorly understood. It has been previously demonstrated that capsular material (a constituent of SAM) eluted from the *Actinobacillus actinomycetemcomitans* inhibited both DNA and collagen synthesis when added to cultured neonatal murine calvaria (Wilson, Meghji and Harvey, 1988). The capsular material used in this study was removed from *A. actinomycetemcomitans* by gentle stirring in saline. This ready solubility of the surface material of this bacterium coupled with its actions on isolated calvaria suggested a possible pathogenic mechanism to explain bone loss associated with bacterial infection. A number of bacteria implicated in the pathology of periapical lesions also have capsules and/or

other surface components (Progulski and Holt, 1980; Yamamoto et al., 1982; Holt et al., 1980). Since it was not known whether the action of the capsular material from *A. actinomycetemcomitans* was unique or was a common property of the exopolymers of these oral bacteria, this study compares the activity of SAM from three species implicated in the pathology of pulpitis: *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* and *Eikenella corrodens*. Figure 7 (page 44) shows that the mild extraction procedure used to obtain the SAM removes ruthenium red-staining material without appreciable lysis of the bacterial cells.

SAM from all three bacteria inhibited, in a dose-dependent manner, both DNA and collagen synthesis when added to cultured calvaria. The most active SAMs in this assay were those from *P. gingivalis* and *E. corrodens* which significantly inhibited collagen and DNA synthesis at a concentration of 10ng/ml. However, all three SAM preparations gave similar dose responses. The activity of SAM from *A. actinomycetemcomitans* was similar to that reported for a purified constituent of SAM (capsular material) in a previous study (Wilson, Kamin and Harvey; 1985). The manner in which the SAM inhibits calvarial collagen and DNA synthesis is not clear. It had been assumed that the target cells in the calvaria were the osteoblasts although this had not been formally demonstrated. This study shows that isolated murine osteoblasts when exposed to graded concentrations of SAM show a dose-dependent inhibition of both collagen and DNA synthesis. This demonstrates that the osteoblast can be directly

acted upon by the SAM but does not rule out the effect of these bacterial constituents on other bone cell populations such as periosteal fibroblasts or osteocytes. Indeed it has been previously shown that SAM from *Actinobacillus actinomycetemcomitans* can inhibit the proliferation (tritiated thymidine incorporation) and collagen synthesis by cultured gingival fibroblasts (Kamin et al., 1986). In this study SAM and LPS from *Actinobacillus actinomycetemcomitans* were compared. While the SAM inhibited both collagen and DNA production the LPS only inhibited tritiated thymidine incorporation. The relationship between the inhibition of DNA and collagen production in calvaria and osteoblasts cultures is not clear. The possibility that it is due to a cytotoxic effect is ruled out by the failure to demonstrate significant release of lactate dehydrogenase in SAM-stimulated osteoblasts or dead cells in stimulated calvaria. The ability of indomethacin to reverse the inhibition also rules out a cytotoxic mechanism. The possibility exists that the inhibition of DNA synthesis may contribute to the decreased collagen synthesis. However, as described above, in LPS stimulated fibroblasts it was possible to demonstrate a decreased tritiated thymidine incorporation with no alteration in collagen production (Kamin et al., 1986). Thus it is likely that the inhibition of DNA synthesis and collagen synthesis are independent phenomena.

There are few reports concerning the effects on osteoblasts of components from oral bacteria. Sismey-Durrant et al. (1987) found that LPS from *P. gingivalis* stimulated collagenase release from mouse osteoblasts in a dose-related manner over the concentration range 0.1 - 10

μg/ml but had no effect on the secretion of tissue inhibitor of metalloproteinase (TIMP). Furthermore, LPS from the same organism was found to induce secretion of plasminogen activator over the same range of concentrations (Hopps and Sismey-Durrant; 1991). In contrast, LPS from *P. gingivalis* was unable to activate osteoclasts (as assayed by release of tartrate-resistant acid phosphatase) nor could it induce bone resorption by osteoclasts in the absence of osteoblasts. These findings suggest that osteoblasts rather than osteoclasts are the target cell for LPS and, although in this study the effect of SAM on osteoclasts was not investigated the results show that the osteoblast is the target cell for another bacterial component, SAM.

Addition of indomethacin to calvarial cultures exposed to SAM was found to block the inhibitory effect of SAM from *P. gingivalis* and *A. actinomycetemcomitans* and partially block the effect of SAM from *E. corrodens*. These results imply that PGE_2 is responsible for mediating the inhibitory effects of SAM on DNA and collagen synthesis by calvaria although, in the case of SAM from *E. corrodens*, additional mediators may be involved.

Although many investigators have reported on the effects of plaque bacteria and bacterial components on bone resorption *in vitro*, there are comparatively few reports of their effects on bone formation. Larjava (1983) and Multanen et al. (1985) reported that plaque extracts containing respectively 50 μg/ml and 1.0 μg/ml of protein significantly inhibited collagen synthesis in rat calvaria. Although the plaque concentrations were

not specified, these can be estimated to be approximately 500 μg/ml and 10 μg/ml respectively based on a plaque protein content of approximately 10 % (Bowen, 1976). However, in neither case was the active constituent of the plaque identified. With regard to the effect of isolated bacterial components on bone formation, Norton et al. (1970) found that LPS from *Escherichia coli* was effective at inhibiting rat bone growth *in vitro* only at very high concentrations (80 μg/ml). Meikle et al. (1982) also reported that a high concentrations (100 μg/ml) of streptococcal cell walls was required to inhibit ^{3}H-proline incorporation into protein in rabbit calvaria by 20 %. Components from these bacteria, however, appear to be far more potent in inhibiting bone formation. For example, LPS from *A. actinomycetemcomitans* significantly inhibits DNA and collagen synthesis in mouse calvaria at concentrations of 0.1 and 1.0 μg/ml respectively (Wilson, Meghji and Harvey; 1986). In the presence of 1.0 μg/ml of LPS, DNA and collagen synthesis were inhibited by 40 % and 30 % respectively. However, this is still significantly less potent than capsular material from the same organism which has been shown to inhibit collagen synthesis in mouse calvaria by 19 % at a concentration of 10 ng/ml (Wilson, Meghji and Harvey, 1988). This is a similar result to that found using SAM from *A. actinomycetemcomitans* in the present study. Millar et al. (1986) have also investigated the effect of LPS from *P. gingivalis* and *Salmonella minnesota* on bone formation by measuring incorporation of ^{14}C-proline into collagenous protein in foetal rat calvaria. These investigators found that a significant reduction (30-40%) in ^{14}C-proline

incorporation was achieved only with LPS concentrations as high as 10 μg/ml. Although comparisons with the present study are difficult in view of use of different strains of *P. gingivalis* and different assay systems, on a weight for weight basis, the SAM of *P. gingivalis* is apparently 1000-fold more potent than LPS from this organism.

Thus, SAM from three oral bacteria have been shown to be potent inhibitors of bone, and bone cell, DNA and collagen production. The inhibition of bone synthetic function appears to be related to the generation of prostanoids. This may be a partial explanation for the finding that non-steroidal anti-inflammatory drugs (NSAIDs), like ibuprofen, can inhibit alveolar bone loss in man and animals (Feldman et al., 1983 ; Williams et al., 1985). It has recently been reported that in experimental periodontitis in the cynomolgus monkey, treatment with ibuprofen or meclofenamic acid inhibited alveolar bone loss without inhibiting gingivitis (Kornman et al., 1990). This suggests that the host inflammatory response is uncoupled from the process of bone resorption and, by implication, that bacterial moieties like the SAM used in this study are important in bone matrix loss.

CHAPTER 5

Effect of Bacterial Surface Associated Material on Bone Resorption

5.1 Introduction

Periapical bone loss is arguably the most severe consequence of periapical lesions. Although the stimulus promoting this bone breakdown is complex, it is likely that bacterial components which are released from the dead tooth are involved in some way. As stated previously one bacterial component that has been considered to be of prime importance in this respect is lipopolysaccharide (LPS) (Schonfeld et al., 1982, Yamasaki et al., 1992). However, it has been demonstrated that capsular material from the periodontopathogenic bacterium *A.actinomycetemcomitans* is a potent stimulator of bone resorption *in vitro* and is significantly more active, on a dry weight basis, than LPS from the same organism (Wilson, Kamin and Harvey, 1985). The capsular material used in these studies was prepared by gently stirring the bacterial cells in saline for 1h at 4°C. This completely removed the capsule while leaving the bacteria intact as ascertained by electron microscopy. The ready solubility of the outer capsule coupled with its potent osteolytic activity supports the hypothesis that components of this capsular material are involved in promoting bone resorption in periodontal disease (Wilson, Kamin and Harvey (1985). However, *A. actinomycetemcomitans* is only one of a number of oral bacteria which have capsules or surface-associated materials (Slots and Genco, 1984; Progulski and Holt, 1980). The possibility exists that capsular components (or other surface-associated materials) of other bacteria found in periapical

lesions may have bone resorbing activity.

In this study, surface-associated material (SAM) has been extracted from three Gram-negative oral pathogens considered to be active in periapical inflammation: *A. actinomycetemcomitans*, *P. gingivalis*, *E. corrodens* and one Gram-positive organism *S. aureus* and their capacity to induce bone resorption in vitro has been compared. An attempt has been made to define, by use of selective inhibitors, antagonists or neutralizing antibodies, the mechanism(s) by which these materials stimulate bone resorption in the calvarial assay system used to screen osteolytic activity.

5.2 Materials and Methods

5.2.1 Calvarial bone resorption assay

Bone resorption was assayed by measuring calcium release from 5-day old mouse calvaria *in vitro* (Zanelli, Lea and Nisbet, 1969). Halved calvaria were cultured singly on stainless steel grids in 30mm dishes (5 per group), with 1.5ml BGJ medium (Flow Laboratories, Irvine, Scotland) supplemented with 5% complement-inactivated rabbit serum (Gibco, Paisley, Scotland) and 50μg/ml ascorbic acid (Sigma). After 24h the media were changed and media containing various concentrations of SAM or SAM plus various inhibitors were added. In all assays, prostaglandin (PG)E_2 was added at 10^{-6}M as a positive control. The calvaria were cultured for a further 48h and then the calcium content of the media was measured by automated colorimetric analysis (Gitelman, 1967). The statistical

significance of the results was determined by use of the two tailed Student's t-test.

5.2.2 Inhibitor studies

To determine whether endotoxin was contributing to the bone resorbing activity, polymyxin B (Sigma), which binds and inactivates endotoxin, was added to the culture media. The role of prostanoids in bone resorption was tested by adding indomethacin (Sigma) at 1μM. The role of interleukin-1 (IL-1) was assessed by adding the IL-1 antagonist protein (IL-1ra - Synergen, Boulder, Colorado USA). The involvement of TNF in bone destruction was determined by adding the neutralizing monoclonal antibody TN3 19.12 (manufactured by Celltech, Slough, England). The role of another potential osteolytic cytokine - interleukin-6 (IL-6) was assessed by use of a neutralizing rabbit polyclonal antibody to murine IL-6 (supplied by Immunex, Seattle, USA). Antibodies or IL-1ra were added to bone cultures at various concentrations along with the SAM preparations.

5.3 Results

3.3.1 Composition of SAM

The protein content of the SAM from *P. gingivalis* was 50 % (w/w) compared to 17% (w/w) for the SAM from *A. actinomycetemcomitans* and 16% (w/w) for the SAM from *E. corrodens* and 30% (w/w) of SAM from *S. aureus.* The endotoxin content of the SAM preparations was measured by a chromogenic limulus assay. A 1mg/ml solution of the SAM from each bacteria was used for assay. The level of endotoxin present was at or below the sensitivity limit of the assay.

5.3.2 Bone Resorbing Activity of SAM

SAM from all four bacteria reproducibly stimulated release of calcium from cultured murine calvaria. Figure 18 shows the dose responses of the four SAM preparations in one representative assay. The dose range was from 1ng/ml to 10ug/ml. The SAM preparations from both *A. actinomycetemcomitans* and *E. corrodens* showed statistically significant activity at 10ng/ml. *SAM from S. aureus* also stimulated bone resorption: activity was reproducibly seen at concentrations as low as 1ng/ml and there was a linear dose response between 10ng/ml and 10μg/ml. In contrast, the SAM from *P. gingivalis* only demonstrated statistically significant bone resorbing activity at 1ug/ml.

To provide additional evidence that the bone resorption was not due to the action of contaminating LPS, polymyxin B (20μg/ml) was added to cultures along with the various Gram-negative SAM. Polymyxin B binds to

and inactivates LPS and this can be clearly seen in Fig.19, when it was added to bone cultures with LPS from *A. actinomycetemcomitans*. The Polymyxin B completely inhibited the activity of the LPS. In contrast, the same concentration of Polymyxin B had no effect on the bone resorption induced by any of the SAM preparations. Polymyxin B had no effect by itself when added to bone.

5.3.3 The Mechanism of Bone Resorption

(i) Role of prostanoid synthesis: 1μM indomethacin was required to completely inhibit bone resorption stimulated with SAM from *P. gingivalis* (Fig.20). In two experiments 1uM indomethacin inhibited *E. corrodens* SAM activity by 22% and 25%, whereas indomethacin completely inhibited the osteolytic activity of the SAM from *S. aureus* at a concentration as low as 0.1μM (Fig. 20a.) and in one experiment there was 90% inhibition at 0.01μM. In the dose response shown, inhibition is seen at 1nM and 50% inhibition at 10nM. In contrast, indomethacin did not affect bone resorption induced by SAM from *A. actinomycetemcomitans*.

(ii) Role of IL-1: The biological actions of IL-1 can be antagonized by the cloned IL-1 antagonist protein - IL-1ra. Addition of IL-1ra to unstimulated calvarial cultures had no effect on calcium release. In three separate experiments IL-1ra (at 0.1, 1 or 10ug/ml) added to bone cultures stimulated with SAM inhibited, in a dose-dependent manner, the bone

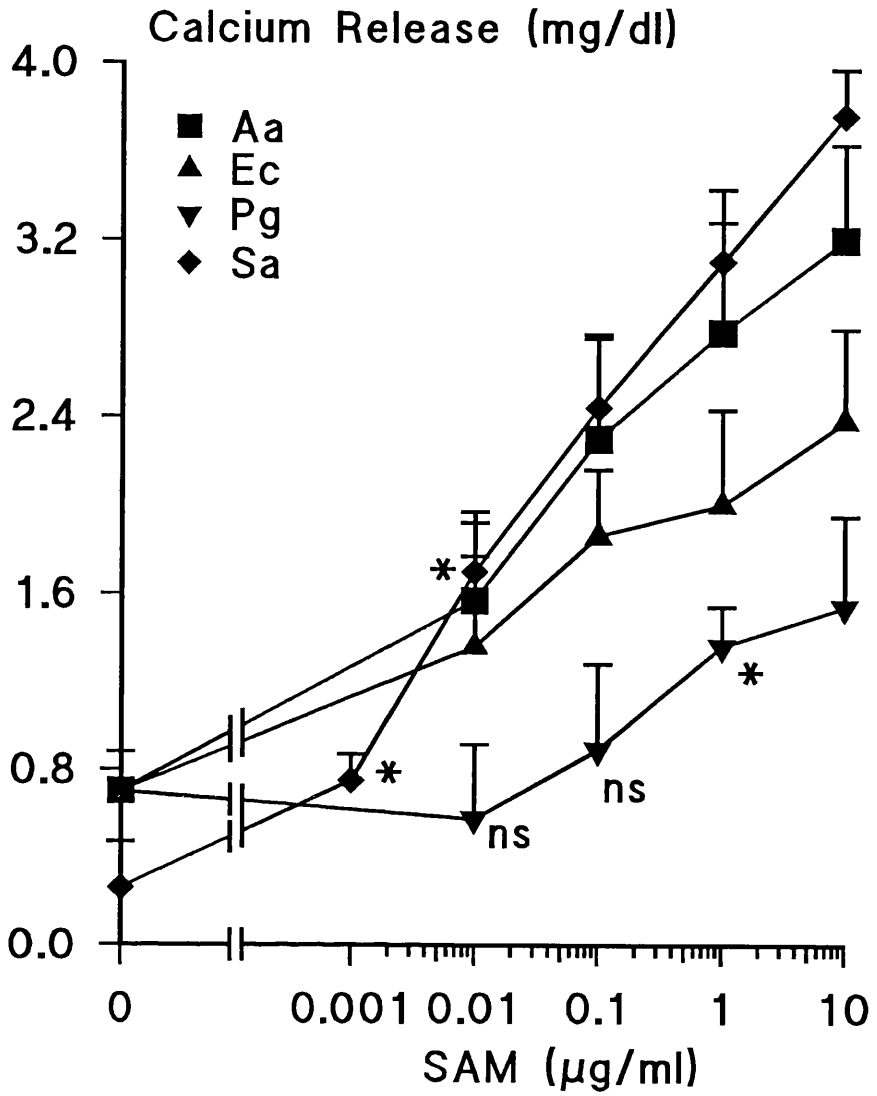


Figure 18. A representative assay showing the comparison of the dose-responses of the bone resorbing activity (measured as calcium release in mg/dl) of capsular material SAM from *A. actinomycetemcomitans (A.a), P. gingivalis (P.g)* or *E. corrodens (E.c)*. Each point represents the mean and standard deviation from 5 cultures. |Sa = *S. aureus*

*** - p<0.05.**

**** - p<0.001.**

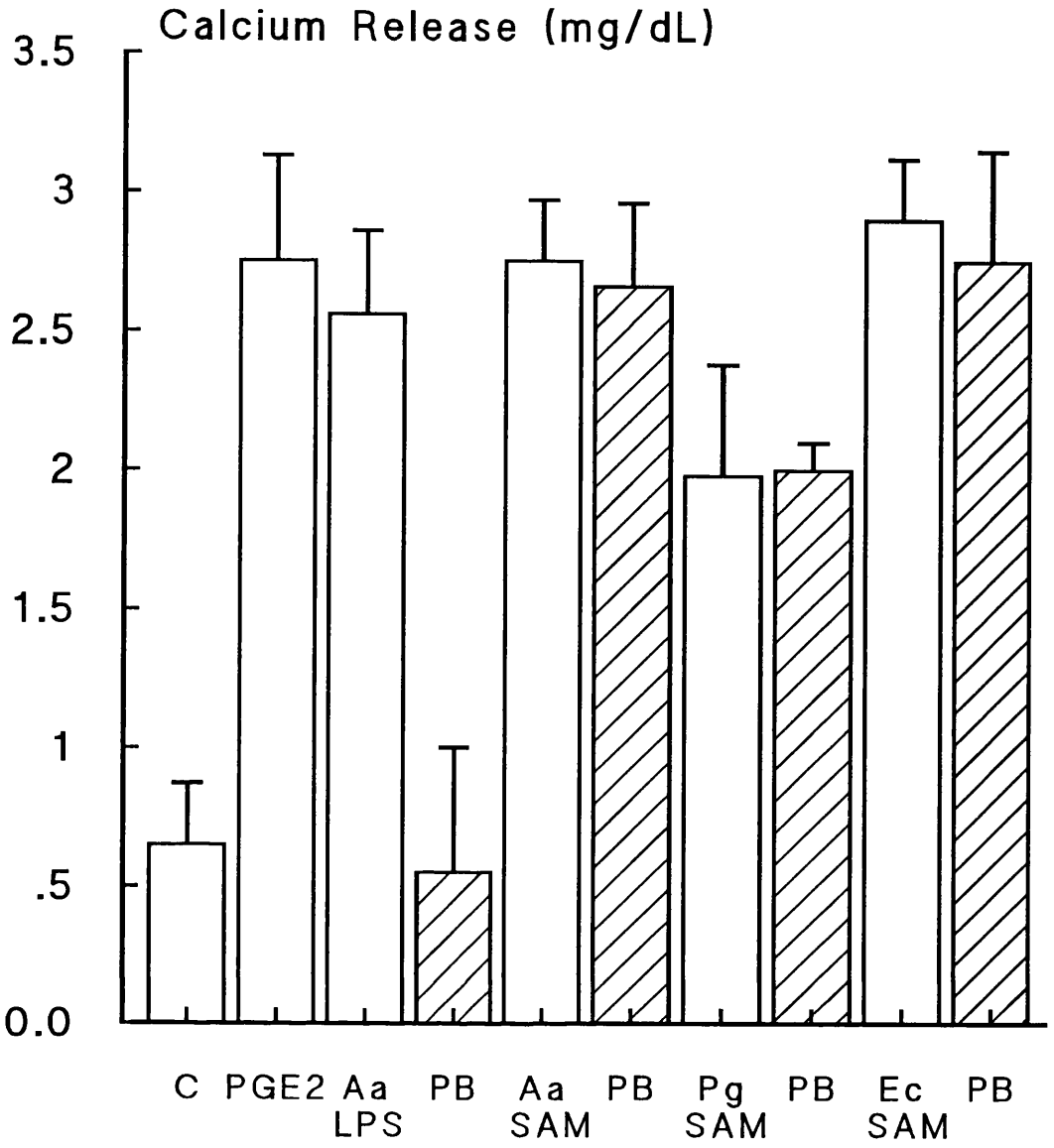


Figure 19. The effect of adding Polymyxin B (PB) to calvarial cultures stimulated by lipopolysaccharide (LPS) from *A. actinomycetemcomitans* or by SAM eluted from *A. actinomycetemcomitans (Aa), P. gingivalis (Pg)* or *E. corrodens* (Ec). The control culture (C) shows the release of calcium from calvaria in the absence of any stimulus. The positive control (PGE_2) is stimulated with 1μM PGE_2 to show that the culture system is working. Each bar represents the mean and standard deviation of 5 cultures. PB significantly inhibited Aa LPS ($p<0.0003$) but had no effect on the SAM preparations.

resorption induced by SAM from *P. gingivalis*. There was statistically significant inhibition with 0.1µg IL-1ra (35-60% in different experiments: $p < 0.001$) with complete inhibition of bone resorption being seen at between 1 and 10µg/ml (Fig.21). In contrast, with SAM from *A. actinomycetemcomitans* or *E. corrodens* inhibition was only observed with 10ug/ml IL-1ra. With both bacteria this concentration of antagonist only produced at the most 25% inhibition of calcium release. IL-1ra had some inhibitory effect on *S. aureus* SAM induced bone resorption but complete suppression of calvarial bone resorption was not seen even at very high concentrations (10-100µg/ml) (Fig.21a.)

(iii) Role of TNF: TNF is another potent activator of bone resorption. To determine the role of TNF in SAM-induced bone resorption a neutralizing antibody to murine TNF (TN3-19.12) was added to bone cultures. Addition of TN3-19.12 to unstimulated bone had no effect on basal calcium release. In three separate experiments it was found that addition of this antibody to bone cultures inhibited resorption induced by SAM from *P. gingivalis* and *E. corrodens* but had no effect on that induced by SAM from *A. actinomycetemcomitans*. At a concentration of antibody of 1µg/ml there was complete inhibition of the activity of the SAM from *P. gingivalis* and approximately 80% inhibition of the activity of SAM from *E. corrodens* (Fig.22.). The addition of TN3-19.12 had very little effect on *S. aureus* induced bone resorption (Fig. 21a).

(iv) Role of IL-6: Addition of various concentrations of a neutralizing antibody to murine IL-6 to calvarial cultures stimulated with SAM from the

four bacteria failed to produce any inhibition of bone resorption.

The results obtained from the use of these various inhibitors and blocking agents is summarized in Table 2.

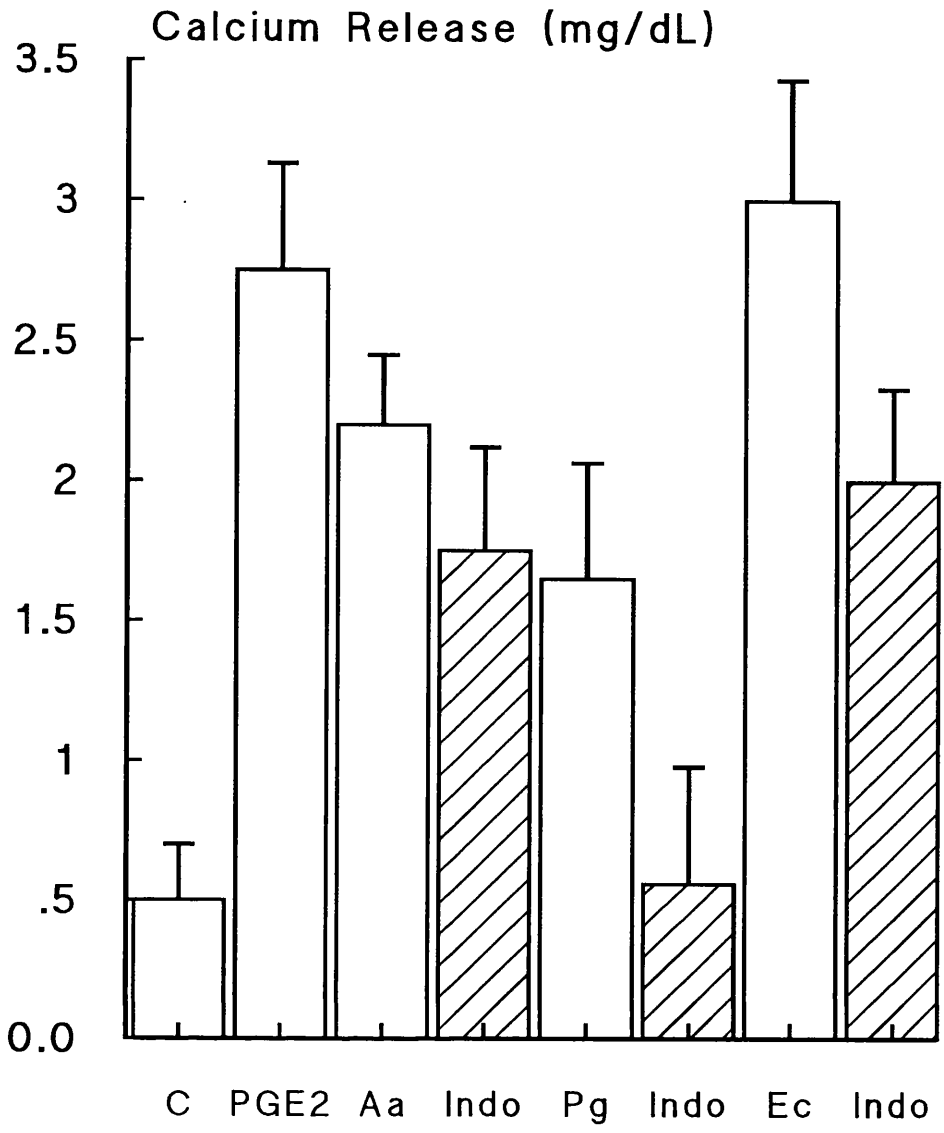


Figure 20. The inhibition of bone resorption induced by SAM from *A. actinomycetemcomitans (Aa), P. gingivalis (Pg)* or *E. corrodens (Ec)* in the absence or presence of 10^{-6}M indomethacin. The control (C) represents the release of calcium in unstimulated cultures and the PGE_2 column is the positive control in which calvaria are stimulated with 1μM PGE_2. Each bar represents the mean and standard deviation of 5 cultures. Indomethacin had no effect on bone resorption induced by SAM from *A. actinomycetemcomitans* (Aa), but significantly reduced that induced by SAM from *P. gingivalis* (Pg) and *E. corrodens* (Ec) ($p < 0.05$).

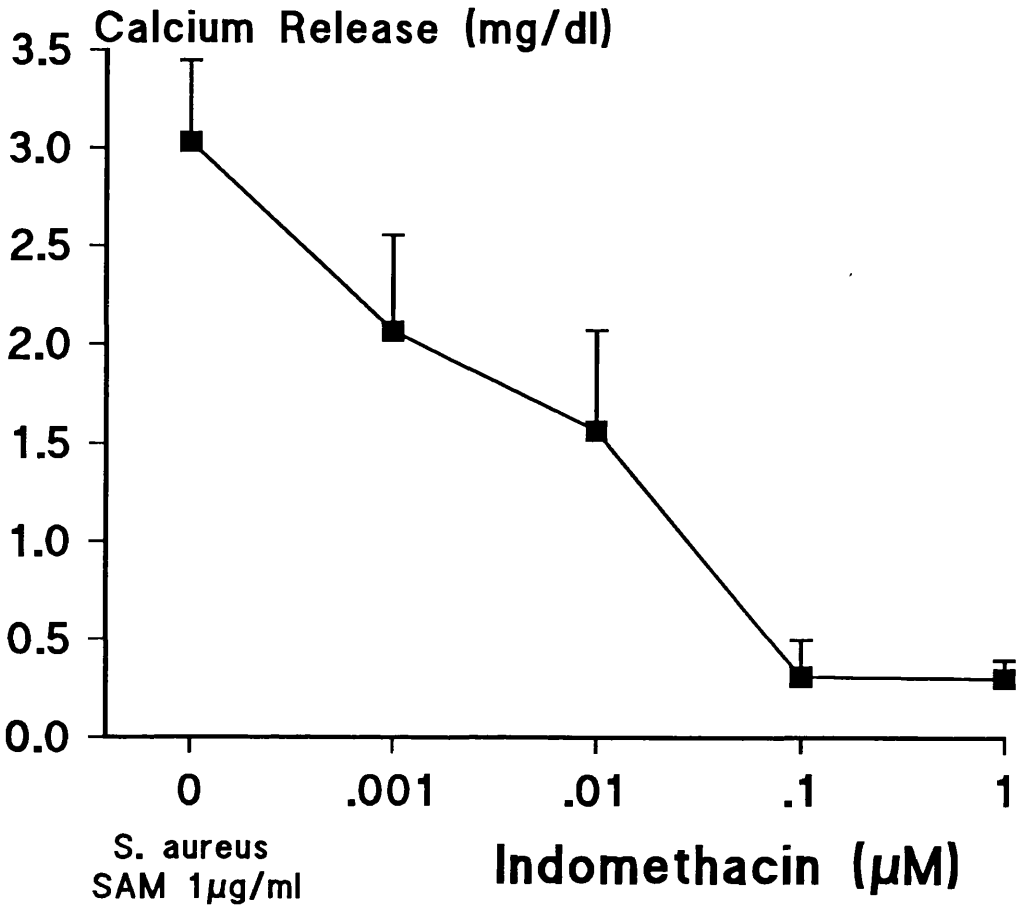


Figure 20a. Inhibition of *S. aureus* SAM-induced calvarial bone breakdown by indomethacin over the dose range 1nM to 1μM. Results are expressed as the mean and standard deviation of 5 replicate cultures.

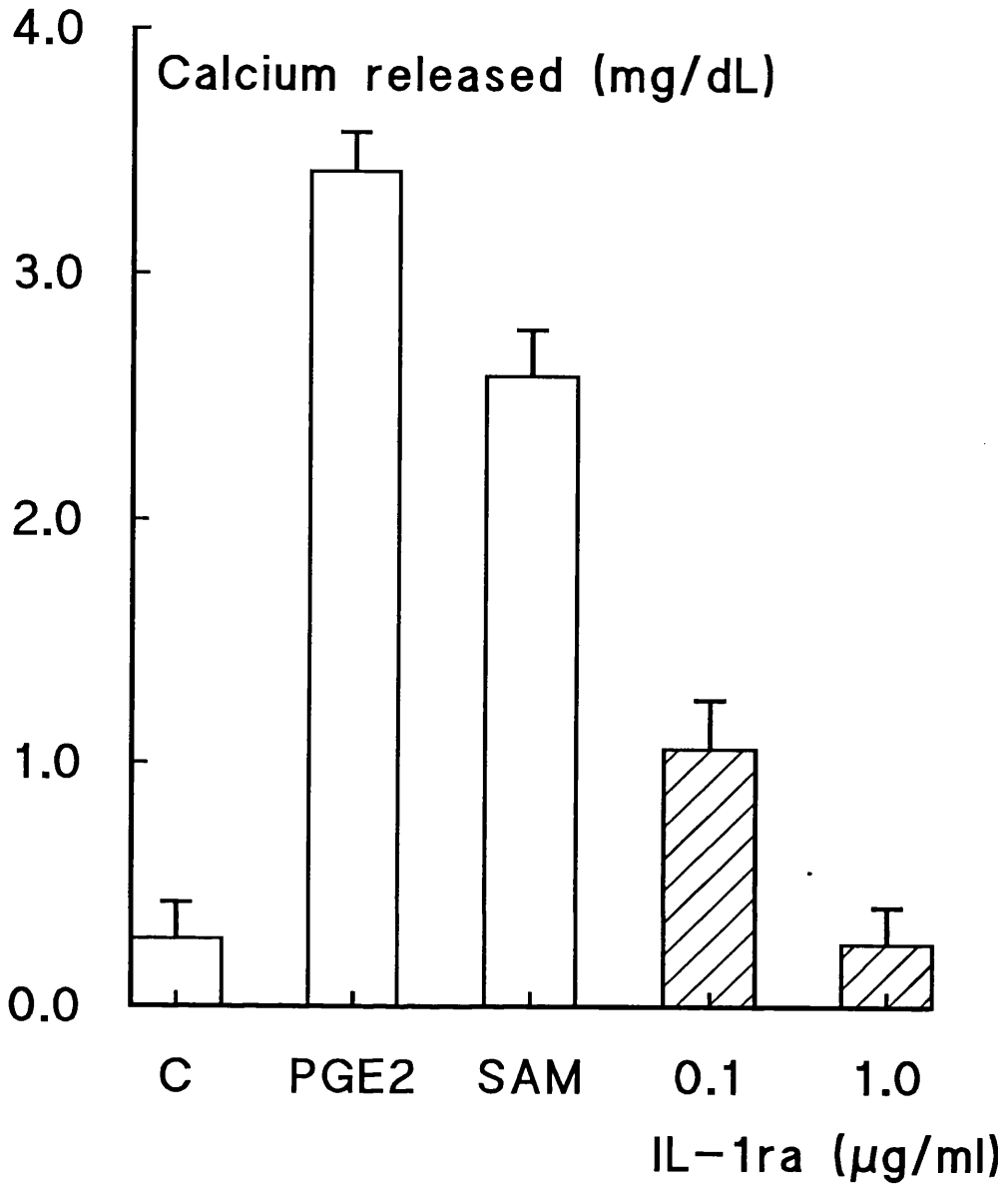


Figure 21. A result from a typical experiment in which the activity of IL-1ra has been tested for its ability to inhibit bone resorption induced by SAM from *P. gingivalis*. The control cultures show the release of calcium from unstimulated cultures and PGE_2 has been added at 1μM to other cultures to act as a positive control. IL-1ra has been used at concentration of 0.1 and 1. Results are expressed as the mean and standard deviation of 5 replicate cultures. 100ng/ml IL-1ra produced significant ($p<0.001$) inhibition of bone resorption.

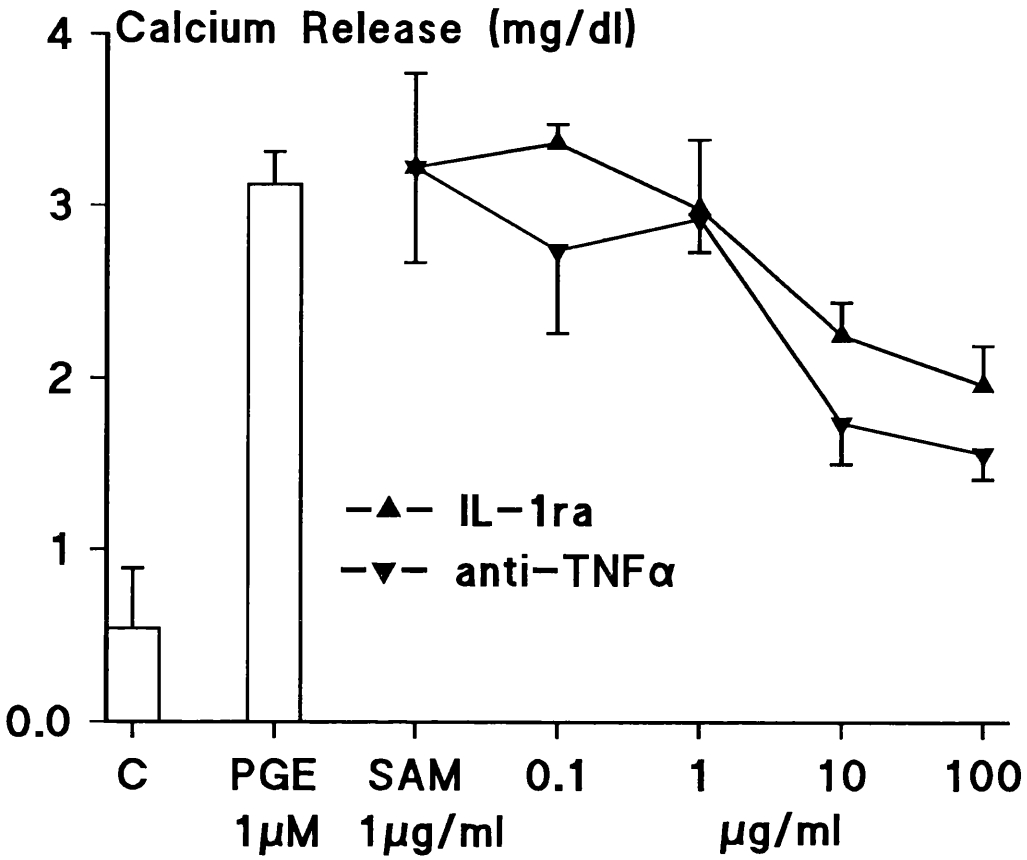


Figure 21a. Graph showing the influence of increasing concentrations of the interleukin-1 receptor antagonist and a neutralizing antibody to murine TNFα on bone resorption induced by 1μg/ml *S. aureus* SAM. The release of calcium from unstimulated calvaria is seen in the column marked (C) and the response of bone to 1μM PGE_2 is shown in the column marked (PGE).

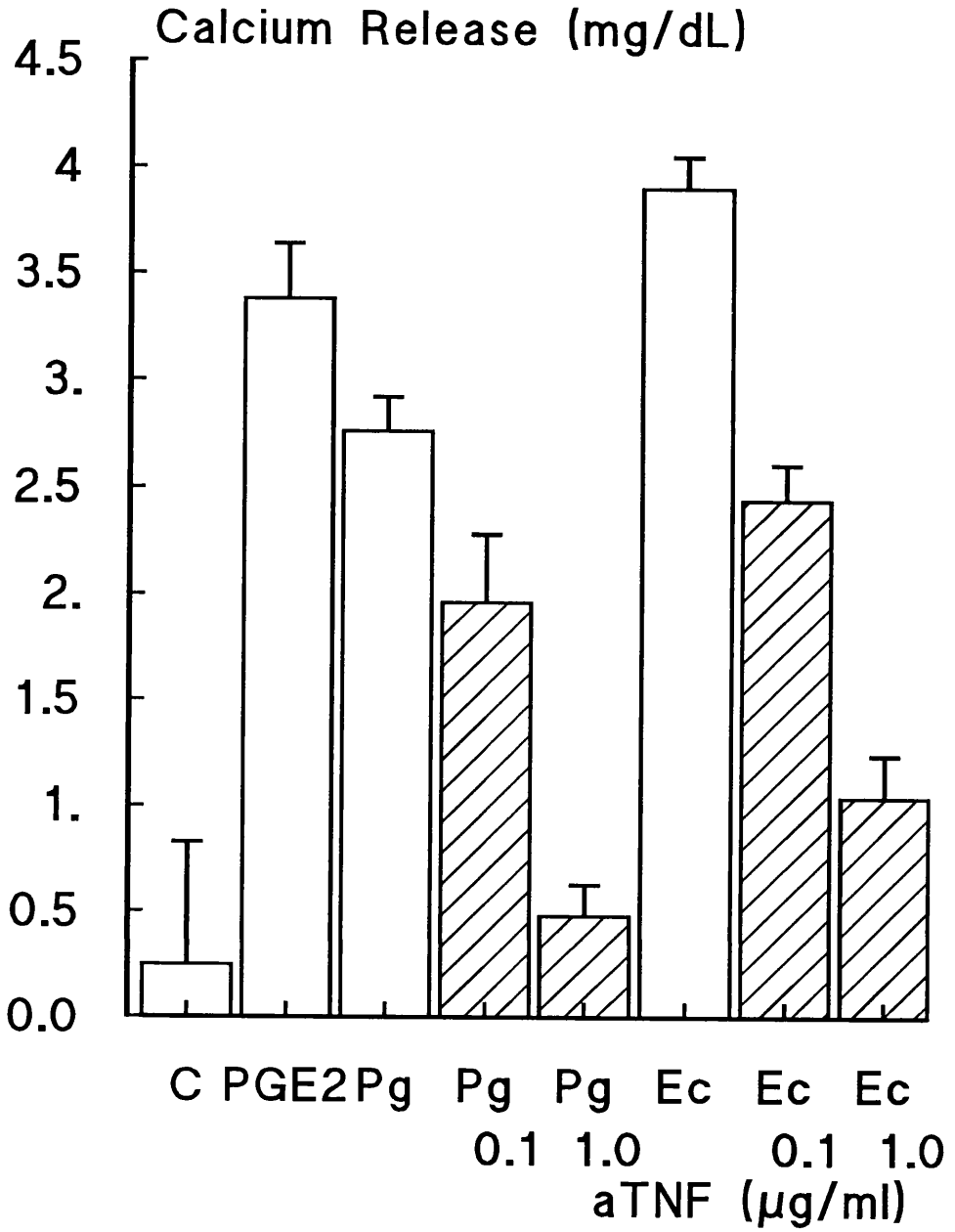


Figure 22. The result from a typical experiment showing the activity of the neutralizing anti-TNF antibody TN3-19.12 in the calvarial bone resorption assay. Antibody has been added at 0.1 or 1 µg/ml to cultures stimulated with SAM from either *P. gingivalis* or *E. corrodens*. The control represents the calcium released by unstimulated cultures and the positive control cultures have been stimulated with 1uM PGE_2. Results are expressed as the mean and standard deviation of 5 replicate cultures. 100ng/ml anti-TNF antibody significantly inhibited ($p = 0.001$) calcium release induced by SAM from both bacteria. The osteolytic activity of SAM from *A. actinomycetemcomitans* was not inhibited by TN3-19.12.

Table 2. Summary of the results obtained from use of indomethacin, IL-1ra and anti-cytokine antibodies on calvaria bone resorption induced by SAM

'Inhibitor'	*Aa*	*Pg*	*Ec*	*Sa*
Indomethacin	-	Complete inhibition	Slight inhibition	Complete inhibition
IL-1ra	Slight inhibition	Complete inhibition	-	slight inhibition
Anti-TNF	-	Complete inhibition	Significant inhibition	slight inhibtion
Anti-IL-6	-	-	-	-

- No or minimal effect

Aa Actinobacillus actinomycetemcomitans

Pg Porphyromonas gingivalis

Ec Eikenella corrodens

Sa Staphylococcus aureus

5.4 Discussion

The inflammation and bone destruction characteristic of periapical lesions are undoubtedly associated with bacteria and their products. Tissue damage may be due to the direct action of bacterial products or they may result from the stimulation of host mechanisms by these products (Stashenk, 1990). In the context of bone breakdown, bacterial substances may directly activate osteoblasts or osteoclasts or they could indirectly activate these cells by stimulating the synthesis and release of prostanoids or cytokines. However, a prerequisite to activation is that the bacterial component actually reaches the target cells. In this respect biologically-active, soluble surface components, especially ones that could be shed during growth of the organism, clearly have a greater "bio-availability" than less soluble components that are an integral part of the cell. Examples of the former would include surface-associated proteins and many components of the bacterial glycocalyx i.e. capsular material, S-layers, slime, fibrils and any other carbohydrate-containing material external to the cell wall (Costerton, Irvin and Cheng, 1981). Such components may be regarded as being "surface-associated" rather than being essential for the integrity of the bacterial cell. LPS, on the other hand, is an example of a structural component in that, although some may be shed during growth, it forms an integral part of the outer membrane of the Gram-negative cell wall. Furthermore, it does not have a high solubility in a physiological milieu. In this investigation surface-associated material (SAM) from four bacteria have been removed by extraction with saline. Electron micrographs

of the cells prior to extraction revealed the presence of extensive ruthenium red-staining material surrounding each organism. Ruthenium red is known to stain many polysaccharides and glycopeptides and has been used to demonstrate the presence of a glycocalyx in the form of a capsule or fibrils on the surfaces of many micro-organisms (Handley, 1991). The existence of a glycocalyx on the surfaces of each of the organisms investigated in the present study has been reported previously (Progulski and Holt, 1980; Yamamoto, et al., 1982; Holt, Tanner and Socransky, 1980). Following saline extraction, the ruthenium red layer was almost entirely removed from the surfaces of each of the organisms and no gross disruption of the cells was evident (Figure 7, page 44). It can therefore be assumed that the saline extracts contained SAM from each of the organisms. Such material is likely to consist mainly of capsular components since other SAM such as fibrils, fimbriae and S-layers were not evident. However, their presence cannot be excluded since it may have been possible to visualise such components had different electron microscopic techniques been employed.

It has been demonstrated previously (Wilson, Kamin and Harvey, 1985) that one particular SAM, i.e. capsular material (CM), from *A. actinomycetemcomitans* is a potent stimulator of bone resorption and is significantly more active than the corresponding LPS. In this study, SAM, which would include CM, from a further three bacteria, *P. gingivalis*, *E. corrodens* and *S. aureus* has been shown to also stimulate calvarial bone resorption. On a weight basis, the SAM from *S. aureus* was the most potent bone resorbing agent, stimulating bone resorption at 1ng/ml. The

SAM from *A. actinomycetemcomitans* and *E. corrodens* were equipotent showing significant activity at a concentration as low as 10ng/ml. The soluble SAM from *P. gingivalis* was less active but still showed bone resorbing activity at 1ug/ml.

These findings have therefore demonstrated that the osteolytic activity of SAM from *A. actinomycetemcomitans* is not a feature unique to this organism and, in fact, may be an inherent activity of the SAM of many bacteria. This strengthens the hypothesis that readily-solubilized surface constituents of bacteria released from the root canals in infected teeth may be play a major role in periapical bone resorption. The possibility that these SAM may interact in a synergistic manner either with themselves or with LPS is under investigation.

The *Limulus* assay demonstrated that the SAM isolated from these various bacteria did not contain significant amounts of endotoxin. However, to be absolutely certain that the bone resorption induced by these materials was not due to either endotoxin or to synergy between endotoxin and SAM the antibiotic Polymyxin B was added to cultures. Polymyxin B is a cationic peptide which inactivates LPS by forming stable molecular complexes with the lipid A region (Vaara et al., 1981), although it does not apparently neutralise some biological activities of LPS from *P. gingivalis* e.g. mitogenicity (Fujiwara et al., 1988). Addition of this antibiotic peptide completely inhibited the bone resorbing activity of LPS from *A. actinomycetemcomitans* but had no influence on bone resorption induced by the three SAM preparations. This suggests, at least in the

cases of *A. actinomycetemcomitans* and *E. corrodens,* that the bone resorbing activity of the SAM cannot be attributed to contamination of the preparations by LPS.

Having established that SAM from these pathogens has the capacity to induce bone resorption *in vitro* it was of interest to determine the mechanism of action. Bone breakdown can be induced or stimulated by a number of mediators of the inflammatory process. In the context of periapical inflammation, perhaps the most important mediators to consider are: (i) prostanoids and (ii) cytokines such as interleukin-1, tumour necrosis factor (TNF) and interleukin-6 (IL-6) (Mundy, 1991).

To determine the role played by prostanoids in SAM-induced bone destruction, indomethacin was added up to a concentration of 1μM. This amount of indomethacin should completely block cyclooxygenase activity without producing serious non-specific effects as can be found with higher concentrations of this drug (Flower, 1974). Addition of indomethacin has been shown to almost completely inhibit the bone resorption induced by LPS from *A. actinomycetemcomitans* at concentrations of between 0.1 and 1uM (Wilson, Kamin and Harvey, 1985).

In the present study indomethacin had little influence on the activity of SAM from *A. actinomycetemcomitans*. However, indomethacin completely inhibited the bone resorption induced by SAM from *P. gingivalis* and *S.aureus,* but had only a moderate effect (22-25% inhibition) on the activity of SAM from *E. corrodens*. This suggests that the bone resorption induced by SAM from *P. gingivalis* and *S. aureus* is mediated solely by the

production of endogenous prostanoids.

IL-1 is probably the most potent activator of bone resorption at least *in vitro* (Gowen and Mundy, 1986). Recently, a natural antagonist of IL-1 (IL-1ra) has been discovered, cloned and expressed (Hannun et al., 1990). This protein binds to the type I IL-1 receptor without triggering an agonist response and thus acts as a true receptor antagonist. IL-1ra has been shown to block the biological responses to IL-1 both *in vitro* (Arend et al., 1990) and *in vivo* (Cominelli et al., 1990; Henderson et al., 1991). It has recently been reported that IL-1ra can inhibit *in vitro* calvarial and long-bone resorption induced by human recombinant IL-1 (Seckinger et al., 1990). We have found that IL-1ra inhibited the bone resorption induced by SAM from *P. gingivalis*. Significant inhibition of bone resorption was found with 100ng/ml IL-1ra and complete inhibition of resorption was produced by either 1 or 10ug/ml IL-1ra in different experiments. This is similar to the results reported by Seckinger et al, (1990) who added exogenous human IL-1 to cultures and suggested that the IL-1 receptor on murine osteoblasts binds to human and murine IL-1 with approximately the same affinity. Although IL-1ra was able to completely inhibit the osteolytic response to SAM from *P. gingivalis*, it had only a minimal effect on bone resorption induced by *E. corrodens*, *A. actinomycetemcomitans* or *S. aureus* and inhibition was only seen with 10-100μg/ml antagonist.

TNF alpha and beta are also cytokines capable of stimulating bone resorption (Thomson, Mundy and Chambers; 1987). If these molecules

were being produced in bone by the action of SAM then the hamster antibody TN3-19.12 (Sheenan, Ruddle and Schreiber; 1989) which neutralizes the biological activity of both mouse TNF alpha and beta should inhibit bone resorption. At a concentration of 100ng/ml TN3-19.12 significantly inhibited, and at 1ug/ml completely inhibited, the activity of SAM from *P. gingivalis.* At a concentration of 1ug/ml TN3-19.12 inhibited the activity of SAM from *E. corrodens* by almost 80%. In contrast the activity of SAM from *A. actinomycetemcomitans* or *S.aureus* was not affected by TN3-19.12.

It has also been suggested that interleukin-6 can stimulate bone resorption (Ishimi et al., 1990; Mundy, 1991) and again, using a neutralizing antibody to murine IL-6 is one way of assessing the role of this cytokine in SAM-induced bone resorption. However addition of the neutralizing antibody to stimulated cultures failed to have any effect on the bone resorption induced by SAM from any of the organisms.

The mechanism of stimulation of bone resorption by the four SAM is clearly very different (Table 2). SAM from *P. gingivalis* stimulates bone resorption and this is completely inhibited by indomethacin, IL-1ra and by neutralizing TNF. The bone-resorbing activity of SAM from *E. corrodens* is slightly inhibited by indomethacin and significantly inhibited by anti-TNF. The bone resorbing activity of SAM from *S. aureus* is completely inhibited by indomethacin. In contrast the osteolytic action of SAM from *A. actinomycetemcomitans* is unaffected by all these treatments. Calcium release by the SAM preparations was unaffected by neutralizing murine

IL-6.

This suggests that SAM from *P. gingivalis* produces bone resorption by stimulating IL-1 and TNF production which then causesthe generation of prostanoids which ultimately are responsible for calcium release. It is likely that IL-1 and TNF synergise in this process thus explaining why inhibition of either one of these cytokines inhibits osteolysis. The SAM from *E. corrodens* appears to work mainly by stimulating TNF synthesis with a slight involvement of prostanoids. The SAM from *S. aureus* appears to work completely via the cyclooxygenase pathway. The mechanism of action of SAM from *A. actinomycetemcomitans* is not clear. It certainly does not work via the synthesis of prostanoids, or the common inflammatory bone resorbing cytokines. It is possible that the components of this SAM can directly activate the osteoclasts. Indeed, SAM from this organism has been shown to have interleukin-1-like activity (Harvey et al., 1987).

Thus, in summary, SAM from all pathogens stimulate calvarial bone resorption and that each SAM appears to cause bone calcium release by a different mechanism.

SECTION 2

Chapter 6

6.1 The Initiation of Epithelial Proliferation and Cyst Formation

Introduction

Radicular cysts are by far the most common cystic lesion in the jaws. The pathogenesis of the radicular cyst can be divided into three phases; (a) initiation, (b) formation, and (c) enlargement. The precise mechanisms involved in these phases are not known. It is generally accepted that this epithelial lining is derived from the epithelial cells rests of Malessez in the periodontal ligament. There is also no doubt that these cells may proliferate, and when they do so, either in vivo or in tissue culture experiments, that there are consistent morphological and histochemical changes (Ten Cate, 1972).

6.2 Cell Rests of Malassez

The epithelial cells rest of Malassez are remnants of the epithelial sheath of Hertwig found as isolated columns of epithelial cells or as an incomplete network between the fibres of the periodontal membrane. Such remnants are numerous at the apices of teeth or at the division of multi-rooted teeth. Ultrastructurally, (Valderhaug and Nylen, 1966; Morgenroth and Morgenroth, 1966), and histochemically (Ten Cate, 1963; 1965), studies indicate that their structure is poorly developed for any synthetic or secretory activity. although the rests appear to reduce in number with age, (Reeve, 1960; Reitan, 1961), significant numbers are still found in persons

of advanced age. This persistence of the rests of Malassez has prompted the view that they must play some functional role, although none has been demonstrated. There is evidence (Spouge, 1980), that in successfully transplanted teeth rests of Malassez are prominent in the reformed periodontal ligament, whereas when transplantation was followed by resorption of the root and ankylosis, no rests were present and the periodontal ligament was disorganised. Such data suggest a relationship between the epithelial and connective tissue components in maintaining the integrity of the periodontium. Such a relationship might also be important where the cyst epithelium is derived from these rests.

Morphologically, the cell rests of Malassez have been divided into resting, proliferating, differentiating, and degenerating types although the evidence for such classification is not great. There is evidence (Lindskog, 1982a; 1982b; Lindskog and Hammarstrom, 1982), that the inner cells of the root sheath displayed modulations in their secretory activity in the same way that ameloblasts do. It could therefore be expected that variations in the degree of quiescence/activity would also exist in the rest of Malassez. Some individuals are more susceptible to radicular cyst formation than others (Haring and Van Dis, 1988; Oehlers, 1970). It has been suggested that such individuals have a defect of their immunological protection mechanisms (Toller, 1970); however it is possible that they may just possess more active rests of Malassez.

These cell rests of Malassez have been demonstrated in periapical granuloma. The similarity in the expression of cytokeratins in the

epithelium of the rests of Malassez in the normal periodontal ligament with that in periapical granulomas and in the lining of radicular cysts gives support to the view that the epithelial lining of radicular cysts is derived from the rests of Malassez (Gao et al., 1988).

The inflammatory radicular cyst is the commonest odontogenic cyst. These have a well characterised development, arising with pulpal necrosis, then periapical inflammation and finally the formation and growth of the cyst. Such cysts are characteristically very inflamed with a pronounced mononuclear cell infiltrate. It is believed that the main stimulus for radicular cyst formation is bacterial endotoxin. Endotoxins or LPS have diverse biological activity, not only acting as mitogens for epithelial cells (Meghji et al. 1992), but are also capable of stimulating the production of cytokines from the surrounding connective tissue and inflammatory cells (Hanazawa et al., 1985). Laboratory investigations of Schonfeld et al., (1982) have shown that 75% inflamed periapical granulomas studied were positive for endotoxin. However, the endotoxin content of mature cysts has not been measured so the endotoxin hypothesis still remains to be established.

The developmental cyst (keratocyst and follicular cyst) occurs less commonly. A role for endotoxin in the formation of such cysts is less likely. The keratocyst (primordial cyst) is an idiopathic intraosseous lesion consisting of a characteristically parakeratinized epithelial lining and a thin fibrous capsule which is usually free from inflammatory cell infiltrate. Similarly the follicular cyst has little or no inflammatory infiltrate, and is

commonly associated with impaired eruption of the tooth.

6.3 Epithelial Proliferation

Proliferating strands of stratified squamous epithelium is a common histological feature of periapical granulomas. The formation and growth of a periapical granuloma is thought to be a direct consequence of periapical infection. This was demonstrated by the studies of Kakehashi et al. (1965); exposure of the pulp from the oral cavity resulted in the development of periapical lesions in rats maintained in a conventional environment, whereas germ-free animals with pulp exposures failed to develop lesions. Bacterial components originating in septic root canals are thought to diffuse into the apical tissues, and indeed Schonfield et al. (1982) showed the presence of endotoxin in chronic inflamed granulomas. Bacterial components are also capable of stimulating inflammatory cells and fibroblasts (which are one of the dominant cell type in a periapical granuloma) to produces eicosanoids and cytokines (Burchett et al., 1988).

6.4 Bacterial and Inflammatory Mediators

Bacterial infection of the dental pulp results in pulpal destruction, and subsequently stimulates an inflammatory cell response and destruction of bone in the periapical region. Bacterial components including lipopolysaccharides, induce the production of many polypeptide mediators, or cytokines, by inflammatory cells (Bailly et al., 1990) These cytokines include interleukin-1 α and β, tumour necrosis factor and interleukin-6.

6.4.1 Endotoxins

Endotoxins from Gram-negative bacterial lipopolysaccharides are made up of a lipid moiety called lipid A and a polysaccharide moiety. The polysaccharide moiety consists of a core region and, in some LPS, repetitive units of oligosaccharides. Endotoxins from different bacteria vary depending upon their bacterial origins (Rietschel et al. 1987). This diversity has an influence on their relative abilities to induce cytokine production. It has been reported that smooth LPS are more potent IL-1 inducers than their corresponding rough mutants (Arend and Masssoni (1986; Männel and Falk, 1989). Among endotoxins from different bacterial species, LPS from *P. gingivalis* is found to be less potent than that from *Eshcerichia coli* (Hanazawa et al.; 1985) and LPS from *Vibrio cholerae* is a very weak IL-1 inducer compared to LPS from either Escherichia coli (Newton, 1986)) or from *Neisseria meningitidis* (Haeffner-Cavaillon and Cavaillon, 1989). Similarly, *Bordetella pertussis* LPS was found to be 3,000-fold less potent than *N. meningitidis* LPS to induce IL-1 production (Cavaillon and Haeffner-Cavaillon, 1987) These differences in IL-1-inducing capacity may be due to biochemical variability with the Lipid A and/or core region. Such variability in the ability to elicit biological functions is also illustrated by the different reactivities of LPS in the *Limulus* assay (Morrison et al., (1987). Interestingly, these differences among the LPS observed in vitro might run parallel to some of the in vivo properties of LPS: thus, it has been shown that in vivo the high adjuvanticity of some LPS is correlated to their capacity to induce IL-1 release (Kido, Nakashima and Kato (1984).

The different IL-1-inducing capacities of LPS from *N. meningitidis* and *B. pertussis* were found to correlate with their abilities to protect rats against colonic peritoneal carcinomatoses in vivo (Cavaillon and Haeffner-Cavaillon, 1990).

6.4.2 Cytokines

The term cytokine was introduced by Cohen in 1974 to describe any soluble substance produced by cells which exerted specific effects on other target cells. This original definition would include all mediators, including prostaglandins, steroid and peptide hormones etc..

A more appropriate definition of a cytokine would be that it is an inducible protein of molecular weight greater than 5,000 dalton which produces specific, receptor-mediated effects on target cells or on the producer cell. These cytokine-mediated effects partly underlie the normal physiological control of (a) mitogenesis (cell division) which is required for cell proliferation and thus tissue development and repair, and (b) cell function which is required for the maintenance of homeostatic and defence mechanisms and the integrated control of different physiological systems. Such a class of mediators therefore includes, in contrast to classical hormones, several directly mitogenic factors such as the soluble proteins labelled as interleukins or colony-stimulating factors and other well-defined protein growth factors.

Interleukin-1 (IL-1)

IL-1 exists as two distinct molecules, termed IL-1α and IL-1ß with only about 25% homology between them. Both forms are synthesized initially as non-glycosylated precursors (or proforms) which are then proteolytically cleaved to produce the active form of the molecule, although the proform of IL-1α does have some biological activity. The source of IL-1 was originally thought to be polymorphonuclear cells, but it is now known that stimulated mononuclear phagocytes are the major source.

The production of IL-1 has been demonstrated in nearly all tissue containing mononuclear phagocytes. These include circulating blood monocytes, pulmonary alveolar macrophages (Simon and Willoughby, 1981), splenic macrophages (Atkins et al., 1967) and peritoneal macrophages (Murphy et al., 1980).

It is also known now that many other cells types produce IL-1, for example: corneal epithelium (Grapbner et al., 1982), epidermal keratinocytes (Sauder et al., 1982), glial cells (Fontana et al., 1982), B lymphocytes, renal mesengial cells, gingival fibroblasts, periosteal fibroblasts (Heath et al., 1985), and osteoblasts (Hanazawa et al., 1987).

The wide distribution and location of IL-1 producing cells illustrates the importance of this molecule in its role as a regulating factor of cellular activity.

Connective tissue destruction including bone resorption are features of chronic inflammatory lesions, such as rheumatoid arthritis, periodontal

disease and dental cysts. Dayer et al., (1977) suggested that in rheumatoid arthritis, these processes were in part related to the high levels of collagenase and prostaglandins present within the synovial fluid. They found the fibroblastic cells cultured from rheumatoid synovium were the source of collagenase and PGE_2 when stimulated by monocyte/macrophage derived, monocyte cell factor (MCF). Subsequently, crude MCF has been shown to stimulate many connective tissue cells to make PGs and collagenase, including rabbit articular chondrocytes (Evequoz et al., 1984), human foreskin fibroblasts (Postlethwaite et al., 1983), and human cyst stromal fibroblasts (Harvey et al., 1984).

Partially purified IL-1 and pure recombinant IL-1 have now been shown to cause bone resorption at picomolar levels (Gowen et al., 1983, Gowen and Mundy, 1986). This effect is not mediated by prostaglandins, leukotrienes or IL-2, and can be inhibited by IFN-gamma (Gowen, Nedwin and Mundy, 1986). IL-1 does not have a direct action on the osteoclast but acts via the osteoblast (Thomson et al., 1986).

IL-1 can stimulate proliferation of osteoblast-like cells (Gowen, Wood and Russell, 1985) and increase DNA synthesis in whole calvaria (Smith et al., 1987). IL-1 also inhibits collagen and non-collagenous protein synthesis (Canalis, 1986), an effect which is not enhanced by IFNγ. Osteoblasts produce PGE2 but not collagenase in response to IL-1 (Gowen et al., 1984; Heath et al., 1984). Recent reports indicate that an osteogenic cell line spontaneously produces IL-1 (Hanazawa et al., 1985). However, reports of constitutive production of IL-1 by normal cells and

cell lines need to be interpreted with caution since this may be attributable to the presence of contaminating stimulants such as endotoxin. Primary cultures of osteoblasts do produce IL-1 in response to exogenous LPS but not PGE_2 or 1,25 $(OH)_2D_3$. The IL-1 activity in reported by Hanazawa et al., (1987) was however found in fractions corresponding to sizes of 15,000 and 31,000 dalton.

IL-1 also stimulates fibroblast proliferation (Schmidt et al., 1982) and articular destruction (Fell and Jubb, 1977) in vitro.

IL-1, therefore, appears to play a major role in mediating connective tissue turnover by stimulating the synthesis of proteinases such as collagenase, and the production of other mediators of catabolic activity such as prostaglandins.

Interleukin-6 (IL-6)

Interleukin 6 (IL-6) is a multifunctional protein produced by variety of cell types including T-cells, monocytes/macrophages, fibroblasts. The production of IL-6 in these various cells is regulated both positively or negatively by a variety of signals, such as mitogens or antigenic stimulation, lipopolysaccharide, IL-1, TNF, PDGF and viruses. On the basis of its various activities, IL-6 was also known as interferon-ß2 (IFN-ß2), 26-kDa protein, B-cell stimulatory factor 2 (BSF-2), hybridoma/plasmacytoma growth factor, hepatocyte stimulating factor cytotoxic T cell differentiation factor, and macrophage-granulocyte inducing factor 2A (MGI-2a) (reviewed by Van Snick, 1990; Hirano et al. 1990 and Hirano et al., 1990b).

The effects of IL-6 on different cells are numerous and varied. The effect

on B-cells is to stimulate differentiation and antibody secretion (Hirano et al., 1986; Okada et al., 1983; Butler et al., 1984). IL-6 exhibits growth factor activity for mature thymic or peripheral T-cells and reportedly enhances the differentiation of cytotoxic T cells in the presence of IL-2 or IFN gamma (Lotz et al., 1988 ; Tosato et al., 1988; Uttenhove et al. 1988).

The various activities of IL-6 described above suggests that this factor will have a major role in the mediation of the inflammatory and immune responses initiated by infection or injury. Although exact functions of IL-6 in vivo are not known, elevated IL-6 levels are reported to be associated with a variety of disease including rheumatoid arthritis (Hirano et al., 1988).

The synthesis of IL-6 by fibroblasts is stimulated by interleukin 1 (IL-1; Content et al., 1985) and tumour necrosis factor (TNF; Kohase et al., 1986).

Tumour Necrosis Factor (TNF)

TNF alpha, (cachectin) is derived from monocytes, and has a molecular weight of 17,800. TNF beta (lymphotoxin) which is derived from lymphocytes has a molecular weight of 18,700.

Both TNF alpha and TNF beta appear to be nearly identical with respect to their biological activities, their main property being the ability to kill transformed cells (Ruff and Gifford, 1980). TNF alpha and TNF beta in purified forms can stimulate bone resorption in vitro in calvarial systems as well as isolated osteoclast assays. The bone resorbing

effect is mediated through osteoblasts and is of similar potency to IL-1 and can be inhibited by IFN-gamma (Thomson et al., 1987; Bertolini et al., 1986; Gowen et al., 1986). Tashjian et al., (1987) have reported that the bone resorbing effect is mediated via prostaglandin production. TNF also inhibits bone collagen and non collagenous protein synthesis, an effect which can be inhibited by indomethacin and enhanced by IFN-gamma. Stimulation of DNA synthesis in whole calvaria is also seen with both forms of TNF.

These results indicate that in addition to IL-1, TNF may have important implications for bone synthesis and resorption.

6.5 OBJECTIVES OF THE STUDY-2

The purpose of the experiments described in this section was to explore the potential role of bacterial endotoxin and cytokines in the initial proliferation of the epithelial cells of Malassez, the formation and enlargement of the cyst. This will be presented in three interrelated investigations.

(i) The investigation for the presence of bacteria, endotoxins and cytokines in the fluid of both inflammatory radicular cysts and the developmental keratocysts and follicular cysts (Chapter 7).

(ii) The immunolocalisation of osteolytic cytokines and adhesion molecules in radicular cysts (Chapter 8).

(iii) The production of the osteolytic cytokines, and immunolocalisation of these cytokines in the developmental keratocysts (Chapter 9).

Chapter 7

Bacterial Endotoxins in the Generation of Odontogenic Cysts:

7.1 Introduction

There are two main groups of odontogenic cysts; the developmental cyst and inflammatory cyst. However all odontogenic cysts arise from the epithelial residues of the tooth-forming organ. The epithelial rests of the dental lamina (Serres) give rise to the odontogenic keratocyst, the reduced enamel epithelium gives rise to follicular and eruption cysts, and the inflammatory radicular cyst arises from the cell rests of Malassez.

In both the developmental and inflammatory cysts, the progressive destruction of bone is dependent both on the growth of the cyst and on its ability to degrade bone. Biomechanical theories of expansion, such as enlargement by hydrostatic pressure, ignore both the cellular aspects of cyst growth and the biochemistry of bone destruction. The production of prostaglandins (Harris et al., 1973), collagenase (Donoff et al., 1972) and interleukin-1 (Meghji et al., 1992) by the keratocyst wall, have helped to clarify the latter. The enlargement of the cyst, must also involve the interaction of the epithelium and the fibrous capsule. This was first demonstrated by Donoff et al., (1972) who showed collagenase activity in cultures of cyst explants only when both the epithelium and fibrous wall were present. We have shown that cyst fibroblasts produced eicosanoids and collagenase when cultured with mononuclear cell factor (Harvey et al., 1984). Furthermore, cyst epithelium synthesises interleukin-

1 and interleukin-6 (Meghji et al, 1992; Bando et al., 1993). Similarly the periapical granuloma is also capable of producing inflammatory mediators such as cytokines (Artese et al., 1991; and eicosanoids (McNicholas et al., 1991). Thus in vivo the epithelial cells and fibroblasts in the connective tissue capsule undoubtedly interact during growth. To explore this further, cyst wall explant media and fluids from 16 radicular cysts, 8 keratocysts and 7 follicular cysts were investigated for the presence of endotoxin and the cytokines, interleukin-1, tumour necrosis factor and interleukin-6. Cyst fluids were also cultured for 72h in anaerobic and aerobic conditions to detect the presence of micro-organisms. The effect of these cytokines, endotoxin and culture media from cyst fibroblasts on epithelial cell proliferation was also investigated.

7.2 Methods and Materials

7.2.1 Preparation of Cyst Explant Media

31 uninfected cysts (sixteen radicular, eight uninflamed keratocyst and seven uninflamed follicular) were enucleated under general anaesthesia. A portion of each was fixed for routine histology, and the remainder transported to the laboratory in sterile Hanks balanced salt solution at 4°C. Samples of cyst wall were minced using scalpels, into fragments approximately 1mm^3. These were placed in 25 cm^2 flasks (Sterilin) with 5 ml of Eagle's Modified Essential Medium (MEM; Gibco) buffered with bicarbonate (3.5g/l), and incubated for three days at 37°C in 5% CO_2, 95% air. On termination of the cultures the medium was

removed, dialysed against fresh MEM and stored at -70°C. The residual fragments of tissue were blotted and weighed. Four samples of normal gingiva, obtained from routine third molar extractions, were also subjected to the same procedures to provide a control.

7.2.2 Cyst Fluids

Cyst fluids were aspirated from 31 cysts centrifuged, aliquots cultured for 72h in anaerobic and aerobic conditions to detect the presence of micro-organisms, the remainder stored at -70°C. Additional aliquots of cyst fluids were also cultured for 72h in Wilkins Chalgren anaerobe agar (Oxoid) supplemented with 10% horse blood (Oxoid) to detect the presence of micro-organisms. Cyst fluid was also assayed for the presence of endotoxins and cytokines.

7.2.3 Endotoxin Assay

A commercially available kit was used for this assay (Alerchek, Portland, ME, USA). This assay is based on the phenomenon that Polymyxin B binds covalently to LPS. Briefly, Polymyxin B is covalently bound to plastic microwell strips; standards, controls and unknown samples were added to each well respectively and allowed to incubate 30 minutes. LPS-horse radish peroxidase conjugate was than added and the plates were further incubated for 5 minutes. Each well was than decanted and washed free of the reactants. Enzyme substrate was added to each well until colour developed (20 minutes). The reaction was stopped with 2M sulphuric

acid. The optical density was determined at 492nm using multichannel spectrophotometer (Titertek). The colour intensity was inversely proportional to the level of the endotoxin present in the standard or samples. The optical density of the standards were plotted against the concentration. By comparing optical density of the samples to this standard curve, the concentrations of endotoxins in the unknown samples was determined.

7.2.4 IL-1α, IL-1ß TNF and IL-6 immunoassay

A commercially available assay was used. (Quantikine, R&D Systems, Minneapolis) This assay is a solid phase ELISA, which employs the quantitative "sandwich" enzyme immunoassay technique. A monoclonal antibody specific for the particular cytokine was coated onto the microtitre plate provided in the kit. Standards and samples were pipetted into the wells so that any cytokine present would bind to the immobilized antibody. After washing away any unbound proteins, an enzyme-linked polyclonal antibody for the cytokine was added to the wells to sandwich the cytokine immobilized during the first incubation. This is followed by a wash to remove any unbound antibody-enzyme reagent. A substrate solution was then added to the wells and colour developed in proportion to the amount of cytokine bound in the initial step. The colour developed was stopped after 10-15 minutes and the intensity of the colour was measured at 450nm using microtitre plate reader (Titertek).

A standard curve was prepared, plotting the Optical Density versus the

concentration of the cytokine in the standard wells. By comparing the Optical Density of the samples to the standard curve, the concentration of the cytokine in the unknown samples was then determined.

7.2.5 Epithelial Cell Culture

A portion of the cyst wall was placed in a sterile tube containing trypsin (O.25) and collagenase (0.5mg/ml) in phosphate buffered saline (PBS) and stirred at 37°C for 20 minutes. The epithelium was carefully detached from the capsule finely chopped and placed in a 25cm^2 flask with 1.5 ml of MEM supplemented with FCS.

Although 20 radicular cysts were cultured in order to grow epithelial cells, it was not possible to do so. 10 out of 20 cysts did have epithelial cell outgrowths from the epithelium explants, but they were rapidly taken over by contaminating fibroblasts. Attempts were made to remove the fibroblasts, but this proved unsuccessful. Recent work by Hume et al., (1990) has shown that epithelial cells from radicular cysts were very difficult to grow. They found that they had more fibroblast growth from radicular cyst cultures compared to follicular cyst or keratocysts. They suggested that the radicular cyst epithelium did not grow well in culture because inflammatory mediators which are present in the cyst wall in vivo, were not present in vitro. This is confirmed by the present study, which shows that cytokines such at IL-1, IL-6 and TNF do stimulate epithelial cell proliferation.

As it was not possible to grow the cyst epithelial cells, it was necessary

to use an epithelial cell line for this study, (GPK, Hola et al., 1987).

7.2.6 Keratinocyte Culture and DNA Synthesis

A guinea pig keratinocyte cell line was kindly provided by Dr. M. Hola, Department of Biochemical Pathology, University College School of Medicine, London. The cells were grown in Eagles' MEM containing 10% foetal calf serum (FCS), 2mM L-glutamine, 2.25g/1 $NaHCO_3$, penicillin and streptomycin (each 100U/ml) and incubated at 37°C in 5% CO_2/air. Keratinocyte suspensions were seeded into 96-well culture plates (Microtitre, Linbro) at concentrations of 20,000 cells/well in 100μl of MEM with 10% FCS and left overnight to attach. The culture media was then replaced with MEM containing 2% FCS and various concentrations of either purified LPS from *P. gingivalis* and *A. actinomycetemcomitans* or cytokines, in groups of 6 wells per concentration. Control cultures received only unsupplemented medium. Cells were incubated for a further period of 24h and then 0.5μCi of 5-^{3}H-thymidine (specific activity 20Ci/mmol: Amersham) was added to each well 6h before termination of cultures. Trichloroacetic acid-insoluble material in each cell layer was dissolved in 0.4M NaOH and radioactivity measured in 3ml of scintillant by scintillation spectrometry (Rackbeta: LKB) with external standardization.

7.2.7 Cyst Fibroblast Culture

Fibroblasts were grown from cyst explants in Eagles' MEM containing 10% foetal calf serum (FCS), 2mM L-glutamine, 2.25g/l $NaHCO_3$, penicillin and

streptomycin (each 100U/ml) and incubated at 37°C in 5% CO_2/air. Cells were subcultured at weekly intervals and used for experiments between passages 6 and 12.

7.2.8 Preparation of Fibroblast Culture Medium

Fibroblast suspensions were seeded into 75cm^2 culture flasks (ICN Flow) at concentrations of 10 million cells in 20 ml of MEM with 10% FCS and left overnight to attach. The culture medium was then replaced with serum free MEM containing 1 μg/ml purified LPS from *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans* or *E. coli* and or indomethacin (1μM). Control cultures received only unsupplemented medium. Cells were incubated for a further period of 96h at 37°C in 5%CO_2/95% air. On termination of the cultures the medium was removed, centrifuged and stored at -70°C.

7.3 RESULTS

7.3.1 Histology of Cysts

In H&E stained sections, the 16 radicular cyst specimens exhibited the typical histology of a radicular cyst with various degrees of epithelial cell proliferation and leukocytic infiltration. 8 Keratocysts, 7 follicular cysts and the 4 specimens of healthy gingiva were uninflamed.

7.3.2 Bacteriology of Cysts

All the cysts investigated were uninfected. Culture of cyst fluid did not

yield any bacteria (aerobes or anaerobes).

7.3.3 Content of Cyst Fluid and Explant Media

7.3.3.1 Endotoxins

The results showed significantly higher concentrations of endotoxin in radicular cyst fluids (mean; 5.5±1.8ng/ml) than in keratocysts (mean; 0.04±0.05ng/ml) or in follicular cyst (mean; 0.06±0.1ng/ml). (Figure 23). Both the cyst explant media and the gingival explant media did not contain any endotoxin.

7.3.3.2 Concentration of Osteolytic Cytokines in Cyst Fluids and Media

The results showed that all the cyst fluids and explants contained immunoreactive cytokines (Table 3). There was no difference between amount of IL-1α in the fluids from the follicular and radicular cysts, however keratocyst fluid contained significantly more IL-1α than the other cysts. Only the radicular cyst fluid contained IL-1β. All three cyst types had significant amount of IL-6. None of the fluids had measurable TNFα. Measurement of the media supporting explants of cyst tissue revealed that the keratocyst explant media contained significantly more IL1α compared to the other cyst types. Only the radicular cyst explant media contained IL1β. All cyst explant media had IL-6 present, however of the results showed that inflammatory radicular cysts produced significantly more IL-6 than did the developmental cysts: follicular cysts and keratocysts. None of the cyst explants contained any TNFα. The gingival explant media contained minimal amounts of cytokines. (Table 3).

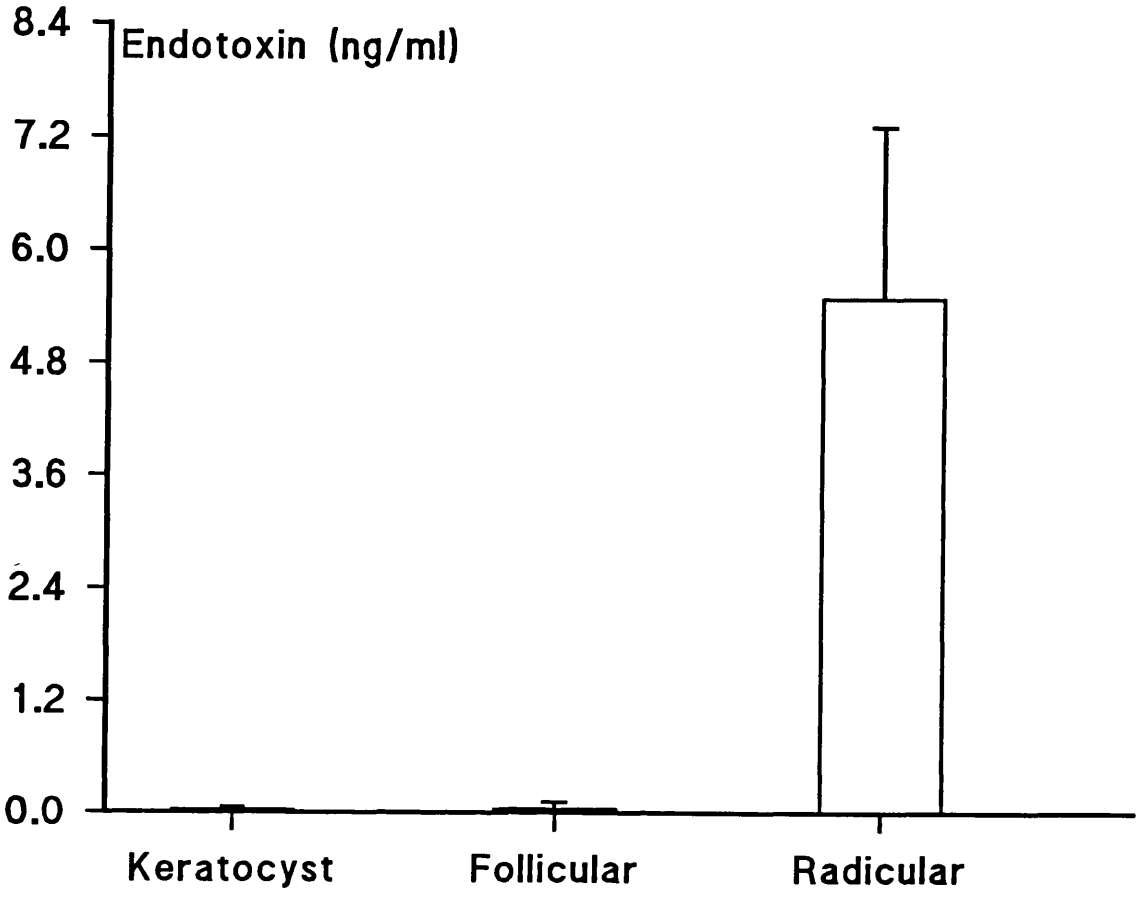


Figure 23 Bar-chart showing the levels of endotoxin in cyst fluid from 8 keratocysts, 8 follicular cysts, 16 radicular cysts.

Table 3: Summary of the Cytokines: levels in Cyst Fluids, Cyst Explant Media and control gingival tissue explant media.

	IL-1β mean (ng/ml$\pm$sd)	IL-1α mean (ng/ml$\pm$sd)	IL-6 mean (ng/ml$\pm$sd)
Keratocyst fluid	0.078 $\pm$0.058	9.60* $\pm$5.42	5.07 $\pm$0.84
Keratocyst explants	0.046 $\pm$0.016	4.18 $\pm$1.32	2.55 $\pm$0.55
Follicular fluid	0.046 $\pm$0.054	5.18 $\pm$1.13	5.31 $\pm$0.68
Follicular explants	0.044 $\pm$0.042	2.81 $\pm$0.47	2.07 $\pm$0.78
Radicular fluid	5.53* $\pm$2.5	5.29 $\pm$0.98	5.12 $\pm$0.85
Radicular explants	2.73* $\pm$1.082	3.10 $\pm$1.15	3.73 $\pm$0.47
Gingival explants	0.032 $\pm$0.048	0.04 $\pm$0.03	0.56 $\pm$0.26

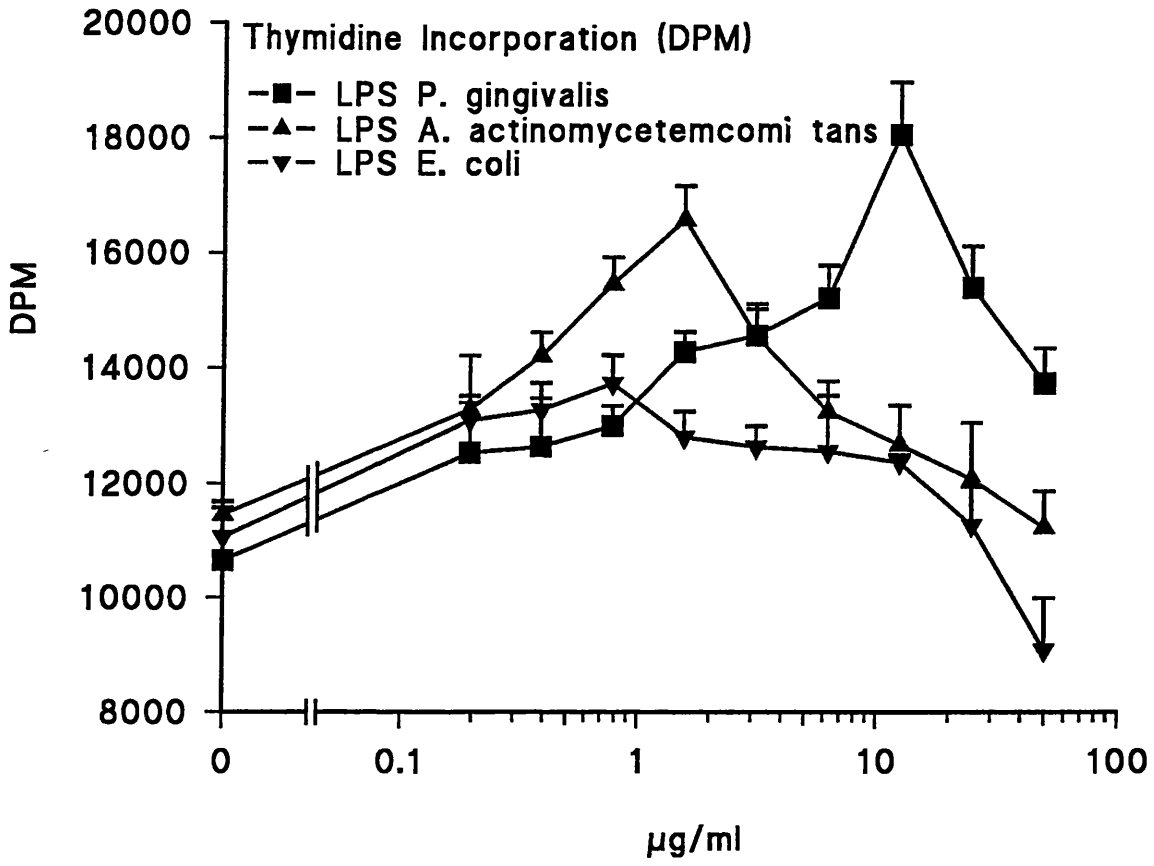


Figure 24 The effect of increasing concentrations of LPS from *A. actinomycetemcomitans (triangle), P. gingivalis (square) or E. coli (inverted triangle)* on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by epithelial cells. Results are expressed as the mean and standard deviation of 6 cultures.

7.3.4 Effect of Endotoxin (LPS) on Keratinocyte Proliferation

LPS from *A. actinomycetemcomitans* and *P. gingivalis*, *E. coli* were tested at concentrations ranging from 0-50μg/ml. All three LPS stimulated keratinocyte proliferation in a dose related manner. LPS from *A. actinomycetemcomitans* significantly stimulated ^{3}H-thymidine incorporation over the concentration range 0.1-10μg/ml with maximal stimulation at 1.5ug/ml (44%). LPS from *P. gingivalis* also stimulated ^{3}H-thymidine incorporation with maximal stimulation at 12.5μg/ml (70%). In contrast LPS from *E. coli* induced a slight stimulation at low concentrations (18% at 1 μg/ml).

7.3.5 The Effect of Cytokines and Prostaglandin E_2

Interleukin-1, interleukin-6, tumour necrosis factor and prostaglandin E_2 were tested on keratinocyte proliferation. All three cytokines stimulated keratinocyte proliferation in a bell shaped dose related manner (Figs. 25a, b and c) Interleukin-1 was tested over the range 0-10ng/ml and gave maximal stimulation at 1.25ng/ml with an increase of 121.55%. (Fig 25a). Tumour necrosis factor alpha was tested over the range of 0-50ng/ml, with maximal stimulation at 3.125ng/ml. (Fig. 25b). Interleukin-6 was tested over the range of 0-500U/ml, with maximal stimulation at 62.5U/ml (145.51%). Significant stimulation was obtained at 0.29U/ml, (Fig. 25c).

In contrast PGE_2 only had no effect on proliferation of keratinocytes, and

actually induced cytotoxicity at 10μM (Fig 26).

7.3.6 The Effect of Fibroblast Culture Media on Keratinocyte Proliferation

Fibroblast culture medium, and media from fibroblasts cultured in the presence of indomethacin (1μM), LPS from *P. gingivalis* (0.1μg/ml) and combination of both indomethacin and LPS were tested for their ability to induce keratinocyte proliferation at concentrations of 0-50%.

Increasing concentrations of fibroblast culture medium had increasing proliferative effect on keratinocytes (Fig 27), addition of indomethacin had a marked increase in the proliferation of these cells, giving 146% increase at 50% dilution of the culture medium compared to an increase of 60% by untreated medium.

Addition of LPS to the culture medium (with indomethacin) supporting the fibroblasts had a marked increase in stimulating the keratinocytes to proliferate, giving an increase of 222% at a dilution of 50% of the culture medium.

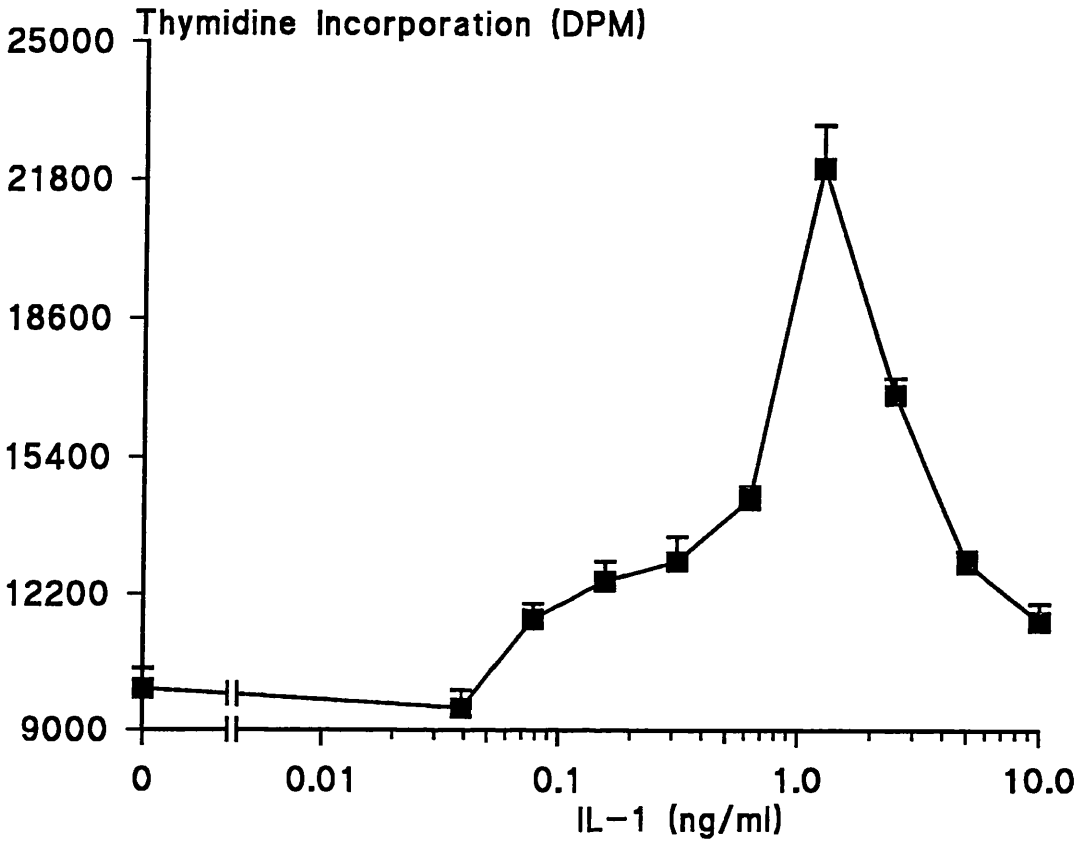


Figure 25a The effect of increasing concentrations of IL-1 on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by epithelial cells.

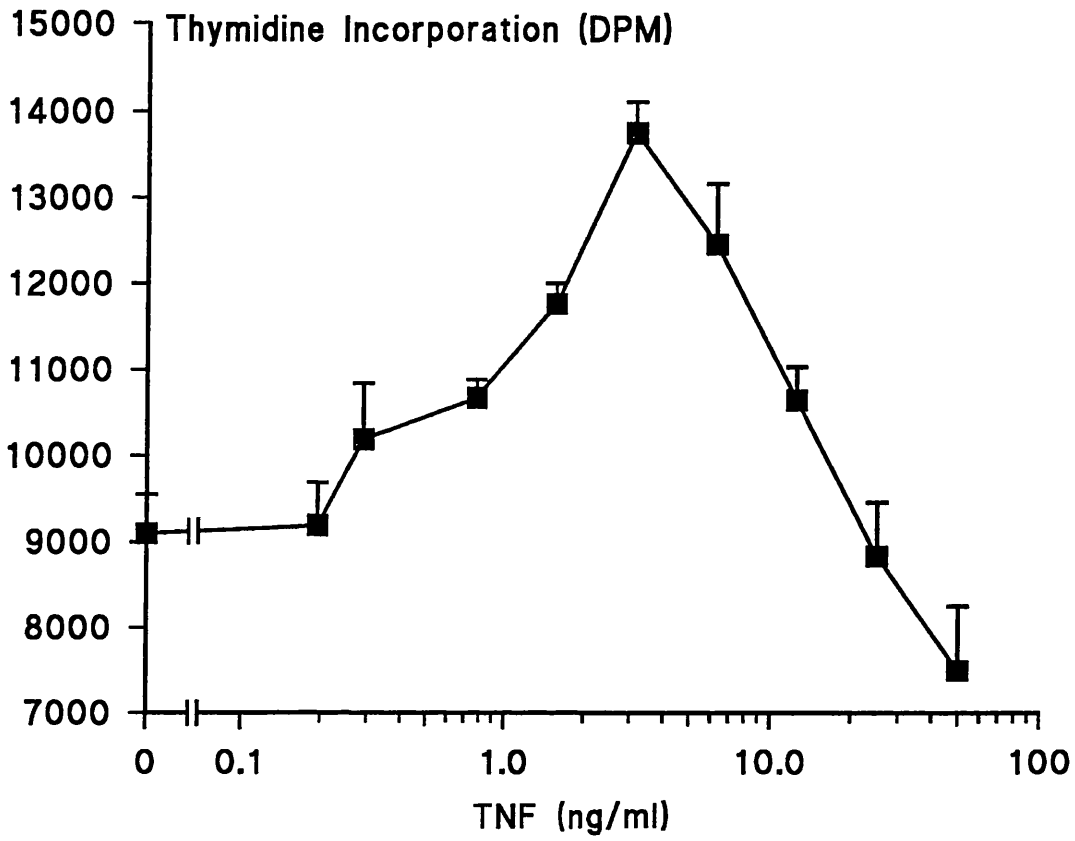


Figure 25b The effect of increasing concentrations of TNF on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by epithelial cells.

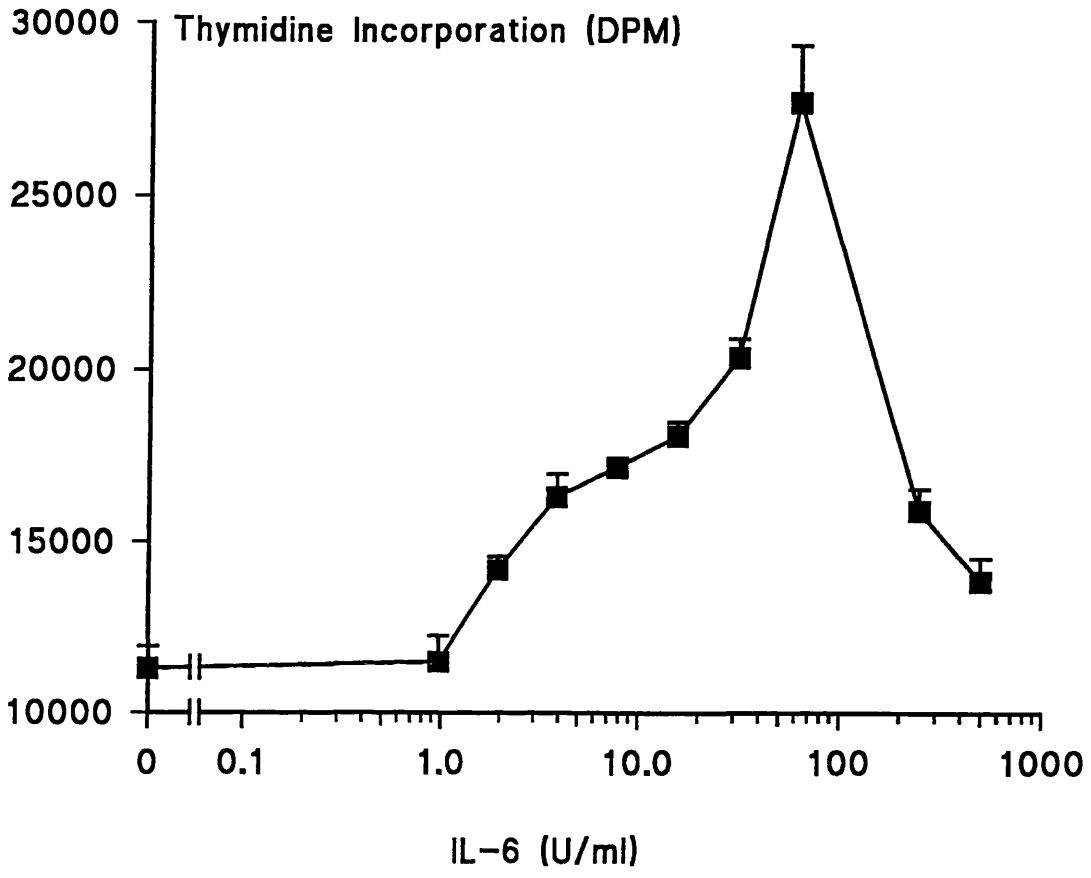


Figure 25c The effect of increasing concentrations of IL-6 on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by epithelial cells.

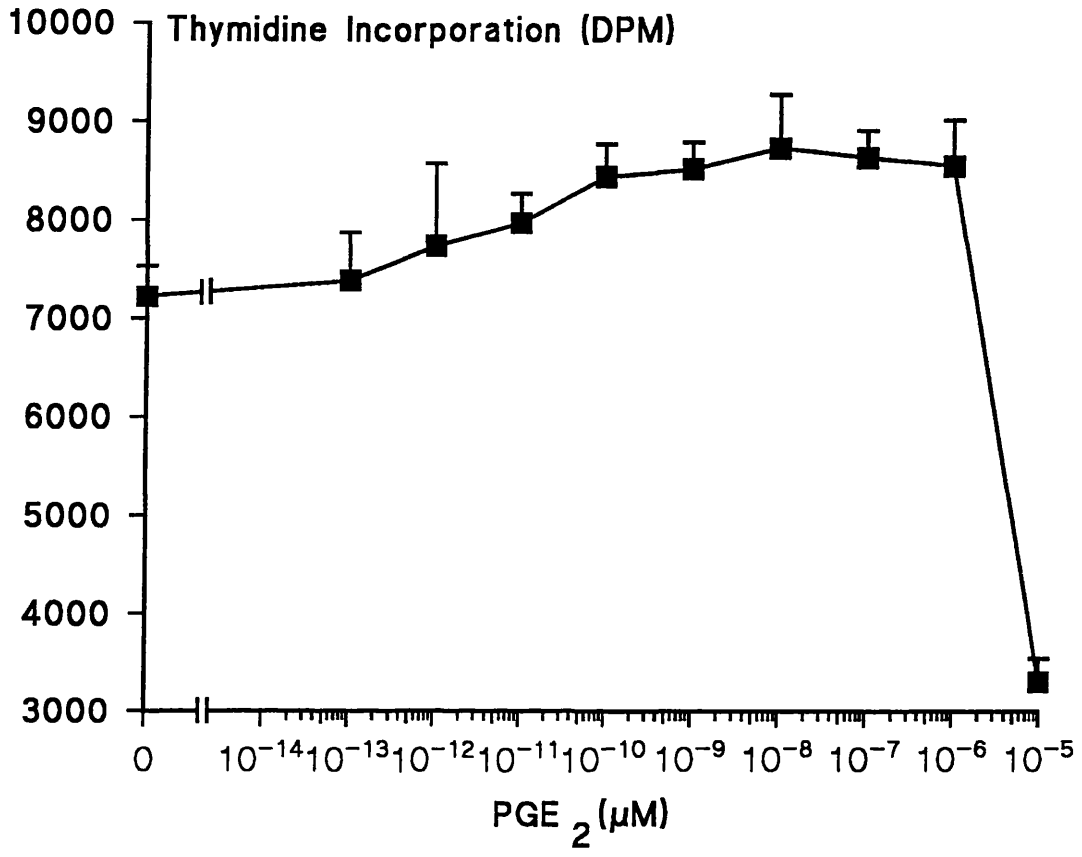


Figure 26

The effect of increasing concentrations of PGE_2 on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by epithelial cells. Results are expressed as the mean and standard deviation of 6 cultures.

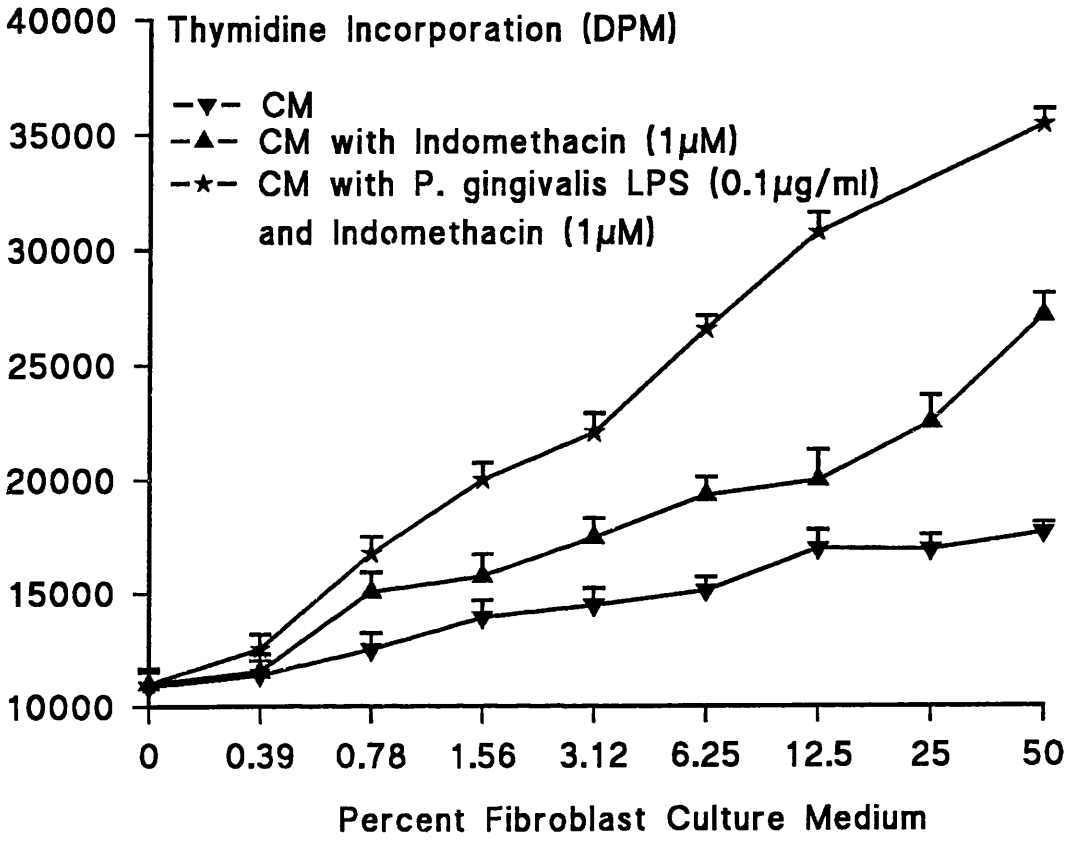


Figure 27

The effect of increasing concentrations of fibroblast culture medium (inverted triangle) and medium from fibroblasts cultured in the presence of indomethacin (1μM; triangle), LPS (0.1 μg/ml; star) on DNA synthesis. Measured as incorporation of tritiated thymidine into DNA by epithelial cells.

7.4 DISCUSSION

In the present studies, endotoxin was found to be present in relatively large quantities only in the radicular cyst fluid. The keratocyst and the follicular cysts fluids did not contain significant levels of endotoxins, nor did the cyst explant and non-inflamed gingival tissue explants. Interestingly all the cysts fluids were microbiologically sterile.

This demonstration of endotoxin in radicular cyst fluid has significant implications for an understanding of the etiology of these lesions, particularly in the light of the finding that these lesions are usually sterile. It has been speculated for many years that endotoxins may be involved in the inflammatory and immunological response of periapical tissue to root canal infection. Indeed, relatively high concentrations of endotoxins have been found in the canals of teeth with necrotic pulp (Schein and Schilder, 1975; Dahlen and Bergenholtz, 1980) and in periapical granuloma (Schonfeld et al., 1982). In the periapical granuloma study the presence of endotoxin was found to be correlated with the presence of inflammation (Schonfeld et al., 1982).

From these results it can proposed that bacterial components play a major initiating role in the pathogenesis of the radicular cyst. What remains unexplained is that the cyst fluid is usually sterile. Indeed Walton and Ardjmand (1992) have shown, in a recent study of induced periapical lesions in monkeys, that bacterial organisms are only present on the tooth surfaces and canals, not in the periapical tissues. This suggests that an important initiating factor is a relatively sparse bacterial

presence within the pulp canal eliminated at the apex by the cyst's "immune system". The endotoxins and other bacterial products however escape into the apical tissues from the root canal, and promote epithelial proliferation, essential for the formation of the cysts, and in doing so get entrapped in its lumen. As to why these endotoxins are not broken down by the host defence mechanism, it is not known.

By contrast, the initiating stimulus in the keratocyst or the follicular cyst is not known.

However, once established, all odontogenic cysts have common metabolic mechanisms; for instance, all cysts are capable of synthesising prostanoids (Harris et al., 1973), leukotrienes (Matejeka et al., 1984, interleukin-1 (Meghji, Harvey and Harris, 1989, interleukin-6 (Meghji et al., 1992, Bando et al., 1993), collagenase (Donoff et al., 1984, Harvey et al., 1984). This is confirmed by the results of this study which also shows that both the developmental and inflammatory cyst fluid appear to contain similar cytokines, and the cyst explants also seem to synthesise the same cytokines. Thus, although the stimuli initiating epithelial proliferation are different the same common growth pathway may be involved.

The results in this study have shown that endotoxin stimulates epithelial cell proliferation. However, except for LPS from *P. gingivalis* are only moderately it potent stimuli for epithelial cells proliferation (Fig.

24). Moreover, high concentrations actually had a inhibitory effect on this proliferation. On the other hand the cytokines IL-1 and IL-6 which are present in all cyst fluids are potent stimulators of epithelial cell proliferation. One important observation is the PGE_2 inhibitory effect (Fig 26) on epithelial growth.

In vivo the epithelial cell lining is undoubtedly under the influence of the adjacent connective tissue wall. This was first explored by studying the effect of LPS on cyst fibroblasts in culture. The fibroblast culture media were then tested for their ability to induce keratinocyte proliferation at concentrations of 0-50%. Increasing concentrations of fibroblast culture media had increasing proliferative effect on keratinocytes. The addition of indomethacin to these fibroblast cultures to eliminate prostaglandins significantly enhanced the proliferation of these cells. This confirmed the anti-proliferative effect of prostanoids on the epithelium. Fibroblasts are capable of synthesising a number of growth factors including; keratinocyte growth factor (KGF), epidermal growth factor (EGF) interleukin-6 (Bartold and Haynes 1991), interleukin-1 and fibroblast growth factor. The most likely candidates for this proliferative effect are likely to be KGF (Rubin et al., 1988) , IL-1, IL-6 and EGF. Indeed the epithelial cells from both follicular cysts and keratocysts are known to have EGF receptors (Shrestha et al., 1992).
Only the radicular cyst fluid and explant contained both IL-1α and IL-1β.

This could be due to the presence of endotoxin in the cyst fluid. Keratocysts contain approximately twice the amount of IL-α, compared to follicular and radicular cysts, this could reflect the benign neoplastic nature of this lesion which tends to recur with greater frequency than other types of odontogenic cyst (Brannon, 1976). It may also be a manifestation of a wider syndrome, the basal cell naevus syndrome (Gorlin and Goltz, 1960). Recent work has suggested that Gorlin's syndrome which includes the formation of multiple keratocysts is due to absence of a gene which controls cell growth (Farndon et al., 1992).

These findings contribute to our understanding of the pathogenesis of odontogenic cysts. The keratocyst has been previously shown to synthesise large quantities of IL-1 (Meghji et al., 1992) but again the mechanism is not known. It could be due to the "benign neoplastic nature" of the keratinocyte epithelium in this lesion.

One major problem in explaining the pathogenesis of the keratocyst and follicular cyst in terms of cytokine expression and action is the question of the stimuli promoting the cytokine synthesis. Both the keratocysts and follicular cysts are classically defined as non-inflammatory developmental cysts, without any overt involvement of bacterial products or inflammatory cells in the process of cyst growth. Their occurrence from the ubiquitous cell rests of the dental lamina and reduced enamel epithelium remain unexplained.

Furthermore, endotoxins commonly cause granuloma formation and

progress to abscess formation. Perhaps the factor that relates the radicular cysts to other odontogenic cysts is again the absence of the tumour suppressor gene in the cell rests of Malassez ie those patients with a protective gene develop granulomas and abscesses rather than the cysts.

Chapter 8

The Immunocytochemical Localization of Inflammatory Cytokines and Vascular Adhesion Receptors in Radicular Cysts

8.1 Introduction

Histologically the cyst lumen is lined by stratified squamous epithelium which arises from the epithelial rests of Ma lasez. The major part of the cyst wall is composed of condensed connective tissue containing a chronic inflammatory cell infiltrate. The growth of odontogenic cysts is accompanied by, and is presumed to be limited by, local bone destruction. The mechanism resulting in this osteolysis is only partially determined. In the last decade it has been established that various cytokines including: interleukin-1 (IL-1; Gowen et al., 1983); tumour necrosis factor (TNF; Bertolini et al., 1986) and interleukin-6 (IL-6; Roodman, 1992) are potent modulators of bone remodelling. Work from this laboratory has demonstrated that cyst explant supernatant, which was undoubtedly a mixture of cytokines, potently promotes wall fibroblast to release prostanoids (Meghji, 1989). It is also known that explants of cysts produce IL-1 (Meghji, Harvey, Harris, 1989). This cytokine-eicosanoid interaction maybe a crucial factor which accounts for the bone destruction and cyst growth. However, the cellular source of IL-1 and other cytokines has not been defined.

In this present study a panel of antibodies to the human cytokines described above have been used to immunolocalize these proteins within radicular cysts removed at operation. The presence of the leukocyte

chemotactic cytokine -interleukin-8 has also been sought. In addition antibodies to the vascular endothelial cell adhesion receptors; ICAM-1 (intercellular adhesion molecule) and ELAM-1 (endothelial leukocyte adhesion molecule: also termed E-selectin) were also used to determine the activation state of cyst blood vessels. These adhesion molecules are responsible for the selective and short-term adherence of blood-borne leukocytes to vascular endothelium and the extracellular matrix. This adherence, transendothelial migration and retention of leukocytes at sites of inflammation and wound healing is upregulated by cytokines.

8.2 Materials and Methods

8.2.1 Tissues

12 dental cysts were obtained from patients undergoing surgery at the Eastman Dental Hospital and were confirmed as radicular cysts on the basis of clinical and histological criteria. In order to determine the specificity of the immunocytochemical staining in lesional tissues, specimens of normal gingiva and normal buccal mucosa were included in this study. Two periapical granulomas were also investigated for comparative purposes.

8.2.1.1 Tissue Preparation

Fresh samples of lesional or normal tissues were snap frozen in liquid nitrogen and then stored at -70°C. Frozen sections were sectioned at 5μm in a Bright cryostat with the cabinet temperature maintained at below -25°C and taken up onto glass slides treated with APES (3-

aminopropyltriethoxy silane). Slides were air dried at room temperature overnight and then fixed in acetone for 20 min prior to use. From each block serial sections were stained with haematoxylin and eosin to determine histology.

8.2.2 Antibodies

Sheep polyclonal antisera to human recombinant IL-1 alpha (S76a BM) and beta (S77b BM, Poole and Gaines Das, 1991) and a goat antiserum to human recombinant IL-6 (G150 BM; Rafferty et al., 1991) were raised by Dr. S. Poole, NIBS). Murine monoclonal antibodies to human IL-8 (14E4, 46E5) were obtained from Dr JM Schroder (Department of Dermatology, Kiel University, FRG). A mouse anti-human recombinant TNF alpha (52B83) was provided by Dr Mark Bodmer (Celltech, Slough). Murine monoclonal antibodies to ICAM-1 (6.5B5) and ELAM-1 (1.2B6) were obtained from Dr Dorian Haskard (Royal Postgraduate Medical School, Hammersmith Hospital, London).

In addition, monoclonal antibodies to: (i) von Willebrand factor (factor VIII); (ii) CD68 (anti-macrophage); (iii) CD14 (anti-monocyte); (iv) fibroblasts (5B5) and CD3 (T cells) were obtained from DAKO (High Wycombe). The optimal dilutions of each antibody were determined and used in all subsequent studies.

8.2.3 Immunocytochemistry

After fixation in acetone, sections were briefly washed in 0.1M Tris buffer pH 7.6 and then treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30min to block endogenous peroxidase. After washing in Tris

buffer, sections were then treated with a 1:50 dilution of normal donkey (Sigma) or normal rabbit serum (Sigma) for 30min to block non-specific binding of conjugated secondary antibodies. After blocking, optimal dilutions of the various antibodies described above were added to sections and incubated for 2h at room temperature. Sections were again washed with Tris buffer and secondary antibodies (biotinylated donkey anti-sheep - Sigma diluted 1:5,000 or biotinylated rabbit anti-mouse -Sigma diluted 1:100) added and left for 30min at room temperature. After a further wash in buffer, a streptavidin-peroxidase complex (Strep ABC: DAKO) was added and sections incubated for a further 30min. After a final wash a solution of 3,3-diaminobenzidine (DAB) plus hydrogen peroxide was added to sections and incubated for 3-5min at room temperature. Sections were counterstained with Mayer's haematoxylin and examined microscopically.

8.2.3.1 Controls

For each specimen, serial sections were incubated either without primary antibody or by substitution of the primary antiserum with pre-immune sera (sheep) or non-immune sera (mouse). Reactivity in the absence of the secondary conjugated antibody was also assessed to determine the specificity of binding. A further check of antibody specificity, where sufficient amount of cytokine was available, was to incubate the specific antibody overnight with the cytokine at 4°C and then use the complex as described above.

Antibody Dilution:

IL-1α: 1/100, IL-1ß: 1/50, IL-6: 1/50, TNFα: 1/20, ICAM: 1/20, E-selectin: 1/25.

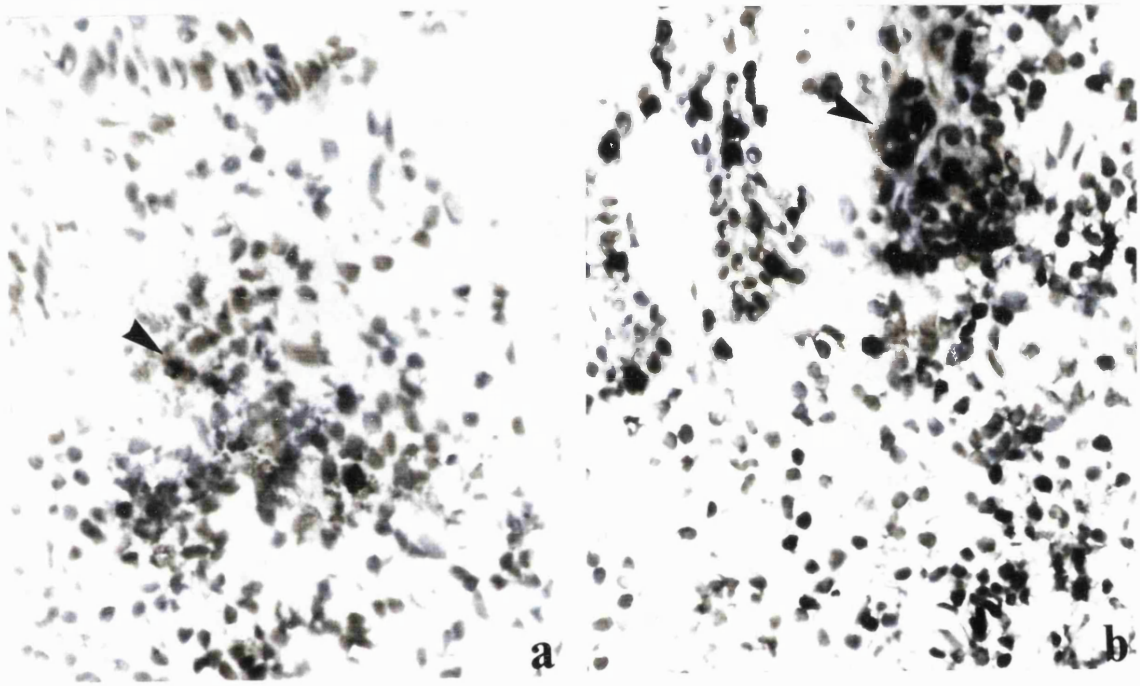


Figure 28

a) CD68 positive macrophages (arrowed) present in the cyst wall near to the epithelial cell layer (magn x 80)

b) CD3 positive T lymphocytes (arrowed) found both within and near to the epithelial cell layer (magn x 80).

8.3 Results

8.3.1 Histology: In H&E stained sections, the 12 cyst specimens exhibited the typical histology of a radicular cyst with various degrees of epithelial cell proliferation and leukocytic infiltration. The antibodies to monocytes (anti-CD14), macrophages (anti-CD68) or T cells (anti-CD3) revealed that the specimens contained few cells expressing CD14 but that, in general, there were significant numbers of CD68 and CD3 expressing cells present (Fig 28).

The two periapical granulomas were composed of fibrous connective tissue with an extensive blood supply and the infiltrate consisted of macrophages, T cells and plasma cells.

Neither specimen showed the presence of epithelial cells, ie. cell-rests of Malassez. Normal gingival tissue was composed of fibrous connective tissue with a scattering of leukocytes covered with stratified squamous epithelium. The normal buccal mucosa specimens were normal in appearance and contained no significant evidence of leukocytic infiltration.

8.3.2 Immunocytochemistry: With each sample of tissue serial sections were reacted without the primary antibody or with substitution of the primary antibody by pre-immune or non-immune sera. In each case this resulted in no positive staining in sections. Preabsorption of the anti-IL-1α antiserum S76A BM with recombinant IL-1α (a kind gift of Dr U. Gubler, Hoffman LaRoche Ltd USA) completely abolished specific IL-1α staining but not IL-1ß or IL-6 staining in specimens.

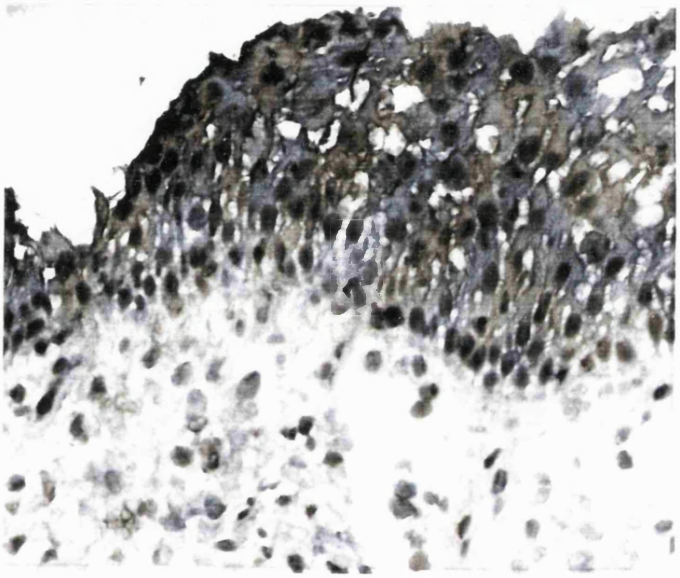


Figure 29 Interleukin-1α staining in a radicular cyst. Strong specific staining is seen confined to the epithelial cell layer (magn x 80).

Figure 30a and b Blood vessels adjacent to the epithelial cell layer (arrowed) showing IL-1α staining in the vascular endothelial cell population. In fig 30a the arrowed blood vessel is surrounded by infiltrating leukocytes (magn x 80)

8.3.2.1 Cytokine Localization

IL-1α - Specific strong immunocytochemical staining for IL-1α was found in the epithelial layer of the cyst wall in all specimens (Fig 29). In 8 of the 12 specimens positive staining was also found in the endothelial cells of some blood vessels (Figs 30a and b). IL-1α-stained blood vessels tended to be associated with infiltrating inflammatory cells or near to the epithelial layer. There was no IL-1α staining in the infiltrating leukocytes or in fibroblast-like cells.

Only small numbers of slightly positive stained blood vessels were noted in the two specimens of periapical granulomas.

Normal gingiva showed slight staining in the surface epithelial cell layer. However, no staining was seen in the epithelium, blood vessels or other cell populations in specimens of normal buccal mucosa

IL-1ß - In a similar manner to IL-1α, strong positive staining was found in the epithelium in all specimens (Fig 31). In 6 of the 12 specimens there was also staining in blood vessels, again only those near the epithelium or surrounded by infiltrating leukocytes. Only one specimen showed positive staining in fibroblast-like cells. Periapical granulomas had a few positively stained blood vessels. Normal gingiva showed slight positive staining in the surface cells of the epithelium but there was no staining in any cell population in normal buccal mucosa.

IL-6 - All 12 specimens demonstrated strong positive staining in the epithelial cell layer (Fig 32a). As with IL-1, positive IL-6 staining was also found in the vascular endothelial cells in 8 specimens (Fig 32b). These

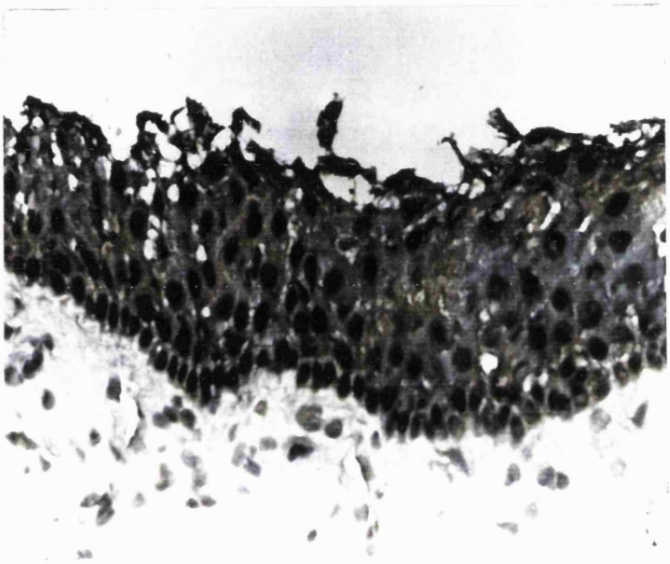


Figure 31 IL-1ß staining in a specimen of radicular cyst. Strong specific staining is found in the epithelial cell layer (magn x 80).

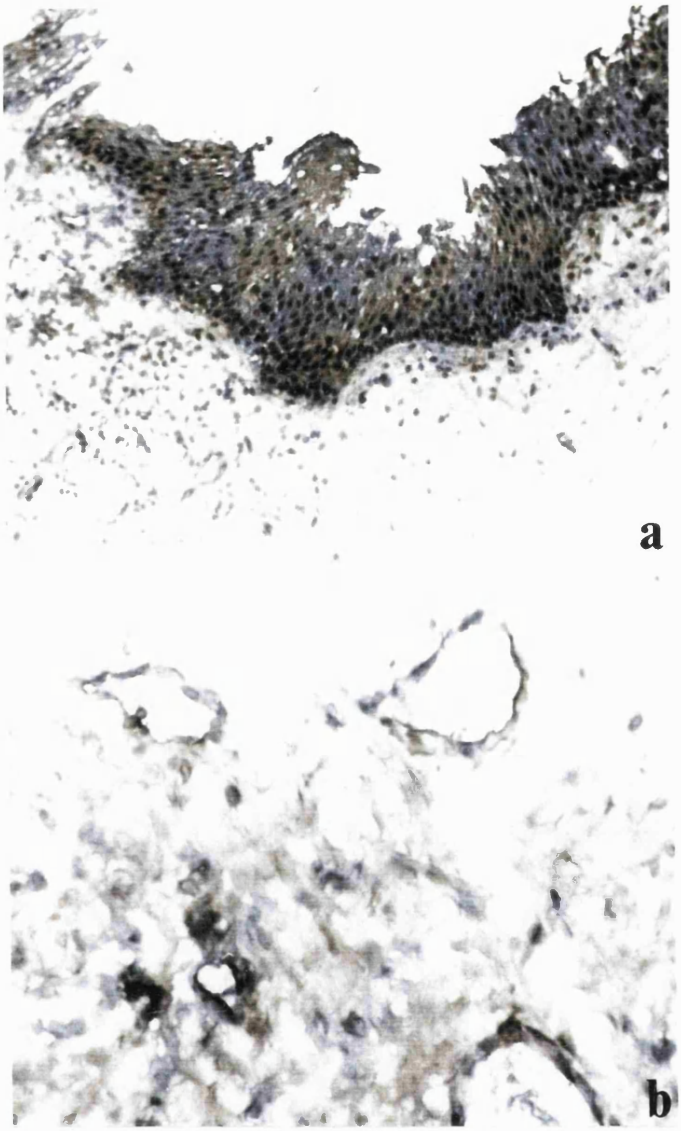


Figure 32

(a) IL-6 staining in a specimen of radicular cyst. Strong specific staining is seen in the epithelial cell layer (magn x 32).

(b) IL-6 staining in vascular endothelial cells in a number of closely spaced blood vessels in a specimen of radicular cyst (magn x 80).

positively-stained vessels tended to be more extensively distributed throughout the cyst than was seen with the IL-1 immunostaining in blood vessels which tended to be more concentrated near to the epithelium. There was no staining in infiltrating leukocytes or in fibroblast-like cells. Periapical granulomas showed positive staining only in a few blood vessels.

Normal gingiva showed slight staining in the surface cell layer of the epithelium and in a few vascular endothelial cell adjacent to the epithelium. Normal buccal mucosa showed no staining in any cell population.

TNFα - Positive staining for TNFα was found only in two cases where macrophage-like cells contained reaction product. These cells were immediately adjacent to the epithelial cell layer (Fig 33). There were no positively stained cells in the periapical granulomas, or in normal gingiva or buccal mucosa.

IL-8 - Positive IL-8 staining was only found in the two specimens above which also showed TNFα staining. Again positive reaction product was found in macrophage-like cells.

8.3.3 Vascular Endothelial Cell Reactivity

ICAM-1 - All cysts contained large numbers of ICAM-1 positive capillaries and venules mainly situated near the epithelial layer. In addition ICAM-1 staining was also found in the infiltrating leukocyte populations (Fig 34a). All specimens also demonstrated specific ICAM-1 staining of the epithelial cell layer (Fig 34b). By contrast small numbers of ICAM-1-positive vessels were noted in periapical granulomas and in normal gingiva and buccal

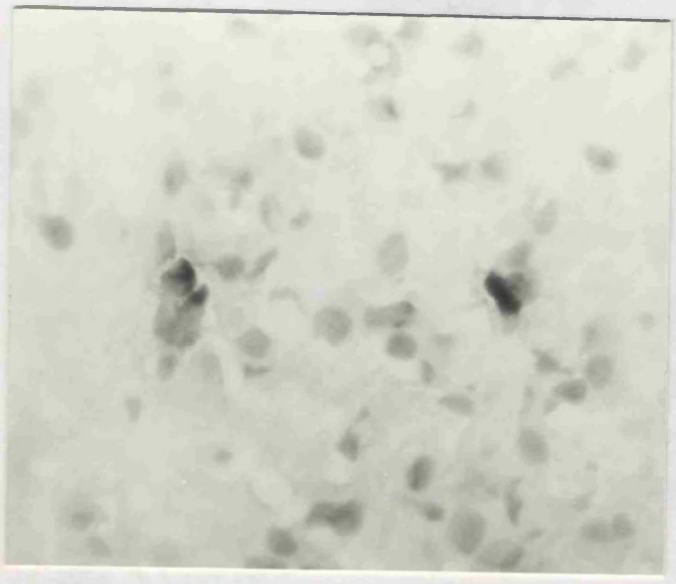


Figure 33 TNF immunostaining in macrophages in a specimen of radicular cyst. These cells were adjacent to the epithelial cell layer (magn x 128).

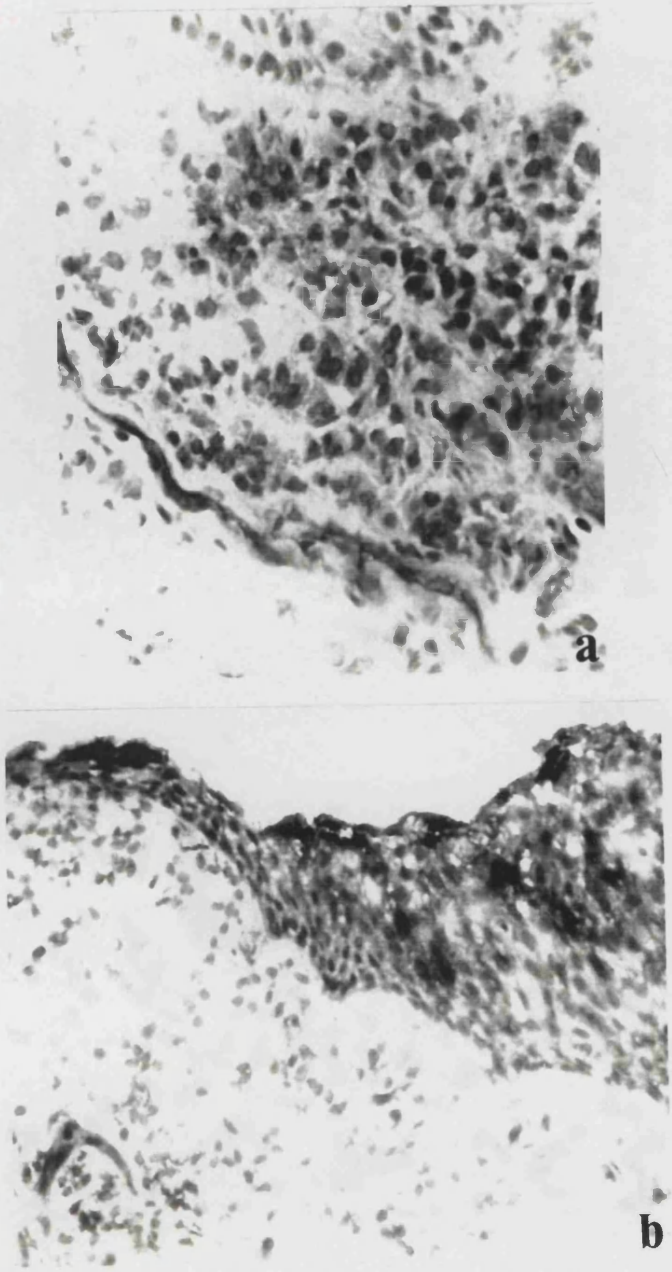


Figure 34
(a) Immunocytochemical localization of ICAM-1 in vascular endothelial cells of small blood vessels (large arrows) and in the infiltrating leukocytes which have presumably extravasated from these vessels (small arrows) in a specimen of radicular cyst (magn x 80)
(b) Strong ICAM-1 positive staining in the epithelial cell layer of a specimen of a radicular cyst (magn x 80).

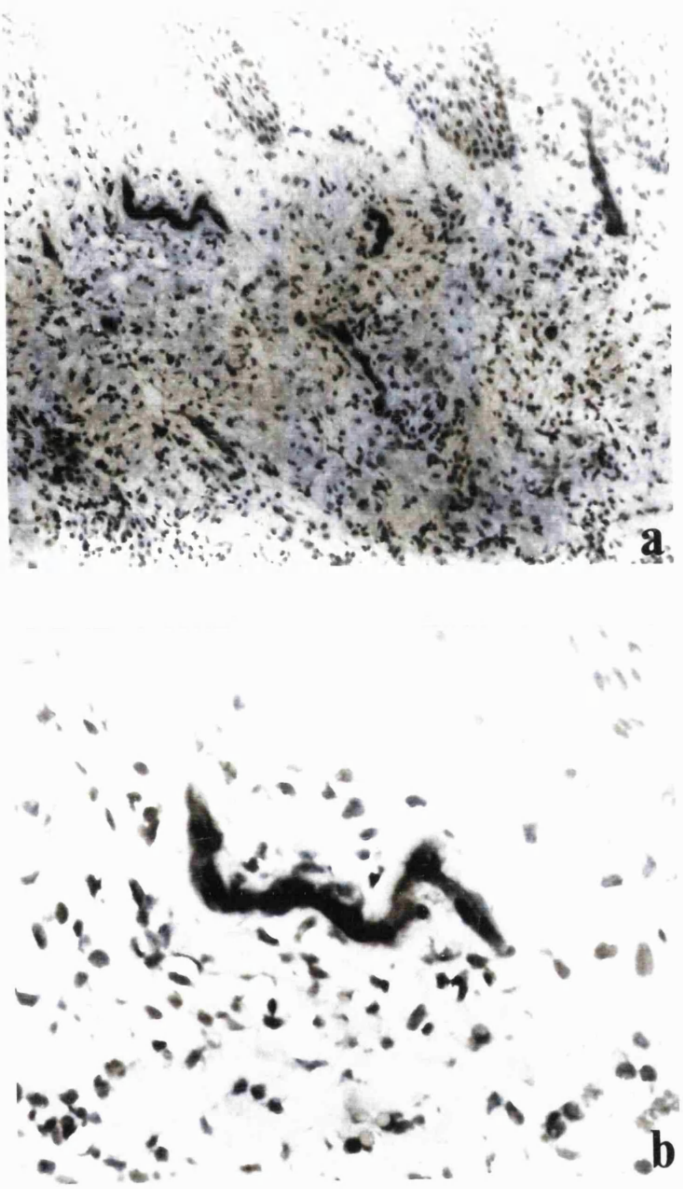


Figure 35a and b Low (x 32) and high (x 80) power micrographs of a radicular cyst showing the location of ELAM-1 positive blood vessels.

mucosa. However the epithelial layer in these normal tissues did not react with the anti-ICAM-1 antibody.

ELAM-1 - As with ICAM-1, large numbers of capillaries and venules adjacent to the epithelial cell layer in all cyst specimens showed positive staining (Figs 35a and b). A few blood vessels in the periapical granulomas and in normal oral tissues also showed positive staining.

The proportion of blood vessels in cysts staining with both antibodies was estimated. The total numbers of blood vessels in sections was determined by counting all factor VIII-positive vessels. The number of ICAM-1 and ELAM staining vessels were then enumerated in serial sections and the percent positive vessels calculated. The proportion of vessels showing positive ICAM-1 staining ranged from 19-32% and for ELAM-1 18-42%.

8.4 Discussion

Histopathological examination of radicular cysts showsthat they contain a number of infiltrating leukocyte populations including macrophages, CD4 and CD8 positive T cells and plasma cells (Rafferty et al., 1988). In some specimens the epithelium is HLA-DR positive (Matthews and Browne, 1987). Thus the radicular cyst has the features of a chronic inflammatory lesion. This pattern of cellular infiltration has been presumed to be the result of localized infection even though bacteria have not been isolated from the cyst lumen.

The signals that turn a periapical granuloma into a cyst which destroys bone have been discussed earlier. It is known that the cysts are associated with the production of prostanoids (Harris et al., 1973). Cyst explants were shown to resorb murine calvarial bone and this could be partially inhibited by indomethacin. We have subsequently shown that explants of radicular cysts produce the potent osteolytic cytokine - IL-1 (Meghji, Harvey and Harris. The cellular source of this cytokine was thought to be the infiltrating macrophages but other cell populations such as fibroblasts, vascular endothelial cells or even epithelial cells could be involved. Other cytokines implicated in the inflammatory response also have osteolytic activity. These include: TNFα and TNFß (lymphotoxin) and IL-6. These may also have been produced by either infiltrating cells or resident cells of the cyst.

To determine the range of cytokines produced by cyst tissue and their cellular source, sections of radicular cysts have been reacted with specific

antibodies to a number of cytokines including : IL-1 (α and ß), TNFα, IL-6 and the small chemotactic cytokine IL-8 (Oppenheim et al., 1991). These cytokines have been immunolocalized using immunoperoxidase cytochemistry in 12 specimens of radicular cysts. The cysts investigated here were all inflamed but with various degrees of leukocytic infiltrate. Immunocytochemical staining of the cellular infiltrate with antibodies to CD14 (monocytes), CD68 (macrophages) or CD3 (pan T cell) showed that all specimens contained infiltrating macrophages and T cells but few, if any, CD14 positive monocytes. This suggests that the radicular cyst is similar to a slow-turnover granuloma in that there is a limited influx of monocytes to replace existing macrophages.

Immunostaining for cytokines showed that IL-1 alpha was present in the epithelial cell layer in all specimens. In 8 of the 12 cysts IL-1 alpha staining was also found in endothelial cells in blood vessels either close to the epithelium or within areas of infiltrate. IL-1 beta staining was also found in the epithelial cell layer in all 12 specimens. In 6 specimens there was some staining of vascular endothelial cells. It was of great interest that apart from the epithelium and endothelial staining all other cell populations were negative for IL-1 staining. This included the numerous infiltrating macrophages and T lymphocytes and the indigenous fibroblast populations.

IL-6 was also localized in all specimens in the epithelial cell layer. In addition, in 8 cysts, IL-6 immunostaining was also found in vascular endothelial cells in vessels scattered throughout the tissue. No other cell

population in the cyst demonstrated IL-6 staining.

In contrast to the universal staining with IL-1 and IL-6 antibodies only two of the 12 cysts gave a positive reaction with antibodies to TNF alpha or IL-8. In both specimens which demonstrated both TNF and IL-8 immunoreactivity, staining was limited to cells which had the appearance of macrophages and which were in areas stained with the anti-CD68 monoclonal in serial sections. There was no particular differences in these two specimens to account for the positive reactivity with the TNF and IL-8 antibodies.

To ensure that the binding of antibodies to the cysts was due to the pathological process and was not a non-specific binding process the reactivity of normal gingiva and normal buccal mucosa was investigated. The epithelial surface cell layer in normal gingiva demonstrated only slight reactivity with antibodies to both IL-1s and to IL-6. This may be have been due to the bacteria normally associated with the gingiva. Normal buccal mucosa showed no positive reactivity in any cell population with the various anti-cytokine antibodies.

It appears from these studies therefore that the main osteolytic cytokines present in dental cysts are IL-1 α and ß and IL-6. However the major cellular source of these osteolytic proteins appears to be the cyst epithelial cells and to a lesser extent the endothelial cells of blood vessels and the fibroblast-likecells. It is envisaged that the cyst epithelial cell population is stimulated to produce these cytokines by the exposure to bacterial constituents including lipopolysaccharide (LPS). In addition to having

effects on bone both IL-1 (Sauder, 1989) and IL-6 (Grossman et al. 1989) have been reported to stimulate keratinocyte cell division. It is therefore possible that the growth of radicular cysts is due to two related processes: (i) the paracrine stimulation of bacterial products and (ii) the autocrine stimulation of cyst epithelial cell division by IL-1 and IL-6. The osteolytic activity of these cytokines causing local bone loss is also essential for cyst growth but the remote "internal" epithelial lining is unlikely to be the direct source. Our earlier experiments showed an interaction of epthelium and wall, which has an obvious logic considering the clinical observation that cyst regress when the stimulus for epithelial growth is removed by root canal therapy. However the failure to localise the cytokines in the capsule fibroblasts remains unexplained.

In addition to localizing cytokines, sections of cysts were also reacted with monoclonal antibodies to the vascular endothelial cell adhesion molecules - ELAM-1 and ICAM-1. The synthesis of these adhesion molecules is stimulated by inflammatory cytokines such as IL-1 and TNF and by LPS (Haskard, 1989). ELAM-1 was found in all cyst specimens with the proportion of blood vessels giving positive staining ranging from 19-32% of the total number of factor VIII positive blood vessels. Likewise, all cysts showed positive staining for ICAM-1. Again the proportion of ICAM-1-positive vessels was counted and found to range from 18-42% of the Factor VIII positive stained blood vessels. The mean number of vessels staining with antibodies to ICAM or ELAM was similar - 25% and 27% respectively. The positively stained vessels tended to be found close to

the epithelial cell layer suggesting that the cytokines produced by this cell population is responsible for the stimulation of these adhesion molecules. Of interest was the finding that all cyst specimens demonstrated ICAM-1 staining in the epithelial cell layer. ICAM-1 is normally expressed at very low level on human keratinocytes but is upregulated in several skin diseases such as psoriasis and lichen planus (Griffiths et al., 1991). The main stimulus for keratinocyte ICAM-1 expression appears to be TNFα (Griffiths et al., 1991). This is clearly not the case with the dental cyst and the signals inducing epithelial ICAM-1 expression in this lesion may be IL-1 and/or IL-6 or some possibly the various bacterial constituents present in the cyst fluid. The contribution that ICAM-1 makes on cyst epithelium or capsule to the overall pathology of this lesion remains to be determined. The inhibition of the synthesis or activity of these cytokines could therefore be of therapeutic benefit and avoid the necessity for surgery. The recent discovery of a natural IL-1 antagonist makes this prospect all the more feasible (Henderson and Blake, 1992). Although this may have little relevance in clinical practice to the radicular cyst, there may be an important application in the elimination of the aggressive keratocyst especially in Gorlin's syndrome (see Chapter 9).

Chapter 9

Interleukin-1 is the principal osteolytic cytokine produced by keratocysts.

9.1 Introduction

The keratocyst (primordial cyst) is an idiopathic intraosseous lesion consisting of a characteristically parakeratinized epithelial lining and a thin fibrous wall which is usually free from inflammatory cell infiltrate. The keratocyst may also be a manifestation of a wider syndrome, the basal cell naevus syndrome (Gorlin and Goltz, 1960). They also tend to recur with greater frequency than other types of odontogenic cyst (Brannon, 1976) and occasionally can extend aggressively into the paranasal sinuses and soft tissues of the face. This may be due to the incomplete removal of the original cyst epithelial lining with its intrinsic growth potential or residual daughter cysts. Both Main (1970) and Toller (1971), have shown that the mitotic activity of keratocyst epithelium was higher than that of other odontogenic cyst epithelium and normal buccal mucosa. Cyst expansion appears to be determined by the growth of the cyst epithelium and by the rate at which the surrounding bone is destroyed. Biomechanical theories of expansion, such as enlargement by hydrostatic pressure, ignore both the cellular aspects of cyst growth and the biochemistry of bone destruction. The production of prostaglandins (Harris et al., 1973) and collagenase (Donoff et al., 1972) by the keratocyst wall, have helped to clarify the latter. The enlargement of the keratocyst, must also involve the interaction of the epithelium and

the fibrous wall. This was demonstrated by Donoff et al., (1972) who showed collagenase activity in cultures of keratocyst explants only when both the epithelium and fibrous wall were present. The demonstration that cyst fibroblasts, when stimulated by mononuclear cell factor (Harvey et al., 1984) and cyst explant media (Meghji et al., 1989), synthesised eicosanoids and collagenase, could imply that the stimulus is from the epithelium. Furthermore, transplanted epithelia from keratocysts form new cysts in nude mice (Vedtofte et al., 1982).

Since the demonstration of soluble resorbing activity produced by mouse fibrosarcoma in vitro (Goldhaber, 1960), tissue culture has been an important tool for investigating the mechanisms of pathological bone resorption. Prostaglandins (PG), notably PGE_2 had been considered prime candidates as local mediators of pathological osteolysis, since they are potent stimulators of osteoclastic bone resorption and are synthesised in increased amounts by dental cysts. However, bone resorption induced in culture by explants of keratocyst is only partially inhibited by the inclusion of indomethacin (Harris et al., 1975). This indicates that in addition to prostaglandins the keratocyst explants release bone resorbing factors whose synthesis and activity are not mediated by the enzyme cyclooxygenase.

It is already known that inflamed radicular cysts produce interleukin 1-like activity in vitro (Meghji et al., 1989). The stimulus for the production of IL-1 in these cysts is presumed to be bacterial products such as lipopolysaccharide (LPS), but this is absent in the developmental

keratocysts. One possible explanation for keratocyst growth is that the odontogenic epithelium produces cytokines constitutively. The possibility that such molecules may be produced by keratocysts and may account for the raised levels of prostaglandin and collagenase synthesis by the uninflamed capsule has been investigated. Therefore the media supporting keratocyst explants have been investigated for the presence of osteolytic cytokine bioactivity and we have directly assessed the bone resorbing activity of these media. The role of IL-1 in the osteolytic activity of these media has been confirmed by the addition of a monospecific neutralizing antibody to IL-1

An attempt has also been made to identify the cellular source of these cytokines using immunocytochemistry.

9.2 Materials and Methods

9.2.1 Preparation of Cyst Explant Media: Six keratocysts and 3 samples of normal gingival tissue were surgically removed and divided into portions for histology or for culture. The latter were transported to the laboratory in sterile Hanks balanced salt solution at 4°C. The tissues were minced, using scalpels, into fragments of approximately 1 mm^3 and placed in 25 cm^2 flasks (Sterilin) with five ml of Dulbecco's Modified Eagles Medium (DMEM; Gibco) buffered with bicarbonate (3.5 g/L), and incubated for three days at 37°C in 5%CO_2/95% air. On termination of the cultures the medium was removed, dialysed using benzoylated dialysis tubing (molecular weight cut-off 2kDa; Sigma) against fresh DMEM to remove prostaglandins, but not large peptides, and stored at -70°C. The residual fragments of tissue were blotted and weighed.

The cyst or gingival culture medium was diluted in additional culture medium to give a final tissue: medium ratio of 5 mg/ml in order to standardise the activity on a tissue weight basis. This dilution was chosen on the basis of earlier studies of the effect of dilution of media on bioactivity (Meghji et al, 1989).

9.2.2 Preparation of Crude Interleukin 1 (Mononuclear cell factor: MCF): Mononuclear cell supernatants containing IL-1 were prepared from peripheral blood mononuclear cells from healthy adult volunteers.

Following sedimentation of whole blood on Histopaque (Sigma), the mononuclear cells were washed twice and cultured for 48 h at a

concentration of $2x10^6$ cells per ml in DMEM, in the presence of phytohaemagglutinin (PHA: 1 μg/mL, Wellcome). The culture medium was dialysed (using benzoylated tubing; Sigma) against fresh medium and stored at -70°C.

9.2.3 Thymocyte proliferation assay (LAF assay): IL-1-like activity was measured by its capacity to enhance the mitogen-stimulated proliferation of mouse thymocytes (the lymphocyte activation factor (LAF) assay: Gery et al, 1972). Briefly, thymocytes from seven-week old mice (C3H-HeJ; Harlan Olac, Blackthorn, U.K.) were plated in 96-well culture plates (Sterilin) at 1.5 x 10^6 per well in 100 μl of RPMI 1640 medium containing 5% complement-inactivated foetal calf serum (FCS), 4mM 2-mercaptoethanol (Sigma), and 0.5 μg/mL of the mitogen concanavalin A (Sigma) and cultured at 37°C in 5%CO_2/95% air. The cells were cultured with 1:2 dilution of MCF as a positive control and 1:2 dilution of the keratocyst explant media for 48 h and 0.5 μCi ^{3}H-thymidine (Amersham International specific activity 28.9 Ci/mmol) added per well for the last 6 hours of culture. Incorporation of ^{3}H-thymidine into 5% trichloroacetic acid-insoluble material was measured by scintillation spectrometry.

9.2.4 Chondrocyte Collagenase Assay: The induction of collagenase production by rabbit chondrocytes in culture has been shown to be specific for IL-1 (Treschel et al., 1982; Evequoz et al., 1984).

Rabbit articular chondrocytes were prepared by a method adapted from Treschel et al, (1982). Slices of articular cartilage from the knee and shoulder joints of two-week old New Zealand White rabbits were sequentially digested with 0.5% hyaluronidase (Sigma) for 20 min at 37°C, followed by 0.25% trypsin and 0.1% bacterial collagenase (Sigma) for 1 hour at 37°C in serum-free DMEM, then 0.1% bacterial collagenase for 2 hours in the presence of 10% FCS.

The cells obtained were washed twice and grown to confluence in DMEM with 10% FCS. Cell suspensions from the cultures were prepared by trypsin digestion (0.25%), and inoculated into 16-mm wells in 24-well culture plates at $2x10^5$ cells per well and the cells were cultured at 37°C in 5% CO_2/95%air. The culture medium was removed when the cells were confluent, and 0.5ml aliquots of test preparations diluted in DMEM were incubated in triplicate wells for 48h. These included dilutions of human recombinant IL-1, MCF and conditioned media supporting cysts or gingival tissues.

The supernatants were assayed for collagenase activity using thermally reconstituted ^{3}H-acetylated rat skin collagen fibrils (Cawston et al., 1979).

Briefly trypsin-resistant collagen was extracted from rat skin taking great care to preserve the native state of the molecule, and acetylated. For the assay, a known amount of acetylated rat skin collagen fibrils, suspended in collagenase buffer (100mM Tris pH 7.8, 150mM NaCl, 2.7mM $CaCl^2$) was incubated with the sample under test. To detect the

presence of latent collagenase (procollagenase) in the samples, O.7mM 4-aminophenylmercuric acetate (APMA) was also added to the assay tubes. Internal standards for each assay were provided by setting-up blank tubes (containing appropriate amounts of tissue culture medium as controls), trypsin-blanks (containing final concentration 25 μg/ml, to check the integrity of the fibrils) and total digest tubes (containing 30μg/ml bacterial collagenase: Clostridial peptidase type II (Sigma)) to demonstrate the average total counts available in each tube. The tubes were incubated at 37°C for 2-5h.

After the incubation period the tubes were centrifuged at 9000 rpm for 15 min so that any remaining undigested ^{3}H-collagen formed a tight pellet at the bottom of the tube. 250 μl of the supernatant containing solubilised collagen fragments was then carefully transferred into disposable scintillation vial inserts. 3 ml of scintillation fluid (Unisolve 1, Koch Light) was added to each vial and counted in a scintillation counter. Since the relationship between percentage collagen lysed and the counts in the supernatant is linear between 20-80% lysis, only values falling between these limits were used. The samples generating counts outside these limits were reassayed with smaller or greater volumes as appropriate. Collagenase activity was expressed as units/ml where 1 unit of collagenase digests 1 μg of collagen per minute at 37°C.

9.3.5 Interleukin-6 Bioassay: IL-6 was measured using a subclone, B9, of the hybridoma cell line B13.29 (a gift from Dr L. Aarden, Leiden), that

proliferates only in the presence of IL-6 Aarden et al, 1985). The assay was performed as described by Aarden et al (1985, 1987). B9 cells (5,000/200μl well in 96 well flat-bottomed plates) were cultured in Iscove's Modified Dulbecco's Medium (IMDM: Gibco) containing 5% heat-inactivated FCS and dilutions of the test material or standard IL-6. Plates were incubated at 37°C for 24h and cells were pulse-labelled for the last 4h with ^{3}H-thymidine, harvested using a Skatron cell harvester and the radioactivity incorporated measured by scintillation spectrometry. Culture media were tested at a range of concentrations in triplicate and were related to a standard dose response curve of human recombinant IL-6 (kindly provided by Dr L. Aarden). 1U/ml of IL-6 is the concentration that produces half-maximal incorporation of ^{3}H-thymidine.

9.2.6 Tumour Necrosis Factor Bioassay: TNF bioactivity was measured using the murine L929 cytotoxicity assay (Flick and Gifford, 1984). Briefly, L929 cells were cultured at 37°C in 5%CO_2/95% air in Eagles MEM with 5% FCS at $4x10^4$ cells/well for 20h in flat bottomed 96-well microtitre plates. The medium was then replaced with fresh medium containing actinomycin D (Sigma: 1μg/ml) and serial dilutions of a standard human recombinant TNF (Genzyme) or culture samples to be tested for TNF bioactivity. Following a further 20h incubation the medium was removed and the cell layer fixed with methanol for 2 minutes. 100μl of 0.1% methylene blue in 0.01 borate buffer, pH 8.5, was added to the wells and left for 30 minutes. The excess dye was then removed and the

plates washed (3X) with borate buffer. The dye was eluted with 100μl of 0.1M HCL with 2% ethanol. The absorbance measured on a Titertek Multiskan spectrophotometer at 650nm. The concentration of TNF that gave 50% maximal cell killing (one bioassay unit) was approximately equivalent to 50pg/ml of recombinant human TNFα.

9.2.7 Bone Resorption

Bone resorption was assayed (as described in Chapter 5) by the measurement of calcium released from five-day-old mouse calvaria in vitro. Dialysed cyst culture medium, gingival culture medium or MCF was introduced at a dilution of 5mg/ml (tissue weight/volume) or at 1:10 for the MCF preparation in fresh BGJ medium. A control group containing just BGJ medium and a positive control group exposed to PGE_2 (1μM) were included in all the bone resorption experiments. In some of the experiments indomethacin (1 μM) was added to the cyst culture media or MCF. In other experiments a monospecific rabbit polyclonal antibody which neutralises the biological activity of both IL-1α and IL-1ß (Genzyme, Suffolk, UK) was incubated with the culture media at 4°C overnight at a dilution of 1:50 before addition to the bone cultures. This dilution was determined from a limited dose response study with MCF or conditioned cyst media. As a control, normal rabbit serum was added to the tissue culture supernatants or the MCF and incubated overnight at 4°C before testing for bone resorbing activity.

9.2.8 Immunocytochemical Localization of Cytokines

9.2.8.1 Tissues

Keratocysts were obtained from patients undergoing surgery and the diagnosis was confirmed by histological examination of paraffin sections of portions of the cysts. Normal buccal mucosa and normal gingiva were obtained at surgery.

Fresh samples of these tissues were snap frozen in liquid nitrogen and then stored at -70°C. Frozen sections were cut at 5μm in a Bright cryostat with the cabinet temperature maintained at below -25°C and taken up onto glass slides treated with APES (3-aminopropyltriethoxy silane). Slides were air dried at room temperature overnight and then fixed in acetone for 20 min prior to use. From each block, serial sections were stained with haematoxylin and eosin to determine the cyst histology.

9.2.8. 2 Antibodies

Sheep polyclonal antisera to human recombinant IL-1 alpha and beta (Poole and Gaines Das, 1991) and a goat antiserum to human recombinant IL-6 (Rafferty et al., 1991) were obtained from Dr Stephen Poole (National Institute of Biological Standards and Control, South Mims). A mouse anti-human recombinant TNF alpha (52B83) was supplied by Dr Mark Bodmer (Celltech, Slough).

9.2.8.3 Immunocytochemistry

After fixation in acetone, sections were briefly washed in Tris buffer

pH 7.6 and were then treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30min to block endogenous peroxidase. After washing in Tris buffer, sections were then treated with a 1:50 dilution of normal donkey or normal rabbit serum for 30min to block non-specific binding of conjugated secondary antibodies. After blocking, optimal dilutions of the various antibodies described above were added to sections and incubated for 2h at room temperature. Sections were again washed with Tris buffer and secondary antibodies (biotinylated donkey anti-sheep; Sigma diluted 1:5,000 or biotinylated rabbit anti-mouse; Sigma diluted 1:100) added and left for 30min at room temperature. After a further wash in buffer a streptavidin-peroxidase complex (Strep ABC) was added and sections incubated for a further 30min. Following a final wash a solution of 3,3-diaminobenzidine (DAB) plus hydrogen peroxide was added to sections and incubated for 3-5min at room temperature. Sections were counterstained with Mayer's haematoxylin and examined microscopically.

9.2.8.3 Controls

For each specimen, serial sections were incubated in the absence of the primary or the secondary antibody to determine the specificity of binding. A further check of antibody specificity, where sufficient amount of cytokine was available, was to incubate the specific antibody overnight with the cytokine at 4°C and then use the complex as described above.

9.2.9 Statistical Analysis: Student's unpaired t-test was applied to all data.

9.3 Results

9.3.1 Interleukin-1 bioactivity (1)

9.3.1.1 LAF Assay - IL-1-activity, as measured by co-mitogenic activity in the LAF assay, was readily detectable in the conditioned medium obtained from the explant cultures of keratocysts. All six keratocysts stimulated thymocytes to proliferate significantly in the LAF assay as shown in Fig. 37. In contrast, culture media supporting normal gingiva explants failed to show significant LAF activity.

9.3.2 Interleukin-1 bioactivity (2)

9.3.2.1 Chondrocyte collagenase assay - IL-1-like activity, measured by its ability to stimulate rabbit chondrocytes to synthesise collagenase was also readily detectable in the conditioned media obtained from keratocysts. The supernatants from all six keratocyst significantly stimulated collagenase synthesis by chondrocytes (Fig. 38). Again, culture supernatants from normal gingival cultures failed to stimulate collagenase release.

9.3.3 Interleukin-6 bioactivity - Keratocysts produced relatively large amounts of IL-6 bioactivity (10,669 +/- 1053 U/ml culture medium, : mean and standard deviation, normal tissue 410 U/ml).

9.3.4 Tumour necrosis factor bioactivity - In contrast to the production of IL-1 and IL-6 bioactivity, keratocysts did not produce measurable amounts of TNF bioactivity as assessed by this cytotoxicity assay.

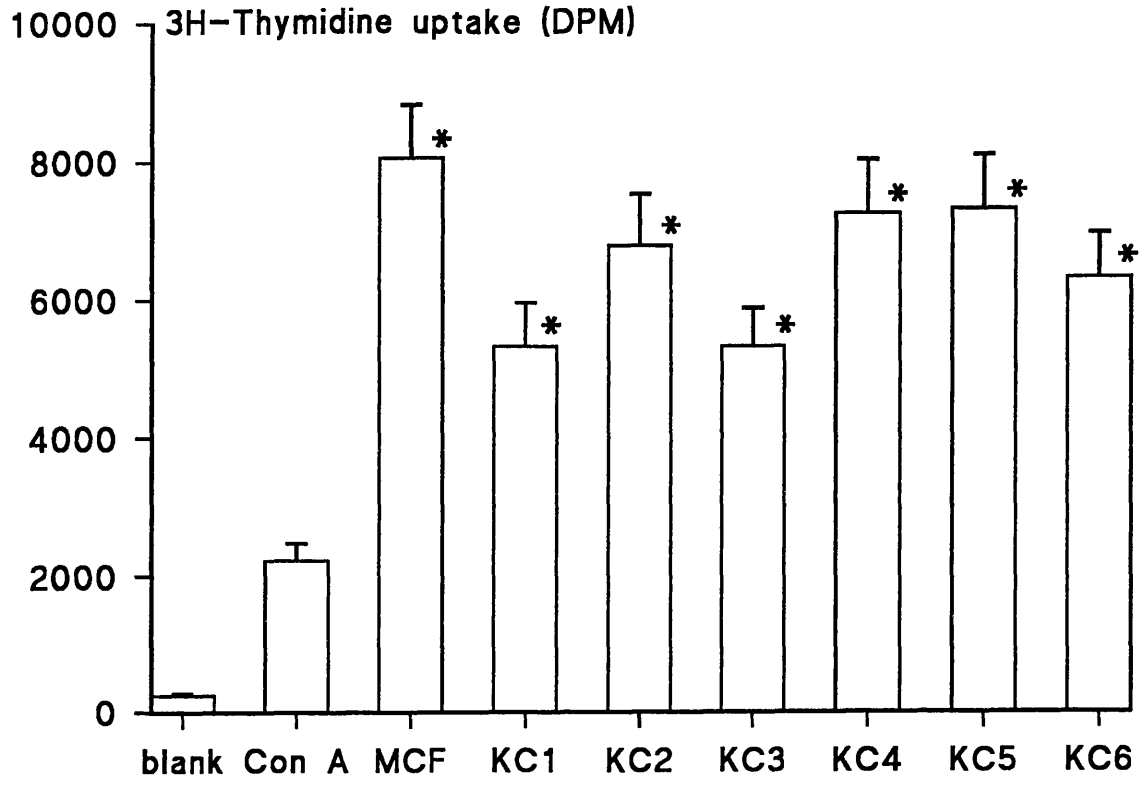


Figure 37. The effects of keratocyst culture media (KC1-KC6; diluted 1:2) on mouse thymocyte proliferation. Thymocytes sub- optimally stimulated with concanvalin A (Con A).

Mononuclear cell factor (MCF; diluted 1:2) was used as a positive control.

The results are shown as the mean + standard deviation of the thymocyte cultures (n = 6).

*$p<0.05$

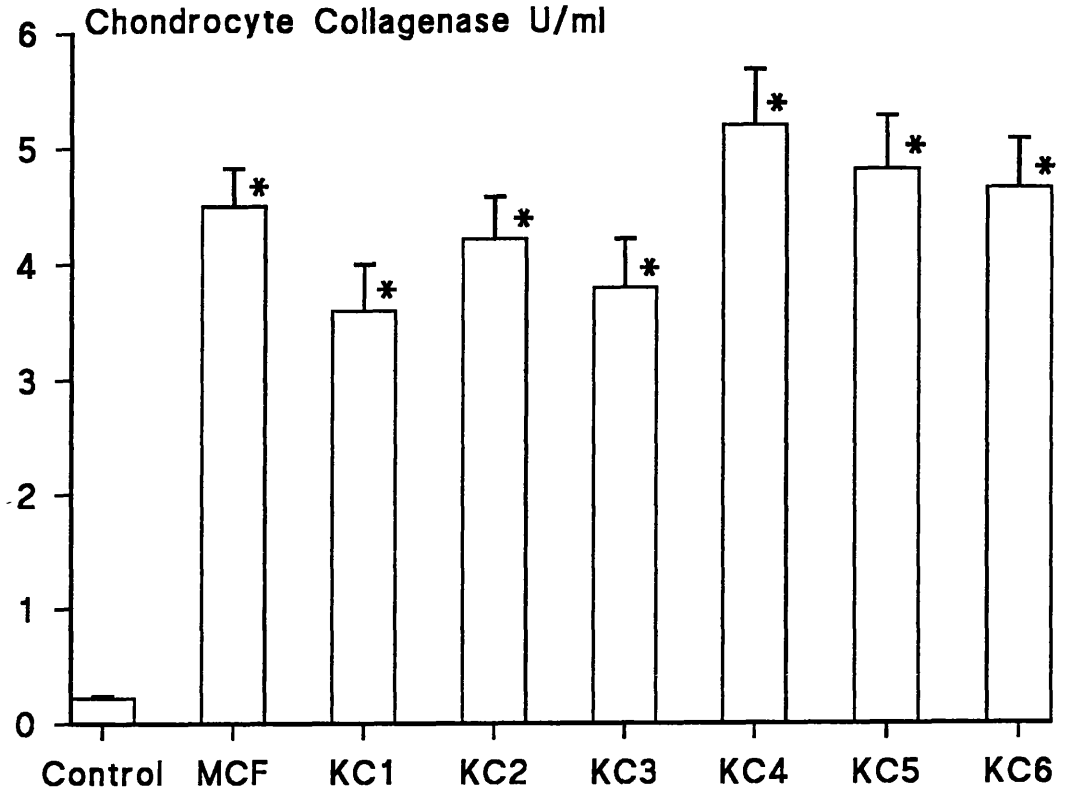


Figure 38. The effects of keratocyst culture media (KC1-KC6; diluted 1:10) on collagenase synthesis by rabbit chondrocytes. Mononuclear cell factor (diluted 1:10) was used as a positive control.

The results are shown as the mean + standard deviation of the chondrocyte cultures (n = 6).

*$p < 0.05$

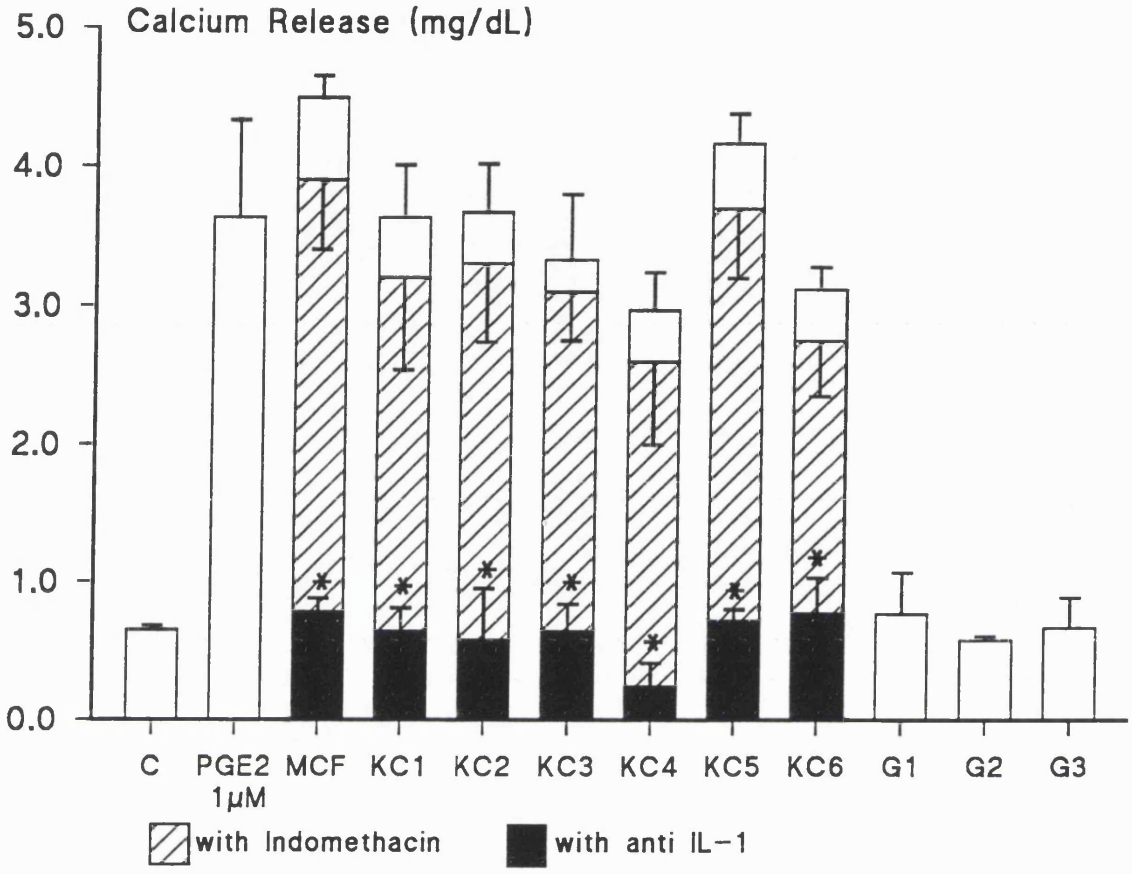


Figure 39. The effects of dialysed keratocyst cyst culture media (KC1-KC6; diluted 1:10; open bars) and dialysed normal gingival tissue culture media (G1-G3; diluted 1:10; open bars), the effect of the same cyst media in the presence of indomethacin (1 μmol) (cross-hatched bars) and the effect of the same media in the presence of anti-human IL1 on bone resorption (closed bars). Showing the amount of calcium released into the culture media during a 48 h incubation of mouse calvaria with the test media.

Mononuclear cell factor (diluted 1:10) and prostaglandin E_2 (PGE_2; 1 μmol) were used positive controls.

The results are shown as the Mean + standard deviation of the bone cultures (n = 5). *$p < 0.05$ resorption inhibited significantly by anti-human IL1

Figure 40. Immunocytochemical staining for cytokines in keratocyst and normal buccal mucosa. A. IL-1α staining in keratocyst epithelium (x160). B. IL-6 staining in keratocyst epithelium (x80). C. IL-1α staining in normal buccal mucosa (x32).

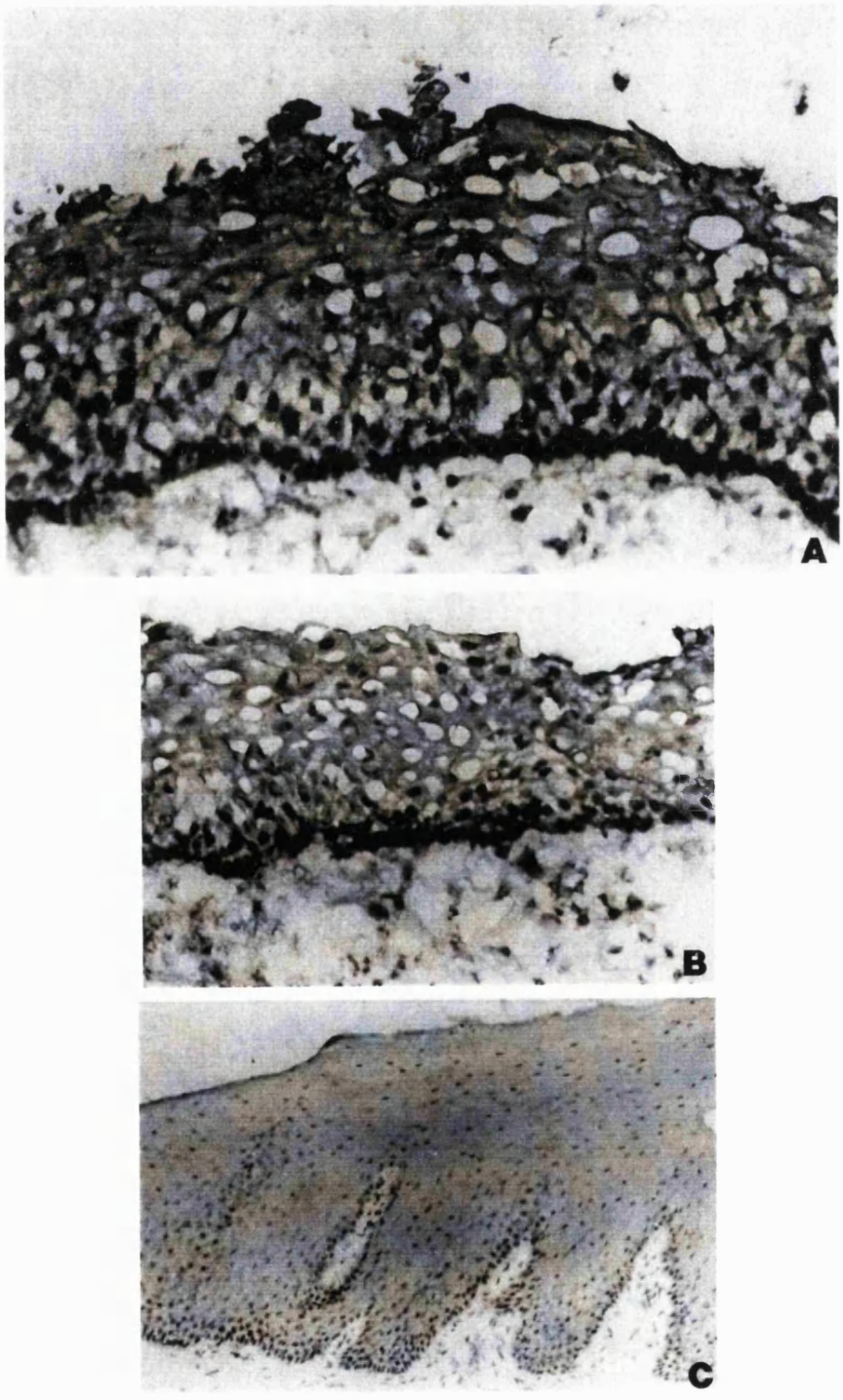
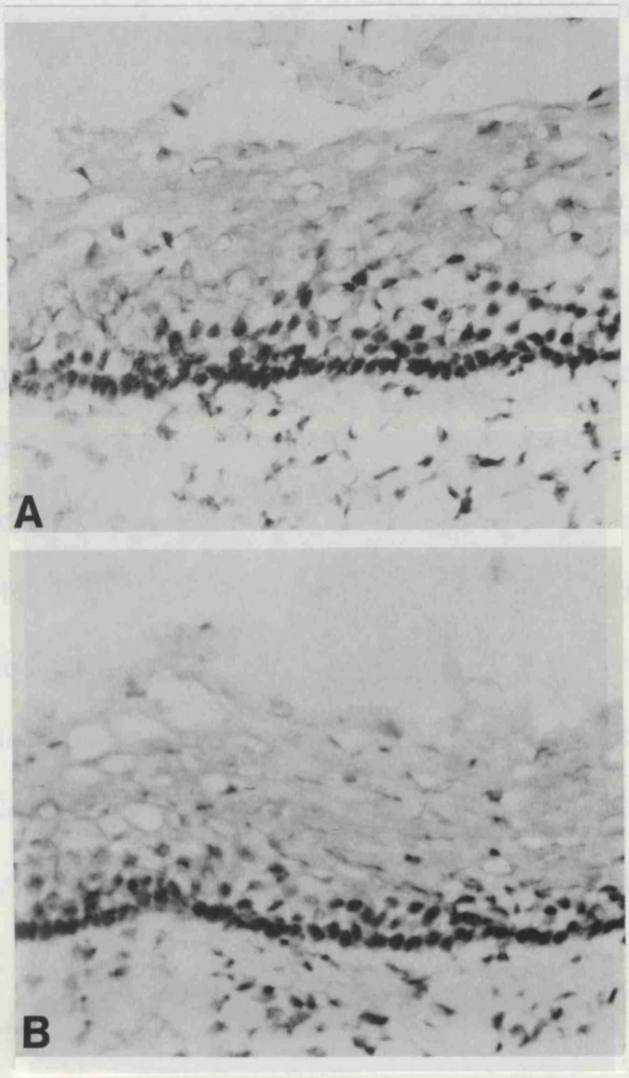


Figure 41. Immunocytochemical staining for cytokines in keratocysts. A. IL-1ß staining showing lack of positive reaction product (x80). B. TNF*α* staining showing lack of positive reaction product (x80).



9.3.5 Bone resorption - Dialysed culture supernatants from all keratocysts significantly stimulated bone resorption. In order to determine whether this osteolytic activity was mediated by prostaglandin synthesis, indomethacin (1 μM) was added to the bone cultures. This did not inhibit the bone resorbing activity of the keratocyst supernatants as shown in Fig.39. In contrast, the bone resorbing activity of all the keratocyst supernatants was completely neutralised by a monospecific polyclonal antibody to human IL-1α and IL-1ß (Fig. 39). Conditioned media from gingival cultures failed to demonstrate bone resorbing activity.

9.3.6 Immunolocalisation of cytokines - Cryostat sections of keratocysts, normal buccal mucosa and normal gingiva were examined.

To control for non-specific binding of antibody, sections from each cyst and from buccal mucosa and gingiva were taken through the immunocytochemical procedure in which either the primary or secondary antibody being omitted. In both control situations sections showed no positive staining. The specificity of IL-1 alpha staining was further assessed by incubating an aliquot of the antisera with an excess of recombinant IL-1 alpha (kindly supplied by Dr Gubler, Hoffman LaRoche Ltd) overnight at 4°C. The antibody was then compared with a similar aliquot of antibody incubated in the same manner but in the absence of IL-1α. Incubation of the IL-1α antiserum with IL-1α completely inhibited the immunocytochemical staining in keratocysts and in specimens of radicular cysts.

9.3.6.1 IL-1 alpha - Immunocytochemical staining for IL-1 alpha was found only in the epithelial layer of the cyst wall and was not seen in sections of normal buccal mucosa or gingiva (Fig 40a).

9.3.6.2 IL-1 beta - No immunocytochemical reaction product was found in any of the sections examined (Fig 41a)

9.3.6.3 IL-6 - Sections of keratocyst showed positive staining for IL-6 in the epithelial layer (Fig 40b) but in no other cell populations. No IL-6 staining was seen in control tissues.

9.6.3.4 TNF alpha - No immunocytochemical reaction product was found in the keratocyst sections (Fig 41b) or in the control tissues.

9.4 Discussion

A number of cytokines including IL-1, IL-6 and TNF have osteolytic activity and can also stimulate fibroblast proliferation. The activities of these cytokines are consistent with them having a role in cyst growth. However, it has not been previously established that cytokines are involved in the pathogenesis of keratocysts. In this study we have tested the hypothesis that IL-1 is a major mediator promoting bone breakdown in keratocysts and that this cytokine facilitates the growth and expansion of these cysts. Both bioassays demonstrated that IL-1 was present in the cyst explant media. The LAF assay is not a particularly selective assay, but the chondrocyte collagenase assay is relatively specific for IL-1 and e.g. will not respond to the other major osteolytic/chondrolytic cytokine - TNF (Schnyder et al 1987). Normal gingival tissue was also cultured in the

same manner and tested for IL-1 bioactivity. In contrast to the keratocysts these gingival samples did not release assayable amounts of IL-1.

Culture supernatants from keratocyst cultures were also tested for the presence of IL-6 or TNF bioactivity. It was possible to measure relatively large amounts of IL-6 in these culture fluids using the specific B9 hybridoma assay. In contrast, the culture fluids did not show bioactivity in the TNF bioassay (the murine L929 cytotoxicity assay).

The osteolytic activity of the culture media supporting the cyst explants, promoted calcium release from murine calvaria. By contrast, the media supporting normal gingival specimens showed no osteolytic activity. The bone resorption induced by cyst explant media was not due to prostanoids as these had been removed by dialysis of the media using benzoylated dialysis tubing (molecular weight cut-off of 2kDa). Furthermore, addition of indomethacin to bone cultures stimulated with cyst explant media failed to inhibit osteolysis. By contrast, the addition of a monospecific neutralizing antibody to human IL-1, which does not neutralize IL-6 or TNF, completely inhibited the osteolytic activity of these conditioned media.

These results were corroborated by immunocytochemical analysis of the cytokines present in keratocysts. Monospecific antisera to human IL-1α, IL-1ß or IL-6 and a monoclonal antibody to human TNF were used. Normal gingiva and buccal mucosa showed no reactivity with any of these antibodies, whereas the keratocysts investigated revealed strong staining for IL-1α and IL-6 in the epithelial cell layer. The IL-1α staining was completely inhibited by incubation of recombinant IL-1α with the antibody

showing the absolute specificity of this reaction. Epithelial cells did not show any reaction with a monospecific antibody to IL-1ß. This contrasts with our studies of the cytokine localization in radicular cysts in which the epithelial cells contain both IL-1α and ß (see Chapter 8). However as with the radicular cyst only the keratocyst epithelium demonstrated IL-1α and IL-6 staining, no other cell population indicated the presence of these cytokines. The monoclonal antibody to TNFα did not show any reactivity with the keratocyst specimens. These findings of cytokine immunolocalization are in complete agreement with the release of cytokines from cyst explants and strongly support the hypothesis that the cytokines released by cultured cyst explants are principally the product of the epithelial cells which may act on the capsular cells to release both cytokines and eicosanoids.

In summary, keratocysts release IL-1 and IL-6 but not TNF in explant culture, and the culture supernatants stimulate calvarial bone breakdown which is completely inhibited by a monospecific neutralizing antibody to IL-1 but not by indomethacin. This supports the hypothesis that the development and growth of keratocysts may be due to the action of IL-1α and IL-6 and the former is the principal osteolytic factor promoting bone breakdown. The role of the IL-6 is less clear. It does not appear to be directly involved in the calvarial bone breakdown but may still influence bone turnover in a more subtle way, and may also have a stimulatory influence on other cells present in the cyst. For example it has been reported that IL-6 stimulates the proliferation of cultured human epidermal

cells (Grossman et al, 1989). There is therefore the possibility that IL-6 may stimulate epithelial cell proliferation in the keratocyst via an autocrine feedback mechanism.

One major problem in explaining the pathogenesis of the keratocyst in terms of cytokine expression and action is the question of the stimuli promoting the cytokine synthesis. Keratocysts are classically defined as non-inflammatory developmental cysts, without any overt involvement of bacterial products (e.g. lipopolysaccharides) or inflammatory cells in the process of cyst growth. Their origin from the ubiquitous cell rests of dental lamina remains unexplained.

Further studies are therefore needed to try to identify exogenous or endogenously altered cytokine gene control factors which could be responsible for cytokine synthesis. The possibility that the defect lies in the control of cytokine inhibitors or growth factors e.g. release of natural cytokine antagonists, soluble receptors or binding proteins is currently under investigation. It may be relevant that patients with Gorlins Syndrome have been shown to be deficient in the gene controlling cell growth located in the region 9q22.3-q31 (Farndon et al. (1992).

Chapter 10

Summary and Conclusions

The two main groups of odontogenic cysts are excellent models for comparing and contrasting the eicosanoid and cytokine profiles of developmental and inflammatory lesions.

The sequence of inflammatory events which lead to pulpal necrosis and periapical inflammation are well recognised clinically. This work is intended to provide a better understanding of the varied outcome by the addition of new information derived from studies of several important stages.

To recapitulate, there are two main consequences of pulpal death and infection; a) *periapical abscess* formation in the case of acute infection, b) *periapical granuloma* and/or a *radicular cyst* where the infection is low grade and chronic.

10. 1 ***The Periapical Abscess***: The periapical abscess not only creates local destruction of the connective tissue and bone but in some cases leads to cellulitis, osteitis and occasionally osteomyelitis. In studying the role of bacteria in connective tissue destruction much attention has focused on the biological activity of one component common to many of the disease-related bacteria, namely the lipopolysaccharides (LPS) of the outer membrane of the cell wall of Gram-negative bacteria. However, the concentration of purified LPS needed to produce a cytotoxic effect on

fibroblasts in vitro is over 100μg/ml. In contrast, the capsular material from oral bacteria was between 100-1000 times more potent than LPS in inhibiting fibroblast cell division. These investigations have also shown that the capsule surface-associated material (SAM) from three Gram-negative bacteria; *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* and *Eikenella corrodens* produced dose-dependent inhibition of thymidine incorporation by human fibroblasts, the human monocytic cell line U937, and guinea pig epidermal cells, whereas LPS from these bacteria was either inactive or substantially less active over the same dose range. One of the constituents of SAM from strains of *P. gingivalis and A. actinomycetemcomitans* formerly considered to be non-leucotoxic was highly toxic to human peripheral blood polymorphonuclear cells with 50% killing at concentrations less than 1ng/ml. The LPS from these bacteria was at least 1000 times less active in neutrophil killing.

The effect of SAM on bone is also important in explaining the outcome of periapical infection, and was therefore tested on osteoblast proliferation, collagen synthesis and bone resorption. SAM from all three pathogens significantly inhibited DNA and collagen synthesis by murine calvaria and isolated osteoblasts at concentrations as low as 10ng/ml. This inhibitory effect was blocked by 1μM indomethacin which suggested that effect was prostanoid mediated.

The ability of SAM from four oral pathogens to induce bone resorption in vitro has been compared. Selective inhibitors were used to define the mechanisms by which these materials stimulate bone resorption.

These were indomethacin to inhibit prostanoid synthesis, interleukin-1 receptor antagonist to antagonise locally-produced IL-1 and neutralising antibodies to murine tumour necrosis factor (TNF) and interleukin -6 (IL-6).

The results showed that the bone resorption induced by SAM from *S.aureus* was mediated by prostaglandins (PGs), osteolytic activity of SAM from *P. gingivalis* appeared to be mediated by a mixture of IL-1, prostaglandins (PGs) and TNF. The activity of E. corrodens SAM was principally TNF and some PGs. This contrasted with the bone resorption induced by SAM from A. actinomycetemcomitans which was not significantly inhibited by any of these inhibitors and therefore has not been identified. However all SAM has potent bone resorbing activity.

These findings show that the easily solubilised expolymers from oral bacteria play a major part in soft and hard tissue breakdown in periapical lesions.

10.2 Chronic Periapical Granuloma: The pathogenesis of the chronic periapical granuloma is best understood as arising where the microbe component is minimal and contained or eliminated by the local immune response. Although the presence of oral bacteria within the necrotic pulp could account for much of the pathosis seen, several studies have shown these lesions to be sterile. Thus the best possible explanation available for the pathogenesis of the chronic periapical granuloma is that it is due to the bacterial products such as endotoxins or lipopolysaccharides (LPS) originating in septic root canals that have limited diffusion into the

periapical tissue. However the transition from granuloma to cyst may also represent a defect in epithelial cell regulation which in its most florid form is seen the the Basal Cell Naevus Syndrome (Gorlin and Goltz, 1960). Such a destruction may be transient or prolonged thus giving rise to either a single cyst or multiple sequential cysts.

10.3 Radicular Cysts

Endotoxins or LPS have diverse biological activity, not only acting as mitogens for epithelial cells, but are also capable of stimulating the production of cytokines from the surrounding connective tissue and inflammatory cells which could explain the radicular cyst pathogenesis.

To test this hypothesis, fluids from radicular cysts, keratocysts and follicular cysts were investigated for the presence of endotoxin and cytokines. The results showed significantly higher concentrations of endotoxin in radicular cyst fluids (mean; 5.5 ± 1.8ng/ml) than in keratocysts (mean; 0.04 ± 0.05ng/ml) or in follicular cyst (mean; 0.06 ± 0.1ng/ml). None of the cyst fluids grew any microorganisms. Immunoassay showed the presence of interleukin 1 alpha and beta in the inflammatory radicular cysts, whereas the developmental keratocyst and follicular cyst only showed the presence of IL-1 alpha. All the cyst fluids contained IL-6, but none of the cyst fluids contained any TNF.

Hence it can be concluded that all cysts fluids and explant media contain cytokines, but only the inflammatory radicular cysts have significantly higher levels of endotoxins and that all the cyst fluids were

microbiologically sterile.

This sets the scene for the pathogenesis of the radicular cysts which can be divided into three phases; (i) initiation, (ii) formation, and (iii) enlargement.

(i) The epithelial lining is derived from the epithelial cells rests of Malassez in the periodontal ligament. Therefore a range of endotoxins, cytokines and prostanoids were tested for their capacity to induce epithelial cell proliferation. These were LPS from *A. actinomycetemcomitans* and *P. gingivalis*, *Escherichia Coli (E. coli),* interleukin-1, tumour necrosis factor, interleukin-6, prostaglandin E_2 and cyst fibroblast culture medium.

The results showed that all three LPS, especially *P. gingivalis* stimulated keratinocyte proliferation in a dose related manner, but inhibited cell proliferation at higher doses.

All three cytokines significantly stimulated keratinocyte proliferation. PGE_2 only had a slight effect on the proliferation of keratinocytes at low concentrations, and actually induced cytotoxicity at 10μM. These observations would suggest that a balance exists between the proliferation influence of these factors in low concentrations beyond which the net effect is inflammation and cell necrosis. However *in vivo* the cell rests also interact with the adjacent connective tissue. This was first explored by studying the effect of LPS on fibroblasts in culture. The fibroblast culture media were then tested for their ability to induce keratinocyte proliferation at concentrations of 0-50%. Increasing concentrations of fibroblast culture media had increasing proliferative effect on keratinocytes. The addition of

indomethacin to these fibroblast cultures to eliminate PGs significantly enhanced the proliferation of these cells. This confirmed the anti-proliferative effect of prostanoids on the epithelium. Fibroblasts are capable of synthesising a number of growth factors including: keratinocyte growth factor (KGF), interleukin-6, interleukin-1 and fibroblast growth factor. The most likely candidates for this proliferative effect are likley to be KGF, IL-1 and IL-6. .

We believe that bacterial endotoxins (LPS) and cytokines may activate the cell rests of Malasez in the same manner as they activate keratinocytes. However inflammatory prostanoids probably inhibit this fundamental stage in cyst development.

(ii) The proliferating cyst epithelium appears to induce, organise and maintain its connective tissue wall. The enlargement of the cyst is dependent on continued epithelial proliferation which appears to be a paracrine process mediated by cytokines.

To confirm this a panel of antibodies against the cytokines IL-1, TNF, IL-6, were used to localise these molecules in radicular cysts. All specimens showed positive staining for IL-1 (alpha and beta) and IL-6 in epithelial and vascular endothelial cells. Immunohistochemical staining of cryostat sections of keratocysts revealed the presence of IL-1α and IL-6 in cyst epithelial cells but not in other cell types. Sections did not react with antibodies to IL-1ß or TNF. Only two of the radicular cyst specimens demonstrated TNF staining which was located in macrophages in the capsule. It was surprising that the fibroblasts, which have previously been

shown to produce both IL-1 and IL-6 were not stained positive. There can be two explanations; either the fibroblasts are not synthesising these cytokines when examined or that the cells release the cytokines as soon as they are synthesised so that none remain in the cell to be stained. This problem can be solved by using molecular biological techniques such as *in-situ* hybridisation.

It appears from these studies therefore that the cytokines present in radicular cysts are IL-1α, IL-1β and IL-6, the cytokines present in keratocysts are IL-1α and IL-6. The major cellular source of these cytokines appears to be the cyst epithelial cells. These cytokines undoubtedly play a role in epithelial cell division. It is therefore postulated that following the initiation of cell rest division the growth of odontogenic cysts is due to two related processes: (a) the autocrine stimulation of cyst epithelial cell division by IL-1 and IL-6 and the paracrine activity of these cytokines on the fibroblast component of the cyst wall to produce osteolytic factors such as the eicosanoids and cytokines.

(iii) The cyst induced bone resorption is essential for expansion and this has already been shown to be the function of the fibroblast under the influence of both cytokines from cyst epithelium and any contained inflammatory cells (Fig 42). The cyst wall agents identified as being capable of inducing bone resorption include PGE_2, PGI_2, LTB_4, C_4, D_4 and E_4 and 5- and 12-HETE and also the osteolytic cytokines IL-1α and β and IL-6.

The keratocyst is explic able by this model with the obvious exception of

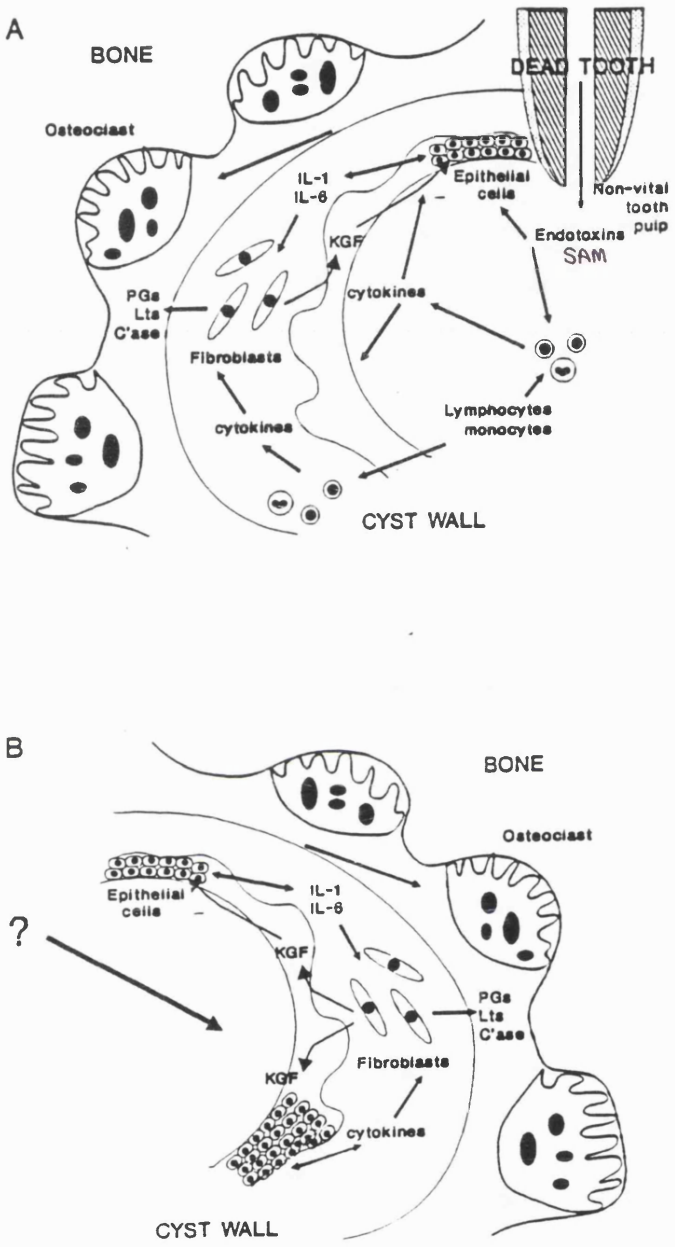


Figure 42 Scheme of proposed cellular interactions leading to bone destruction by (A) radicular cysts and (B) keratocysts and follicular cysts.

(A) Bacterial endotoxins from the non-vital tooth pulp stimulate epithelial cell rests proliferation directly and indirectly by stimulation of cytokine synthesis by lymphocytes and monocytes. This also maintains cyst epithelial growth.

(B) Unknown stimulus stimulates epithelial cell rests proliferation directly and indirectly by stimulation of cytokine synthesis by the epithelial cells and the connective tissue.

Abbreviations: PGs: Prostaglandins Lts: Leukotrienes
C'ase: Collagenase KGF: Keratinocyte Growth Factor

the initiation process which appears to be the result of deficient cellular control mechanism normally able to maintain a quiescent status quo for intraosseous developmental cell rests.

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INTERLEUKIN-1: THE PRINCIPAL OSTEOLYTIC CYTOKINE PRODUCED BY KERATOCYSTS

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Summary—Fragments of keratocysts removed at operation were maintained in explant culture and the media were assayed for the biological activity of the potent osteolytic cytokines— interleukin (IL)-1, interleukin (IL)-6 and tumour necrosis factor (TNF). Media were also assayed for their ability to stimulate bone resorption. All six cysts examined released IL-1 and IL-6 bioactivity but TNF bioactivity was unmeasurable. Dialysed cyst media stimulated bone resorption and this could be completely inhibited by a monospecific antibody which neutralized IL-1α and IL-1β. Immunohistochemical staining of cryostat sections of keratocysts revealed the presence of IL-1α and IL-6 in cyst epithelial cells but not in other cell types. Sections did not react with antibodies to IL-1β or TNF. It is therefore proposed that IL-1α is the major osteolytic cytokine produced by keratocysts and that IL-6 and IL-1 may contribute to keratocyst growth by promoting epithelial cell proliferation and bone resorption, respectively.

Key words: keratocysts, bone resorption, interleukin-1.

INTRODUCTION

The keratocyst (primordial cyst) is an idiopathic intraosseous lesion consisting of a characteristically parakeratinized epithelial lining and a thin fibrous capsule that is usually free from inflammatory cell infiltrate. The keratocyst may also be a manifestation of a wider syndrome, the basal cell naevus syndrome (Gorlin and Goltz, 1960). Keratocysts also tend to recur with greater frequency than other types of odontogenic cyst (Brannon, 1976). This may be due to the incomplete removal of the original cyst epithelial lining with its intrinsic growth potential or residual daughter cysts. Both Main (1970) and Toller (1971) have shown that the mitotic activity of keratocyst epithelium was higher than that of other odontogenic cyst epithelium and normal buccal mucosa. Cyst expansion is determined by the growth of the cyst epithelium and by the rate at which the surrounding bone is destroyed. Biomechanical theories of expansion, such as enlargement by hydrostatic pressure, ignore both the cellular aspects of cyst growth and the biochemistry of bone destruction. The finding of the production of prostaglandins (Harris *et al.*, 1975) and collagenase (Donoff, Harper and Guralnick, 1972) by the keratocyst wall has helped to clarify the biochemical aspects. The enlargement of the keratocyst must also involve the interaction of the epithelium and the fibrous capsule. This was demonstrated by Donoff *et al.* (1972) who showed collagenase activity in cultures of keratocyst explants only when both the epithelium and fibrous wall were present. The demonstration that the cyst fibroblasts, when stimulated by mononuclear cell factor (Harvey *et al.*, 1984) and cyst explant media (Meghji, Harvey and Harris, 1989), synthesized eicosanoids and collagenase could imply that the stimulus is from the epithelium. Furthermore, transplanted epithelia from keratocysts forms new cysts in nude mice (Vedtofte, Holmstrup and Dabelsteen, 1982).

Since the demonstration of soluble resorbing activity produced by mouse fibrosarcoma *in vitro* (Goldhaber, 1960), tissue culture has been an important tool for investigating the mechanisms of pathological bone resorption. Prostaglandins, notably PGE_2, had been considered prime candidates as local mediators of pathological osteolysis, as they are potent stimulators of osteoclastic bone resorption and are synthesized in increased amounts by dental cysts. However, bone resorption induced in culture by explants of keratocyst is only partially inhibited by the inclusion of indomethacin (Harris *et al.*, 1975). This indicates that in addition to prostaglandins the keratocyst explants release bone-resorbing factors whose synthesis and activity are not mediated by the enzyme cyclooxygenase.

Several macromolecular bone-resorbing factors have been identified recently as cytokines, including IL-1 (Gowen *et al.*, 1983), TNF (Bertolini *et al.*, 1986) and IL-6 (Ishimi *et al.*, 1990). It has become clear that IL-1 and TNF account for much of the bone-resorbing activity attributed to 'osteoclast activating factor' (OAF) produced by mononuclear leucocytes (Horton *et al.*, 1972) and myeloma cells (Mundy *et al.*, 1974). Although many cells types are capable of synthesizing these cytokines, they are particularly associated with chronic inflammatory lesions; rheumatoid arthritis (Nouri, Panayi and Goodman, 1984)

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IL, interleukin; LAF, lymphocyte activation factor; MCF, mononuclear cell factor; PG, prostaglandins; TNF, tumour necrosis factor.

and periodontal disease (Charon *et al.*, 1982). IL-1 in particular is a potent stimulator of prostaglandin and collagenase synthesis by connective tissue cells (Dayer, Robinson and Krane, 1977) and is thought to be the principal mediator of their synthesis in inflamed connective tissues.

We have already shown that inflamed radicular cysts produce IL-1 like activity *in vitro* (Meghji *et al.*, 1989). The stimulus for the production of IL-1 in these cysts is presumed to be bacterial products such as lipopolysaccharide. This is absent in the developmental keratocysts. One possible explanation for keratocyst growth is that the odontogenic epithelium produces cytokines constitutively. Considering the potent bone-resorbing activity of cytokines such as IL-1, TNF and IL-6 and the fact that keratinocytes can synthesize IL-1 and IL-6 (Luger *et al.*, 1981; Sauder, 1989; Barker *et al.*, 1991), we have now investigated the possibility that such molecules may be produced by keratocysts and may account for the raised levels of prostaglandin and collagenase synthesis by the uninflamed capsule. Therefore we have assayed the media supporting keratocyst explants for the presence of osteolytic cytokine bioactivity and have directly assessed the bone-resorbing activity of these media. The role of IL-1 in the osteolytic activity of these media has been confirmed by the addition of a monospecific neutralizing antibody to IL-1. We have also identified the cellular source of these cytokines using immunocytochemistry.

MATERIALS AND METHODS

Preparation of cyst explant media

Six keratocysts and three samples of normal gingival tissue were surgically removed and divided into portions for histological examination or for culture. The portions were transported to the laboratory in sterile Hanks balanced salt solution at 4°C. The tissues were minced, using scalpels, into fragments of approx. 1 mm^3 and placed in 25 cm^2 flasks (Sterilin) with 5 ml of DMEM (Gibco, Grand Island, N.Y., U.S.A.) buffered with bicarbonate (3.5 g/l), and incubated for 3 days at 37°C in 5% CO_2/95% air. On ending the cultures the medium was removed, dialysed using benzoylated dialysis tubing (molecular weight cut-off 2kDa; Sigma, St Louis, MO, U.S.A.) against fresh DMEM to remove prostaglandins but not large peptides, and stored at −70°C. The residual fragments of tissue were blotted and weighed.

The cyst or gingival culture medium was diluted in additional culture medium to give a final tissue: medium ratio of 5 mg/ml in order to standardize the activity on a tissue weight basis. This dilution was chosen on the basis of earlier studies of the effect of dilution of media on bioactivity (Meghji *et al.* 1989).

Preparation of crude IL-1 (MCF)

Mononuclear cell supernatants containing IL-1 were prepared from peripheral blood mononuclear cells from healthy adult volunteers. After sedimentation of whole blood on Histopaque (Sigma), the mononuclear cells were washed twice and cultured for 48 h at a concentration of 2×10^6 cells per ml in DMEM, in the presence of phytohaemagglutinin (1 μg/ml, Wellcome, Crewe, U.K.). The culture medium was dialysed (using benzoylated tubing; Sigma) against fresh medium and stored at −70°C.

Thymocyte proliferation assay (LAF assay)

IL-1 like activity was measured by its capacity to enhance the mitogen-stimulated proliferation of mouse thymocytes [the lymphocyte activation factor (LAF) assay: Gery, Gershan and Waksman (1972)]. In brief, thymocytes from 7-week-old mice (C3H-HeJ; Harlan Olac, Blackthorn, U.K.) were plated in 96-well culture plates (Sterilin) at 1.5×10^6 per well in 100 μl of RPMI 1640 medium containing 5% complement-inactivated (FCS), 4 mM 2-mercaptoethanol (Sigma) and 0.5 μg/ml of the mitogen concanavalin A (Sigma), and cultured at 37°C in 5% CO_2/95% air. The cells were cultured with 1:2 dilution of MCF as a positive control and 1:2 dilution of the keratocyst explant media for 48 h and 0.5 μCi [^{3}H]-thymidine (Amersham International specific activity 28.9 Ci/mmol) added per well for the last 6 h of culture. Incorporation of [^{3}H]-thymidine into 5% trichloroacetic acid-insoluble material was measured by scintillation spectrometry.

Chondrocyte collagenase assay

The induction of collagenase production by rabbit chondrocytes in culture has been shown to be specific for IL-1 (Trechsel *et al.*, 1982; Evequoz *et al.*, 1984). Rabbit articular chondrocytes were prepared by a method adapted from Trechsel *et al.* (1982). Slices of articular cartilage from the knee and shoulder joints of 2-week-old New Zealand White rabbits were sequentially digested with 0.5% hyaluronidase (Sigma) for 20 min at 37°C, followed by 0.25% trypsin and 0.1% bacterial collagenase (Sigma) for 1 h at 37°C in serum-free DMEM, then 0.1% bacterial collagenase for 2 h in the presence of 10% FCS.

The cells obtained were washed twice and grown to confluence in DMEM with 10% FCS. Cell suspensions from the cultures were prepared by trypsin digestion (0.25%), and inoculated into 16-mm wells in 24-well culture plates at 2×10^5 cells per well; the cells were cultured at 37°C in 5% CO_2/95% air. The culture medium was removed when the cells were confluent, and 0.5 ml samples of test preparations diluted in DMEM were incubated in triplicate wells for 48 h. These included dilutions of human recombinant IL-1, MCF and conditioned media supporting cysts or gingival tissues.

The supernatants were assayed for collagenase activity using thermally reconstituted [^{3}H]-acetylated rat skin collagen fibrils (Cawston and Barrett, 1979). In brief, trypsin-resistant collagen was extracted from rat skin taking great care to preserve the native state of the molecule, and acetylated. For the assay, a known amount of acetylated rat skin collagen fibrils, suspended in collagenase buffer (100 mM tris ph 7.8, 150 mM NaCl, 2.7 mM $CaCl^2$), was incubated with the sample under test. To detect the presence of latent collagenase (procollagenase) in the samples, 0.7 mM 4-aminophenylmercuric acetate was also added to the assay tubes. Internal standards for each assay were provided by setting-up blank tubes (containing appropriate amounts of tissue culture medium as controls), trypsin-blanks (containing final

concentration 25 μg/ml, to check the integrity of the fibrils) and total digest tubes [containing 30 μg/ml bacterial collagenase: Clostridial peptidase type II (Sigma)] to demonstrate the average total counts available in each tube. The tubes were incubated at 37°C for 2–5 h.

After the incubation period the tubes were centrifuged at 9000 rev/min for 15 min so that any remaining undigested [^{3}H]-collagen formed a tight pellet at the bottom of the tube; 250 μl of the supernatant containing solubilized collagen fragments were then carefully transferred into disposable scintillation vial inserts. Three ml of scintillation fluid (Unisolve 1, Koch Light) were added to each vial and counted in a scintillation counter. As the relation between percentage collagen lysed and the counts in the supernatant is linear between 20–80% lysis, only values falling between these limits were used. The samples generating counts outside these limits were reassayed with smaller or greater volumes as appropriate. Collagenase activity was expressed as units/ml where one unit of collagenase digests 1 μg of collagen/min at 37°C.

IL-6 bioassay

IL-6 was measured using a subclone, B9, of the hybridoma cell line B13.29 (a gift from Dr L. Arden, Leiden, The Netherlands) that proliferates only in the presence of IL-6 (Arden, Lansdorp and de Groot, 1985). The assay was as described by Arden *et al.* 1985, 1987). B9 Cells (5,000/200 μl well in 96-well, flat-bottomed plates) were cultured in Iscove's modified Dulbecco's medium (Gibco) containing 5% heat-inactivated FCS and dilutions of the test material or standard IL-6. Plates were incubated at 37°C for 24 h and cells were pulse-labelled for the last 4 h with [^{3}H]-thymidine, harvested using a Skatron cell harvester and the radioactivity incorporated measured by scintillation spectrometry. Culture media were tested at a range of concentrations in triplicate and were related to a standard dose–response curve of human recombinant IL-6 (kindly provided by Dr L. Arden). One U/ml of IL-6 is the concentration that produces half-maximal incorporation of [^{3}H]-thymidine.

TNF bioassay

TNF bioactivity was measured using the murine L929 cytotoxicity assay (Flick and Gifford, 1984). In brief, L929 cells were cultured at 37°C in 5% CO_2/95% air in Eagles MEM with 5% FCS at 4×10^4 cells/well for 20 h in flat bottomed 96-well microtitre plates. The medium was then replaced with fresh medium containing actinomycin D (Sigma: 1 μg/ml) and serial dilutions of a standard human recombinant TNF (Genzyme, Suffolk, U.K.) or culture samples to be tested for TNF bioactivity. After a further 20 h incubation the medium was removed and the cell layer fixed with methanol for 2 min. One-hundred μl of 0.1% methylene blue in 0.01 borate buffer, pH 8.5, were added to the wells and left for 30 min. The excess dye was then removed and the plates washed (3X) with borate buffer. The dye was eluted with 100 μl of 0.1 M HCL with 2% ethanol. The absorbance measured on a Titertek Multiskan spectrophotometer at 650nm. The concentration of TNF that gave 50% maximal cell killing (one bioassay unit) was approximately equivalent to 50 pg/ml of recombinant human TNFα.

Bone resorption

Bone resorption was assayed by the measurement of calcium released from 5-day-old mouse calvariae *in vitro* (Zanelli, Lea and Nisbet *et al.*, 1969). Halved calvariae were cultured singly on stainless-steel grids in 30-mm dishes (five per group), with 1.5 ml BGJ medium (Flow Laboratories, Scotland, U.K.), supplemented with penicillin and streptomycin (100 u/l each Gibco), L-glutamine (2mM; Gibco), 5% complement-inactivated rabbit serum (Gibco) and 100 μg/ml ascorbic acid (Sigma). After 24 h the media were changed, and dialysed cyst culture medium, gingival culture medium or MCF was introduced at a dilution of 5 mg/ml (tissue weight/volume) or at 1 : 10 for the MCF preparation in fresh BGJ medium. A control group containing just BGJ medium and a positive control group exposed to PGE_2 (1μM) were included in all the bone-resorption experiments. In some of the experiments, indomethacin (1 μM) was added to the cyst culture media or MCF. In other experiments a monospecific rabbit polyclonal antibody which neutralizes the biological activity of both IL-1α and IL-1β (Genzyme) was incubated with the culture media at 4°C overnight at a dilution of 1 : 50 before addition to the bone cultures. This dilution was determined from a limited dose–response study with MCF or conditioned cyst media. As a control, normal rabbit serum was added to the tissue culture supernatants or the MCF and incubated overnight at 4°C before testing for bone resorbing activity. The cultures were incubated for a further 48 h and the calcium content of the media was measured by automated colorimetric analysis (Gitelman, 1967).

Immunocytochemical localization of cytokines

Tissues. Two keratocysts were obtained from patients undergoing surgery and the diagnosis was confirmed by histological examination of paraffin sections of portions of the cysts. Normal buccal mucosa and normal gingiva were obtained at surgery.

Fresh samples of these tissues were snap frozen in liquid nitrogen and then stored at −70°C. Frozen sections were cut at 5 μm in a Bright cryostat with the cabinet temperature maintained at below −25°C and taken up onto glass slides treated with 3-aminopropyltriethoxysilane. Slides were air dried at room temperature overnight and then fixed in acetone for 20 min before use. From each block, serial sections were stained with haematoxylin and eosin to determine the cyst type.

Antibodies. Sheep polyclonal antisera to human recombinant IL-1α and IL-1β (Poole and Gaines Das, 1991) and a goat antiserum to human recombinant IL-6 (Rafferty *et al.*, 1991) were obtained from Dr Stephen Poole (National Institute of Biological Standards and Control, South Mims). A mouse anti-human recombinant TNF α (52B83) was supplied by Dr Mark Bodmer (Celltech, Slough, U.K.).

Immunocytochemistry. After fixation in acetone, sections were briefly washed in tris buffer pH 7.6 and then treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to block endogenous peroxidase.

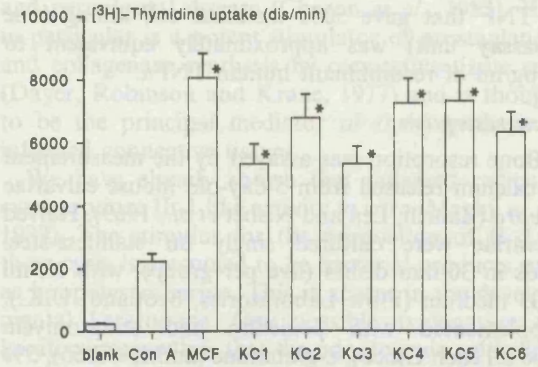

Fig. 1. The effects of keratocyst culture media (KC1-KC6; diluted 1 : 2) on mouse thymocyte proliferation. Thymocytes sub-optimally stimulated with concanavalin A (Con A). Mononuclear cell factor (MCF; diluted 1 : 2) was used as a positive control. The results are shown as the mean ± SD of the thymocyte cultures ($n = 6$). $*p < 0.05$.

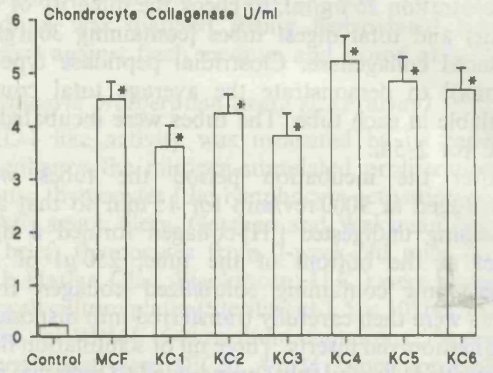

Fig. 2. The effects of keratocyst culture media (KC1-KC6; diluted 1 : 10) on collagenase synthesis by rabbit chondrocytes. Mononuclear cell factor (diluted 1 : 10) was used as a positive control. The results are shown as the mean ± SD of the chondrocyte cultures ($n = 6$). $*p < 0.05$.

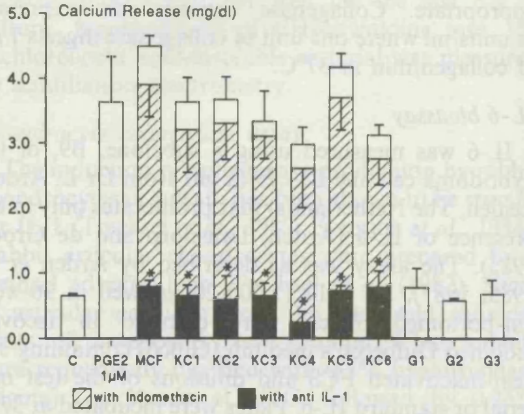

Fig. 3. The effects of dialysed keratocyst culture media (KC1–KC6; diluted 1:10; open bars) and dialysed normal gingival tissue culture media (G1-G3; diluted 1:10; open bars), the effect of the same cyst media in the presence of indomethacin (1 μmol) (cross-hatched bars) and the effect of the same media in the presence of anti-human IL-1 on bone resorption (solid bars). Showing the amount of calcium released into the culture media during a 48 h incubation of mouse calvariae with the test media. Mononuclear cell factor (diluted 1:10) and PGE_2 (1 μmol) were positive controls. The results are shown as the mean ± SD of the bone cultures ($n = 5$). $*P < 0.05$ resorption inhibited significantly by anti-human IL-1.

After washing in tris buffer, sections were then treated with a 1 : 50 dilution of normal donkey or normal rabbit serum for 30 min to block non-specific binding of conjugated secondary antibodies. After blocking, optimal dilutions of the various antibodies described above were added to sections and incubated for 2 h at room temperature. Sections were again washed with tris buffer and secondary antibodies [biotinylated donkey anti-sheep (Sigma) diluted 1 : 5,000 or biotinylated rabbit anti-mouse (Sigma) diluted 1 : 100] added and left for 30 min at room temperature. After a further wash in buffer a streptavidin–peroxidase complex was added and sections incubated for a further 30 min. After a final wash a solution of 3,3-diaminobenzidine plus hydrogen peroxide was added to sections and incubated for 3–5 min at room temperature. Sections were counterstained with Mayer's haematoxylin and examined microscopically.

Controls. For each specimen, serial sections were incubated in the absence of the primary or the secondary antibody to determine the specificity of binding. A further check of antibody specificity, where enough cytokine was available, was to incubate the specific antibody overnight with the cytokine at 4°C and then use the complex as described above.

Statistical analysis. Students unpaired *t*-test was applied to all data.

RESULTS

IL-1 bioactivity

LAF Assay. IL-1 activity, as measured by comitogenic activity in the LAF assay, was readily detectable in the conditioned media obtained from the explant cultures of keratocysts. All six keratocysts stimulated thymocytes to proliferate significantly in the LAF assay as shown in Text Fig. 1. In contrast, culture media supporting normal gingiva explants failed to show significant LAF activity.

Chondrocyte collagenase assay. IL-1 like activity, measured by its ability to stimulate rabbit chondrocytes to synthesize collagenase, was also readily detectable in the conditioned media obtained from keratocysts. The supernatants from all six keratocyst significantly stimulated collagenase synthesis by chondrocytes (Text Fig. 2.). Again, culture supernatants from normal gingival cultures failed to stimulate collagenase release.

Plate 1

Fig. 4. Immunocytochemical staining for cytokines in keratocyst and normal buccal mucosa. (A) IL-1α staining in keratocyst epithelium. × 160 (B) IL-6 staining in keratocyst epithelium. × 80 (C) IL-1α staining in normal buccal mucosa. × 32

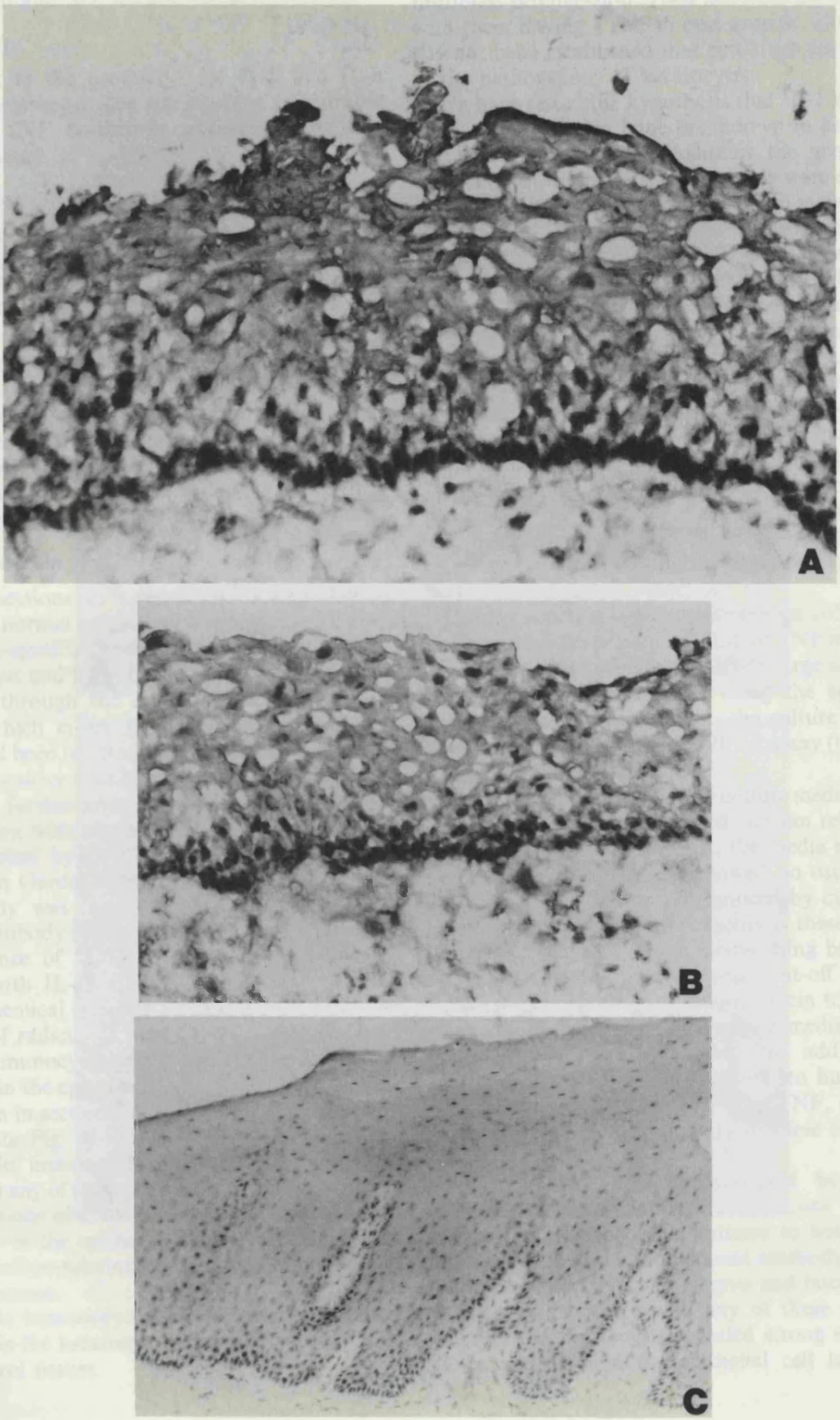

Plate 1

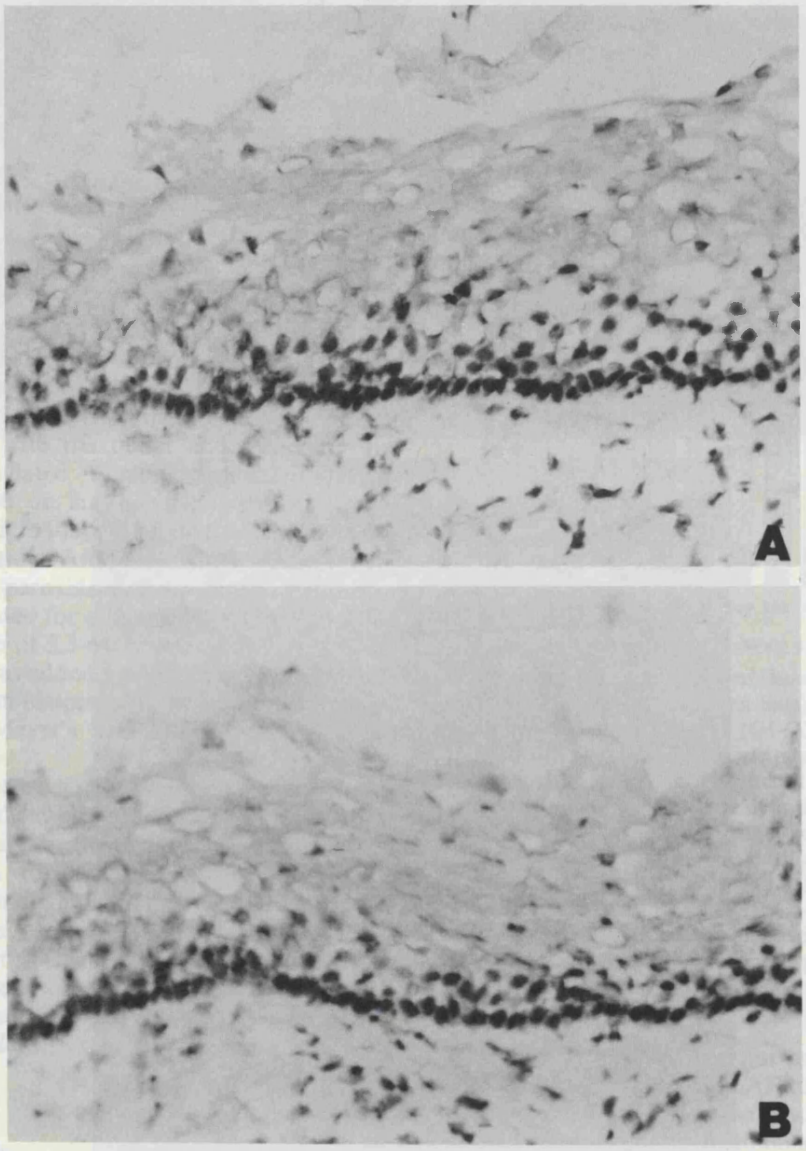

Plate 2

IL-6 bioactivity

Keratocysts produced relatively large amounts of IL-6 bioactivity (10,669 ± 1053 U/ml culture medium, mean and SD; normal tissue 410 U/ml).

TNF bioactivity

In contrast to the production of IL-1 and IL-6 bioactivity, keratocysts did not produce measurable amounts of TNF bioactivity as assessed by this cytotoxicity assay.

Bone resorption

Dialysed culture supernatants from all keratocysts significantly stimulated bone resorption. In order to determine whether this osteolytic activity was mediated by PG synthesis, indomethacin (1 μM) was added to the bone cultures. This did not inhibit the bone-resorbing activity of the keratocyst supernatants, as shown in Text Fig. 3.In contrast, the bone-resorbing activity of all the keratocyst supernatants was completely neutralized by a monospecific polyclonal antibody to human IL-1α and IL-1β (Text Fig. 3). Conditioned media from gingival cultures failed to demonstrate bone resorbing activity.

Immunolocalization of cytokines

Cryostat sections of keratocysts, normal buccal mucosa and normal gingiva were examined. To control for non-specific binding of antibody, sections from each cyst and from buccal mucosa and gingiva were taken through the immunocytochemical procedure in which either the primary or secondary antibody had been omitted. In both controls, sections showed no positive staining. The specificity of IL-1α staining was further assessed by incubating a sample of the antisera with an excess of recombinant IL-1α (kindly supplied by Dr Gubler, Hoffman LaRoche Ltd, Welwyn Garden City, U.K.) overnight at 4°C. The antibody was then compared with a similar sample of antibody incubated in the same manner but in the absence of IL-1α. Incubation of the IL-1α antiserum with IL-1α completely inhibited the immunocytochemical staining in keratocysts and in specimens of radicular cysts.

IL-1α. Immunocytochemical staining for IL-1α was found only in the epithelial layer of the cyst wall and was not seen in sections of normal buccal mucosa or gingiva (Plate Fig. 4).

IL-1β. No immunocytochemical reaction product was found in any of the sections examined (Plate Fig. 5).

IL-6. Sections of keratocyst showed positive staining for IL-6 in the epithelial layer (Plate Fig. 4) but in no other cell populations. No IL-6 staining was seen in control tissues.

TNFα. No immunocytochemical reaction product was found in the keratocyst sections (Plate Fig. 5) or in the control tissues.

DISCUSSION

A number of cytokines including IL-1, IL-6 and TNF have osteolytic activity and can also stimulate fibroblast proliferation. Their activities are consistent with them having a role in cyst growth. However, it has not been established that cytokines are involved in the pathogenesis of keratocysts.

We have tested the hypothesis that IL-1 is a major mediator promoting bone breakdown in keratocysts and that this cytokine facilitates the growth and expansion of these cysts. Keratocysts were explanted immediately after surgical removal and maintained in culture to allow them to produce and release the cytokines they would presumably synthesize *in vivo*. Two bioassays were used to measure the biological activity of the IL-1 present in the culture media supporting the explants. Both assays demonstrated that IL-1 was present in these media. The LAF assay is not particularly selective but the chondrocyte collagenase assay is relatively specific for IL-1 and, for example, will not respond to the other major osteolytic/chondrolytic cytokine TNF (Schnyder, Payne and Dinarello, 1987). Normal gingival tissue was also cultured in the same manner and tested for IL-1 bioactivity. In contrast to the keratocysts these gingival samples did not release assayable amounts of IL-1.

Culture supernatants from keratocyst cultures were also tested for the presence of IL-6 or TNF bioactivity. It was possible to measure relatively large amounts of IL-6 in these culture fluids using the specific B9 hybridoma assay. In contrast, the culture fluids did not show bioactivity in the TNF bioassay (the murine L929 cytotoxicity assay).

The osteolytic activity of the culture media supporting the cyst explants promoted calcium release from murine calvariae. By contrast, the media supporting normal gingival specimens showed no osteolytic activity. The bone resorption induced by cyst explant media was not due to prostanoids as these had been removed by dialysis of the media using benzoylated dialysis tubing (molecular weight cut-off of 2kDa). Furthermore, addition of indomethacin to bone cultures stimulated with cyst explant media failed to inhibit osteolysis. By contrast, the addition of a monospecific neutralizing antibody to human IL-1, which does not neutralize IL-6 or TNF, completely inhibited the osteolytic activity of these conditioned media.

These results were corroborated by immunocytochemical analysis of the cytokines present in keratocysts. Monospecific antisera to human IL-1α, IL-1β or IL-6 and a monoclonal antibody to human TNF were used. Normal gingiva and buccal mucosa showed no reactivity with any of these antibodies, whereas the keratocysts revealed strong staining for IL-1α and IL-6 in the epithelial cell layer. IL-1α

Plate 2

Fig. 5. Immunocytochemical staining for cytokines in keratocysts. (A) IL-1β staining showing lack of positive reaction product (B) TNFα staining showing lack of positive reaction product. ×80

staining was completely inhibited by incubation of recombinant IL-1α with the antibody, showing the absolute specificity of this reaction. Epithelial cells did not show any reaction with a monospecific antibody to IL-1β. This contrasts with our unpublished studies of the cytokine localization in radicular cysts in which the epithelial cells contain both IL-1α and IL-1β. Only the keratocyst epithelium demonstrated IL-1α and IL-6 staining, no other cell population indicated the presence of these cytokines. The monoclonal antibody to TNFα did not show any reactivity with the keratocyst specimens. These findings of cytokine immunolocalization are in complete agreement with the release of cytokines from cyst explants and strongly support the hypothesis that the cytokines released by cultured cyst explants are a product of the epithelial cells.

In summary, keratocysts release IL-1 and IL-6 but not TNF in explant culture, and the culture supernatants stimulate calvarial bone breakdown which is completely inhibited by a monospecific neutralizing antibody to IL-1 but not by indomethacin. Immunocytochemical localization of cytokines in tissues reveals the presence of IL-1α and IL-6 but not IL-1β or TNF. The cellular source of the immunochemically determined cytokine is the epithelial cell. This supports the hypothesis that the development and growth of keratocysts may be due to the action of IL-1α and IL-6. IL-1α is the principal osteolytic factor promoting bone breakdown; the role of the IL-6 is less clear. It does not appear to be directly involved in the calvarial breakdown but may still influence bone turnover in a more subtle way, and may also have a stimulatory influence on other cells present in the cyst. For example, it has been reported that IL-6 stimulates the proliferation of cultured human epidermal cells (Grossman *et al.*, 1989). There is therefore the possibility that IL-6 may stimulate epithelial cell proliferation in the keratocyst via an autocrine feedback mechanism.

One major problem in explaining the pathogenesis of the keratocyst in terms of cytokine expression and action is the question of the stimuli promoting the cytokine synthesis. Keratocysts are classically defined as non-inflammatory developmental cysts, without any overt involvement of bacterial products (e.g. lipopolysaccharides) or inflammatory cells in the process of cyst growth. Their origin from the ubiquitous cell rests of dental lamina remains unexplained.

Further studies are therefore needed to try to identify exogenous or endogenously altered cytokine gene control factors that could be responsible for cytokine synthesis. The possibility that the defect lies in the control of cytokine inhibitors, e.g. release of natural cytokine antagonists, soluble receptors or binding proteins is currently under investigation.

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ANTI-PROLIFERATIVE AND CYTOTOXIC ACTIVITY OF SURFACE-ASSOCIATED MATERIAL FROM PERIODONTOPATHOGENIC BACTERIA

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Summary—The easily solubilized surface-associated material from three bacterial species associated with periodontal diseases, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Eikenella corrodens*, produced dose-dependent inhibition of thymidine incorporation by human fibroblasts, the human monocytic cell line U937 and guinea pig epidermal cells. In contrast, lipopolysaccharides from *A. actinomycetemcomitans* and *P. gingivalis* were either inactive or substantially less active over the dose range tested. One of the constituents of surface-associated material from a 'non-leucotoxic' strain of *A. actinomycetemcomitans* was highly cytotoxic to human peripheral blood polymorphonuclear cells, with 50% killing from less than 1 ng/ml. A constituent of the surface-associated material from *P. gingivalis* was approximately one log order less active. The lipopolysaccharides from these bacteria were at least three log orders less active in neutrophil killing. These findings add weight to the hypothesis that easily solubilized exopolymers from periodontopathogens play a major part in the pathology of periodontal diseases.

Key words: periodontopathogens, surface-associated material, lipopolysaccharide, periodontal disease.

INTRODUCTION

That there is a close association between dental plaque and the pathogenesis of the periodontal diseases is now widely accepted (Slots, 1979). However, the exact nature of this association is still unclear. In attempting to ascertain the role of bacteria in these diseases, attention has focused on the biological activity of one component common to many of the disease-related bacteria, namely lipopolysaccharide. One particular activity associated with periodontopathogenic lipopolysaccharides is the inhibition of mammalian cell division. Numerous studies have shown that lipopolysaccharides from various bacteria inhibit the growth of murine and human fibroblasts (Larjava *et al.*, 1987; Layman and Diedrich, 1987). However, in studies with *Actinobacillus actinomycetemcomitans* we have shown that its purified lipopolysaccharide inhibit the division of fibroblasts only at high concentrations (10–50 μg/ml). In contrast, its capsular material, obtained by gentle solubilization in saline at 4°C, was between one and two log orders more potent in inhibiting their division (Kamin *et al.*, 1986).

Fibroblasts are only one of the cell populations in the inflamed gingiva that are exposed to bacteria and their components. The response of epithelial cells, macrophages, polymorphonuclear leucocytes and others to lipopolysaccharides and/or other bacterial components has not been investigated in a systematic manner. We now report on the response of cultured fibroblasts, epithelial cells, the macrophage cell line U937, and peripheral blood polymorphonuclear leucocytes to surface-associated material from three periodontopathogenic bacteria.

Abbreviations: FCS, fetal calf serum; MEM, minimum essential medium; PBS, phosphate-buffered saline; RPMI, Rosswell Park Memorial Institute (medium); SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Isolation of surface-associated material

All bacteria were grown on solid media to promote the production of capsules.

A. actinomycetemcomitans Y4 (a leucotoxic strain) and *A. actinomycetemcomitans* NCTC 9710 (a non-leucotoxic strain) were grown at 37°C under anaerobic conditions on brain–heart infusion agar (Oxoid, Basingstoke, U.K.).

Porphyromonas gingivalis W50 was grown at 37°C under anaerobic conditions on a medium consisting of 5 g trypticase, 5 g proteose peptones (Oxoid), 2.5 g glucose, 2.5 g sodium chloride, 0.375 g cysteine HCl (BDH), 2.5 g yeast extract, 0.25 g haemin and 0.05 g menadione per litre of medium (Oxoid).

Eikenella corrodens NCTC 10596 was grown at 37°C under anaerobic conditions on a medium consisting of brain–heart infusion (37 g/l; Oxoid), 0.375 g cysteine HCl (BDH), 0.25 g haemin and 0.05 g menadione (Oxoid).

All bacteria were grown on solid media to enhance production of surface-associated material. After 72 h all cultures were inspected visually for contamination, Gram-stained, removed from the plate with sterile saline and then centrifuged at 30,000 *g* for 30 min at 4°C. Bacteria were then resuspended in saline and recentrifuged. They were then lyophilized and the surface-associated material was obtained by saline extraction, as described by Wilson, Kamin and Harvey (1985).

In brief, lyophilized bacteria were gently stirred at 4°C in sterile saline for 1 h and then sedimented by centrifugation. The surface-associated material was precipitated by addition of acetone, centrifuged, dialysed and then lyophilized. The protein content of the precipitated material was assayed by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as standard. The extent of contamination by endotoxin was tested in a chromogenic limulus amoebocyte assay (Pyrogent; Byk-Mallinckrodt, U.K.).

The surface-associated material of *A. actinomycetemcomitans* and *P. gingivalis* was fractionated by gel filtration and ion-exchange chromatography, as described by Wilson *et al.* (1985). In each case, the major component obtained was a protein- and carbohydrate-containing material which was used in experiments with polymorphonuclear leucocytes.

Preparation of lipopolysaccharides

After extraction of the surface-associated material from *A. actinomycetemcomitans*, *P. gingivalis* and *E. corrodens*, lipopolysaccharides were extracted from the resulting cells as described by Kiley and Holt (1980). In brief, cells were extracted three times with phenol/water at 68°C and the resulting aqueous phases were lyophilized after dialysis against distilled water. The crude lipopolysaccharide obtained was purified by treatment with RNase, DNase and pronase, and then ultracentrifuged. The purity of the lipopolysaccharide was assessed by SDS–PAGE.

Fibroblast culture and DNA synthesis

Human gingival fibroblasts were grown from explants obtained during minor oral surgical procedures. The cells were grown in Eagles' MEM containing 10% FCS, 2 mM L-glutamine, 27 mmol/l $NaHCO_3$, penicillin and streptomycin (each 100 U/ml) and incubated at 37°C in 5% CO_2/air. Cells were subcultured at weekly intervals and used for experiments between passages 6 and 12.

Fibroblast suspensions were seeded into 96-well culture plates (Microtitre, Linbro, U.K.) at concentrations of 15,000 cells/well in 100 μl of MEM with 10% FCS and left overnight to attach. The culture medium was then replaced with MEM containing 2% FCS and various concentrations of either purified lipopolysaccharide or surface-associated material, in groups of six wells per concentration. Control cultures received only unsupplemented medium. Cells were incubated for a further 24 h and then 18.5 kBq of [5-^{3}H]-thymidine ([^{3}H]-TdR: specific activity 740 kBq/mmol, Amersham, Arlington Heights, IL, U.S.A.) was added to each well 6 h before ending the cultures. Trichloro-acetic acid-insoluble material in each cell layer was dissolved in 0.4 M NaOH and radioactivity measured in 3 ml of scintillant by scintillation spectrometry (Rackbeta: Pharmacia, Sweden) with external standardization.

Keratinocyte culture and DNA synthesis

Keratinocytes (GPK cell line, derived from guinea pig ear epidermis) were obtained from M. Hola, Department of Biochemical Pathology, University College School of Medicine, London, U.K. They were grown in Eagles' MEM containing 10% FCS, 2 mM L-glutamine, 27 mmol/l $NaHCO_3$, penicillin and streptomycin (each 100 U/ml) and incubated at 37°C in 5% CO_2/air. Keratinocyte suspensions were seeded into 96-well culture plates (Microtitre, Linbro) at concentrations of 20,000 cells per well in 100 μl of MEM with 10% FCS and left overnight to attach. The culture medium was then replaced with MEM containing 2% FCS and various concentrations of either purified lipopolysaccharide or surface-associated material, in groups of six wells per concentration. Control cultures received only unsupplemented medium. Cells were incubated for a further period of 24 h and then 18.5 kBq of [5-^{3}H]-thymidine (specific activity 740 kBq/mmol; Amersham) was added to each well 6 h before ending the cultures. The incorporation of tritiated thymidine was measured as described above.

Macrophage cell-line culture and DNA synthesis

U937, a monocytic cell line derived from a human histiocytic lymphoma was used in these experiments. The cells were seeded into 96-well culture plates (Microtitre, Linbro) at a concentration of 500,000 cells per well in 100 μl of RPMI medium (Gibco, Grand Island, NY, U.S.A.) with 5% heat-inactivated FCS containing various concentrations of either purified lipopolysaccharide or surface-associated material, in groups of six wells per concentration. Control cultures received only unsupplemented medium. Cells were incubated for 48 h and then 18.5 kBq of [5-^{3}H]-thymidine (specific activity 740 kBq/mmol; Amersham) was added to each well 6 h before ending the cultures. The cells were collected using a cell harvester (Skatron) and radioactivity was measured in 3 ml of scintillant by scintillation spectrometry (Rackbeta; Pharmacia) with external standardization.

Preparation of polymorphonuclear leucocytes and cytotoxicity assay

Preparation of neutrophil monolayers. Peripheral venous blood was collected in plastic tubes containing 20 U/ml heparin, mixed 1:4 with Dulbecco's PBS and separated on a cushion of Ficoll–Hypaque according to Boyum (1968). Pelleted red blood cells and neutrophils were resuspended in fresh lysing buffer (0.155 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 M EDTA) to disrupt the red blood cells, washed twice with PBS, and then in RPMI medium. Cells pelleted after the final wash were resuspended in RPMI 1640 containing 10% FCS, diluted to 2×10^5 cells per ml and stored on ice until required. Glass coverslips (13 mm dia) were placed in sterile cluster trays and overlaid with 1 ml of leucocyte suspension.

Neutrophil cytotoxicity assay. After a 60 min incubation at 37°C to allow the neutrophils to adhere

to coverslips, the monolayers were washed twice with PBS and then various concentrations of lipopolysaccharide or the protein/carbohydrate material purified from the surface-associated material were added. Dose- and time-response experiments were made for each of the four microbial extracts. After incubation the coverslips were washed with PBS and viability was tested by adding 0.1% trypan blue and counting, by phase-contrast microscopy, the number of cells capable of excluding the dye. Assays were made in triplicate and for each coverslip 200 neutrophils were counted. The number of cells taking up trypan blue gave the percentage of cells killed by the specific concentration of microbial extract after the appropriate incubation time.

RESULTS

Preparation of surface-associated material

Saline extraction of the three periodontopathogens released the surface-associated material without causing damage to the cells, as assessed by electron microscopy (Wilson, Kamin and Harvey, 1985; Meghji, unpublished). The endotoxin content of these extracts was in the range 1–10 ng/mg dry weight.

Inhibition of fibroblast DNA synthesis

The surface-associated material from the three periodontopathogens inhibited the incorporation of [^{3}H]-TdR into human gingival fibroblasts in a dose-dependent manner over the range 10 ng/ml to 10 μg/ml (Fig. 1). At 10 ng/ml the preparations had very similar potencies, achieving a reduction in thymidine incorporation of between 17 and 18%. At the highest concentration tested, 10 μg/ml, surface-associated material from *P. gingivalis* was the most effective inhibitor of incorporation, achieving a 50% reduction compared with control cultures.

In contrast, lipopolysaccharide from *A. actinomycetemcomitans* had no significant effect on fibroblast proliferation, even at concentrations as high as 10 μg/ml.

Inhibition of keratinocyte DNA synthesis

Over the concentration range of 10 ng/ml to 10 μg/ml, surface-associated material from *A. actinomycetemcomitans*, *P. gingivalis* and *E. corrodens* inhibited thymidine uptake into cultured guinea pig keratinocytes in a dose-dependent manner. At 10 ng/ml, that from both *A. actinomycetemcomitans* and *P. gingivalis* achieved a statistically significant degree of inhibition, amounting to 18% in both cases. At higher concentrations, the inhibition achieved by all three preparations was significantly different from that of control cultures. In contrast, addition of lipopolysaccharide from *A. actinomycetemcomitans* or *P. gingivalis* to keratinocytes resulted in a dose-dependent stimulation of thymidine incorporation over the concentration range 0.01–10 μg/ml (Fig. 2).

Inhibition of U937 DNA synthesis

Surface-associated material from the three periodontopathogens was added to U937 cells over the dose range 100 ng/ml to 50 μg/ml. Again, all three preparations were able to inhibit thymidine incorporation by these cells (Fig. 3). The material from *A. actinomycetemcomitans* and *E. corrodens* were most active, producing 50% inhibition at between 200–400 ng/ml. The surface-associated material from *P. gingivalis* was less active, causing 50% inhibition at approx. 2–3 μg/ml.

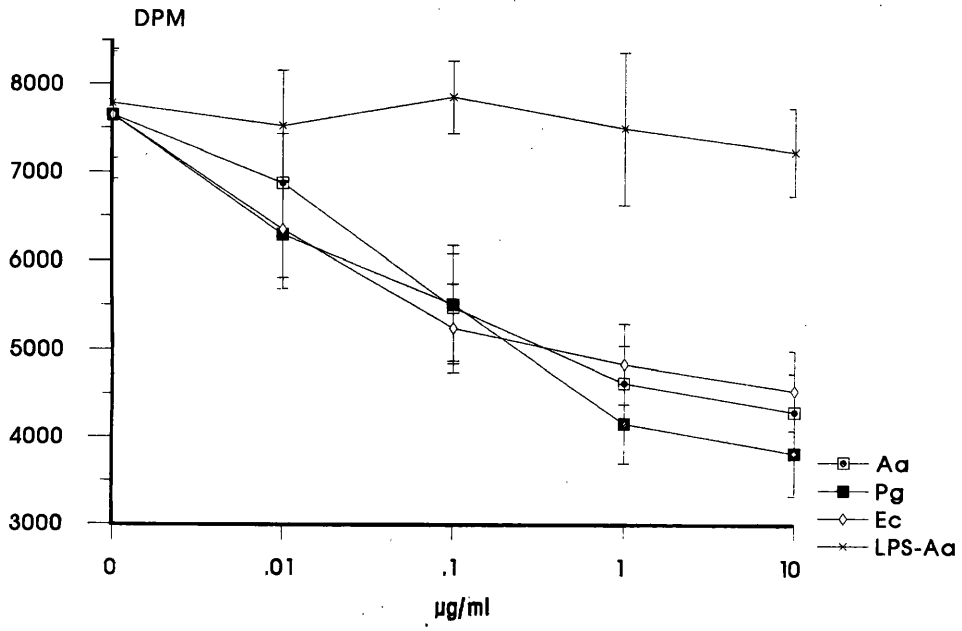

Fig. 1. Inhibitory effect of increasing concentrations of surface-associated material from *A. actinomycetemcomitans* (Aa; open square), *P. gingivalis* (Pg; solid square), *E. corrodens* (Ec; open diamond) and lipopolysaccharide from *A. actinomycetemcomitans* (LPS-Aa; cross) on DNA synthesis, measured as incorporation of tritiated thymidine into DNA by human gingival fibroblasts. The results are expressed as mean and SD of six cultures. The significance of the results was calculated by Student's *t*-test.

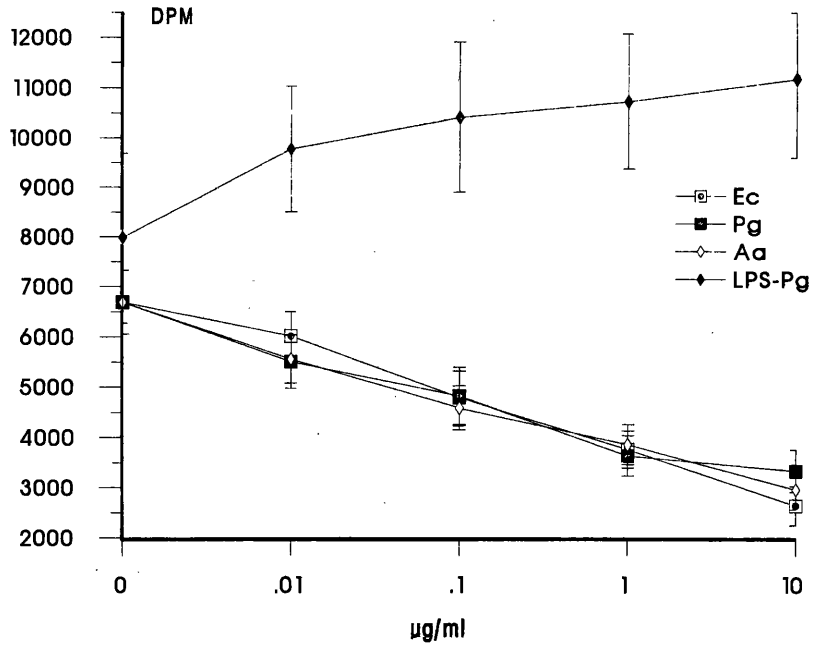

Fig. 2. Inhibitory effect of increasing concentrations of surface-associated material from *A. actinomycetemcomitans* (Aa; open diamond), *P. gingivalis* (Pg; solid square), *E. corrodens* (Ec; open square) and lipopolysaccharide from *P. gingivalis* (LPS-Pg; solid diamond) on DNA synthesis, measured as incorporation of tritiated thymidine into DNA by keratinocytes. The results are expressed as mean and SD of six cultures. The significance of the results was calculated by Student's *t*-test.

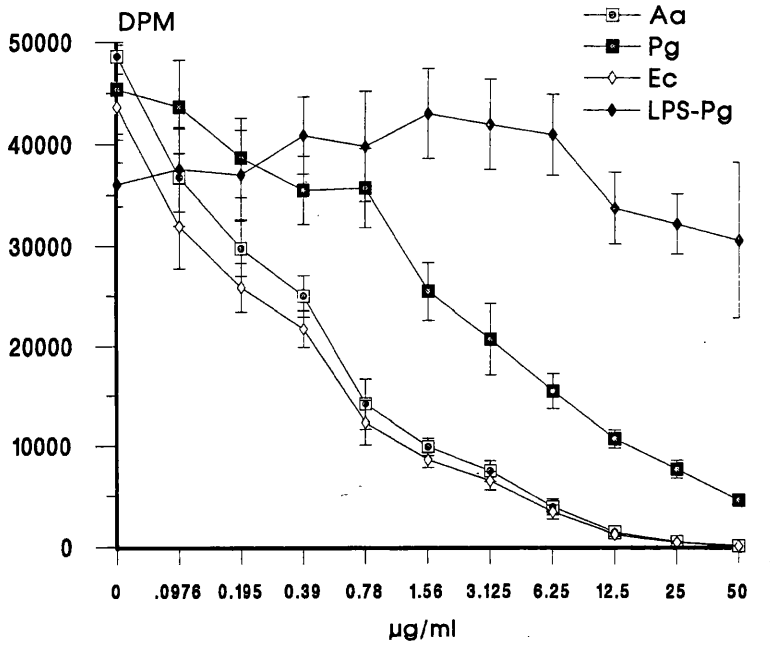

Fig. 3. Inhibitory effect of increasing concentrations of surface-associated material from *A. actinomycetemcomitans* (Aa; open square), *P. gingivalis* (Pg; solid square), *E. corrodens* (Ec; open diamond) and lipopolysaccharide from *P. gingivalis* (LPS-Pg; solid diamond) on DNA synthesis, measured as incorporation of tritiated thymidine into DNA by U937 cells. The results are expressed as mean and SD of six cultures. The significance of the results was calculated by Student's *t*-test.

In contrast lipopolysaccharide from *P. gingivalis* failed to inhibit [^{3}H]-TdR incorporation even at 50 μg/ml (Fig. 3).

Neutrophil cytotoxicity

Preliminary experiments had indicated that maximum cytotoxicity could be achieved by 5.0 and 50.0 μg/ml of lipopolysaccharide from *A. actinomycetemcomitans* and *P. gingivalis*, respectively, whereas with the substance isolated from the crude preparations of surface-associated materials the respective concentrations were 0.6 and 12.5 ng/ml. These concentrations were used to determine the time course of cytotoxicity for the four bacterial extracts (Fig. 4); at these concentrations all neutrophils are killed in 60 min. The dose response for these four bacterial constituents was determined in assays lasting for 30 min. This showed that lipopolysaccharide from *A. actinomycetemcomitans* was much more active than that from *P. gingivalis*, although even with 100 μg/ml lipopolysaccharide from *A. actinomycetemcomitans* there was only 80% neutrophil cytotoxicity. In contrast, as little as 12.5 ng/ml of the substance isolated from the crude preparations of surface-associated material from *A. actinomycetemcomitans* produced 100% cytotoxicity in 30 min, with 50% killing at less than 1 ng/ml. The preparation purified from the surface-associated material of *P. gingivalis* produced 100% killing at 12.5 μg/ml, with 50% activity at 12.5 ng/ml (Fig. 5).

DISCUSSION

Many of the bacterial species associated with periodontal disease are capable of exerting a cytotoxic effect on a number of mammalian cells (Boehringer, Taichman and Shenker, 1984; Stevens and Hammond, 1988; Fotos *et al.*, 1990). Most studies in this field have been concerned with the effect of bacterial cell sonicates on human gingival fibroblasts; sonicates from *A. actinomycetemcomitans*, *P. gingivalis*, *Fusobacterium nucleatum*, *E. corrodens* (Stevens and Hammond, 1988) *Prevotella intermedia* (Fotos *et al.*, 1990) and *Treponema denticola* (Boehringer *et al.*, 1984) all show some degree of toxicity towards fibroblasts. Comparatively few studies have been directed at evaluating the potential of periodontopathogens to adversely affect growth of other mammalian cell types such as epithelial and endothelial cells, lymphocytes and monocytes, the exception to this generalization being the many investigations concerning the leucotoxicity of *A. actinomycetemcomitans* (Zambon *et al.*, 1988). Despite the demonstrable cytotoxicity of crude bacterial extracts, few investigators have attempted to characterize the active constituents or utilized purified bacterial components in their assays. The few such studies have invariably involved the leucotoxin from *A. actinomycetemcomitans* or lipopolysaccharide from a number of species. Indeed, a review of the literature leaves the impressions that 'cytotoxicity' is attributable solely to lipopolysaccharides of Gram-negative periodontopathogens and, in the case of *A. actinomycetemcomitans*, a proteinaceous leucotoxin (Tsai *et al.*, 1984).

We have here prepared saline extracts of three periodontopathogenic bacteria: *A. actinomycetemcomitans*, *P. gingivalis* and *E. corrodens*. These bacteria have a number of surface features, including capsules and fibrils (Handley, 1991), and our procedure was sufficient to remove such material, which we have termed surface-associated material. The extraction was gentle, and electron microscopic examination

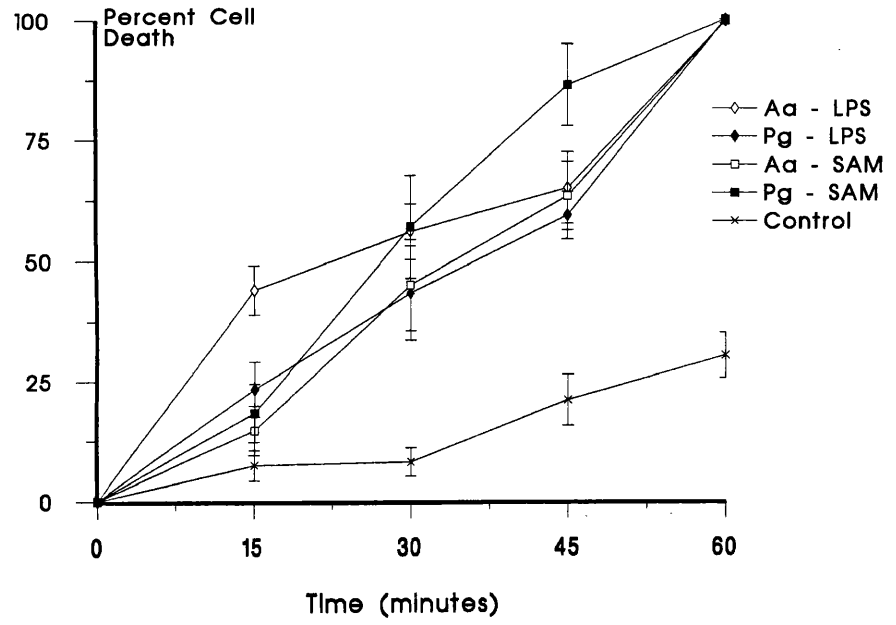

Fig. 4. Cytotoxicity of surface-associated material from *A. actinomycetemcomitans* (0.6 ng/ml; Aa-SAM; open square) and *P. gingivalis* (12.5 ng/ml; Pg-SAM; solid square) and lipopolysaccharide from *A. actinomycetemcomitans* (5 μg/ml; Aa-LPS; open diamond) and *P. gingivalis* (50 μg/ml; Pg-LPS; solid diamond) over 60 min.

revealed that, after extraction, the surface-associated material had been almost totally removed but the bacteria were intact (Wilson *et al.*, 1985; Meghji, unpublished). This was reinforced by the finding of very low levels of endotoxin in the surface-associated material.

We had earlier demonstrated that one of the components of surface-associated material from *A. actinomycetemcomitans* was 1–2 log orders more active than the corresponding lipopolysaccharide in inhibiting thymidine incorporation into human fibroblasts (Kamin *et al.*, 1986). This inhibition of cell function could contribute to the pathology of periodontal disease by compromising repair processes. It was therefore of interest to determine if this anti-proliferative activity of surface-associated material from *A. actinomycetemcomitans* was unique to this bacterium and to the fibroblast. It was also of interest

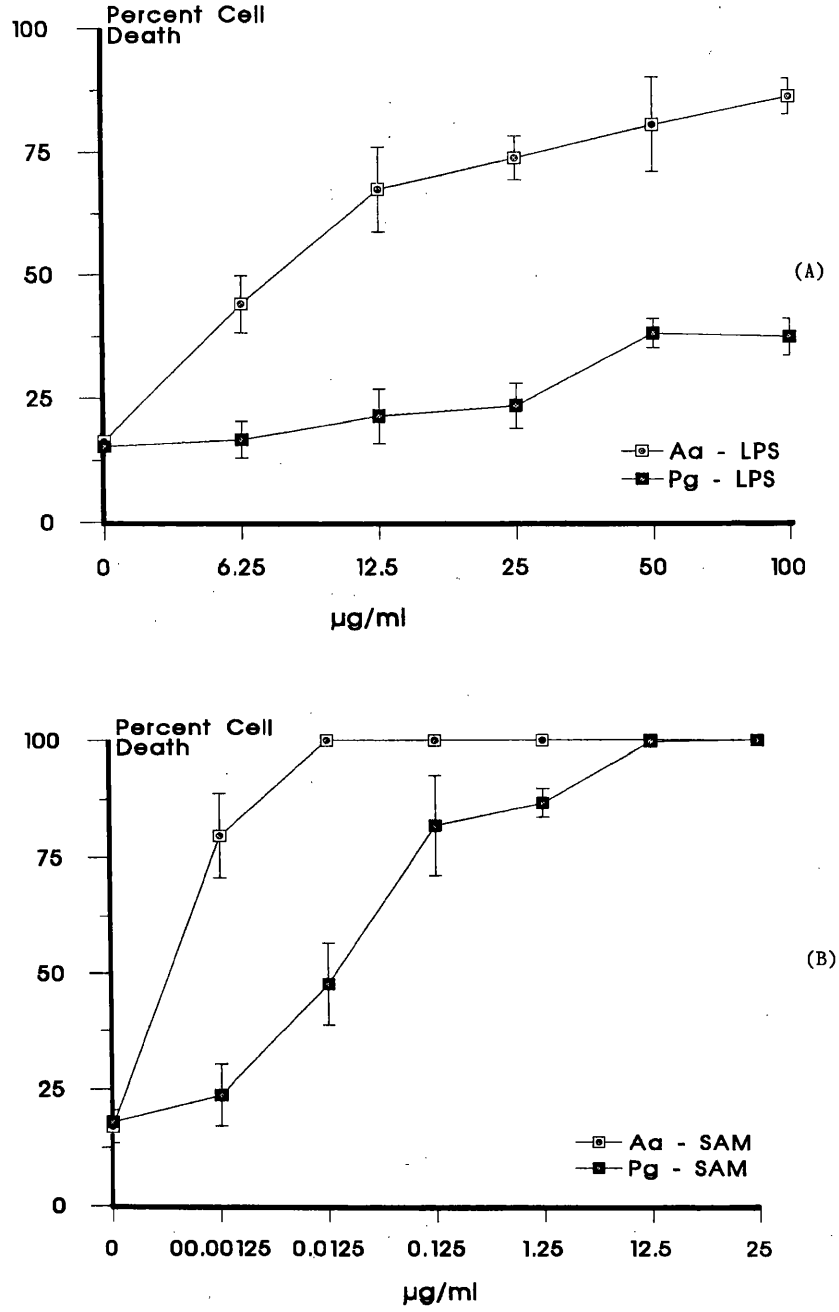

Fig. 5. (A) Increasing percentage cell death with increasing concentrations of lipopolysaccharide from *A. actinomycetemcomitans* (Aa-LPS; open square) and *P. gingivalis* (Pg-LPS; solid square) over 30 min. Results are expressed as the mean and SD of three cultures. (B) Increasing percent cell death with increasing concentrations of surface-associated material from *A. actinomycetemcomitans* (Aa-SAM; open square) and *P. gingivalis* (Pg-SAM; solid square) over 30 min. Results are expressed as the mean and SD of three cultures.

to determine the relative activities of surface-associated material and lipopolysaccharide from the same organism.

With regard to fibroblast cytotoxicity, we show that surface-associated material from *P. gingivalis*, *A. actinomycetemcomitans* and *E. corrodens* is a potent inhibitor of fibroblast proliferation, achieving statistically significant inhibition (approx. 17% in each case) at concentrations as low as 10 ng/ml, whereas lipopolysaccharide from one of these organisms, *A. actinomycetemcomitans*, had little effect on fibroblast proliferation at concentrations as high as 10 μg/ml.

In the case of *A. actinomycetemcomitans*, several studies have indirectly implicated surface-associated material as the component(s) responsible for fibroblast inhibition. For example, Shenker, Kushner and Tsai (1982) and Stevens, Gatewood and Hammond (1983) demonstrated that the component responsible for fibroblast toxicity was neither endotoxin nor the leucotoxin. Furthermore, Stevens *et al.* (1983) showed that the inhibitory activity was present in a phosphate-buffer extract of the organism, implying that the factor responsible was a surface component.

P. gingivalis sonicates are apparently toxic to human gingival fibroblasts, although the concentrations required to achieve a statistically significant effect were very high, amounting to between 10 and 50 μg/ml of protein (Larjava *et al.*, 1987; Fotos *et al.*, 1990). Once again, lipopolysaccharide was considered to be the active component responsible. However, our results imply that surface components other than lipopolysaccharide are the prime agents responsible for the fibroblast cytotoxicity of *P. gingivalis*. There has been only one report of the toxicity of *E. corrodens* towards fibroblasts (Stevens and Hammond, 1988). These investigators found that sonic extracts containing 50 μg/ml of protein inhibited thymidine incorporation by human gingival fibroblasts but only to the extent of approx. 10%. The component(s) responsible for the cytotoxic effect was not identified.

There are few reports of the *in vitro* effects of periodontopathogenic bacteria on epithelial cells. Kamen (1981) found that sonic extracts of *P. gingivalis* inhibited thymidine uptake by human foreskin keratinocytes in a dose-related manner over the concentration range 0.25–25 μg/ml. However, a statistically significant degree of inhibition was obtained only at a concentration of 2.5 μg/ml of sonicate, and this amounted to between 47 and 66% depending on the length of exposure. Kamen (1983) also found that a sonic extract of *A. actinomycetemcomitans* inhibited keratinocyte proliferation. The keratinocyte-inhibitory factor in these sonicates was not identified, but it was suggested that lipopolysaccharide may have been responsible (Kamen, 1983). Our finding that surface-associated material from *A. actinomycetemcomitans*, *E. corrodens* and *P. gingivalis* inhibited keratinocyte proliferation at concentrations as low as 10 ng/ml, while lipopolysaccharide from these organisms actually stimulated proliferation, would suggest that the inhibitory component of the bacterial sonicates may have been surface-associated material rather than lipopolysaccharide.

With the exception of *A. actinomycetemcomitans*, few organisms have been investigated for their potential cytotoxicity towards monocytes. The toxic effects shown by *A. actinomycetemcomitans* towards these cells have been attributed to its leucotoxin (Tsai *et al.*, 1979), which Simpson, Berthold and Taichman (1988) have shown to be effective against the monocytic cell line (U937) used in our investigation at doses as low as 9 ng. Although we used a leucotoxic strain (Y4) of *A. actinomycetemcomitans*, PAGE of the surface-associated material showed that no protein-containing band with a molecular weight of the order of 115 kDa was present (Meghji, unpublished), implying the absence of the leucotoxin. Current attempts at purification of surface-associated material from this organism and identification of its constituents should reveal the nature of the cytotoxic component(s). Surface-associated material from both *P. gingivalis* and *E. corrodens* also showed appreciable toxicity towards our monocytic cell line, that from *E. corrodens* being considerably more potent than that of *P. gingivalis* and very similar in potency to that from *A. actinomycetemcomitans*, achieving 27% inhibition at a concentration of approx. 100 ng/ml.

The adverse effects of *A. actinomycetemcomitans* on neutrophils have been attributed to a 115 kDa outer membrane-associated leucotoxic protein (Tsai *et al.*, 1984). We now show that one of the constituents of surface-associated material, a protein- and carbohydrate-containing material, from a "non-leucotoxic" strain of this organism (Ohta and Kato, 1991) is a potent leucocidal agent. At concentrations as low as 1.25 ng/ml this material killed 80% of neutrophils in a suspension containing 2×10^5 cells/ml. PAGE of this material revealed that no protein-containing band with a molecular weight corresponding to the leucotoxin (115 kDa) was present (S. Kamin, unpublished). Purified surface-associated material of *P. gingivalis*, although exhibiting a considerable degree of cytotoxicity, was far less potent, requiring a concentration 100-fold greater to achieve a similar degree of killing. In the case of both organisms, the substance purified from the surface associated material had a potency at least three log orders greater than that of the lipopolysaccharide from the corresponding organism.

Our studies have shown that the easily solubilized surface components of three periodontopathogens are able to inhibit thymidine incorporation by a number of mammalian cells and have potent cytotoxic activity against human neutrophils. The corresponding lipopolysaccharide from each bacterium was either inactive or several orders of magnitude less active. Even during heatlh, periodontal tissues undergo resorptive and formative phases with tissue turnover. Thus anti-proliferative activity could contribute to the loss of periodontal tissue and may play a part in modulating the function of gingival epithelial cells. The likely outcome of inhibiting monocyte cell division is unclear but it would be of interest to determine if the surface-associated material can inhibit maturation of monocytes into macrophages. It must be emphasized that we used continuous cell lines to determine the effects of surface-associated material on keratinocytes and monocytes. It should be borne in mind that, whilst these are commonly used in biological research as convenient sources of relevant cells, their response to stimuli may be qualitatively or quantitatively

different from that of primary cultures. However, it was clear that with both cell lines there was a definite response to the surface-associated material but that lipopolysaccharides were relatively inactive. The lethal effect of surface-associated material, particularly from *A. actinomycetemcomitans*, on neutrophils may have a profound role in pathogenesis. The main function of these cells will be removal of living and dead bacteria through opsonization and phagocytosis, and disruption of this activity could have profound inflammatory effects.

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Inhibition of Bone DNA and Collagen Production by Surface-Associated Material From Bacteria Implicated in the Pathology of Periodontal Disease

Sajeda Meghji, Brian Henderson,* Sean Nair,* and Michael Wilson†*

Gentle extraction of oral bacteria implicated in the pathogenesis of periodontal disease, *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis*, or *Eikenella corrodens*, with saline removes the extracellular components while leaving the bacteria intact. This readily-solubilized surface-associated material (SAM) has been demonstrated to significantly inhibit DNA and collagen synthesis by murine calvaria at concentrations as low as 10 ng/ml. DNA and collagen synthesis in isolated calvarial osteoblasts were also inhibited by these SAM preparations with similar dose responses. The inhibitory effect of these bacterial expolymers was blocked by 1uM indomethacin. The potent inhibitory actions on bone synthesis of the SAM from these bacteria may contribute to the alveolar bone loss found in patients with periodontal disease. *J Periodontol 1992; 63:736–742.*

Key Words: Periodontal diseases/pathogenesis; periodontal diseases/microbiology; *Actinobacillus actinomycetemcomitans; Porphyromonas gingivalis; Eikenella corrodens;* DNA; collagen; calvaria; osteoblasts.

Chronic inflammatory periodontal disease (CIPD) is the most common chronic inflammatory lesion afflicting man. Inflammation is confined to the gingiva and is associated with the destruction of the alveolar bone in which the teeth are located. The progressive destruction of this bone leads to the eventual loss of the teeth.[1] At present there are no clinical therapies which will inhibit the destruction of the extracellular matrix of the alveolar bone. A better understanding of the cellular mechanisms involved in this disease is needed, therefore, in order to develop effective drug-based therapies.

CIPD is believed to be caused, either directly or via the host response, by certain oral bacteria such as *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Eikenella corrodens*, and *Campylobacter rectus* (formerly *Wolinella recta)*.[2] With regard to the bone loss characteristic of advanced forms of the disease, most attention has been focused on the mechanisms of bone destruction and the role played in this by lipopolysaccharide (LPS). However, it is possible that bone loss in CIPD is the result of the inhibition of bone formation or of a combination of increased breakdown and decreased synthesis. Indeed, there is evidence to suggest that inhibition of bone synthesis contributes to alveolar bone loss in CIPD.[3,4]

Many of the bacteria associated with the pathology of CIPD have capsules and/or other surface components.[5-7] We have shown previously that solubilized capsular components from *Actinobacillus actinomycetemcomitans*, when added to cultured murine calvaria, inhibited both DNA and collagen synthesis.[8] The bone cell population responsive to the capsular material is believed to be the osteoblast, but this has not been formally demonstrated. It is not known if this inhibitory activity is unique to the capsular components of this organism or is a general action of the outer, easily solubilized, components of periodontopathogenic bacteria. Therefore, in the present study we have examined solubilized surface-associated material (SAM) from 3 periodontopathogenic bacteria for their ability to inhibit DNA and collagen synthesis in cultured murine calvaria. Furthermore, the mechanism of inhibition has been assessed by use of the cyclo-oxygenase inhibitor, indomethacin. In order to assess the effects of these bacterial components on their potential target cells, osteoblasts, these cells have been isolated from murine calvaria and exposed to graded concentrations of SAM.

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MATERIALS AND METHODS

Preparation of SAM

Actinobacillus actinomycetemcomitans Y4 was grown at 37°C under anaerobic conditions on Brain Heart Infusion agar.‡ *Porphyromonas gingivalis* W50 was grown at 37°C under anaerobic conditions on a medium consisting of 5g Trypticase,§ 5g proteose peptose,‡ 2.5g glucose,‖ 2.5g sodium chloride,‖ 2.5g yeast extract,‡ 0.375g cysteine HCl,‖ 0.25g haemin, and 0.05g menadione‡ per liter. *Eikenella corrodens* NCTC 10596 was grown at 37°C under anaerobic conditions on a medium consisting of Brain Heart Infusion (37g/l),‡ 0.375g cysteine HCl,‖ 0.25g haemin, and 0.05g menadione‡ per liter.

All bacteria were grown on solid media to enhance capsule production. After 72 hours all cultures were inspected visually for contamination, Gram-stained, removed from the plate with sterile saline, and then centrifuged at 30,000xg for 30 minutes at 4°C. Bacteria were washed once with saline. They were then lyophilized and the SAM was obtained by saline extraction as described previously.[9] Briefly, lyophilized bacteria were very gently stirred in sterile saline at 4°C for 1 hour and then the bacteria were sedimented by centrifugation. The solubilized SAM was then precipitated by addition of acetone, centrifuged, dialysed, and then lyophilized. The protein content of the extracted SAM was determined by the Lowry assay[10] and the endotoxin content was assayed by a chromogenic *Limulus* amoebocyte lysate assay.#

Collagen Synthesis by Calvaria

Calvaria from 5 day-old mice were halved along the sagittal suture and incubated in groups of 6 for 18h in 50mm culture dishes containing BGJ medium¶ supplemented with 50μg/ml ascorbic acid,** 2mM glutamine†† 5% heat-inactivated rabbit serum,‡‡ and 50μg/ml beta-aminopropionitrile fumarate** to inhibit collagen cross-linking. ^{3}H-proline (specific activity 31.5 Ci/mmol)§§ was added at 2μCi/ml for the last 6 hours of a 24-hour incubation period with various concentrations of SAM.

The incorporation of tritiated proline into collagen was measured by a modification[8] of the pepsin extraction method of Webster and Harvey.[11] Briefly, collagen was extracted from each halved calvaria by limited pepsin digestion (0.5mg/ml pepsin in 0.5M acetic acid for 16 hours at 4°C). Insoluble debris was removed by centrifugation and the collagen was precipitated from solution by the addition of 5% (w/v) NaCl. The precipitate was centrifuged, redissolved in 0.5M acetic acid, and then reprecipitated with 5% NaCl. The final collagen pellet was dissolved in 0.5M acetic acid, mixed with 3 ml scintillant‖ ‖ and the radioactivity measured by scintillation spectrometry on an LKB Rackbeta scintillation counter with external standardization.

DNA Synthesis by Calvaria

DNA synthesis was measured by adding tritiated thymidine (specific activity 20Ci/mmol§§) at 0.5μCi/ml to halved calvaria (in groups of 6 prepared as above) for the last 6 hours of a 24-hour culture period in the presence of SAM. The calvaria were individually extracted with 5% trichloroacetic acid (TCA) to remove free isotope, and then dissolved in 150 μl hyamine hydrochloride for 2 hours at 60°C and acidified with 1M HCl prior to addition of scintillant. Radioactivity was measured as described above.

Histology of Calvaria

Calvaria from unstimulated or SAM-stimulated cultures were fixed in glutaraldehyde, embedded in Epon, and sectioned on a Reichert Jung microtome at 1 μm. Sections were stained with toluidine blue and examined by light microscopy. With some specimens ultrathin sections were prepared and examined by electron microscopy using a JEOL 100CXII microscope.

Actions of Indomethacin

To assess the role of prostanoids in the inhibition of collagen and DNA synthesis, indomethacin was added at 1 μM to control osteoblast cultures or to cultures exposed to various concentrations of SAM.

Preparation of Murine Osteoblasts

Osteoblasts were released from calvaria of 5-day old mice using a sequential enzyme digestion method as described previously.[12] The purity of the final osteoblast preparation was ascertained by a number of criteria. Cell morphology was determined by phase contrast microscopy and alkaline phosphatase positivity was determined by a standard histochemical method using alpha-naphthyl phosphate.[12] The ability of cells to accumulate cyclic AMP (cAMP) was also assessed. Primary cultures of osteoblasts at 10^5 cells/30 mm dish were exposed to 5×10^{-9}M parathyroid hormone (PTH) for 5 minutes. cAMP was extracted by extraction in ethanol-HCl at −20°C overnight and measured by commercial radioimmunoassay.§§

Collagen Synthesis by Isolated Osteoblasts

Trypsinized osteoblast suspensions were seeded into 96-well culture plates## at concentrations of 15,000 cells/well in 100 μl of MEM with 10% FCS and left overnight to attach. The culture media were then replaced with fresh

‡Oxoid Ltd., Basingstoke, UK.
§BBL, Basingstoke, UK.
‖BDH, Poole, UK.
#Pyrogent, Byk-Mallinckrodt, UK.
¶Gibco, Paisley, UK.
**Sigma, Poole, UK.
††Flow Laboratories, High Wycombe, UK.
‡‡Wellcome Foundation, Crew, UK.
§§Amersham, Amersham, UK.
‖ ‖Unisolve 1:Koch Light, Haverhill, UK.
##Microtitre, Linbro, Flow Laboratories, High Wycombe, UK.

J Periodontol
September 1992

MEM supplemented with 10% FCS and containing various concentrations of SAM, in groups of 6 wells per concentration. Control cultures received only unsupplemented medium. The cells were incubated with 0.5μCi of ^{3}H-proline for a further period of 24 hours.

The incorporation of tritiated proline into collagen was measured by the pepsin extraction method of Webster and Harvey.[11] Briefly, culture media were placed in LP3 tubes with 100 μl of 1mg/ml pepsin in 1 M acetic acid. The tubes were incubated for 16 hours at 4°C. At the end of the incubation period 100 μl of rat acid-soluble collagen (RASC) was added as a carrier and the volume in each tube was made up to 1 ml with 0.5M acetic acid. Collagen was precipitated from solution by the addition of 5% (w/v) NaCl and incubated for 2 hours at 4°C. The precipitate was centrifuged, redissolved in 0.5M acetic acid, and then reprecipitated with 5% NaCl. The final collagen pellet was dissolved in 0.5M acetic acid, mixed with 3 ml scintillant‖ and the radioactivity measured by scintillation spectrometry on an LKB Rackbeta scintillation counter with external standardization.

DNA Synthesis by Isolated Osteoblasts

Osteoblast suspensions were seeded into 96-well culture plates## at 15,000 cells per well in 100 μl of MEM with 10% FCS. These were incubated overnight to allow the cells to attach. The culture media were then replaced by MEM with 2% FCS containing various dilutions of SAM in groups of 6 wells per dilution. Control cultures received only unsupplemented medium. The culture plates were incubated for 18 hours, and then 0.5 μCi of 5-^{3}H-thymidine added to each well and the incubation continued for a further 6 hours. Trichloroacetic acid-insoluble material in each cell layer was dissolved in 0.5M NaOH and radioactivity measured in 3 ml of scintillant‖ by scintillation spectrometry on an LKB Rackbeta scintillation counter with external standardization.

Cell Viability

The cytotoxic activity of bacterial SAM on isolated osteoblasts was assessed by measurement of lactate dehydrogenase release into the media by a commercial assay.**

Statistics

The significance of results was determined by student *t*-test.

RESULTS

Endotoxin Content of SAM

The endotoxin content of SAM preparations was measured by the chromogenic Limulus assay. Solutions of SAM at a concentration of 1 mg/ml were assayed to determine the endotoxin content. The amount of endotoxin in these SAM preparations was in the range 1 to 10 ng/ml.

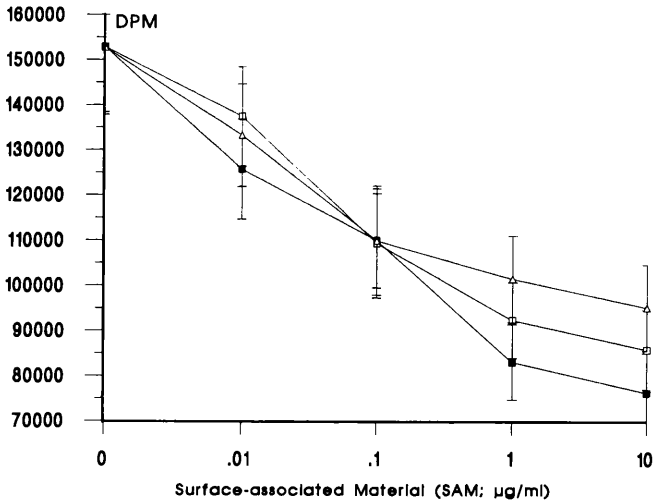

Figure 1. Inhibitory effect of increasing concentrations of SAM from A. actinomycetemcomitans *(Aa; open square),* P. gingivalis *(Pg; closed square) or* E. corrodens *(Ec; triangle) on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by murine calvaria. Results are expressed as the mean and standard deviation of 6 cultures. The significance of the results has been calculated by student's* t*-test. Both SAM from* P. gingivalis *(P <0.01) and* E. corrodens *(P <0.05) significantly inhibited thymidine incorporation at 10 ng/ml. At a concentration of 100 ng/ml all SAM preparations significantly inhibited thymidine incorporation (P <0.001).*

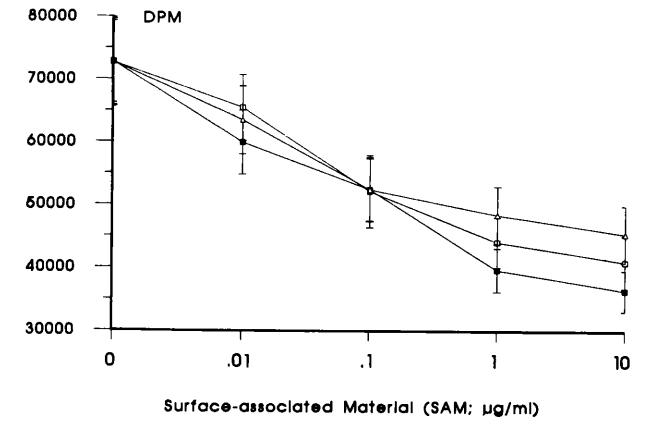

Figure 2. Inhibitory effect of increasing concentrations of SAM from A. actinomycetemcomitans *(Aa; open square),* P. gingivalis *(Pg; closed square) or* E. corrodens *(Ec; triangle) on radiolabeled proline incoporation into collagen of cultured murine calvaria. Results are expressed as mean and standard deviation of 6 replicate cultures. Both SAM from* P. gingivalis *(P <0.01) and* E. corrodens *(P <0.05) significantly inhibited tritiated proline incorporation. At a concentration of 100 ng/ml all SAM preparations significantly inhibited proline incorporation (P <0.001).*

Effect of SAM on Calvarial DNA and Collagen Synthesis

Lyophilized SAM was dissolved in medium and added to calvarial cultures at concentrations ranging from 10 ng/ml to 10 μg/ml (w/v). The SAM from all 3 bacteria had a dose-dependent inhibitory effect both on DNA synthesis (Fig. 1) and collagen synthesis (Fig. 2). SAM from *P. gingivalis* and *E. corrodens* were most potent showing sig-

Table 1. Blocking Effects of 1μM Indomethacin on the Inhibition of DNA and Collagen Synthesis by Murine Calvarial Explants

Treatment	*P*	DNA Synthesis DPM $\times 10^4$	*P*	Collagen Synthesis DPM $\times 10^4$
Control		1.33+/−0.15		7.32+/−0.84
Aa		0.95+/−0.11		5.23+/−0.60
Aa + Indomethacin	0.01	1.17+/−0.13	0.01	6.44+/−0.74
Pg		0.96+/−0.11		5.30+/−0.61
Pg + Indomethacin	0.001	1.3 +/−0.15	0.002	7.16+/−0.82
Ec		0.96+/−0.11		5.33+/−0.60
Ec + Indomethacin	0.049	1.11+/−0.13	0.068	6.15+/−0.70

The *P* value gives the statistical significance between the activity in the presence of 100 ng bacterial SAM and with the bacterial SAM plus 1μM indomethacin. Results are expressed as the mean and standard deviation.

nificant inhibition of both DNA and collagen synthesis at 10 ng/ml. In the case of SAM from *P. gingivalis* both DNA and collagen synthesis were inhibited by 18% at a concentration of 10 ng/ml. At a concentration of 100 ng/ml, SAM from all bacteria caused significant inhibition of collagen and DNA synthesis.

Histology of Calvaria

Calvaria were examined by both light and electron microscopy. In control calvaria the bone was intact with a surrounding periosteal lining. In SAM-stimulated cultures there was clear evidence of osteolysis with the appearance of osteoclasts in areas of resorption. Detailed examination of sections exposed to the various SAMs failed to detect evidence of cytotoxicity due to these bacterial constituents.

Role of Prostanoids in the Action of SAM

To ascertain whether products of cyclo-oxygenase activity were contributing to the inhibition of calvarial DNA and collagen synthesis induced by SAM, indomethacin (1μM) was added to cultures containing 100 ng/ml of each SAM. Indomethacin had no stimulatory effect on collagen synthesis in control calvaria but almost completely blocked the inhibitory effect on DNA and collagen synthesis of SAM from *P. gingivalis* (Table 1). In the case of SAM from *A. actinomycetemcomitans,* indomethacin restored DNA and collagen synthesis to levels that were not statistically significantly different from those in control cultures. The inhibitory effect of SAM from *E. corrodens* on DNA and collagen synthesis was only partially blocked by indomethacin.

Effect of SAM on DNA and Collagen Synthesis by Cultured Osteoblasts

Cells isolated by sequential enzyme digestion from murine calvaria and identified as osteoblasts on the basis of morphology, alkaline phosphatase staining, and their response to PTH were used in these studies.

Isolated osteoblasts were cultured in the presence of graded concentrations of the 3 bacterial SAM preparations. All three SAM preparations inhibited DNA and collagen synthesis in a dose-dependent fashion (Figs. 3 and 4 respectively). As with whole calvaria, the SAM from *P. gingivalis* significantly inhibited collagen and DNA synthesis at 10 ng/ml to the extent of 31.5% and 31.8% respectively. SAM from *E. corrodens* significantly inhibited DNA synthesis but not collagen synthesis at 10 ng/ml, whereas SAM from *A. actinomycetemcomitans* did not significantly inhibit either collagen or DNA synthesis at this concentration. At 100

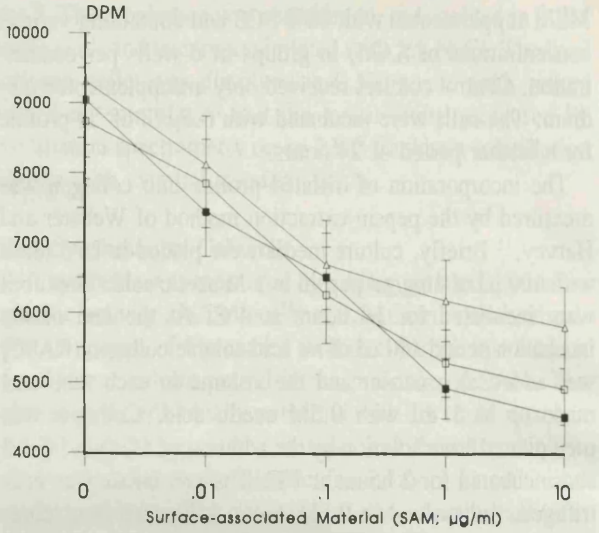

Figure 3. Inhibitory effect of increasing concentrations of SAM from A. actinomycetemcomitans *(Aa; open square),* P. gingivalis *(Pg; closed square) or* E. corrodens *(Ec; triangle) on DNA synthesis (measured by tritiated thymidine incorporation) by isolated murine calvarial osteoblasts. Results are expressed as the mean and standard deviation of 6 replicate cultures. SAM from* P. gingivalis *and* E. corrodens *significantly* (P *<0.05) inhibited thymidine incorporation at 10 ng/ml. At 100 ng/ml all SAM preparations significantly inhibited thymidine incorporation* (P *<0.001).*

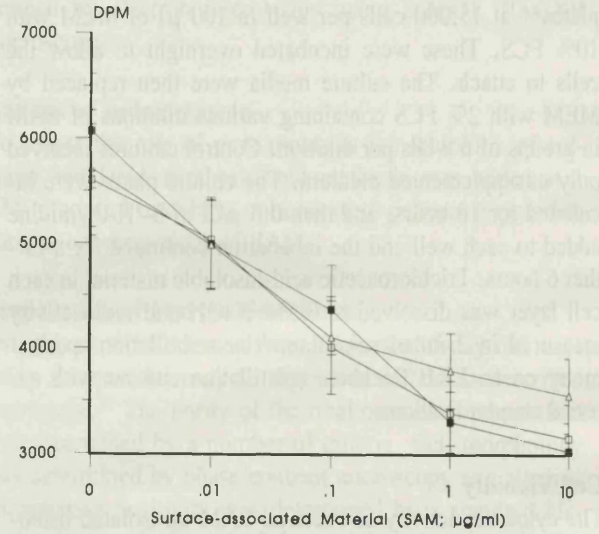

Figure 4. Inhibitory effect of SAM from A. actinomycetemcomitans *(Aa; open square),* P. gingivalis *(Pg; closed square) or* E. corrodens *(Ec; triangle) on incorporation of tritiated proline into collagen by isolated murine calvarial osteoblasts. Results are expressed as mean and standard deviation of 6 replicate cultures. SAM from* P. gingivalis *significantly* (P *<0.01) inhibited proline incorporation at 10 ng/ml. At 100 ng/ml all SAM preparations significantly inhibited proline incorporation* (P *<0.001).*

ng/ml SAM from all 3 bacteria significantly inhibited DNA and collagen synthesis.

Measurement of lactate dehydrogenase release from osteoblasts exposed to various concentrations of SAMs failed to demonstrate any significant degree of cytotoxicity.

DISCUSSION

A number of major human diseases demonstrate systemic or localized progressive loss of bone matrix. Among such diseases are rheumatoid arthritis, osteoporosis, and the most common lesion involving bone loss, periodontal disease. Research into the mechanisms of bone matrix loss has invariably concentrated on bone destruction. However, as bone homeostasis is a balance between formation and removal of matrix, a decrease in bone synthesis without any change in the rate of formation would result in a reduction in bone mass.

In chronic inflammatory periodontal disease (CIPD) there is loss of the alveolar bone to which the teeth are attached. The mechanism of this bone loss is obscure and one favored hypothesis is that it is the direct result of the action of oral bacteria or bacterial constituents.[13] We have previously demonstrated that capsular material (a constituent of SAM) eluted from the periodontopathogenic bacterium *Actinobacillus actinomycetemcomitans* inhibited both DNA and collagen synthesis when added to cultured neonatal murine calvaria.[8] The capsular material used in this study was removed from *A. actinomycetemcomitans* by gentle stirring in saline. This ready solubility of the surface material of this bacterium coupled with its actions on isolated calvaria suggested a possible pathogenic mechanism to explain bone loss in CIPD. A number of the bacteria implicated in the pathology of CIPD also have capsules and/or other surface components.[5-7] Since it was not known whether the action of the capsular material from *A. actinomycetemcomitans* was unique or was a common property of the exopolymers of these oral bacteria, we have compared the activity of SAM from 3 species implicated in the pathology of CIPD: *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis*, and *Eikenella corrodens*. In a previous study[9] we have shown that the mild extraction procedure used to obtain the SAM removes ruthenium red-staining material without appreciable lysis of the bacterial cells.

SAM from all 3 bacteria inhibited, in a dose-dependent manner, both DNA and collagen synthesis when added to cultured calvaria. The most active SAMs in this assay were those from *P. gingivalis* and *E. corrodens*, which significantly inhibited collagen and DNA synthesis at a concentration of 10 ng/ml. However, all 3 SAM preparations gave similar dose responses. The activity of SAM from *A. actinomycetemcomitans* was similar to that reported for a purified constituent of SAM (capsular material) in our previous study.[9]

The manner in which the SAM inhibits calvarial collagen and DNA synthesis is not clear. It had been assumed that the target cells in the calvaria were the osteoblasts although this had not been formally demonstrated. We have now shown that isolated murine osteoblasts when exposed to graded concentrations of SAM show a dose-dependent inhibition of both collagen and DNA synthesis. This demonstrates that the osteoblast can be directly acted upon by the SAM, but does not rule out the effect of these bacterial constituents on other bone cell populations such as periosteal fibroblasts or osteocytes. Indeed we have reported previously that SAM from *Actinobacillus actinomycetemcomitans* can inhibit the proliferation (tritiated thymidine incorporation) and collagen synthesis by cultured gingival fibroblasts.[14] In this study SAM and LPS from *Actinobacillus actinomycetemcomitans* were compared.

While the SAM inhibited both collagen and DNA production, the LPS only inhibited tritiated thymidine incorporation. The relationship between the inhibition of DNA and collagen production in calvaria and osteoblasts cultures is not clear. The possibility that it is due to a cytotoxic effect is ruled out by the failure to demonstrate significant release of lactate dehydrogenase in SAM-stimulated osteoblasts or dead cells in stimulated calvaria. The ability of indomethacin to reverse the inhibition also rules out a cytotoxic mechanism. The possibility exists that the inhibition of DNA synthesis may contribute to the decreased collagen synthesis. However, as described above, in LPS stimulated fibroblasts it was possible to demonstrate a decreased tritiated thymidine incorporation with no alteration in collagen production.[14] Thus it is likely that the inhibition of DNA synthesis and collagen synthesis are independent phenomena.

There are few reports concerning the effects on osteoblasts of components from oral bacteria. Sismey-Durrant and Hopps[15] found that LPS from *P. gingivalis* stimulated collagenase release from mouse osteoblasts in a dose-related manner over the concentration range 0.1 to 10 μg/ml but had no effect on the secretion of tissue inhibitor of metalloproteinase (TIMP). Furthermore, LPS from the same organism was found to induce secretion of plasminogen activator over the same range of concentrations.[16] In contrast, LPS from *P. gingivalis* was unable to activate osteoclasts (as assayed by release of tartrate-resistant acid phosphatase), nor could it induce bone resorption by osteoclasts in the absence of osteoblasts. These findings suggest that osteoblasts rather than osteoclasts are the target cells for LPS and, although we have not investigated the effect of SAM on osteoclasts, our results show that the osteoblast is the target cell for another bacterial component, SAM.

Addition of indomethacin to calvarial cultures exposed to SAM was found to block the inhibitory effect of SAM from *P. gingivalis* and *A. actinomycetemcomitans* and partially block the effect of SAM from *E. corrodens*. These results imply that PGE_2 is responsible for mediating the inhibitory effects of SAM on DNA and collagen synthesis by calvaria although, in the case of SAM from *E. corrodens*, additional mediators may be involved.

Although many investigators have reported on the effects of plaque, bacteria, and bacterial components on bone re-

sorption in vitro, there are comparatively few reports of their effects on bone formation. Larjava[17] and Multanen et al.[18] reported that plaque extracts containing respectively 50 μg/ml and 1.0 μg/ml of protein significantly inhibited collagen synthesis in rat calvaria. Although the plaque concentrations were not specified, these can be estimated to be approximately 500 μg/ml and 10 μg/ml respectively based on a plaque protein content of approximately 10%.[19] However, in neither case was the active constituent of the plaque identified. With regard to the effect of isolated bacterial components on bone formation, Norton et al.[20] found that LPS from *Escherichia coli* was effective at inhibiting rat bone growth in vitro only at very high concentrations (80 μg/ml). Meikle et al.[21] also reported that a high concentration (100 μg/ml) of streptococcal cell walls was required to inhibit ^{3}H-proline incorporation into protein in rabbit calvaria by 20%. Components from periodontopathogenic bacteria, however, appear to be far more potent in inhibiting bone formation. For example, we have shown previously that LPS from *A. actinomycetemcomitans* significantly inhibits DNA and collagen synthesis in mouse calvaria at concentrations of 0.1 and 1.0 μg/ml respectively.[22]

In the presence of 1.0 μg/ml of LPS, DNA and collagen synthesis were inhibited by 40% and 30% respectively. However, this is still signficantly less potent than capsular material from the same organism which has been shown to inhibit collagen synthesis in mouse calvaria by 19% at a concentration of 10 ng/ml.[8] This is a similar result to that found using SAM from *A. actinomycetemcomitans* in the present study. Miller et al.[23] have also investigated the effect of LPS from *P. gingivalis* and *Salmonella minnesota* on bone formation by measuring incorporation of ^{14}C-proline into collagenous protein in fetal rat calvaria. These investigators found that a significant reduction (30 to 40%) in ^{14}C-proline incorporation was achieved only with LPS concentrations as high as 10 μg/ml. Although comparisons with the present study are difficult in view of use of different strains of *P. gingivalis* and different assay systems, on a weight for weight basis, we have shown that the SAM of *P. gingivalis* is apparently 1000-fold more potent than LPS from this organism.

Thus, SAM from 3 bacteria implicated in the pathology of CIPD have been shown to be potent inhibitors of bone and bone cell, DNA, and collagen production. The inhibition of bone synthetic function appears to be related to the generation of prostanoids. This may be a partial explanation for the finding that non-steroidal anti-inflammatory drugs (NSAIDs), like ibuprofen, can inhibit alveolar bone loss in man and animals.[24,25] It has recently been reported that in experimental periodontitis in the cynomolgus monkey, treatment with ibuprofen or meclofenamic acid inhibited alveolar bone loss without inhibiting gingivitis.[26] This suggests that the host inflammatory response is uncoupled from the process of bone resorption and, by implication, that bacterial moieties like the SAM used in this study are important in bone matrix loss.

Further studies are planned to define the mechanism of inhibition of collagen production. For example, the possibility that bacterial SAMs are inhibiting mRNA transcription will be assessed by Northern blotting analysis.

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Biological activities of surface-associated material from *Porphyromonas gingivalis*

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Abstract: Surface-associated material (SAM) from *Porphyromonas gingivalis* was tested for in vitro biological activities that may be relevant to the pathogenesis of chronic periodontitis. SAM was found to stimulate bone resorption at a concentration of 1.0 μg/ml and this was inhibited by indomethacin, interleukin-1 receptor antagonist protein and anti-tumour necrosis factor antibody. At a concentration of 10 ng/ml, the SAM inhibited DNA and collagen synthesis in osteoblasts and murine calvaria and DNA synthesis in fibroblasts, monocytes and epidermal cells. Therefore, easily solubilised surface components from *P. gingivalis* could play a role in the pathogenesis of chronic periodontitis if these activities operate in vivo.

Key words: Porphyromonas gingivalis; Periodontitis; Bone resorption; Surface-associated material

Introduction

Chronic periodontitis is an inflammatory condition characterised by destruction of the supporting structures of the tooth, and *Porphyromonas gingivalis* is one of several organisms considered to be important in the pathogenesis of this disease [1]. Since bacterial invasion of the periodontium is not a characteristic feature of chronic periodontitis, it is likely that tissue destruction is initiated by bacterial products or components. Lipopolysaccharides (LPS) from periodontopathogenic bacteria have received considerable attention as possible mediators of the tissue destruction accompanying chronic periodontitis [1,2]. However, we have demonstrated that LPS-free, surface-associated material (SAM) obtained by gentle saline extraction of the periodontopathogenic bacterium *Actinobacillus actinomycetemcomitans* has in vitro biological activities relevant to the pathogenesis of the disease. These include the ability to stimulate bone resorption [3], inhibit bone collagen synthesis [4] and inhibit fibroblast proliferation [5]. It is possible that SAM of other bacteria believed to be involved in the pathogenesis of chronic periodontitis may adversely affect cells and tissues of the periodontium. The purpose of the present study was to test this hypothesis with SAM from *P. gingivalis*.

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Materials and Methods

Extraction of SAM

P. gingivalis strain W50 was grown at 37°C under anaerobic conditions on a medium consist-

ing of (/l): agar 12 g, trypticase 5 g, proteose peptose 5 g, glucose 2.5 g, sodium chloride 2.5 g, yeast extract 2.5 g, cysteine HCl 0.375 g, haemin 0.25 g and menadione 0.05 g. After 3–4 days, all cultures were inspected visually for contamination, harvested with sterile saline and then centrifuged at 30 000 × *g* for 30 min at 4°C. The cells were resuspended in saline, recentrifuged and then lyophilized. An extract containing surface-associated components from the cells was obtained by washing in saline as previously described [3]. Briefly, lyophilized bacteria were gently stirred at 4°C in sterile saline for 1 h and then the bacteria were sedimented by centrifugation. This was repeated twice more. SAM was precipitated from the combined aqueous phases by addition of acetone. The precipitate was then centrifuged, dialysed and lyophilized.

Electron microscopy

Bacteria were stained with ruthenium red and examined by transmission electron microscopy before and after saline extraction, as described previously, to check on the efficiency of extraction and the integrity of the resultant cells [3].

Calvarial bone resorption assay

Bone resorption was assayed by measuring calcium release from 5-day-old mouse calvaria in vitro as described previously [3]. The role of prostanoids in bone resorption was tested by adding 1 μM indomethacin (Sigma). The role of interleukin-1 (IL-1) was assessed by adding the IL-1 receptor antagonist protein (IL-1ra; Synergen, Boulder, CO). The involvement of tumour necrosis factor (TNF) in bone destruction was determined by adding the neutralizing antibody TN3 19.12 (Celltech, Slough, UK). The role of another potential osteolytic cytokine, interleukin-6 (IL-6), was assessed by use of a neutralizing antibody to murine IL-6 (Immunex, Seattle, WA). The antibodies or IL-1ra were added to bone cultures at various concentrations along with the SAM at a concentration of 1.0 μg/ml.

Fibroblast culture and DNA synthesis

Human gingival fibroblasts were grown from explants obtained during minor oral surgical procedures. The cells were grown in Eagles' MEM containing foetal calf serum (FCS) 10%, 2 mM L-glutamine, $NaHCO_3$ 2.25 g/l, penicillin and streptomycin (each 100 U/ml) and incubated at 37°C in CO_2 5% in air. Cells were subcultured at weekly intervals and used for experiments between passages 6 and 12.

Fibroblast suspensions were seeded into 96-well culture plates (Microtitre, Linbro) at concentrations of 15 000 cells/well in 100 μl of MEM with FCS 10% and left overnight to attach. The culture medium was then replaced with MEM containing FCS 2% and various concentrations of SAM, in groups of six wells/concentration. Control cultures received only unsupplemented medium. Cells were incubated for a further period of 24 h and then 0.5 μCi of 5-^{3}H-thymidine (specific activity 20 Ci/mmol; Amersham International) was added to each well 6 h before termination of cultures. Trichloroacetic acid-insoluble material in each cell layer was dissolved in 0.4 M NaOH and radioactivity was measured in 3 ml of scintillant by scintillation spectrometry (Rackbeta: LKB) with external standardization.

Keratinocyte culture and DNA synthesis

Keratinocytes (GPK cell line, derived from guinea pig ear epidermis) were obtained from M. Hola, Department of Biochemical Pathology, University College School of Medicine, London. The cells were grown in MEM containing FCS 10%, 2 mM L-glutamine, $NaHCO_3$ 2.25 g/l, penicillin and streptomycin (each 100 U/ml) and incubated at 37°C in CO_2 5% in air. Keratinocyte suspensions were seeded into 96-well culture plates at concentrations of 20 000 cells/well in 100 μl of MEM with FCS 10% and left overnight to attach. The culture medium was then replaced with MEM containing FCS 2% and various concentrations of SAM, in groups of six wells/concentration. Control cultures received only unsupplemented medium. Cells were incubated for a further period of 24 h and then 0.5 μCi of 5-^{3}H-thymidine was added to each well 6 h before termination of cultures. The incorporation of tritiated thymidine was measured as described above.

Macrophage cell line culture and DNA synthesis

U937, a monocytic cell line derived from a human histiocytic lymphoma was used in these experiments. The cells were seeded into 96-well culture plates at a concentration of 500 000 cells/well in 100 μl of RPMI (Gibco) with heat-inactivated FCS 5% containing various concentrations of SAM, in groups of six wells/concentration. Control cultures received only unsupplemented medium. Cells were incubated for 48 h then 0.5 μCi of 5-^{3}H-thymidine was added to each well 6 h before termination of cultures. The incorporation of tritiated thymidine was measured as described above.

Collagen synthesis by calvaria

Calvaria from 5-day-old mice were halved along the sagittal suture and incubated in groups of six for 18 h in 50-mm culture dishes containing BGJ medium (Gibco) supplemented with ascorbic acid (Sigma) 50 μg/ml, 2 mM glutamine (Flow), heat-inactivated rabbit serum (Wellcome Foundation) 5% and β-aminopropionitrile fumarate (Sigma) 50 μg/ml to inhibit collagen cross-linking. ^{3}H-proline (specific activity 31.5 Ci/mmol; Amersham International) was added at 2 μCi/ml for the last 6 h of a 24-h incubation period with various concentrations of SAM.

The incorporation of tritiated proline into collagen was measured by a modification [4] of the pepsin extraction method of Webster and Harvey [6]. Briefly, collagen was extracted from each halved calvaria by limited pepsin digestion (pepsin 0.5 mg/ml in 0.5 M acetic acid for 16 h at 4°C). Insoluble debris was removed by centrifugation and the collagen was precipitated from solution by the addition of NaCl 5% w/v. The precipitate was centrifuged, redissolved in 0.5 M acetic acid and then reprecipitated with NaCl 5%. The final collagen pellet was dissolved in 0.5 M acetic acid, mixed with 3 ml of scintillant (Unisolve 1, Koch Light) and the radioactivity was measured by scintillation spectrometry on an LKB Rackbeta scintillation counter with external standardization.

DNA synthesis by calvaria

DNA synthesis was measured by adding 5-^{3}H-thymidine 0.5 μCi/ml to halved calvaria (in groups of six prepared as above) for the last 6 h of a 24-h culture period in the presence of SAM. The calvaria were individually extracted with trichloroacetic acid 5% to remove free isotope, and then dissolved in 150 μl of hyamine hydrochloride for 2 h at 60°C and acidified with 1 M HCl before addition of scintillant. Radioactivity was measured as described above.

Preparation of murine osteoblasts

Osteoblasts were released from calvaria of 5-day-old mice by a sequential enzyme digestion method described previously [7]. The purity of the final osteoblast preparation was ascertained by a number of criteria. Cell morphology was determined by phase-contrast microscopy and alkaline phosphatase positivity was determined by a standard histochemical method with α-naphthyl phosphate [7]. The ability of cells to accumulate cyclic AMP (cAMP) was also assessed. Primary cultures of osteoblasts at 10^5 cells/30-mm dish were exposed to 5×10^{-9} M parathyroid hormone (PTH) for 5 min. cAMP was extracted in ethanol-HCl at -20°C overnight and measured by commercial radioimmunoassay (Amersham International).

Collagen synthesis by isolated osteoblasts

Trypsinized osteoblast suspensions were seeded into 96-well culture plates at concentrations of 15 000 cells/well in 100 μl of MEM with FCS 10% and left overnight to attach. The culture medium was then replaced with fresh MEM with FCS 10% and containing various concentrations of SAM, in groups of six wells/concentration. Control cultures received only unsupplemented medium. The cells were incubated with 0.5 μCi of ^{3}H-proline for a further period of 24 h. The incorporation of tritiated proline into collagen was measured by a pepsin extraction method [6]. Briefly, culture media were placed in LP3 tubes with 100 μl of pepsin 1 mg/ml in 1 M acetic acid. After incubation for 16 h at 4°C, 100 μl of rat acid-soluble collagen was added as a carrier and the volume of each tube was made up to 1 ml with 0.5 M acetic acid. Collagen was precipitated from solution by the addition of NaCl 5% w/v and incubated for 2 h at 4°C. The

precipitate was centrifuged, redissolved in 0.5 M acetic acid and then reprecipitated with NaCl 5%. The final collagen pellet was dissolved in 0.5 M acetic acid, mixed with 3 ml of scintillant (Unisolve 1, Koch Light) and the radioactivity was measured by scintillation spectrometry on a LKB Rackbeta scintillation counter with external standardization.

DNA synthesis by isolated osteoblasts

Osteoblast suspensions were seeded into 96-well culture plates at 15 000 cells/well in 100 μl of MEM with FCS 10%. These were incubated overnight to allow the cells to attach. The culture media were then replaced by MEM with FCS 2% containing various dilutions of SAM in groups of six wells/dilution. Control cultures received only supplemented medium. The culture plates were incubated for 18 h, and then 0.5 μCi of 5-^{3}H-thymidine was added to each well and incubation was continued for a further 6 h. The incorporation of tritiated thymidine was measured as described above.

Results

Electron microscopy

Before saline extraction, a ruthenium red-staining layer external to the cell wall could be seen. There was no evidence of fimbriae, fibrils or S-layers. Most of the ruthenium red-staining layer was removed by the extraction procedure which appeared to have had little effect on the integrity of the cells.

Bone resorbing activity of SAM

SAM stimulated release of calcium from cultured murine calvaria in a dose-related manner over the concentration range 100 ng–10 μg/ml (Fig. 1) although statistically significant release occurred only at concentrations of 1 and 10 μg/ml. Addition of 1 μM indomethacin to the cultures stimulated with SAM completely inhibited bone resorption (Fig. 2).

The biological actions of IL-1 can be antagonized by the cloned IL-1 antagonist protein IL-1ra. Addition of IL-1ra to unstimulated calvarial

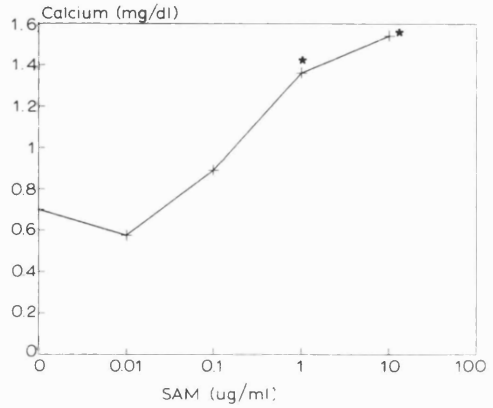

Fig. 1. Bone resorbing activity of SAM from *P. gingivalis*. Each point represents the mean of five cultures; * denotes that the Ca release was significantly different from that of control cultures without SAM (Student's *t*-test; $P < 0.05$).

cultures had no effect on calcium release but, at concentrations of 0.1 or 1.0 μg/ml, it inhibited the bone resorption induced by SAM (Fig. 3). Bone resorption was completely inhibited at 1.0 μg/ml.

TNF-α is another potent activator of bone resorption. To determine the role of TNF in SAM-induced bone resorption a neutralizing antibody to murine TNF was added to bone cul-

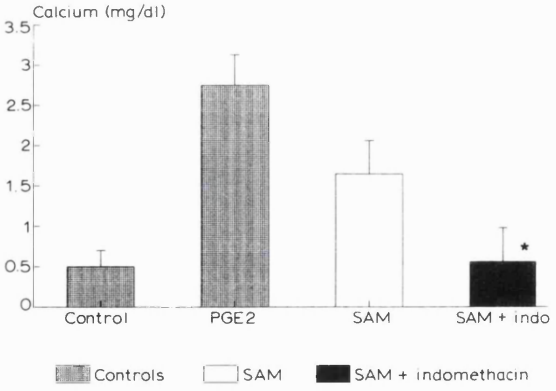

Fig. 2. The effect of 10^{-6} M indomethacin on bone resorption induced by SAM from *P. gingivalis*. The control represents the release of calcium in unstimulated cultures and the PGE_2 column is the positive control in which calvaria were stimulated with 1 μM PGE_2. Each bar represents the mean and standard deviation of five cultures; * denotes that the Ca release was significantly different from that of control cultures with SAM ($P < 0.05$).

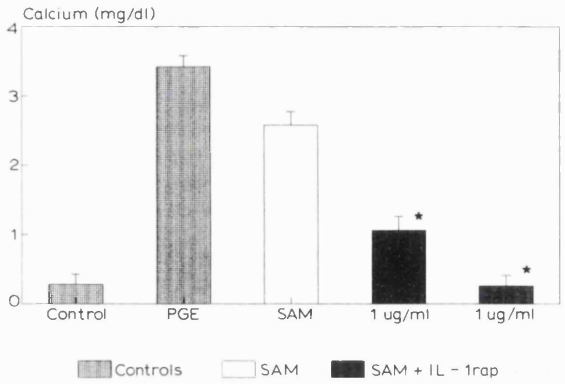

Fig. 3. The effect of IL-1ra on bone resorption induced by SAM from *P. gingivalis*. The control cultures show the release of calcium in unstimulated cultures and the PGE_2 has been added at 1 μM to another set of cultures to act as a positive control. IL-1ra has been used at concentrations of 0.1, 1 or 10 μg/ml. Results are expressed as the mean and standard deviation of five replicate cultures; * denotes that the Ca release was significantly different from that of control cultures with SAM ($P < 0.05$).

tures. It had no effect on basal calcium release from unstimulated bone but it inhibited resorption induced by SAM; complete inhibition was achieved at a concentration of 1.0 μg/ml (Fig. 4).

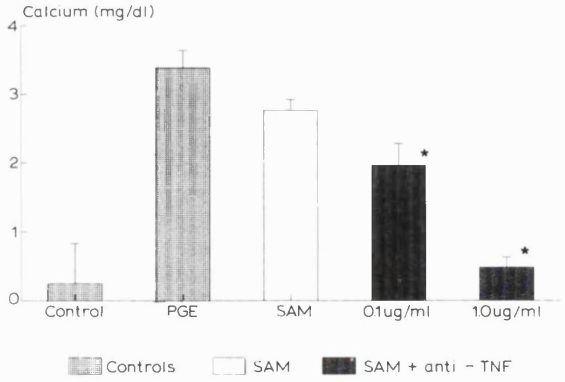

Fig. 4. The effect of anti-TNF antibody (TN3–19.12) on bone resorption induced by SAM from *P. gingivalis*. Antibody has been added at 0.1 or 1 μg/ml to cultures stimulated with SAM. The control represents the calcium released by unstimulated cultures and the positive control cultures have been stimulated with 1 μM PGE_2. Results are expressed as the mean and standard deviation of five replicate cultures; * denotes that the Ca release was significantly different from that of cultures with SAM ($P < 0.05$).

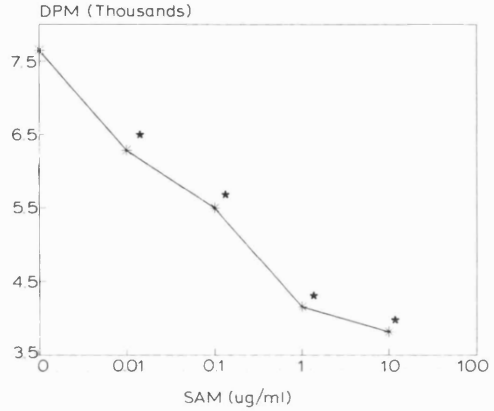

Fig. 5. Inhibitory effect of increasing concentrations of SAM from *P. gingivalis* on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by human gingival fibroblasts. Results are expressed as the mean of six cultures; * denotes that the incorporation of tritiated thymidine was significantly different from that found in control cultures without SAM ($P < 0.05$).

In two experiments, a neutralizing antibody to murine IL-6 was added to bone cultures stimulated with SAM, but in neither case was bone resorption inhibited.

Inhibition of fibroblast DNA synthesis

SAM significantly inhibited the incorporation of ^{3}H-thymidine into human gingival fibroblasts in a dose-dependent manner over the range 10 ng–10 μg/ml (Fig. 5). The degree of inhibition increased from 17 to 50% over the concentration range used.

Inhibition of keratinocyte DNA synthesis

Thymidine incorporation by keratinocytes was inhibited by SAM in a dose-dependent manner at concentrations of 10 ng-10 μg/ml (Fig. 6). Statistically significant inhibition (18%) was achieved by SAM at 10 ng/ml, the lowest concentration tested.

Inhibition of U937 DNA synthesis

Addition of SAM to U937 cells resulted in a statistically significant decrease in thymidine incorporation over the concentration range 0.2–50 μg/ml (Fig. 7); 50% inhibition was achieved at a concentration of approx. 2–3 μg/ml.

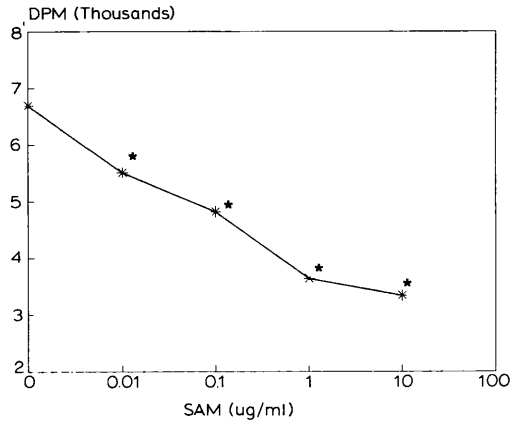

Fig. 6. Inhibitory effect of increasing concentrations of SAM from *P. gingivalis* on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by keratinocytes. Results are expressed as the mean of six cultures; * denotes that the incorporation of tritiated thymidine was significantly different from that found in control cultures without SAM ($P < 0.05$).

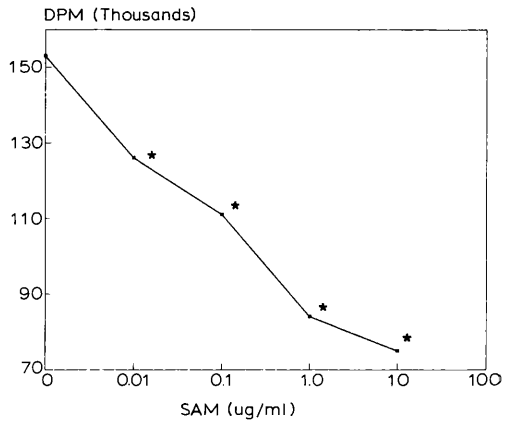

Fig. 8. Inhibitory effect of increasing concentrations of SAM from *P. gingivalis* on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by murine calvaria. Results are expressed as the mean of six cultures; * denotes that the incorporation of tritiated thymidine was significantly different from that found in control cultures without SAM ($P < 0.05$).

Effect of SAM on calvarial DNA and collagen synthesis

SAM had a dose-dependent inhibitory effect on both DNA synthesis (Fig. 8) and collagen synthesis (Fig. 9) in mouse calvaria. Statistically significant inhibition of both was achieved at a concentration of 10 ng/ml.

Effect of SAM on DNA and collagen synthesis by cultured osteoblasts

Cells isolated by sequential enzyme digestion from murine calvaria and identified as osteoblasts on the basis of morphology, alkaline phosphatase staining and their response to PTH were used in these studies. SAM inhibited DNA and collagen

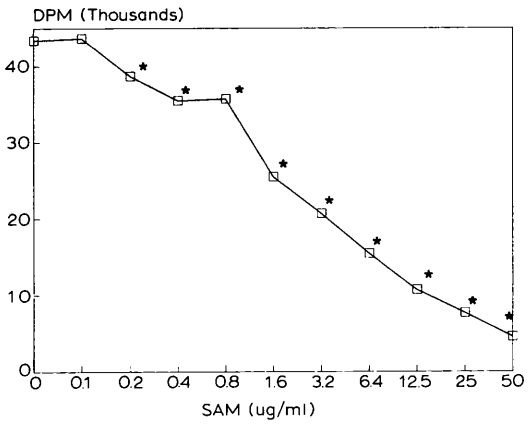

Fig. 7. Inhibitory effect of increasing concentrations of SAM from *P. gingivalis* on DNA synthesis, measured as incorporation of tritiated thymidine into DNA, by monocytes. Results are expressed as the mean of six cultures; * denotes that the incorporation of tritiated thymidine was significantly different from that found in control cultures without SAM ($P < 0.05$).

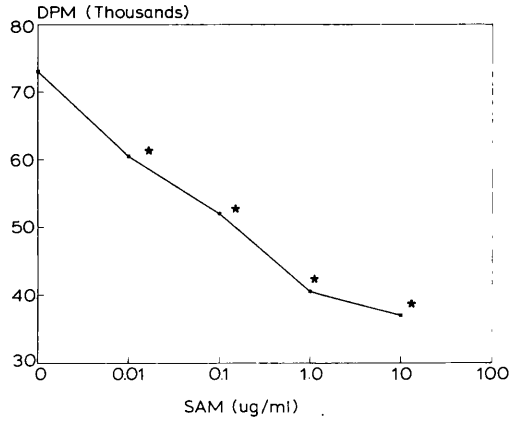

Fig. 9. Inhibitory effect of increasing concentrations of SAM from *P. gingivalis* on proline incorporation into collagen by cultured murine calvaria. Results are expressed as the mean of six replicate cultures; * denotes that the incorporation of proline was significantly different from that found in control cultures without SAM ($P < 0.05$).

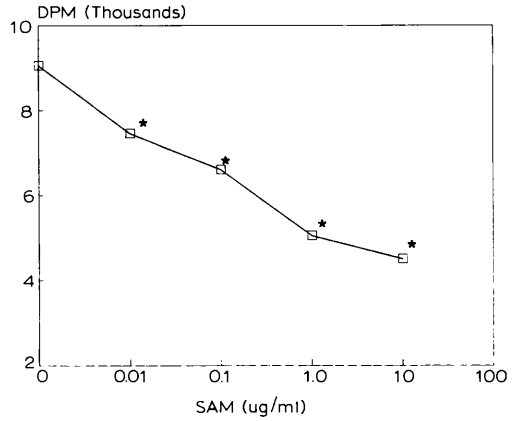

Fig. 10. Inhibitory effect of increasing concentrations of SAM from *P. gingivalis* on DNA synthesis (measured by tritiated thymidine incorporation) by isolated murine calvarial osteoblasts. Results are expressed as the mean of six replicate cultures; * denotes that the incorporation of thymidine was significantly different from that found in control cultures without SAM ($P < 0.05$).

synthesis in a dose-dependent fashion over the concentration range 10 ng–10 μg/ml (Figs. 10 and 11). Statistically significant inhibition of both DNA and collagen synthesis was achieved at 10 ng/ml.

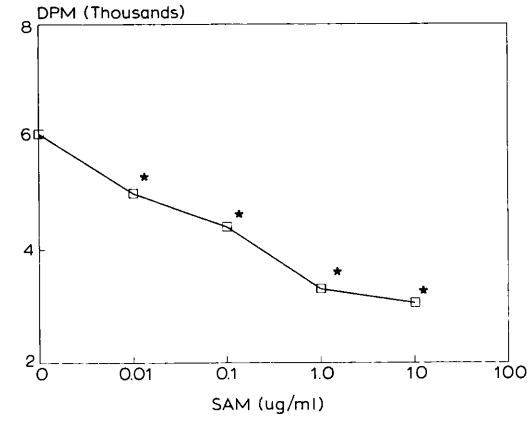

Fig. 11. Inhibitory effect of SAM from *P. gingivalis* on incorporation of tritiated proline into collagen by isolated murine calvarial osteoblasts. Results are expressed as the mean of six replicate cultures; * denotes that the incorporation of proline was significantly different from that found in control cultures without SAM ($P < 0.05$).

Discussion

The inflammation and bone destruction characteristic of chronic periodontitis are associated with subgingival plaque bacteria and their products. Disease symptoms may be due to the direct action of bacterial components or products or they may result from the stimulation of host mechanisms by these substances [8]. However, a prerequisite for activation is that the bacterial component actually reaches the target cells. In this respect biologically active, soluble surface components, especially ones that could be shed during growth of the organism, clearly have a greater 'bio-availability' than less soluble components that are an integral part of the cell. Examples of the former would include surface-associated proteins and many components of the bacterial glycocalyx, i.e. capsular material, S-layers, slime, fibrils, etc. [9]. Such components may be regarded as being 'surface-associated' rather than as being essential for the integrity of the bacterial cell. In this investigation we have removed SAM from *P. gingivalis*. Electron micrographs of the cells before extraction revealed the presence of extensive ruthenium red-staining material surrounding each cell. Ruthenium red stains many polysaccharides and glycopeptides and has been used to demonstrate the presence of a glycocalyx in the form of a capsule or fibrils on the surfaces of many microorganisms [10]. Following saline extraction, the ruthenium red layer was almost entirely removed from the surface of the cells and no gross disruption was evident.

SAM stimulated bone resorption in a mouse calvarial system although its potency was approx. 100-fold lower than that of the SAM from *A. actinomycetemcomitans* [3]. The bone resorption induced by the SAM was inhibited by indomethacin, IL-1ra and an antibody to TNF-α but not by an antibody to IL-6. This implies that the SAM causes bone resorption by stimulating the release of IL-1 and TNF which then induce the production of prostanoids, which are ultimately responsible for calcium release. The IL-1 and TNF probably act synergically in this process which would explain why inhibition of either cytokine inhibited SAM-induced bone resorption.

As well as stimulating bone resorption, the SAM was also a potent inhibitor of bone formation in terms of DNA and collagen synthesis in mouse calvaria and isolated osteoblasts. There are few published reports concerning the effect on bone synthesis of components from periodontopathogenic bacteria. Millar et al. [11] found that LPS from *P. gingivalis* inhibited proline incorporation into rat calvaria although this was significant only at a concentration of 10 μg/ml. LPS from *A. actinomycetemcomitans* also inhibited synthesis in mouse calvaria at a concentration of 1.0 μg/ml [12]. From the results of the present study, SAM from *P. gingivalis* would appear to be a more potent inhibitor of bone collagen synthesis than LPS, significant inhibition being achieved at a concentration of 10 ng/ml. Its potency was similar to that of capsular material from *A. actinomycetemcomitans* [4]. The manner in which the SAM inhibits bone formation in calvaria is unknown. However, its ability to inhibit DNA and collagen synthesis in osteoblasts would suggest that these cells are involved.

Bone loss may result from the stimulation of bone resorption, the inhibition of bone formation or a combination of both. The results of this investigation have demonstrated that SAM from *P. gingivalis* can inhibit bone formation as well as stimulate bone resorption. Therefore, the SAM of *P. gingivalis* could be an effective mediator of bone loss in chronic periodontitis if these activities were operative in vivo.

Many of the bacterial species associated with inflammatory periodontal diseases have been shown to be capable of exerting a cytotoxic effect on a number of mammalian cells [13–15]. The majority of such studies have been concerned with the effect of bacterial cell sonicates on human gingival fibroblasts. Hence, it has been shown that sonicates from *A. actinomycetemcomitans*, *P. gingivalis*, *Fusobacterium nucleatum*, *Eikenella corrodens* [15], *Prevotella intermedia* [14] and *Treponema denticola* [13] all exhibit some degree of toxicity towards fibroblasts. Larjava et al. [16] and Fotos et al. [14] have demonstrated that high concentrations (10–50 μg/ml of protein) of ultrasonicates of *P. gingivalis* can inhibit the proliferation of these cells in vitro. In the present study we have shown that SAM from *P. gingivalis* inhibited fibroblast proliferation at much lower concentrations (10 ng/ml).

Comparatively few studies have been directed at evaluating the potential of periodontopathogens to affect adversely other mammalian cell types such as epithelial cells and monocytes. With regard to the in vitro effects of such bacteria on epithelial cells, Kamen [17] found that sonic extracts of *P. gingivalis* inhibited thymidine uptake by human foreskin keratinocytes, a statistically significant degree of inhibition being obtained at a concentration of 2.5 μg/ml. In the present study, SAM inhibited keratinocyte proliferation at concentrations as low as 10 ng/ml. With the exception of *A. actinomycetemcomitans*, few periodontopathogenic bacteria have been investigated for their potential cytotoxicity towards monocytes. The toxic effects of *A. actinomycetemcomitans* for these cells have been attributed to its leukotoxin [18] which Simpson et al. [19] have shown to be effective against the monocytic cell line (U937) used in the present investigation at doses as low as 9 ng. In the present study, SAM was toxic for the monocytic cell line, achieving statistically significant inhibition of thymidine incorporation at a concentration of 200 ng/ml.

The results of this investigation have demonstrated that easily solubilised SAM from *P. gingivalis* has a range of biological activities which, if operative in vivo, could have a profoundly deleterious effect on the periodontal tissues. Thus, the material was able to stimulate bone resorption, inhibit bone formation and inhibit the proliferation of osteoblasts, fibroblasts, keratinocytes and monocytes.

Acknowledgement

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High-titer antisera from patients with periodontal disease inhibit bacterial capsule-induced bone breakdown

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Meghji S, Henderson B, Wilson M: High-titer antisera from patients with periodontal disease inhibit bacterial capsule-induced bone breakdown.

Solubilized surface-associated material (SAM) from a number of periodontopathogenic bacteria have been shown to be potent stimulators of bone resorption *in vitro* in the murine calvarial bone culture assay. Antibodies to the constituents of SAM are also found in patients with periodontal diseases. Serum from patients with severe generalized periodontitis (SGP) containing high titers of antibodies to the SAM of *Porphyromonas gingivalis* completely inhibited the bone resorption induced by SAM from this organism. In contrast, serum from patients with low titers of antibodies to SAM from *P. gingivalis* failed to inhibit bone resorption. High-titer sera (containing antibodies to SAM from *Actinobacillus actinomycetemcomitans*) from patients with localized juvenile periodontitis (LJP) were added to calvarial cultures stimulated with SAM from *A. actinomycetemcomitans*. Of 6 high-titer sera tested, only 4 inhibited bone breakdown, the other 2 sera having no effect on resorption. Low-titer sera were also ineffective at blocking bone resorption. This suggests that the antibody response to SAM may have a protective effect in patients with periodontal disease.

Key words: surface-associated material – antibodies – periodontal disease – bone

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Gentle saline extraction of the periodontopathogenic bacteria *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* removes surface-associated material (SAM) from these organisms. The solubilized SAM from these bacteria are potent stimulators of bone resorption (1, 2) and significant titers of IgG antibodies to constituents of the SAM are found in the serum of patients with periodontal diseases (1).

The role that antibodies to periodontopathogenic bacteria (or their constituents) play in the pathogenesis or pathology of the periodontal diseases is unclear. Antibodies in the serum may play a role in removing oral bacteria, or constituents of these bacteria, from the bloodstream. Antibodies in the gingival pocket may induce pathology by activation of type I, II or III hypersensitivity immune reactions. Alternatively, if antibodies to oral bacteria, or their constituents, had a neutralizing action they could, in theory, inhibit the bacterially-induced tissue pathology. In this study we have investigated the activity of sera from patients with periodontal diseases in the calvarial bone resorption assay to determine if antibodies to SAM components stimulate or inhibit bone resorption induced by the corresponding SAM antigens.

Material and methods

Isolation of bacterial surface-associated material

Actinobacillus actinomycetemcomitans Y4 was grown at 37°C under anaerobic conditions on Brain Heart Infusion (Oxoid) agar. *Porphyromonas gingivalis* W50 was grown at 37°C under anaerobic conditions on a medium consisting of 5 g Trypticase (BBL), 5 g proteose peptone (Oxoid), 2.5 g glucose (BDH), 2.5 g sodium chloride (BDH), 2.5 g yeast extract (Oxoid), 0.0375 g cysteine HCl

(BDH), 0.25 g hemin (Oxoid) and 0.05 g menadione (Oxoid).

All bacteria were grown on solid media to enhance production of SAM. After 72 h all cultures were inspected visually for contamination, removed from the plate with sterile saline and then centrifuged at 30000 *g* for 30 min 4°C. Bacteria were resuspended in saline and recentrifuged. They were then lyophilized and the SAM was obtained by saline extraction as previously described (2). Briefly, lyophilized bacteria were gently stirred at 4°C in sterile saline for 1 h and then the bacteria were sedimented by centrifugation. The solubilized SAM was precipitated by addition of acetone, centrifuged, dialyzed and then lyophilized. The endotoxin content of the SAM was measured by the chromogenic *Limulus* amebocyte lysate assay (Pyrogent, Byk-Mallinckrodt, U.K.) according to the manufacturer's instructions. The endotoxin content of the SAM was in the range 1–10 ng/mg dry weight.

Serum samples

The sera used in this study and the antibody titers to SAM preparations from the two test organisms are shown in Table 1.

Enzyme-linked immunosorbent assay (ELISA) for antibodies to SAM

IgG serum antibodies were measured using a direct antigen-binding assay. The optimal coating conditions (concentration, buffer, etc.) for each SAM preparation were determined and the standard coating protocol chosen for each SAM was to coat at 10 μg/ml in PBS using flat-bottomed 96-well plates (Immulon) overnight at 4°C. The wells were

Table 1. Serum IgG antibody titers against *A. actinomycetemcomitans* (Aa) or *P. gingivalis* (Pg) in LJP or SGP patients

	Antibody Titers from ELISA	
Titer Group	*Aa*	*Pg*
Low titer	600	38
	800	17
	8000	460
	1500	430
	5000	450
	1200	44
Mean and SD	2850 ± 2998	240 ± 227
High titer	100000	15000
	100000	6000
	62000	2400
	62000	8000
	125000*	10000
	125000*	8000
Mean and SD	95666 ± 28373	8233 ± 4196

* Sera did not inhibit bone resorption induced by SAM.

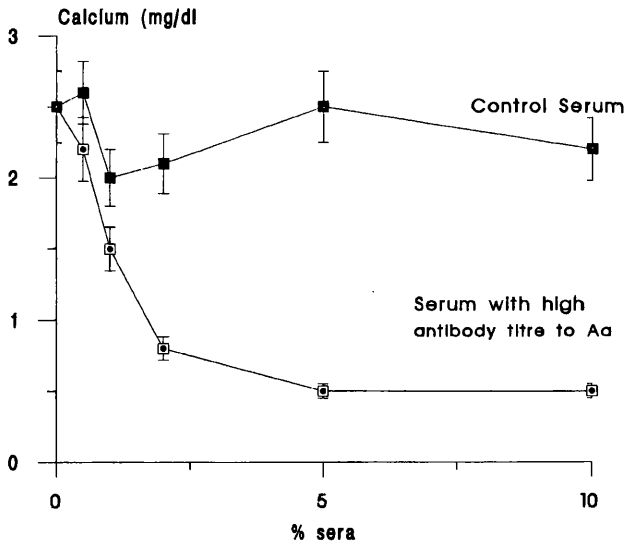

Fig. 1. Titration curves showing the effect of human sera in the calvarial bone resorption assay. Murine calvaria were stimulated to resorb by addition of 100 ng of SAM from *A. actinomycetemcomitans*. This graph shows that normal sera was unable to block the calcium release induced by SAM from this organism. In contrast, a high-titer serum from a patient with LJP was capable of dose-dependently inhibiting this bone destruction. Results are expressed as the mean and standard deviation of 5 replicate cultures per point.

then thoroughly washed with PBS containing 0.05% Tween (PBST) to remove unbound antigen and then they were blocked to inhibit non-specific binding by incubation for 1h with PBST containing 1% dried skimmed milk (Sainsbury, U.K.) at 37°C. After blocking, 50 μl of serial serum dilutions were added to each well and the plates were incubated for 1 h at 37°C. Following four washes with PBST, 50 μl of a 10^{-3} goat anti-human IgG peroxidase conjugate (Sigma) was added and the plate incubated for a further 1 h at 37°C. After washing the plate thoroughly, the substrate (tetramethyl benzidine plus hydrogen peroxide) in 0.1 M citrate buffer pH 5.1 was added. The enzyme reaction was stopped after 10 min by the addition of 50 μl of 0.5 M H_2SO_4. The absorbance was subsequently read on a Titertek Multiskan spectrophotometer (Flow) at 450 nm.

The percentage binding of each serum at each dilution was calculated relative to a standard 100% control binding (produced by optimally coating one column of each plate with human IgG) and to a non-specific binding control (in which antigen was omitted). The serum binding values giving 30% binding value (ABT_{30}) were then calculated.

Murine calvarial bone resorption assay

Bone resorption was assayed by the measurement of calcium release from 5-day-old mouse calvaria

in vitro (3). Halved calvaria were cultured singly on stainless steel grids in 30 mm dishes (5 per group), with 1.5 ml of BGJ medium (Flow Laboratories, Irvine, U.K.) supplemented with 5% complement-inactivated rabbit serum (Gibco, Paisley, U.K.) and 50 g/100 ml ascorbic acid (Sigma). After 24 h, the media were changed and media containing SAM from *P. gingivalis* (10 μg/ml) or *A. actinomycetemcomitans* (100 ng/ml), with or without dilutions of sera from individuals with no evidence of periodontal disease or from those with clinically diagnosed disease, were added. The sera from patients with SGP were divided into high- or low-titer groups (6 per group) and were tested for their ability to block bone resorption induced by SAM from *P. gingivalis*. Patients with LJP were similarly divided into low- or high-titer groups (6 per group) and were tested against SAM from *A. actinomycetemcomitans*. In all assays, PGE_2 was added at 1 μM to calvarial explants as a positive control and explants were cultured in unsupplemented media to act as the negative control. The calvaria were cultured for a further 48 h and then the calcium content of the media was measured by automated colorimetric analysis (4). The significance of the results was calculated by use of Student's t-test.

Results

Influence of serum concentration on SAM-induced bone resorption

Initial studies were undertaken to ascertain the effects of adding graded amounts of serum to calvaria stimulated to resorb by SAM from *A. actinomycetemcomitans*. This showed that even high concentrations of serum from individuals with no evidence of periodontal disease had no significant effect on SAM-stimulated bone resorption. Addition of various dilutions of high-titer sera from a patient with LJP to calvarial cultures stimulated to resorb with 100 ng/ml of SAM from *A. actinomycetemcomitans* demonstrated a dose-dependent inhibition of calcium release. In contrast, normal sera were without effect in this assay (Fig. 1). Based upon these initial studies, a serum dilution of 1/50 was chosen to examine the influence of larger groups of sera in this assay.

Test of "high" and "low" titer sera in the calvarial bone resorption assay

Twelve sera were chosen from each group of patients – the 6 highest titer and the 6 lowest titer

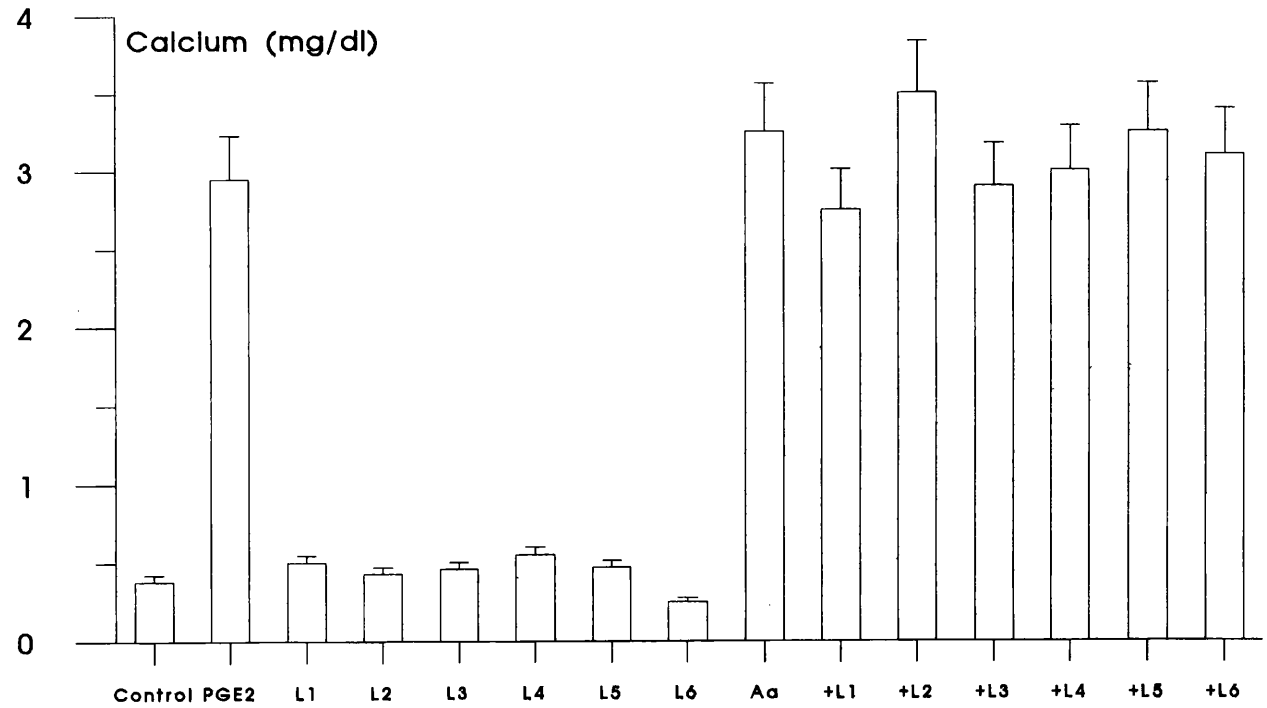

Fig. 2. The influence of 6 low-titer LJP sera (L1 to L6) on the calvarial bone resorption assay. The control (C) cultures shows the calcium release from unstimulated calvaria. The PGE2 control demonstrates the calcium release from calvaria stimulated with 1 μM of the potent osteolytic agent PGE_2. The next six columns show the effect of adding 1:50 dilutions of these 6 sera to the calvaria. The next column (Aa) is the calcium release stimulated by adding 100 ng SAM from *A. actinomycetemcomitans*. The final 6 columns show the effect on SAM-induced osteolysis of adding the 6 sera (L1 to L6). Results are expressed as the mean and standard deviation of 5 replicate cultures. None of the sera inhibited calcium release induced by *A. actinomycetemcomitans*.

sera (Table 1). The LJP sera were tested to see if they would all block bone resorption induced by SAM from *A. actinomycetemcomitans* and the SGP sera were tested for inhibition of bone resorption induced by SAM eluted from *P. gingivalis*.

Influence of LJP sera on bone resorption induced by SAM from *A. actinomycetemcomitans*

The 6 low-titer LJP sera (L1 to L6) when added to unstimulated calvarial cultures had no stimulatory or inhibitory action. When added to calvarial cultures stimulated by 100 ng/ml of SAM from *A. actinomycetemcomitans*, none of these sera inhibited calcium release from the calvaria (Fig. 2). The 6 high-titer sera (L7 to L12) were also added to unstimulated calvarial cultures. Sera L11 and L12 appeared to inhibit the basal release of calcium which occurs in this assay. When these 6 sera were added to cultures stimulated to resorb with 100 ng/ml of SAM from *A. actinomycetemcomitans*, sera L7 to L10 completely inhibited the release of calcium ($p < 0.01$). In contrast, sera L11 and L12 did not inhibit calvarial calcium release (Fig. 3).

Influence of SGP sera on bone resorption induced by SAM from *P. gingivalis*

Six low-titer sera from patients with SGP (S1 to S6) were added at a dilution of 1/50 to unstimulated calvarial cultures. None of the sera had any significant influence on basal calcium release. When these sera were added to cultures stimulated to resorb by addition of 10 μg/ml of SAM from *P. gingivalis*, only S5 produced a slight inhibition of calcium release (Fig. 4). Addition of 6 high-titer SGP sera to calvaria produced no effect on basal calcium release. When added to calvaria stimulated to resorb by addition of 10 μg/ml of SAM from *P. gingivalis*, all sera significantly inhibited ($p < 0.01$) calcium release bringing levels of release almost to the basal control level (Fig. 5).

Discussion

The role of the immune response to bacteria in the pathology of periodontal diseases has been under active study for almost four decades (5). It has been established that antibodies to a wide variety

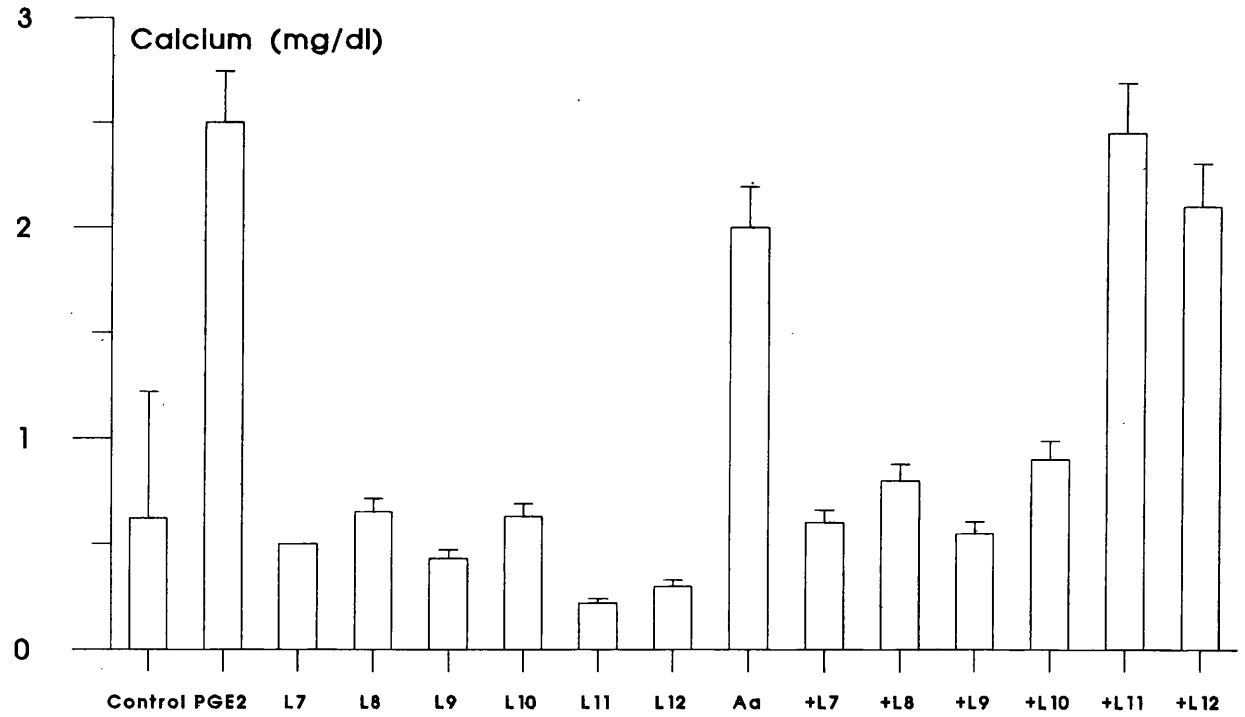

Fig. 3. The influence of 6 high-titer LJP sera (L7 to L12) on the calvarial bone resorption assay. The control (C) culture shows the release of calcium from unstimulated bone. The PGE2 column is the positive control and shows calcium release in the presence of 1 μM PGE_2. The next six columns (L7–L12) show the effect of adding a 1:50 dilution of these sera to calvarial cultures. The next column (Aa) is the calcium release when 100 ng of SAM from *A. actinomycetemcomitans* is added to cultures. The next six columns show the effect of these 6 sera on the osteolysis induced by SAM from *A. actinomycetemcomitans*. Each column represents the mean and standard deviation of 5 replicate cultures.

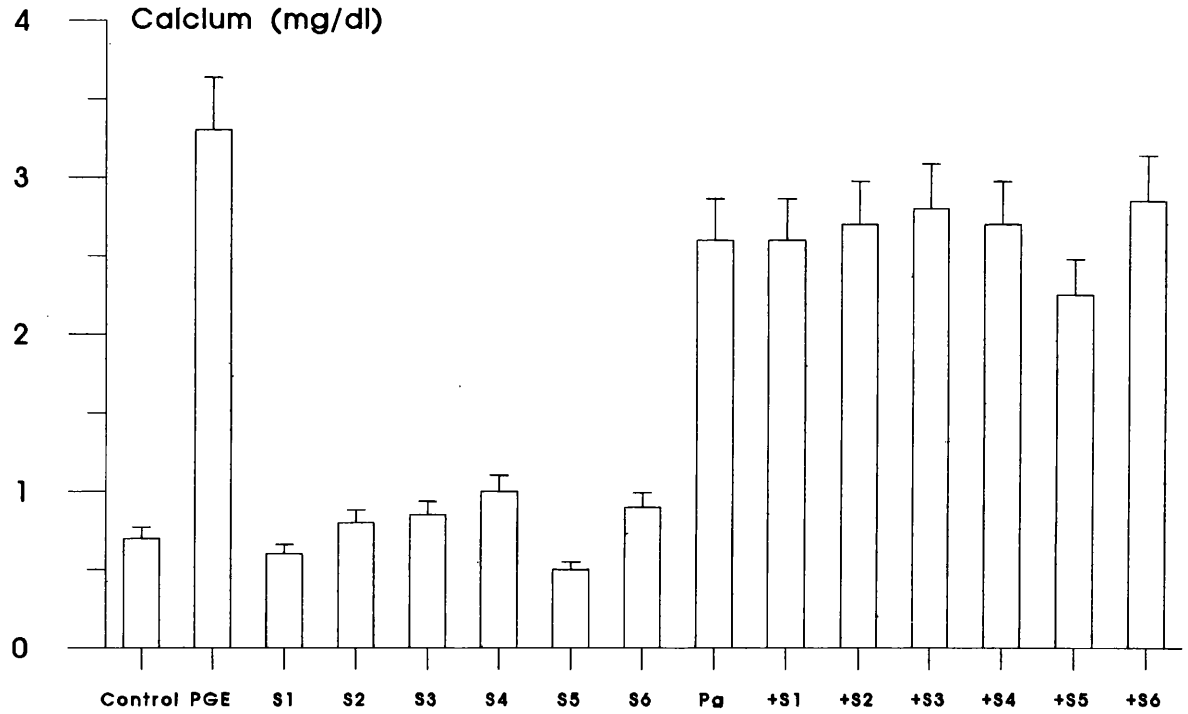

Fig. 4. The influence of 6 low-titer SGP sera (S1 to S6) on the calvarial bone resorption assay. The control (C) column shows the calcium release from unstimulated cultures and the PGE column that from calvaria cultured in the presence of 1 μM PGE_2. The next six columns (S1 to S6) are calvarial cultures to which have been added 1:50 dilutions of individual sera. The next column (Pg) is the calcium release from calvarial bone stimulated with 10 μg/ml SAM from *P. gingivalis*. The last six columns show the calcium release from calvaria cultured in the presence of 10 μg/ml SAM and the individual sera. Each column represents the mean and standard deviation of 5 replicate cultures.

of periodontopathogenic bacteria are present in the sera of patients with various forms of periodontitis (6). The role played by antibodies to bacteria such as *P. gingivalis* and *A. actinomycetemcomitans* in the pathogenesis or pathology of the periodontal diseases is not fully established.

The protective role of antibodies in conventional extracellular bacterial infections takes four major forms: opsonization, direct bactericidal action, toxin neutralization and interference with bacterial adhesion. These humoral mechanisms and the associated cellular responses are largely responsible for the curtailment of bacterial infections. This is not the case in the periodontal diseases where the putative inciting bacteria appear to be able to resist the actions of these humoral host responses. The failure of humoral mechanisms to remove periodontopathogenic bacteria is not understood but may be due to the large numbers of bacteria present subgingivally which simply swamp the antibodies. The enormous concentration of bacteria subgingivally may also limit access of antibodies to these microorganisms.

As we have shown previously (1, 2), the SAM from three well-studied periodontopathogenic bacteria are potent stimulators of bone resorption in the murine neonatal calvarial bone resorption assay. SAM from these bacteria also inhibit bone collagen synthesis ((7) and unpublished studies). These constituents are solubilized from the bacterial surface by nothing more than very gentle stirring in saline at 4°C. This implies that these materials could be equally readily shed from the bacterial surface in the periodontal pocket, giving rise to a variety of biological consequences including stimulation of alveolar bone resorption.

By use of a direct binding ELISA assay, we have also shown that patients with periodontal disease have IgG antibodies to these materials in their sera. Patients with LJP had higher titers of antibodies to SAM from *A. actinomycetemcomitans* than patients with SGP. The converse was true with patients suffering SGP who had higher titers of antibodies to SAM from *P. gingivalis* (1).

The possibility existed that these antibodies could interact with the SAM to inhibit or exacerbate their biological activities. We have now demonstrated that addition of serum, from patients with SGP who have high serum antibody titers to SAM from *P. gingivalis*, can completely inhibit the bone resorbing activity of SAM from this organism. Serum from individuals with no clinical evidence of periodontal disease or from SGP patients with low titers of antibodies to SAM from *P. gingivalis* had no effect on bone resorption induced by the corresponding SAM. However, of 6 high-titer

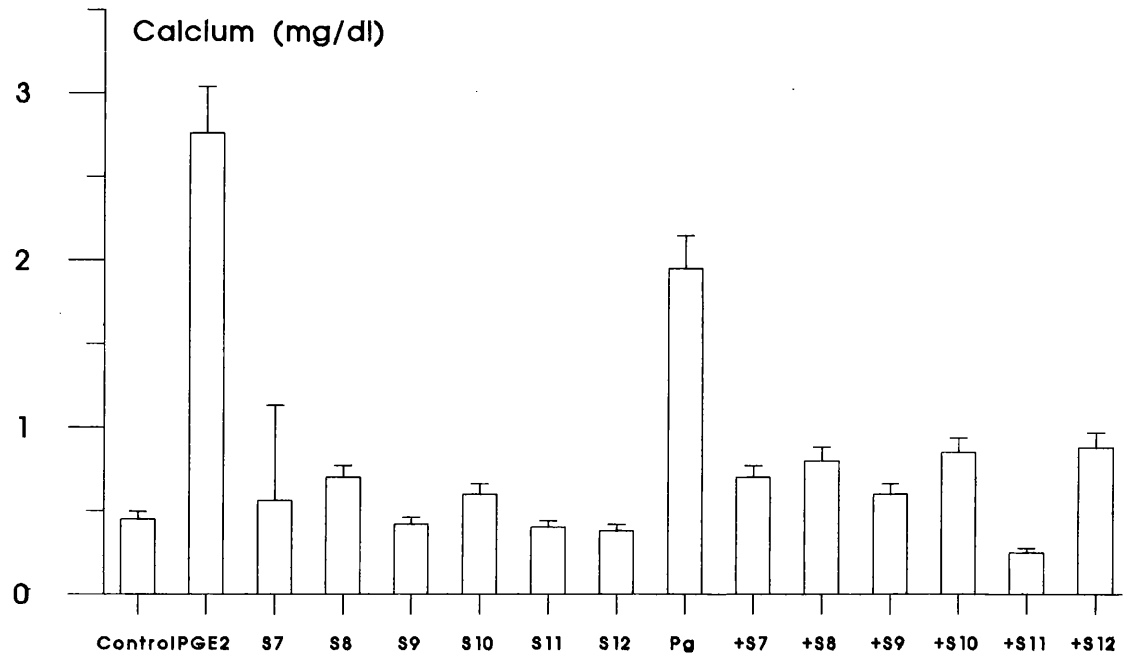

Fig. 5. The influence of 6 high-titer SGP sera (S7 to S12) on the calvarial bone resorption assay. The control (C) column shows the calcium release from unstimulated cultures and the PGE2 column that from calvaria cultured in the presence of 1 μM PGE_2. The next six columns (S7 to S12) are calvarial cultures to which have been added 1:50 dilution of individual high-titer sera. The next column (Pg) is the calcium release stimulated by 10 μg/ml SAM from *P. gingivalis*. The last six columns show the calcium release from calvaria stimulated with SAM from this organism in the presence of 1:50 dilutions of individual high-titer sera.

serum samples from patients with LJP, only 4 inhibited bone resorption induced by SAM from *A. actinomycetemcomitans*. The low-titer sera from LJP patients had no effect.

Although the sera of patients with LJP and SGP have been shown to have significant levels of antibodies against a variety of antigens from *A. actinomycetemcomitans* and *P. gingivalis*, respectively (6), few investigators have studied the actual biological functions of these antibodies. Tsai *et al.* (8) have shown that more than 90% of sera from patients with juvenile periodontitis neutralized the leukotoxic activity of sonic extracts of *A. actinomycetemcomitans* whereas most sera from periodontally-healthy individuals and patients with adult periodontitis had no such activity. All of the sera with leukotoxin-neutralizing activity had antibodies against the leukotoxin as demonstrated by immunoprecipitation. Baker and Wilson (9) have also demonstrated that sera from patients with high levels of antibodies to *A. actinomycetemcomitans* enabled complement-dependent killing of the organism by neutrophils. Neither pooled sera from periodontally-healthy individuals nor serum from an LJP patient with a low antibody titer to *A. actinomycetemcomitans* exhibited such activity.

In the case of *P. gingivalis*, Cutler *et al.* (10) have shown that sera from adult periodontitis patients with high level of IgG to this organism exhibited opsonic activity against it. However, only 3 of 18 such sera were found to possess this opsonizing ability. Complement-mediated killing of *P. gingivalis* by human serum also appears to be dependent on the presence of high levels of IgG to the bacterium (11). Sera from periodontally-healthy patients and those with adult periodontitis but with low levels of IgG against *P. gingivalis* exhibited little bactericidal activity. On the basis of the above reports, it would appear that specific antibodies may enhance host defences against periodontopathogenic bacteria by virtue of their involvement in bacterial killing and their ability to neutralize toxins.

With regard to one of the characteristic features of advanced periodontitis, bone loss, the only report of the effect of human serum on bone resorbing activity of bacterial components from periodontal pathogens has been that of Iino and Hopps (12). These authors found that human serum inhibited bone resorption induced by LPS from *A. actinomycetemcomitans* and *Capnocytophaga ochracea* but had no effect on that induced by LPS from *P. gingivalis*. However, the authors suggested that inhibition was unlikely to have been antibody-mediated in view of the fact that the serum used had similar levels of antibodies against all three organisms. The results of the present study suggest that antibodies to the osteolytic constituents of the SAM from *A. actinomycetemcomitans* and *P. gingivalis* can inhibit the effects of these substances

on bone. Interestingly, 2 of the 6 serum samples from LJP patients did not inhibit bone destruction even though the antibody titers were very high. This suggests that, although the serum concentration of antibody is important, another important parameter must be the epitope specificity of the antibodies. Thus in the sera which failed to block bone resorption the antibodies must be binding to constituents of the SAM which are not involved in stimulating bone resorption. This recalls the findings of Cutler *et al.* (10) regarding the lack of opsonizing activity of certain sera with high antibody titers to *P. gingivalis.*

The clinical information available from the patients under study does not allow us to determine if there is less alveolar bone damage in patients displaying SAM-neutralizing antibodies. This will require a prospective study of a larger group of patients. This study is currently being planned. If it can be shown that there is a relationship between serum-neutralizing antibody titer and alveolar bone destruction then this would suggest that antibodies to SAM could be used in a therapeutic manner. Monoclonal antibodies to the osteolytic constituents of SAM could be applied locally to inhibit bone loss.

Acknowledgements

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Stimulation of bone collagen and non-collagenous protein synthesis by products of 5- and 12-lipoxygenase: determination by use of a simple quantitative assay

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Summary

The influence of 5- and 12-lipoxygenase products on the rate of collagen and non-collagenous protein (NCP) synthesis by murine calvarial explants has been investigated using a new assay based on the resistance of native collagen to degradation by pepsin. The reproducibility and simplicity of this assay allows the quantitative estimation of the rate of bone formation in large numbers of cultures.

Hydroxyeicosatetraenoic acids (HETEs) stimulated both the rate of collagen and NCP synthesis with maximal stimulation occurring at 10–100 p*M*. All leukotrienes stimulated collagen synthesis. LTB_4, C_4 and D_4 showed similar dose-responses with maximal activity occurring at 100 p*M*. LTE_4 was less potent only showing activity at 1–10 n*M*. Only LTD_4 demonstrated the capacity to stimulate NCP synthesis with significant stimulation being seen at 10 n*M*.

The extreme sensitivity of bone collagen and NCP synthesis to lipoxygenase products suggests that these mediators may play a physiological role in bone remodelling.

Key words: Bone remodelling; Calvaria; Collagen synthesis; Non-collagenous proteins

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Introduction

There is a substantial body of literature documenting the actions of the products of arachidonate cyclo-oxygenase activity on bone cells and bone in culture [1–3]. Most work has been done using prostaglandins (PGs) such as PGE_2. The finding that PGE_2 is capable of stimulating bone breakdown in vitro has suggested that the prostanoids are mediators of bone destruction in inflammatory diseases such as rheumatoid arthritis [4], periodontal disease [5,6], osteolytic tumours [7,8] and in dental cysts [9]. However, it is now established that non-steroidal anti-inflammatory drugs, which inhibit cyclo-oxygenase activity, have no inhibitory action on the bone loss seen in rheumatoid arthritis [10] or in hypercalcaemia of malignancy [11].

Arachidonic acid is also metabolized by cellular lipoxygenases to produce several families of lipid mediators, the best known being the products of 5-lipoxygenase – the leukotrienes. We have previously shown [12] that 5- and 12-HETEs and leukotrienes are potent stimulators of bone resorption. Indeed leukotriene B_4 shown to be 3–4 log orders more potent than PGE_2 in stimulating murine calvarial bone resorption. Leukotrienes have been found both in rheumatoid joint fluids [13] and gingival tissues from patients with periodontal disease [14]. Whether the leukotrienes found in these lesional sites contribute to bone damage has still to be established.

Paradoxically, PGE_2 has also been demonstrated to increase bone matrix synthesis. Clinical use of PGE_2 to maintain patency of the ductus arteriosus in infants with congenital heart disease was found to produce significant periosteal new bone formation [15]. This finding has been reproduced in rats [16,17]. In in vitro cultures prostaglandins have been shown to stimulate collagen synthesis in chicken embryonic tibia [18] and foetal rat calvaria [19].

In the light of the dual action of prostaglandins it was of interest to determine if leukotrienes had any effect on bone protein synthesis. In this investigation a range of leukotrienes and HETEs have been tested to see if they are capable of stimulating the synthesis of collagen and non-collagenous protein by neonatal murine calvarial explants. A new method for estimating collagen and NCP synthesis has been used in this study. This is a modification of the conventional assay in which ^{3}H-proline-labelled calvaria are exposed to highly purified bacterial collagenase to release labelled collagen. In the modified assay, pepsin is used to lyse the calvaria allowing the separation of intact collagen from the NCP. One advantage of this assay is its simplicity allowing large numbers of samples to be analysed.

Methods and Materials

Leukotrienes: The HETEs and leukotrienes were a gift from Dr J. Rokach, Merck Frost, Canada Inc. They were dissolved in phosphate-buffered saline (PBS) prior to dilution in culture medium. Parathyroid hormone (PTH) was

obtained from the National Institute of Biological Standards and Control (NIBSC, Potters Bar).

Tissue culture: Calvaria were removed aseptically from 5-day-old BALB/c mice. The fronto-parietal bones were trimmed of any adhering connective tissue, and divided along the sagittal suture. The calvarial segments were cultured in groups of 5–10 in 50-mm dishes (Sterilin) with 5 ml of BGJ medium (Flow), supplemented with 5% complement-inactivated rabbit serum (Wellcome) and containing 50 μg/ml L-ascorbic acid (Sigma) and 100 U/ml penicillin and streptomycin (Gibco). Bone explants were incubated for 24 h at 37°C in 5% CO_2 and then the media were replaced with media containing dilutions of the HETEs or leukotrienes over the dose range 10^{-11} to 10^{-6} *M*. Explants were cultured for a further 24 h and [5-^{3}H]-proline (0.5 μCi/ml: 23 Ci/mmol; Amersham; 1 Ci = 37 GBq) was added for the last 6 h.

The effect of PTH on bone collagen synthesis was tested over the dose range 0.01 to 100 mU/ml. Each agonist was tested at least twice and all gave consistent dose-responses.

Collagen and non-collagenous protein synthesis: Cultures were terminated after the 6-h labelling period by transferring the bones to a solution of 5% trichloroacetic acid (TCA, BDH) for 2 h. This step also removes unbound isotope and small peptides from the bones. The procedure for extraction and purification of the radioactively-labelled collagen was a modification of the method of Webster and Harvey [20] for monolayer cell cultures, and is shown schematically in Fig. 1. Following TCA extraction, the bones were rinsed in Hank's solution and each bone was placed in a 3-ml polystyrene tube (LP3;

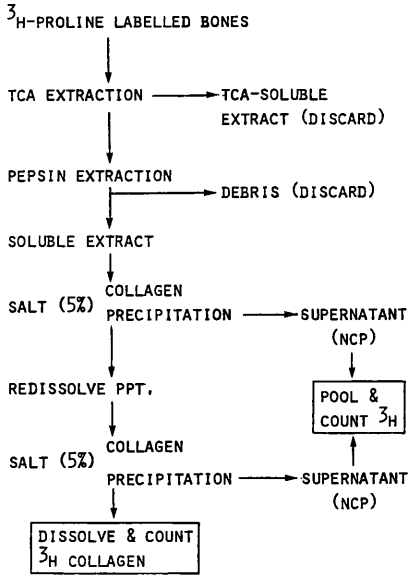

Fig. 1. Extraction and purification scheme for the measurement of collagen and NCP synthesis by murine calvaria.

Luckham). Collagen was extracted with 1 ml of 0.5 *M* acetic acid containing pepsin (0.5 mg/ml; EC 3.4.4.1, Sigma) for 16 h at 4°C. Insoluble bone debris was removed by centrifugation (5000 *g* for 30 min) and 200 μg of acid-soluble rat skin collagen was added to each tube as a carrier for the labelled collagen during subsequent purification. Collagen was precipitated by addition of NaCl (25% *w/v* in 0.5 *M* acetic acid) to a final concentration of 5% (*w/v*). After 3 h the tubes were centrifuged at 5000 *g* for 30 min, the supernatant removed and stored and the pellet redissolved in 1 ml 0.5 *M* acetic acid. The collagen was re-precipitated as described and the second supernatant removed and stored. The final precipitate was dissolved in 250 μl of 0.5 *M* acetic acid and transferred to scintillation tubes and radioactivity measured by scintillation spectrometry (Rackbeta, LKB) with external standardization.

The rate of NCP synthesis was estimated by measurement of the ^{3}H-proline activity in the pooled supernatants removed during collagen purification (Fig. 1).

Standardization: The assay was standardized in terms of the extractability of collagen and NCP, their recovery during purification and the specificity, reproducibility and sensitivity of the assay.

The extractability of labelled collagen was measured by determination of hydroxy ^{3}H-proline (^{3}H-hypro) activity in the residue and the extract of the pepsin digest. The recovery of collagen during purification was determined by measuring ^{3}H-hypro in the salt supernatants and the final precipitate. The fate of the NCP was examined by measuring radioactivity in similar fractions from parallel bone cultures which were pulsed with ^{3}H-tryptophan (2 μCi/ml, specific activity 3.1 Ci/mmol; Amersham) in place of ^{3}H-proline (tryptophan is not incorporated into collagen, but is a common amino acid in NCP).

The specificity of the assay for collagen was established by the following analyses of the final collagen precipitate: (i) the ratio of ^{3}H-hypro to ^{3}H-pro; (ii) carboxymethyl cellulose (CM) chromatography; (iii) SDS-PAGE and the extent of contamination by ^{3}H-tryptophan. The specificity of the assay for NCP was determined from the extent to which the salt supernatants were contaminated with ^{3}H-hypro. In addition, the collagen and NCP synthesis by calvaria was assessed both by the conventional bacterial collagenase assay and the pepsin-based assay.

(i) ^{3}H-pro and ^{3}H-hypro assay: Measurement of these amino acids was performed after chromatographic separation on a column of 'Amberlite' resin (CG-120 Na, BDH), 15 cm × 0.5 cm. Samples were hydrolysed for 16 h at 110°C in 6 *N* HCl, evaporated to dryness and dissolved in 250 μl of eluting buffer (0.1 *M* citrate, pH 2.55) containing proline (1 mg/ml) and hydroxyproline (2 mg/ml) as carriers. Radioactivity was measured in 1-ml fractions of eluate.

(ii) CMC chromatography: Approximately 40 000 dpm of labelled sample collagen was mixed with 10 mg acid-soluble rat skin collagen and chromatographed on a 1.6 × 5-cm column of CM45 (Whatman) as described previously [20], with the modifications that the volume of elution buffer was increased to 200 ml containing a linear gradient of 0–0.15 *M* NaCl and the fraction volume collected was 1.6 ml.

(iii) SDS-PAGE: Approximately 40 000 dpm aliquots of the sample collagen was applied to tube gels (0.5 × 10 cm, 5% acrylamide) and electrophoresed in Tris/borate buffer (100 m*M*, pH 8.6) containing 0.1% SDS [20]. Mercaptoethanol (20 μl of a 10% solution in sample buffer) was added to some gels 30 min after the start of electrophoresis in order to reduce type III collagen and separate the chains from α_1(I) [21].

To compare the estimation of collagen and NCP synthesis by the bacterial collagenase and pepsin sensitivity methodologies, a group of whole calvaria were divided in half after pulse-labelling with ^{3}H-pro, thus ensuring even labelling of both halves. One half was then assayed by the pepsin sensitivity method and the other by the bacterial collagenase assay which was described by Peterkofsky and Diegelman [22] and modified for neonatal mouse calvaria by Dietrich et al. [23]. The collagenase used in this assay was obtained by sequential fractionation of crude bacterial collagenase (Sigma, type II) on Ultra gel ACA 34, ACA 44 and ACA 54. The final preparation had no proteolytic activity on casein as a substrate. The collagenolytic activity was 400 units/ml. This material was a gift from Mr Compton, Guy's Hospital, London.

Statistics: The significance of the results was estimated by use of Student's *t*-test for unpaired observations.

Results

1. Assessment of assay

Extractability and recovery of protein: Limited digestion with pepsin caused partial disintegration of the calvaria and the debris was easily separated from the extract by centrifugation. Analysis of ^{3}H-hypro and ^{3}H-tryptophan in four separate experiments (Table 1) showed that approximately 90% of collagen and 80% of NCP were extracted into solution by pepsin digestion. During subsequent purification, loss of collagen into the supernatants was approximately 12% and loss of NCP by co-precipitation with collagen was

Table 1

The fate of hydroxy ^{3}H-proline and ^{3}H-tryptophan during extraction and purification procedures for the collagen and NCP assays, expressed as a percentage of the starting activity in the TCA-extracted bones

	Hydroxy ^{3}H-proline		^{3}H-Tryptophan	
	Exp 1	Exp 2	Exp 3	Exp 4
TCA-extracted bone	100	100	100	100
Pepsin extract	93	89	81	81
Supernatant 1	9	10	75	75
Supernatant 2	3	3	4	5
Final collagen ppt	81	76	2	1

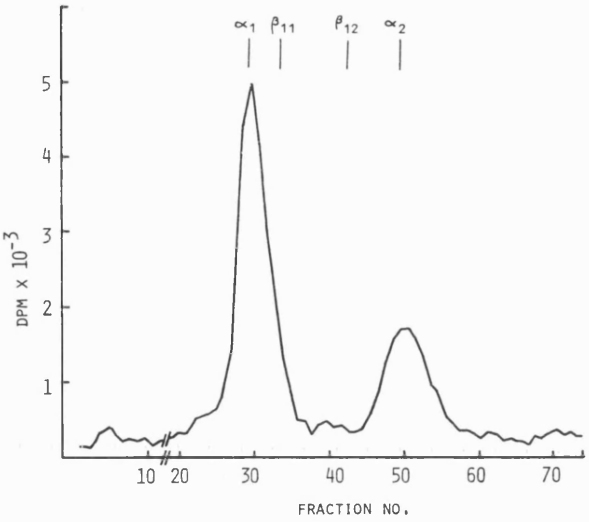

Fig. 2. Ion exchange chromatography of the purified collagen precipitates on carboxymethyl cellulose. The elution positions of carrier rat skin collagen chains are shown.

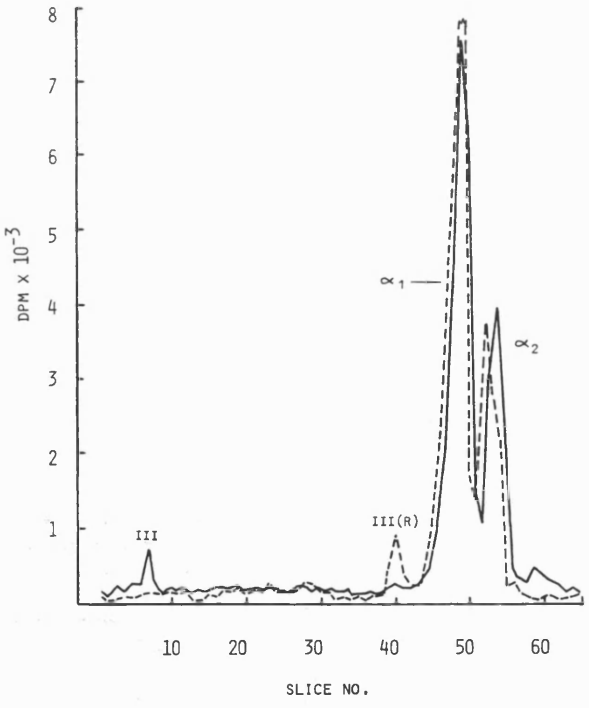

Fig. 3. SDS PAGE of the purified collagen precipitate, with (– – –) or without (——) delayed reduction of the sample with mercaptoethanol. The position of the standard collagen chains are indicated.

less than 2% (Table 1).

Specificity of protein labelling: The final collagen precipitate had a ^{3}H-hypro:^{3}H-pro ratio of 0.78 (mean of eight separate experiments; range 0.73–0.84). Analysis by CMC chromatography (Fig. 2) showed that 94% of radioactivity eluted in the position of α1 and α2 collagen chains. Electrophoresis of the final precipitates revealed that 3% of the radioactivity was in type III collagen, identified by its mobility and susceptibility to reduction by mercaptoethanol (Fig. 3).

Contamination of the NCP fraction by collagen, calculated from ^{3}H-hypro analysis, was 8.4% or 9.8% of the total supernatant activity in two separate experiments.

Reproducibility of protein synthesis: The reproducibility of the assay was very good. The coefficients of variation (cv) were 2.4% and 5.4% in two separate experiments where collagen was purified from aliquots of a pooled pepsin extract of ^{3}H-pro-labelled calvaria. The variation between calvaria with respect to the synthesis of collagen and NCP was also small, the coefficients of variation averaging 17.9% and 19.7% respectively, for groups of five bones.

Comparison between collagenase and pepsin extraction: In three experiments prelabelled calvaria were halved and the collagen and NCP content estimated by the standard bacterial collagenase extraction technique or the alternative pepsin extraction method. As can be seen in Table 2 both extraction techniques gave similar estimates of collagen and NCP synthesis. The percent collagen synthesis estimated by the pepsin method was 12.1 ± 2.6 (mean and standard deviation; $n=3$) compared with 14.2 ± 2.8 for the collagenase technique.

2. Effect of lipoxygenase products

Influence of 5- and 12-HETEs: 5- and 12-HETEs stimulated both collagen and NCP synthesis over the dose range 10 p*M* to 10 n*M* when compared to unstimulated bone. At higher concentrations there was a decline in the stimulation of protein synthesis (Figs. 4 and 5).

Influence of leukotriene B_4: Leukotriene B_4 had a pronounced stimulatory effect on calvarial bone collagen synthesis with maximal stimulation being seen

Table 2

Comparison of the incorporation of ^{3}H-proline into collagen and NCP in cultured calvaria as assessed by the bacterial collagenase and pepsin extraction assays

Extracted material (cpm)			
Pepsin extraction		Collagenase extraction	
Collagen	NCP	Collagen	NCP
17669 ± 3785	23591 ± 710	16870 ± 3930	19242 ± 2648

Results are expressed as the mean and standard deviation of three experiments.

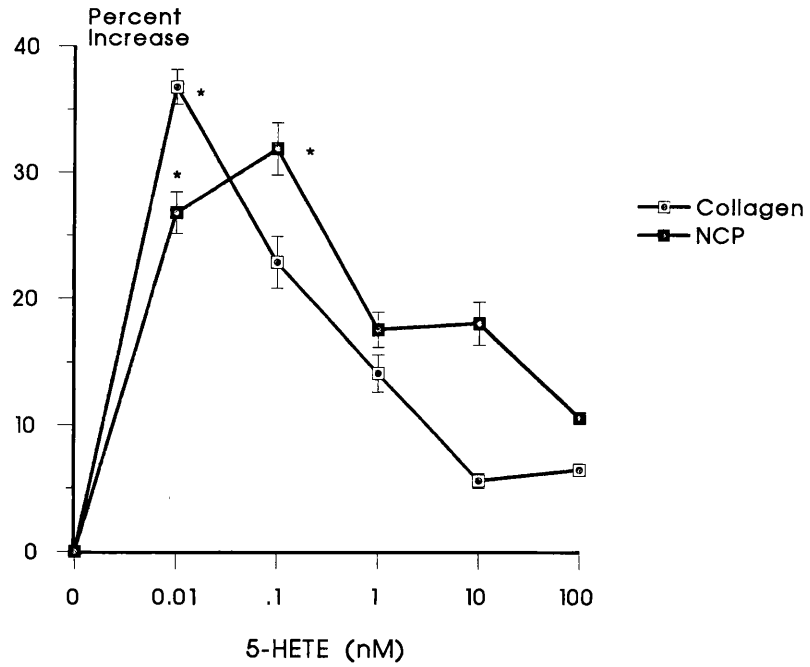

Fig. 4. The stimulation of collagen or NCP synthesis by murine calvaria exposed to concentrations of 5-HETE over the dose range 10 p*M* to 100 n*M*. Results are expressed as the mean percentage increase (± SEM) compared to unstimulated cultures. The number of culture replicates per point was five and the * represents a significant result compared with unstimulated control cultures ($P<0.05$).

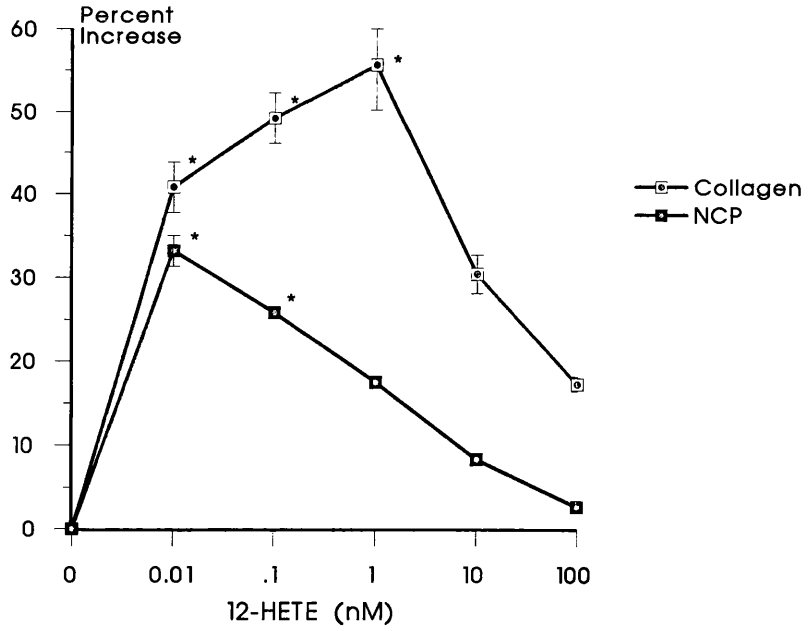

Fig. 5. The stimulation of collagen or NCP synthesis by murine calvaria exposed to concentrations of 12-HETE over the dose range 10 p*M* to 100 n*M*. Results are expressed as the mean percentage increase (± SEM) compared to unstimulated cultures. The number of culture replicates per point was five and the * represents a significant result compared with the unstimulated control cultures ($P<0.05$).

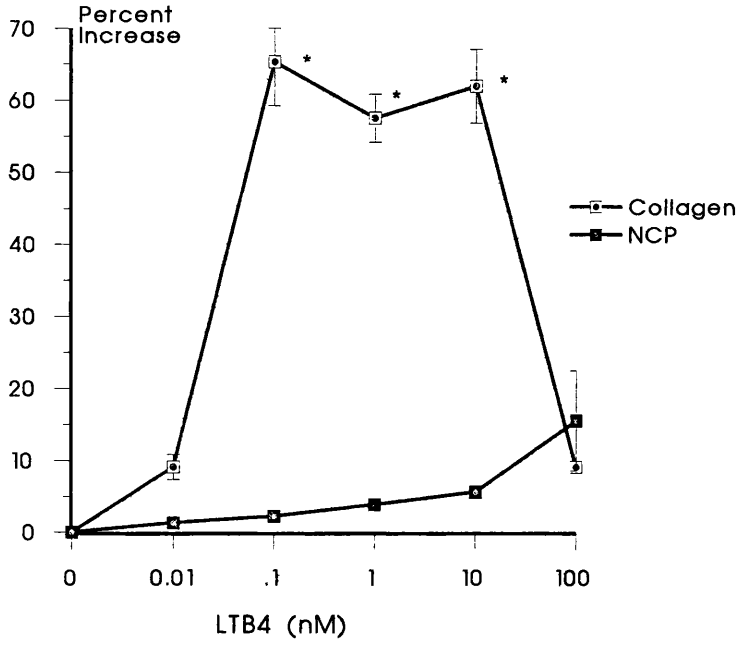

Fig. 6. The influence of LTB_4 on collagen and NCP synthesis by cultured murine calvaria. LTB_4 was added to calvarial cultures over the dose range 10 p*M* to 100 n*M*. Results are expressed as the mean percentage increase (± SEM) compared to unstimulated cultures. The number of culture replicates per point was five and the * represents a significant result compared to unstimulated control cultures ($P<0.05$).

at between 100 p*M* and 10 n*M* compared with unstimulated control cultures. Of interest was the finding that at the highest concentration tested there was no significant stimulation. In contrast to collagen synthesis this leukotriene did not significantly stimulate NCP synthesis over the dose range tested (Fig. 6).

Activity of the peptidoleukotrienes: All three peptidoleukotrienes (LTC_4, LTD_4 and LTE_4) stimulated calvarial bone collagen synthesis over the basal rate in control cultures. LTC_4 was the most potent of all the leukotrienes tested, showing significant stimulation at 10 p*M* and reaching a plateau of stimulation between 100 p*M* and 100 n*M* (Fig. 7). At 100 p*M* both LTC_4 and LTD_4 (Fig. 8) produced 80–90% stimulation of collagen synthesis. In contrast, LTE_4 (Fig. 9) only produced stimulation of collagen synthesis at concentrations of 1 n*M* and above. At the highest concentration of LTD_4 tested there was a decline in the stimulation noted at lower concentrations. The peptidoleukotrienes were also relatively inactive with regard to stimulation of NCP synthesis. Only LTD_4 showed any significant stimulation of NCP synthesis.

3. Effect of PTH

Parathyroid hormone (0.01–100 mU/ml) was added to cultured calvaria and the collagen synthesis assessed. There was a dose-dependent inhibition of synthesis over the range 1–100 mU PTH (results not shown).

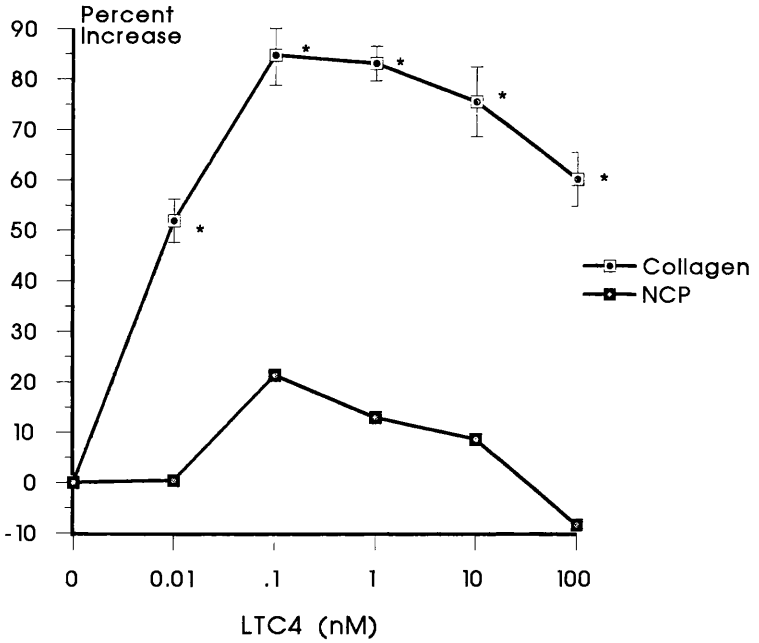

Fig. 7. The influence of the peptidoleukotriene LTC_4 on collagen and NCP synthesis by cultured murine calvaria. LTC_4 was added to calvaria in culture over the dose range 10 p*M* to 100 n*M*. Results are expressed as the mean percentage increase (± SEM) compared to unstimulated cultures. The number of replicate cultures per point was five and the * represents a significant increase compared to the unstimulated control cultures ($P<0.05$).

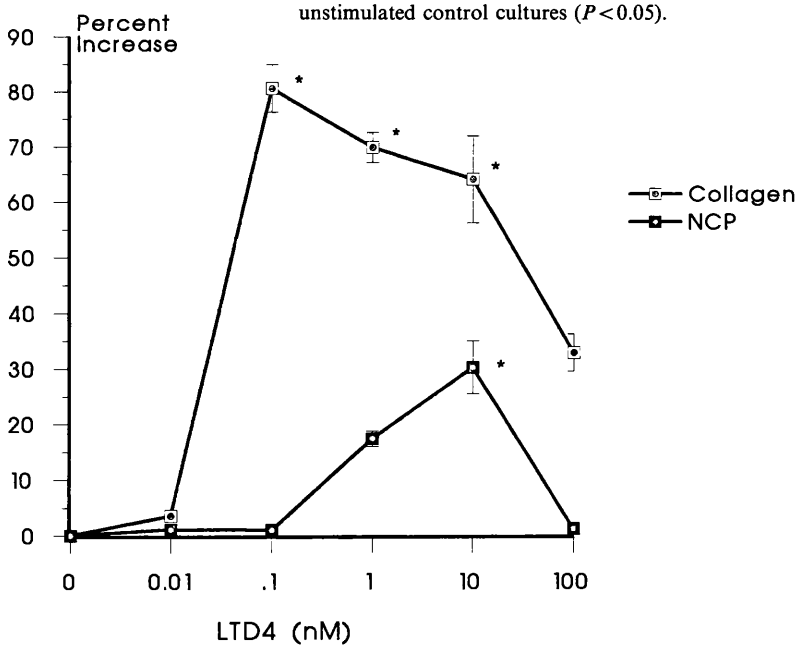

Fig. 8. The influence of LTD_4 on collagen and NCP synthesis by cultured murine calvaria. LTD_4 was added to calvarial cultures at concentrations ranging from 10 p*M* to 100 n*M*. Results are expressed as the mean percentage increase (± SEM) compared to unstimulated cultures. The number of replicate cultures per point was five and the * represents a significant increase compared to the unstimulated control cultures ($P<0.05$).

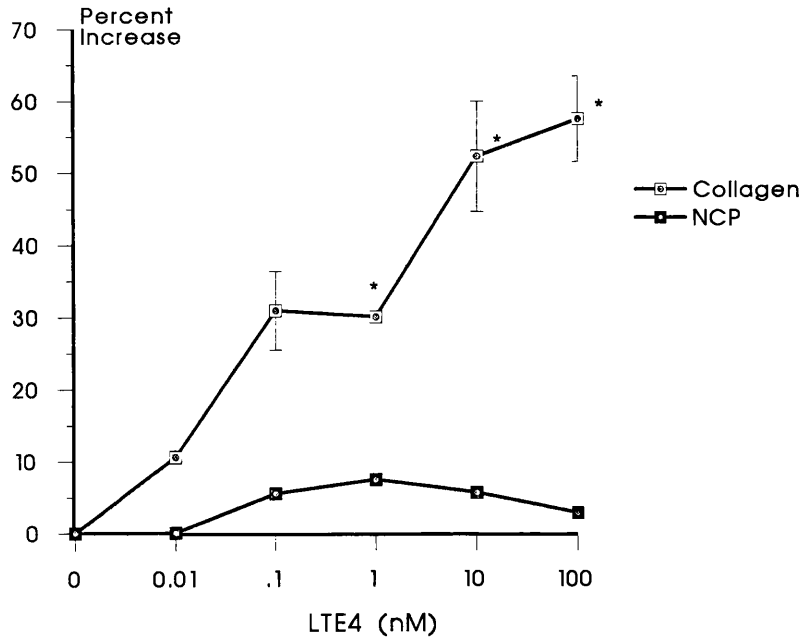

Fig. 9. The influence of LTE_4 on collagen and NCP synthesis by cultured murine calvaria. LTE_4 was added to calvarial cultures at concentrations ranging from 10 p*M* to 100 n*M*. Results are expressed as the mean percentage increase (± SEM) compared to unstimulated cultures. The number of replicate cultures per point was five and the * represents a significant increase compared to the unstimulated control cultures ($P < 0.05$).

Discussion

The pepsin extraction assay described in this paper is a simple method which allows the rapid and direct estimation of collagen and NCP synthesis in bone cultures. The advantages over the classic bacterial collagenase extraction assay is that there is no need for enzyme (i.e. collagenase) purification and there are fewer steps needed to assay the bone collagen. Thus large numbers of samples (up to 100) can be assayed at any one time. The pepsin assay is based on the measurement of radioactively-labelled collagen present in bones after a 6-h pulse with ^{3}H-proline. This is achieved by extraction of native collagen using limited pepsin digestion, purification by salt precipitation and measurement of the radioactivity in the final preparation. NCP is largely susceptible to degradation by pepsin and does not co-precipitate with the collagen. In turn the NCP is not contaminated with collagen. This assay therefore gives an estimate of the 'net' rates of collagen and NCP synthesis over the duration of the proline pulse. Care has been taken to ensure the specificity of the labelling both of the collagen and of the NCP. Comparison of this pepsin extraction assay with the established bacterial collagenase extraction assay of Peterkofsky and Diegelman [22] showed that both assays gave similar results for collagen and NCP synthesis. The purified radiolabelled collagen was shown to be predominantly type I collagen with only 3% of the label being found in type III collagen. This small amount of type III collagen

may have come from the activity of the periosteal fibroblasts which are known to synthesize this collagen.

The pepsin extraction assay has been used to determine the effects of 5- and 12-lipoxygenase products on both collagen and NCP synthesis in mouse calvarial cultures. In a previous report from this laboratory it had been shown that HETEs and leukotrienes were potent stimulators of bone resorption in the calvarial bone resorption assay [12]. The maximal responses occurred at pico- to nano-molar concentrations and the dose-response graphs exhibited marked biphasic responses with these mediators having decreasing effects at higher concentrations. Compared with the response of PGE_2 these lipoxygenase products are 2–4 log orders of magnitude more potent. Two recent reports have supported these initial findings. Leukotriene B_4 has been reported to modulate the mitotic activity of isolated rat calvarial osteoblasts and human osteoblast-like cell lines with the dose-responses showing pronounced biphasic effects [24]. This same leukotriene has also been shown to stimulate inositol phosphate metabolism and intracellular calcium release in isolated osteoblasts [25]. These findings suggest that bone cells respond to leukotrienes by a receptor-mediated mechanism.

The studies of the effect of lipoxygenase products on bone protein synthesis were prompted by reports that prostaglandins can stimulate bone formation in vivo [15–17] and in cultured foetal calvaria [19]. In this latter study, short-term (24-h) cultures of rat calvaria in the presence of 100 n*M* PGE_2 failed to stimulate collagen or NCP synthesis, but with prolonged culture (96 h) the calvaria showed a significant increase in collagen and NCP synthesis. The explanation offered for this finding was that the PGE_2 was not acting directly on the osteoblasts but was stimulating the maturation of pre-osteoblasts into osteoblasts which then synthesized the collagen and NCP and accounted for increased incorporation of ^{3}H-proline [19].

In the present study murine calvaria have been cultured in the presence of various 5- and 12-lipoxygenase products for 24 h with both collagen and NCP synthesis being measured by addition of ^{3}H-proline for the final 6 h of culture. All lipoxygenase metabolites stimulated collagen synthesis over the dose range 10 p*M* to 100 n*M*. The most potent compounds were the HETEs and LTC_4 which produced significant elevation of collagen synthesis at 10 p*M*. Leukotriene E_4 was the least potent lipoxygenase product tested and showed significant activity only at 1 n*M* and above. Measurement of NCP synthesis was also made. The HETEs significantly stimulated NCP synthesis at concentrations as low as 10 p*M*. In contrast, of the leukotrienes tested, only leukotriene D_4 was able to stimulate NCP synthesis and then only at relatively high concentrations (1–10 n*M*).

The dose-response relationship for the HETEs, and to a lesser extent for leukotrienes B_4 and D_4, had a biphasic shape. At low concentrations of agonist there was a dose-dependent stimulation of the incorporation of ^{3}H-proline which reached a maximum, or in the case of 12-HETE and LTB_4, plateaued. With increasing concentration of these agonists there was a decline in the degree of stimulation of proline incorporation. However, none of these eicosanoids produced any inhibition of the basal rate of collagen or NCP synthesis and therefore

could not be considered to be toxic to the calvaria. The reason for this biphasic dose-response is not immediately clear. The possibility exists that increasing concentrations of agonist leads to the synthesis of mediators by bone cells which act to inhibit protein synthesis. Alternatively, as the agonists are in contact with the bone for relatively long periods, the loss of response could be due to receptor desensitization.

All the agents tested in this study stimulated collagen synthesis. As an additional check on the assay, calvaria were incubated in the presence of PTH which has been demonstrated to inhibit calvarial collagen synthesis in vitro [23]. PTH was indeed found to inhibit calvarial collagen synthesis over the dose range 1–100 mU/ml.

It is not possible to define which cells within the calvaria are responding to these lipoxygenase products. The fact that stimulation is seen within 24 h is in contrast with the results of Chyun and Raisz [19] who showed that PGE_2 only stimulated calvarial collagen and NCP synthesis after a 96-h culture period. This was interpreted as being due to the stimulation of pre-osteoblasts to mature into collagen-synthesizing osteoblasts. Based upon this study it is therefore possible that the lipoxygenase products are directly stimulating osteoblasts in the calvaria. However, other possibilities cannot be ruled out.

The extreme sensitivity of murine calvaria to lipoxygenase products and the demonstration of lipoxygenase activity by primary cultures of murine osteoblasts [26] and a human osteosarcoma cell line [27] suggests that these mediators may have a physiological role in bone remodelling. With the development of potent, selective inhibitors of 5-lipoxygenase this hypothesis can now be tested.

Acknowledgements

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Bone remodelling

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Bone is a specialised connective tissue that, together with cartilage, makes up the skeleton. These tissues serve three functions: (a) mechanical support and site of muscle attachment for locomotion; (b) protection for vital organs and bone marrow and (c) a metabolic reserve of ions for the entire organism, especially calcium and phosphate. The fundamental constituents are the cells and a calcified extracellular matrix. The latter is particularly abundant and is composed of collagen fibres and a ground substance rich in glycosaminoglycans. The osteoclasts and osteoblasts have evolved to regulate the growth and turnover of bone and mediate ion fluxes between the bone and blood. The osteoclasts solubilise the mineralised organic matrix, and the osteoblasts are capable of forming bone and synthesising the various structural proteins and growth factors. Except during growth, a balance between bone resorption and formation is maintained. The activities of the bone formation and resorbing cells are regulated by local and systemic humoral factors, such as parathyroid hormone, cytokines, 1,25, dihydroxy vitamin D_3 and the eicosanoids. Any dysfunction of either the local or systemic regulatory systems will lead to pathological changes in the rate of bone formation or resorption and ultimately a clinical disease of the skeleton.

Bone is a highly dynamic connective tissue with a capacity for continuous remodelling. It is composed of a variety of cell types and an extracellular organic matrix which has become calcified.

The two principal cell types, the osteoclast and the osteoblast, are the major effectors in the turnover of bone matrix. The osteoblast produces the matrix, which in a well regulated process becomes calcified to form initially the partially calcified osteoid and ultimately fully mineralised bone. This mineral matrix can be dissolved by the activity of the osteoclast when activated. At any given time, the processes of bone synthesis and bone breakdown are going on simultaneously and the status of the bone represents the net result of a balance between the two processes. This phenomenon is now widely called 'coupling' of bone resorption and formation.

The extracellular organic matrix of bone consists predominantly of type I collagen (85–95%). The non-collagenous constituents are composed of proteins and proteoglycans which are specific to bone and the dental hard connective tissues (fig. 1). Two-dimensional electrophoretic analysis of the non-collagen proteins of bone suggest that there may well be over 200 of them.[1] Many of these proteins are identified as originating from plasma or other non-bone sources, for example alpha-2-glycoprotein and beta-2-microglobulin. The non-collagenous protein products of the bone cells include osteonectin, proteoglycan I, proteoglycan II, osteopontin (sialoprotein I), sialoprotein II, osteocalcin and matrix GLA protein, all of which appear to contribute to the structure and functional qualities of bone.

Bone resorption

Resorption is the removal of the mineral and organic components of the extracellular matrix of bone under the action of osteolytic cells, of which the most important is the osteoclast. The osteocyte may function as a local bone resorber, ensuring the natural renewal of bone. The process involves three steps, each of which may be individually regulated by physiological, pathological or pharmacological mediators.

The first involves the formation of osteoclast progenitors in the haematopoeitic tissues, followed by their vascular dissemination and the generation of resting pre-osteoclasts and osteoclasts in the bone itself. The second phase consists of osteoclast activation at the surface of the mineralised bone. Here, osteoblasts appear to play a major role, by not only 'retracting' to expose the mineral to the osteoclast and pre-osteoclasts, but also by releasing a soluble factor that activates these cells (fig. 2). The third step involves the activated osteoclast resorbing the bone. During bone resorption, the clasts create for themselves cavities known as Howship's lacunae, which correspond closely in size to the area of bone surface enclosed by the cell. This active surface is described as having a ruffled border which consists of many infoldings of the cell membrane, resulting in finger-like projections of the cytoplasm. Thus, an extensive surface is created which is

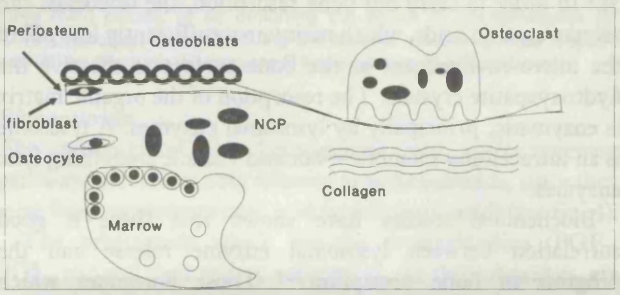

Fig. 1 Bone is composed of a variety of cell types and an extracellular organic matrix. The cells include osteoblasts, osteocytes, osteoclasts and periosteal fibroblasts. The matrix consists of collagen (85–95%) and non-collagenous proteins such as osteonectin, proteoglycan I, proteoglycan II, osteopontin, osteocalcin, sialoprotein II and matrix GLA protein.

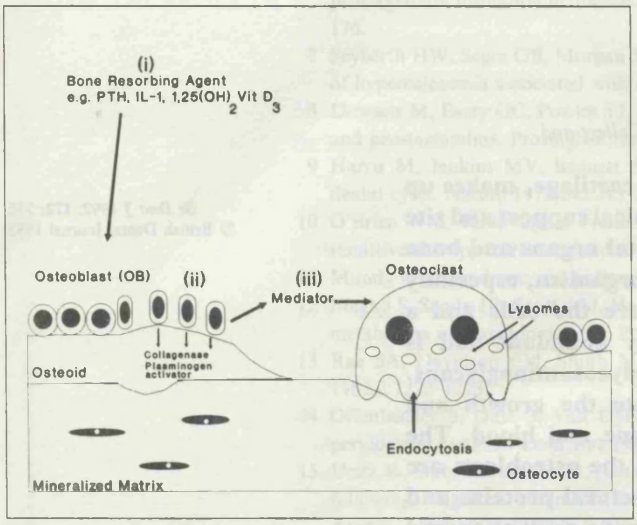

Fig. 2 The role of the osteoblast in bone resorption: Three steps are involved: (i) The bone resorbing agent, PTH, induces a change of shape of the osteoblast, thus facilitating the access of osteoclasts to the bone surface, (ii) at the same time the blasts synthesise and release collagenase and plasminogen activator which digests the osteoid, exposing the mineralised matrix which may be chemotactic to the osteoclast, (iii) the osteoblast releases a short-range soluble activator for the osteoclast.

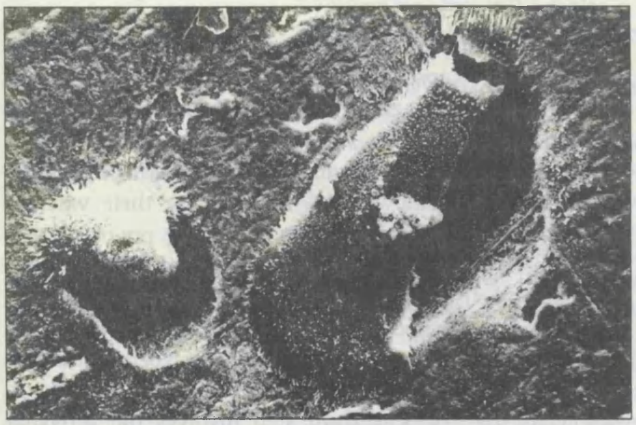

Fig. 3 Electron micrograph of two osteoclasts on a surface of a dentine slice, showing resorptive pits.

well suited for an intensive exchange between the cell and the bone, and is effectively sealed off from the extracellular environment by a tight junction between the bone and the periphery of the osteoclast. Osteoclasts can also move about on the surfaces of bone and dentine slices *in vitro* and leave discontinuous tracks of resorptive activity behind them (fig. 3).[2] In order to carry out bone resorption, the osteoclast can secrete organic acids, which maintain a sufficiently low pH in the micro-environment at the bone surface to dissolve the hydroxyapatite crystals. The resorption of the organic matrix is enzymatic, principally by lysosomal enzymes. A lysosome is an intracellular membrane-enclosd vacuole containing lytic enzymes.

Biochemical studies have shown that there is good correlation between lysosomal enzyme release and the progress of bone resorption.[3,4] Those hormones which stimulate bone resorption also appear to increase the synthesis and secretion of various lysosomal enzymes. The osteoclast lysosomes and their lytic enzymes participate in a continuous process of exocytosis and endocytosis at the ruffled border. Enzymes are released into the extracellular bone matrix (exocytosis) and the solubilised matrix components are taken up into the cell within vacuoles termed phagosomes (endocytosis) for further intracellular digestion. The introduction of endocytosed materials to the lysosomal digestive enzymes occurs by the fusion of the phagosomes and lysosomes.[5]

Other enzymes involved are metalloproteinases, such as collagenase and stromelysins, which are dependent on the presence of the metal ion zinc at the active site, and serine proteinases such as elastase. All these proteinases would not retain much activity in the low pH of the ruffle border. The metalloproteinases are secreted into tissues predominantly in the inactive pro-collagenase form. It is also known that osteoblasts release collagenase, especially in response to resorbing agents such as parathyroid hormone.[6,7] This synthesis of collagenase by osteoblasts suggests that they also contribute to matrix degradation, by digesting the non-mineralised layer of osteoid on the bone surface to facilitate osteoclast activity and also by degrading the demineralised matrix when exposed by osteoclastic activity.

The mediators of bone remodelling

Parathyroid hormone

Parathyroid hormone is an 84 amino acid single chain polypeptide secreted by the four parathyroid glands. It has a direct effect on bone turnover, increasing rates of both new bone formation and bone resorption. Physiological concentrations appear to promote bone formation and increase the distal tubular reabsorption of calcium by the kidney, but decrease reabsorption of phosphate. Increased concentrations promote osteoclastic bone resorption. It stimulates bone resorption both *in vivo* and *in vitro*.[8,9] PTH can also indirectly increase the absorption of calcium by the gut, through the stimulation of 1,25-dihydroxyvitamin D_3 synthesis in the kidneys.

In high concentrations, PTH inhibits osteoblastic collagen synthesis.[10]

Parathyroid hormone related peptide (PTHrP)

A number of tumours associated with hypercalcaemia have been found to produce factors that are clearly not PTH, but appear to bind to PTH receptors and so mimic some of the hormone's actions. This factor has now been purified, and the responsible gene has been cloned.[11-14] In each case, there is considerable homology between the PTHrP and PTH within the N-terminal region (8 of the first 13 residues), while the remaining sequence differs substantially. An N-terminal fragment of PTHrP, (PTHrP-1-34), NH_2, has recently been synthesised and its biological properties evaluated.[15,16] When administered *in vivo* this peptide produces hypercalcaemia, reduces serum phosphate, elevates circulating levels of 1,25-dihydroxyvitamin D_3 and increases the excretion of cAMP and phosphate. Similarly, Sato *et al.*[17] showed that PTHrP stimulates bone resorption *in vitro* and increases the serum calcium levels in mice. The biological profile displayed by this peptide suggests that PTH antagonists could inhibit all or most of its actions. In this way the hypercalcaemia produced by malignant tumours, with its debilitation, anorexia, nausea, diarrhoea, polyuria, polydipsia and depression, can be controlled by corticosteroids and bisphosphonates.

Vitamin D metabolites

The vitamin D metabolites are steroid-like compounds which are derived from the plant ergosterol, fish oils and ingested in the diet, or synthesised in the skin by exposure of epidermal cells to ultraviolet light. The cholecalciferol is hydroxylated in the kidney and then further hydroxylated in the liver to produce the active hormone.

Thus, the major active metabolite of vitamin D is 1,25-dihydroxycholecalciferol. This has been shown to affect bone formation and also to cause bone resorption.[18] It's effect on bone resorption appears to be by the differentiation of committed progenitor cells into mature cells.[19]

The cytokines

Cytokines are defined as short range soluble mediators, released from cells which modulate the activity of other cells. The first ones identified were non-antibody mediators of cellular immunity produced by lymphocytes and called lymphokines. It was thought that lymphocytes were the only cells that could produce such factors, hence the term lymphokines. It is now known that many different cell types can produce these molecules, and the term cytokine is more appropriate.

Several of these cytokines are potent mediators of bone metabolism (Table I). Bone resorbing activity in the supernatants of mononuclear cell cultures was originally described by Horton *et al.*,[20] and this activity was described as 'osteoclast activating factor' (OAF). Much effort was subsequently given to characterising OAF and its mode of action. It has gradually become apparent that OAF activity is due to several mediators produced by activated mononuclear cells, notably interleukin-1 (IL-1) and tumour necrosis factor (TNF). Indeed, a purified preparation of 'OAF' from peripheral blood lymphocytes has recently been shown to be identical to IL-1β.[21] Therefore, the name OAF is best used as a collective term for cytokines which can mediate bone resorption. An example of this would be in myeloma, where OAF has been identified as TNF-beta (lymphotoxin).[22]

IL-1 does not have a direct action on the osteoclast, but like PTH acts via the osteoblast.[23] It has been shown to cause hypercalcaemia when infused or injected into normal, intact mice. This experimental hypercalcaemia can be inhibited by indomethacin, which suggests that it is mediated by prostaglandin synthesis.[24,25] Furthermore, it has a direct promotional effect on osteoclast formation.[26] Interleukin-1 has been associated with the bone destruction by pathological lesions such as odontogenic cysts,[27] and solid tumours such as oral squamous cell carcinoma,[28] and an ameloblastoma associated with hypercalcaemia.[29]

TNF alpha and TNF beta in purified forms can also stimulate bone resorption *in vitro*. The bone resorbing effect is mediated through osteoblasts, is of similar potency to IL-1 and can be inhibited by interferon-gamma (IFN-gamma).[30-32] Tashjian *et al.*[33] have reported that the bone resorbing effect is mediated via prostaglandin production. TNF also inhibits bone collagen and non-collagenous protein synthesis, an effect which can be inhibited by indomethacin and enhanced by INF-gamma.

Another cytokine involved in cytokine-mediated osteoclastic bone resorption is interleukin-6 (IL-6). This is a factor produced by a variety of cell types, including osteoblasts.

Table I Bone resorbing factors

Systemic factors	Parathyroid hormone
	Parathyroid related peptide[a]
	Vitamin D_3 (1,25 dihydroxycholecalciferol)
	Thyroid hormone
Local factors[a]	Prostanoids
	Lipoxygenase metabolites
	Cytokines: Interleukin-1 alpha
	Interleukin-1 beta
	Tumour necrosis factor alpha (cachectin)
	Tumour necrosis factor beta (lymphotoxin)
	Interleukin 6
Growth factors	Epidermal growth factor
	Transforming growth factor alpha*
	Transforming growth factor beta
	Platelet-derived growth factor
Bacterial factors	Lipopolysaccharide
	Muramyl dipeptides
	Capsular material
	Peptidoglycans
	Lipoteichoic acids

[a]Released by inflammation and neoplasms such as carcinomas, ameloblastomas and cysts.

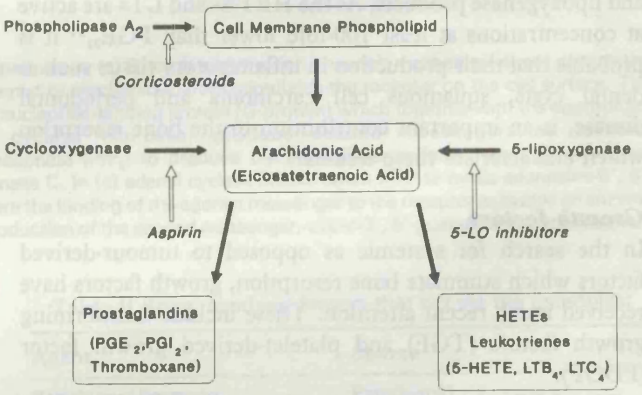

Fig. 4 Arachidonic acid metabolism. Arachidonic acid (eicosatetraenoic acid) is synthesised from cell membrane phospholipids by the action of phospholipase A. It can be metabolised by two major enzymes, cyclooxygenase to give rise to the prostanoids, and by the enzyme, lipoxygenase to give rise to leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs). This cascade can be inhibited in three main places, (i) by inhibiting the action of phospholipase A2 (corticosteroids), (ii) by inhibiting the enzyme cyclooxygenase (aspirin) and (iii) by blocking the enzyme 5-lipoxygenase (5-LO inhibitors).

Eicosanoids

The products of the lipoxygenase and cyclooxygenase pathways are collectively referred to as eicosanoids, since they arise from eicosatetraenoic acid (arachidonic acid) (see fig. 4).

The prostanoids which are the prostaglandins (PGE_2, PGI_2, etc) and their related substances, the thromboxanes, are synthesised from membrane phospholipids by the action of phospholipase A2 to produce arachidonic acid. This is then metabolised by the cyclooxygenase pathway to give the prostanoids or by the lipoxygenase pathway to give leukotrienes.

Prostanoids (PGs)

The first demonstration that PGs could modify bone metabolism was by Klein and Raisz,[34] who showed that PGE_2 stimulated bone resorption *in vitro* in foetal rat calvaria.

The PGs are local pathological mediators of bone destruction, in particular where there is inflammation. High concentrations of PGs have been demonstrated in dental cysts,[35] inflamed gingival tissues,[36] synovial fluids from rheumatoid joints,[37] and breast carcinoma.[38]

Lipoxygenase metabolites

Although the involvement of PGs in bone physiology has been studied for almost 20 years, the other major pathway of arachidonate metabolism, the lipoxygenase pathway, has received very little attention. The first evidence that leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs) stimulate bone resorption was recently shown by Meghji *et al.*,[39] demonstrating a potency *in vitro* several orders of magnitude greater than PGs. The possibility of lipoxygenase metabolite involvement in localised bone destruction is strengthened by reports of their synthesis in inflamed and malignant tissues. Indeed, Sidhagen *et al.*[40] found that lipoxygenase metabolism of ^{14}C-arachidonate in explanted inflamed gingival tissue exceeded cyclooxygenase activity by approximately ten-fold. Similarly, Matejka *et al.*[41] found hydroxy-fatty acids (predominantly 5-HETE) to be a major product of odontogenic cysts. Porteder *et al.*[42] showed that samples from human oral squamous cell carcinoma synthesised approximately equal amounts of cyclooxygenase and lipoxygenase products. As the HETEs and LTs are active at concentrations at least 100-fold lower than PGE_2,[39] it is probable that their production in inflammatory tissue such as dental cysts, squamous cell carcinoma and periodontal disease, is an important contribution to the bone resorption which characterises these lesions.

Growth factors

In the search for systemic as opposed to tumour-derived factors which stimulate bone resorption, growth factors have received much recent attention. These include transforming growth factors (TGF) and platelet-derived growth factor (PDGF).

The TGFs are a family of polypeptides with biological properties similar to those of epidermal growth factors (EGF). Two major classes of TGF have been identified, TGF alpha and TGF beta.

The TGFs have been shown to be potent bone resorbing factors.[43,44] This bone resorbing activity of TGF alpha, TGF beta, EGF and PDGF has also been reported to be mediated entirely by prostaglandins.[43,45,46] TGF alpha is produced by many tumours,[47] especially solid tumours associated with hypercalcaemia (squamous cell carcinoma of lung, oral cavity, kidney and breast). Some tumours also produce TGF alpha and PTH-rp simultaneously.

Bacterial products

Bacterial products such as lipopolysaccharide,[48] capsular material,[49] lipoteichoic acids,[50] muramyl dipeptide[51] and peptidoglycans[52] have been shown to stimulate bone resorption *in vitro*. The implication of bone resorption induced by bacterial products is particularly important for the pathology of periodontal diseases. These bacterial products may also act as foreign antigens and induce monocyte macrophages and then bone cells to produce prostaglandins and cytokines such as IL-1, leading to bone resorption.

Mechanical stress

The relationship between mechanical forces and bone remodelling has been known for a century.[53] This is particularly apparent in cancellous bone, where immobilisation increases bone resorption and decreases formation.[54,55] This shows that mechanical stress is necessary for the normal coupling of bone remodelling.

Mechanical bone remodelling is utilised by orthodontists, where forces exerted on tooth roots enable teeth to move through alveolar bone. Such remodelling involves the activation of bone cells and the simultaneous breakdown and reformation of the bone matrix. Unlike hormones which act as agonists to a specific receptor, there is no agonist–receptor binding when the lining periosteal fibroblasts, osteoblasts on the matrix surface and osteocytes detect mechanical forces.[56] One of the major unanswered questions is how these bone cells respond. The cells may communicate with each other across gap junctions[57] by the production of appropriate factors when their relationship is disturbed. However, each cell is also attached to its surrounding matrix via specialised membrane receptors for extracellular matrix proteins called intergrins.[58] In this way, transmembrane receptors provide a direct link between extracellular matrix deformation and cell function.[59]

The intracellular response

The transduction of mechanical stress into a biochemical signal can occur via stretch sensitive ion channels which have been described in the osteoblast cell membrane[60] (fig. 5a).

The other accepted model for transmembrane stimulation of the osteoblast involves the agonist hormone (or mechanical stimulus) activating a specific receptor on the cell surface.[61] This in turn activates intracellular guanine nucleotide-binding protein (G-protein) which together with the appropriate enzymes (phosphoinositidase C or adenylate cyclase) produces intracellular second messengers (fig. 5b and c). The G protein–receptor link is a key amplification step in intracellular signalling. The second messengers for phosphoinositidase C are diacylglycerol (DAG) and inositol trisphosphate (IP_3). In the case of adenylate cyclase the second messenger is cyclic adenosine-3′, 5′-monophosphate (cAMP) (fig. 5c). The end result of second messenger activity is a physiological event. For example, bone resorption by PTH is mediated by cAMP. Sandy *et al.*[62] have also shown an elevation of inositol trisphosphates (IP_3) by the short-term mechanical stimulation of osteoblasts *in vitro* and that prolonged stress released bone resorbing factors.[63]

Finally, another model for transmembrane signalling involves receptors with their own built-in enzyme activity. Here the binding of the messenger on the ectoplasmic domain of the receptor activates an enzyme on the cytoplasmic domain of the receptor polypeptide (fig. 5d). This results in the production of the second messenger, cyclic-3′, 5′-guanosine monophosphate (cGMP).

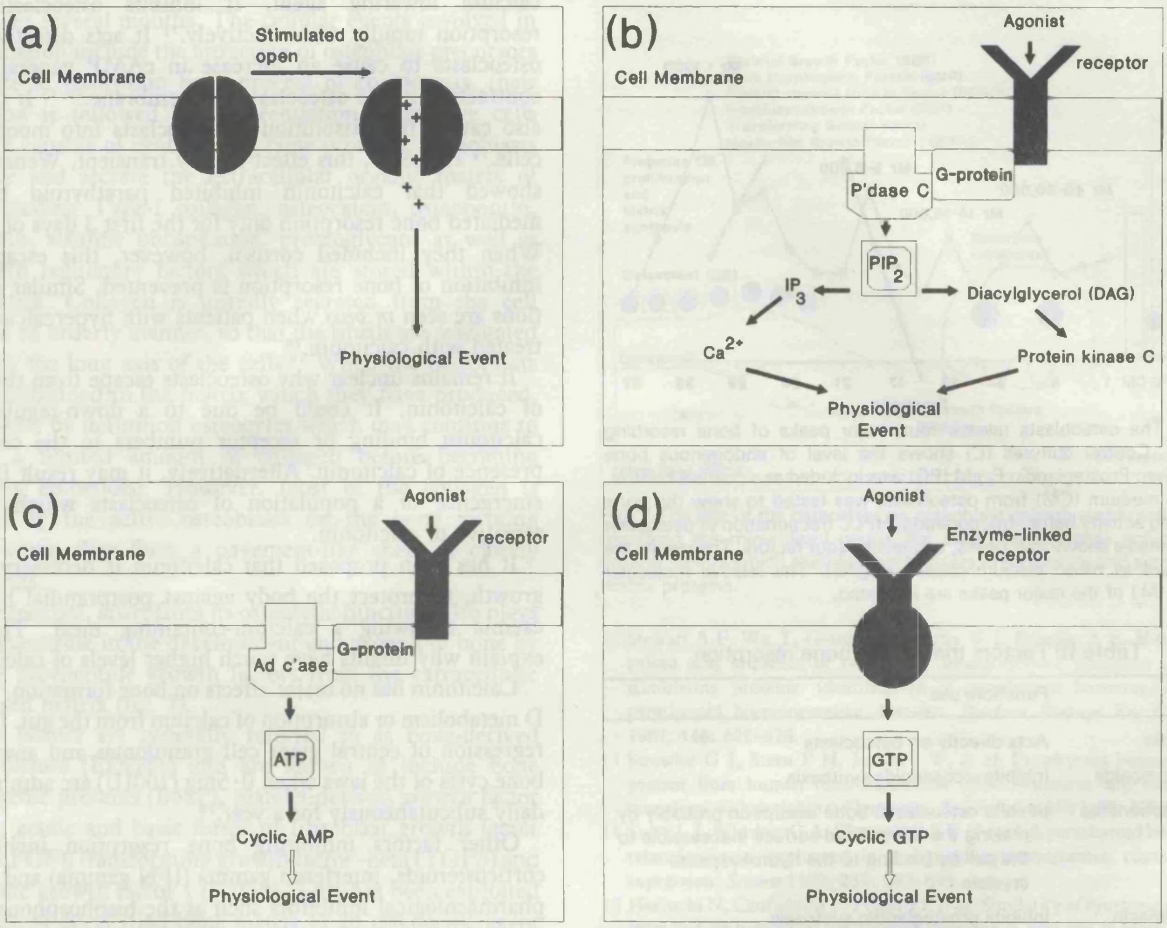

Fig. 5 This schematic diagram shows the major transmembrane signalling pathways. (a) Stretch sensitive ion channel: mechanical stress causes the opening of the ion channel. (b) and (c) Second messenger systems: an agonist or mechanical stress stimulates the receptor on the cell surface. The hormone-receptor complex or the mechanical receptor activates a guanine nucleotide-binding protein (G-protein) which together with the appropriate enzymes — phosphoinositidase C (P'dase C) or adenylate cyclase (Ad'case) produce second messengers as follows; In (b) phosphoinositidase C breaks down phosphatidylinositol 4,5,-bisphosphate (PIP_2) to produce the intracellular second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG). DAG activates the protein kinase C. In (c) adenyl cyclase breaks down ATP to cyclic adenosine-3', 5'-monophosphate (cyclic AMP). (d) Receptor with built-in enzyme activity. Here the binding of the agonist messenger to the receptor activates an enzyme in the cytoplasmic domain of the receptor polypeptide. This results in the production of the second messenger, cyclic-3', 5'-guanosine monophosphate (cGMP).

The osteoblast-osteoclast relationship (Table II)

Although the osteoclast is the principal bone resorbing cell, it is the osteoblast that contains the receptors for the major bone resorbing agents such as parathyroid hormone (PTH), the eicosanoids (PGE_2, PGI_2, LTB4, etc), 1,25-dihydroxy vitamin D_3 and cytokines such as interleukin 1 (IL-1) and tumour necrosis factor (TNF). The osteoblasts, having recognised the resorptive signal, somehow transmit it to the osteoclast. Rodan and Martin[64] hypothesised that bone resorbing agents, such as PTH, may induce a change of shape in the osteoblast which would facilitate the access of osteoclasts to the bone surface. Thus, at low concentrations parathyroid hormone promotes bone formation, but at high concentrations will facilitate bone destruction.

An additional theory has been put forward, that in response to PTH, osteoblasts secrete a short-range soluble activator for osteoclasts.[65] As yet these osteoblast–osteoclast factors have not been characterised and reports have suggested substances with diverse molecular weights are involved. McSheehy and Chambers [66,67] showed that the osteoclast activating factor produced by osteoblasts had a relative molecular weight (M_r) of 500–1000 daltons and was inhibited by lipoxygenase inhibitors, implying that the factor is a lipoxygenase metabolite of arachidonic acid (fig. 4). This is possible since lipoxygenase metabolites of arachidonic acid also stimulate bone resorption *in vitro*.[39] However, Perry *et al.*[68] reported that the osteoclast-activating factor is bigger than 25 000 daltons. Meghji *et al.*[69] confirmed that the activity is heterogeneous, and not a single factor and that osteoblasts spontaneously produce four major peaks of bone resorbing activity when fractionated by gel permeation on high performace liquid chromatography (HPLC). The first peak at an M_r of 40–50 000, the second at an M_r of 14–16 000, the

Table II Bone resorbing factors that act via the osteoblast

Agent	Source
Parathyroid hormone	Parathyroid
Vitamin D_3	Liver via kidney from skin
Interleukin-1	Monocytes (major source) Many other cell types including keratinocytes, fibroblasts and tumour cells
Tumour necrosis factor	Monocytes

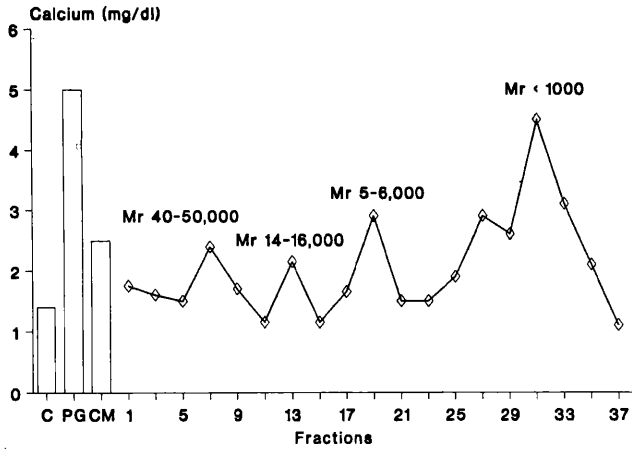

Fig. 6 The osteoblasts release four major peaks of bone resorbing activity. Control cultures (C) shows the level of endogenous bone resorption. Prostaglandin E_2 μM (PG) was included as a positive control. Culture medium (CM) from osteoblasts was tested to show the bone resorbing activity before fractionation. HPLC fractionation of osteoblast culture media shows four peaks, suggesting four factors. The results are expressed as mean calcium release (mg/dl). The relative molecular weight (M_r) of the major peaks are indicated.

Table III Factors that inhibit bone resorption

Agent	Function/use
Calcitonin	Acts directly on osteoclasts
Glucocorticoids	Inhibits eicosanoids synthesis
Bisphosphonates	Inhibits osteoclastic bone resorption probably by making the mineralised surface inaccessible to the cell by binding to the hydroxyapatite crystals
Indomethacin and aspirin	Inhibits prostaglandin synthesis
Interferon-gamma	Inhibits both proliferation and differentiation of osteoclast progenitors
TGF-beta	Inhibits osteoclast formation and differentiation
Interleukin-1 receptor Antagonist (IL-1ra)	Binds to IL-1 receptors. Effective against TNF as well

third peak at an M_r of 5–6000 and the fourth peak below 1000 (fig. 6). These peaks of activity were also seen when PTH and crude IL-1 were used to stimulate osteoblasts, with increased activity at a molecular weight of less than 1000 daltons. Furthermore, increased bone resorbing activity was shown in the lipid extracts of the osteoblast culture media to which PTH and IL-1 had been added. This indicates that the osteoblasts respond to bone resorbing agents by synthesising a low molecular weight, lipid soluble factor(s).

Osteoblasts therefore appear to play a 'pivotal role' in the regulation of bone resorption.

Inhibitors of bone resorption (Table III)

These include calcitonin, cortisol, interferon gamma (IFN-gamma), bisphosphonates, plicamysin and actinomycin D.

Calcitonin (CT)

Calcitonin is synthesised by C cells in the thyroid gland; it has a molecular weight of 3500 daltons and is composed of 32 amino acids.

The precise biological role of calcitonin remains unclear. It was first recognised in 1962 by Copp *et al.*[70] as a serum calcium lowering agent. It inhibits osteoclastic bone resorption rapidly and effectively.[71] It acts directly on the osteoclasts to cause an increase in cAMP generation and contraction of the osteoclast cell membrane.[72,73] It probably also causes the dissolution of osteoclasts into mononuclear cells.[74] However, this effect is only transient. Wener *et al.*[75] showed that calcitonin inhibited parathyroid hormone mediated bone resorption only for the first 3 days of culture. When they included cortisol, however, this escape from inhibition of bone resorption is prevented. Similar observations are seen *in vivo* when patients with hypercalcaemia are treated with calcitonin.[76]

It remains unclear why osteoclasts escape from the effects of calcitonin. It could be due to a down-regulation of calcitonin binding or receptor numbers in the continued presence of calcitonin. Alternatively, it may result from the emergence of a population of osteoclasts which are not sensitive to calcitonin.

It has been proposed that calcitonin is necessary during growth, to protect the body against postprandial hypercalcaemia following a calcium-containing meal. This may explain why infants have much higher levels of calcitonin.[77]

Calcitonin has no major effects on bone formation, vitamin D metabolism or absorption of calcium from the gut. It causes regression of central giant cell granulomas and aneurysmal bone cysts of the jaws when 0·5mg (100IU) are administered daily subcutaneously for a year.[78]

Other factors inhibiting bone resorption include the corticosteroids, interferon gamma (IFN gamma) and various pharmacological inhibitors such as the bisphosphonates and cytotoxic drugs such as plicamycin and actinomycin D. The bisphosphonates and calcitonin are used to retard bone turnover in Paget's disease. The bisphosphonate, sodium etidronate is invaluable in preventing reankylosis of the temporomandibular joint following surgery for ankylosis. IFN gamma has attracted a significant research effort. It is a multifunctional cytokine which is produced by immune cells and inhibits both proliferation and differentiation of osteoclast progenitors. Gowen *et al.*[32] demonstrated *in vitro* that IFN gamma specifically inhibits bone resorption induced by TNF and IL-1. Another cytokine which inhibits osteoclastic bone resorption is IL-1 receptor antagonist.[79] Interleukin-1 receptor antagonist is a cytokine produced by monocytes and monocytoid cell lines. It is a very effective inhibitor of osteoclastic bone resorption stimulated by IL-1 and TNF.

Bone formation

The formation of bone by mesenchymal cells can occur by one of two routes: (1) a direct development of bone from mesenchymal cells, as in the calvarium, called intramembranous ossification, and (2) an intervening cartilage model precedes the formation, ie the proliferation of mesenchymal cells is followed by their differentiation into chondrocytes which become hypertrophied and calcified. The calcified cartilage is replaced by bone which is then remodelled. Formation of bone by this route occurs in long bones and is called endochondral ossification. However, these two modes of ossification do not produce significantly different kinds of bone.[80]

Whatever the means, osteogenesis is a prolonged process

which takes several months. The cellular events involved in bone formation include the attraction of osteoblast precursors to the site of resorption by a process of chemotaxis, their stimulation is followed by differentiation to mature cells which are capable of synthesising bone proteins. Osteoblasts synthesise and secrete the extracellular organic matrix of bone, including Type-I collagen, osteocalcin, osteopontin, osteonectin, alkaline phosphatase, proteoglycans as well as the growth regulatory factors which are stored within the bone matrix. Collagen is initially secreted from the cell surface in an orderly manner, so that the fibrils are orientated parallel to the long axis of the cells.[81] When the osteoblasts become embedded in the matrix which they have produced, they become by definition osteocytes which may continue to produce a limited amount of collagen before becoming completely embedded. However, most of the collagen is produced by the active osteoblasts on the bone forming surface where they form a pavement-like sheet of closely packed cells.

The osteoclast, apart from its osteolytic function, also plays an important role in the development and growth of bone by releasing polypeptide growth factors from the extracellular mineralised matrix (fig. 7).

These factors are generally referred to as bone-derived growth factors (BDGFs) and are known to include bone morphogenic proteins (BMP), platelet-derived growth factor (PDGF), acidic and basic forms of fibroblast growth factor (aFGF, bFGF), transforming growth factor -beta (TGFβ) and insulin-like growth factor 1 (IGF-1). This has been exploited by the use of decalcified bone matrix as an osteogenic agent in clinical practice.[82]

In summary, despite its apparently solid and inert form, bone is probably the most dynamic and complex of body tissues. We are only just beginning to discover the many factors which influence the interactions of its formation and destruction.

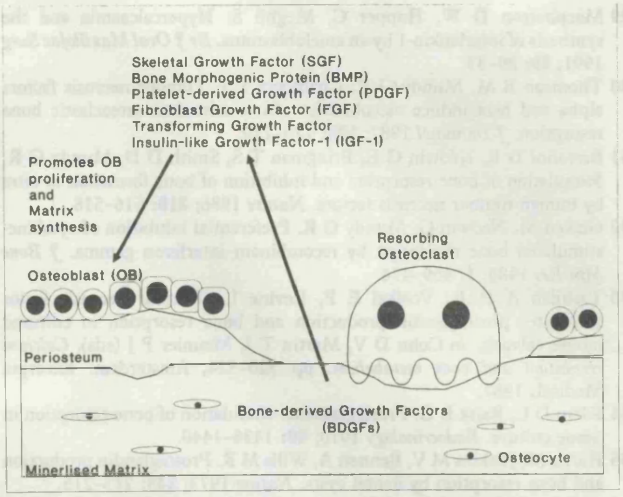

Fig. 7 The role of the osteoclast on osteoblast growth and function: on resorbing the mineralised matrix the osteoclast releases polypeptide growth factors which stimulate osteoblasts to proliferate and synthesise matrix proteins.

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