ROLE OF THE SURFACE ASSOCIATED MATERIAL OF *EIKENELLA CORRODENS* IN BONE RESORPTION ASSOCIATED WITH PERIODONTAL DISEASE

A research thesis submitted in fulfilment
of the requirements for the
Degree of Master of Science in Dentistry

by

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This research thesis is dedicated to my parents,
Behroze & Minocher Irani,
for their unfailing love and support.

SIGNED STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that, to the best of the candidate's knowledge and belief, the thesis contains no material previously published or written by any other person, except where due reference is made in the text of the thesis. The author consents to the thesis being made available for photocopying and loan if applicable, if accepted for the award of the degree.

Dilshad M. Irani
29th May, 1998.

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CHAPTER 1 SUMMARY

Inflammatory periodontal diseases are one of the most prevalent diseases of the skeleton and a major cause of tooth loss. Gram-negative bacteria are implicated as causative agents of the gingival inflammation and connective tissue and bone destruction which characterise the pathology of chronic inflammatory periodontal diseases. A number of bacterial species are frequently isolated from periodontal lesions in chronic adult and localised juvenile periodontitis. The most common are *Actinobacillus actinomycetem-comitans, Porphyromonas gingivalis, Eikenella corrodens, Prevoltella intermedia* and *Camphulobacter rectus.* The causes of the alveolar (jaw) bone loss which is a feature of this disease are poorly understood. Since bacteria are rarely found within the diseased periodontal tissues, it is believed that bacterial products enter the periodontium from subgingival bacterial plaque accumulations and stimulate the bone resorption observed clinically.

A number of recent studies have investigated the biological actions of surface components of periodontopathic bacteria. These surface components consist of loosely adherent surface associated material (SAM) such as capsule, pili and fibrils. The abilities of SAMs from several periodontopathic bacteria to induce bone resorption in a murine calvarium model have been compared. The results indicated that SAM from *A. actinomycetemcomitans* and *E. corrodens* were extremely potent stimulators of bone loss in the murine calvarium model, being active at concentrations as low as 0.001 μg/ml. In comparison, SAMs from the other bacteria were approximately 100 times less active.

One of the major drawbacks of using the murine calvarium model of bone resorption is that, being an organ culture system, it does not allow analysis of the cellular events causing bone resorption. Recent *in vitro* cell culture

systems have been developed that permit the study of the molecular and cellular events of bone remodelling. These models allow us to study the mechanisms by which the SAMs of periodontopathogenic bacteria cause bone loss.

This project was designed to test the following **hypothesis:**

> *That bacterial products such as SAM are released from the subgingival plaque and activate macrophages and stromal osteoblastic cells in the gingiva and periodontium to synthesise and release pro-inflammatory cytokines, some of which may be responsible for the pathologic bone resorption seen in periodontal disease. Alternatively, bacterial products like SAM could directly stimulate formation of osteoclasts (OCs) from precursor cells and/or activate mature OCs to further stimulate bone resorption.*

The findings gained from testing this hypothesis using *in vitro* models of osteoclastogenesis should provide insight into the mechanisms of bone loss associated with periodontal disease.

Accordingly, the specific **aims** of this project were:

Aim 1: To determine whether *Eikenella corrodens* SAM (EC SAM) stimulates human monocytes to release cytokines that induce osteoclastogenesis.

Pro-inflammatory cytokines are major pathological mediators of inflammatory diseases. It has now become apparent that they also have a significant role in regulating bone remodelling. These cytokines can act systemically but it is generally believed that they act locally to regulate bone metabolism and have crucial roles in both normal and pathologic bone cell function. A variety of

cytokines, originally identified by their ability to regulate immune and haematopoietic cells, are known to be produced normally, and in response to, specific stimuli in or near bone.

Recent studies have shown that products of some periodontopathogenic bacteria induce release of the pro-inflammatory cytokines interleukin-1β (IL-1β), tumour necrosis factor α (TNFα), and IL-6. Our results confirm this with SAM from *E. corrodens* and *P. gingivalis* stimulating macrophages to release these cytokines.

Aim 2: To establish if there are any direct effects of EC SAM on the activation of mature OCs or their formation from precursors.

OCs arise from the cells of haematopoietic origin. Studies in which OCs are generated *in vitro* show that cells of the monocyte/macrophage lineage have the potential to become OCs under appropriate conditions. Large numbers of TRAP positive OCs were isolated from the marrow of long bones of neonate mice. These cells were capable of forming resorption pits on mineralised whale dentine slices. *E. corrodens* SAM (EC SAM) was added at various concentrations to these cultures and a positive direct effect was found on the development of OCs.

The increased numbers of OCs stimulated by periodontopathogenic bacterial SAMs could account for the extensive loss of bone observed in periodontal disease and in organ culture systems.

Aim 3: To determine whether EC SAM induces stromal osteoblastic cells to release osteoclastogenic cytokines.

Osteoclastogenesis requires the regulated combination of signals from stromal or osteoblastic cells, extracellular matrix and locally produced

humoral factors. Cytokines such as IL-1, IL-6, and soluble IL-6 receptor (sIL-6R), TNFα and colony stimulating factors (M-CSF, GM-CSF and G-CSF) are known to enhance OC development. These cytokines may be released by stromal or osteoblastic cells and are likely to be stimulated by SAM from periodontopathogenic bacteria. Being a potential cellular source for the release of cytokines, EC SAM was added at various concentrations to osteoblastic cells and the release of IL-6 was measured. Although a positive response was observed, levels of IL-6 released by activated macrophages was much higher.

It was **concluded** that:

- The SAMs of *E. corrodens* and *P. gingivalis* have the capacity to stimulate peripheral blood mononuclear cells to produce osteolytic cytokines. EC SAM was the more potent of the two at stimulating the release of IL-1β, TNFα, IL-6 & GM-CSF.
- The fact that the lipopolysaccharide (LPS) of *E. corrodens* produced minimal stimulation compared to that of EC SAM may mean that some constituent other than LPS is responsible for the observed biological activity of EC SAM.
- Heat and trypsinisation of SAM also indicated that the active cytokine-stimulating components in the SAM were largely proteinaceous in nature.
- Even at low concentrations of 0.005 μg/ml, EC SAM stimulated cytokine release. This may mean that low numbers of this organism present in subgingival plaque could be capable of inducing a pathological response in the periodontium.
- Although results of the preliminary mouse coculture studies were somewhat inconclusive, they indicated that the SAMs of both *E. corrodens* & *P. gingivalis* may directly stimulate OC formation and possibly function.

- Whereas both EC SAM and *P. gingivalis* SAM (PG SAM) stimulated IL-6 release from OB-like cells, monocytes could be stimulated to release most of the osteolytic cytokines.
- Specific periodontopathic bacteria may induce different cytokine responses, and have different effects on the destruction of bone as seen in periodontitis. EC SAM would appear to be a more potent stimulator of macrophages, while PG SAM may be a more potent stimulator of OB mediated bone resorption.
- In the future, bacterial cytokine-inducing components such as EC and PG SAM could be targets for the prevention and treatment of periodontal diseases. If one could determine which were the key components, then the possibility of immunization to prevent the induction of the periodontal diseases takes a step nearer reality.

CHAPTER 2 INTRODUCTION & AIMS

The periodontium is a complex, highly specialized, shock-absorbing and pressure-sensing system consisting of four interrelated tissues supporting the teeth: cementum, periodontal membrane, alveolar bone, and gingiva (Figure 1).

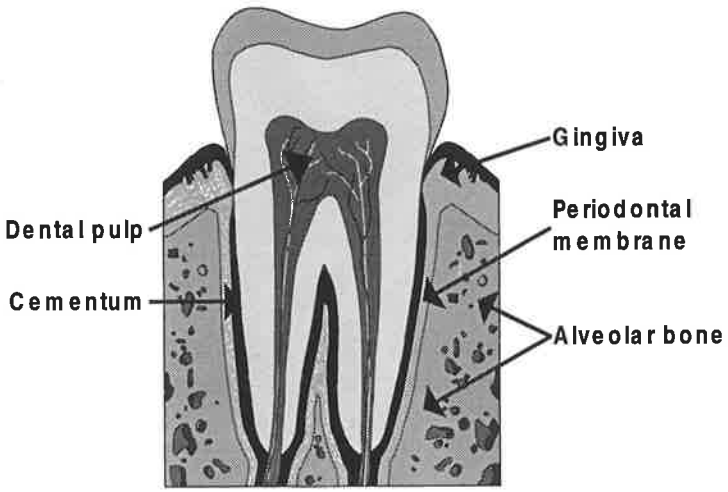

Figure 1: Schematic longitudinal section of a healthy tooth and periodontium.

2.1 PERIODONTAL DISEASE

According to Ismail & Lewis (1993), periodontal diseases are the most prevalent chronic diseases, accounting for over 95% of cases in children, adolescents, adults and elderly people. Gingivitis, inflammation of the gingival tissue surrounding a tooth, is often a precursor to periodontitis, inflammation of the periodontium. Once gingivitis has progressed to involve the periodontal ligament and the bone, the lesion is defined by Priestland (1995) as periodontitis. The pathognomonic sign of periodontitis is loss of alveolar bone and the subsequent formation around the tooth of a pocket, which varies in

depth depending on host responses and the severity and duration of the inflammation. The pocket acts as a reservoir favouring the growth of anaerobic bacteria (Ismail & Lewis 1993). Conversely, Johnson *et al.* (1992) believe that gingivitis does not necessarily progress to periodontitis. Many individual subjects can be shown to have long standing gingivitis without any progression to involve pocketing or loss of attachment of bone. Furthermore, national trends in the United States over the past decade or so indicate a reduction in the prevalence of gingivitis accompanied by, if anything, a slight increase in the prevalence of destructive periodontitis (Douglass *et al.* 1983, Johnson *et al.* 1992).

Adult onset periodontitis is one of the forms of periodontal disease which occurs with increasing prevalence from about thirty five years of age. In this condition, the distribution of periodontal destruction in an individual largely follows the distribution of the retained bacterial plaque and is not associated with immuno-deficiency (Priestland 1995). Hence, this most common form of periodontitis results from a polymicrobial infection characterized by continuous and slowly progressing loss of alveolar bone. Although chronic adult periodontitis is generalized, some tooth sites experience more advanced bone loss than others (Ranney 1991; Ismail & Lewis 1993). The gingivae exhibit varying degrees of inflammation, with recession in some areas and fibrotic regions elsewhere. Exacerbations occur at rather lengthy intervals, with the disease leading to tooth loss only in later life, if at all (Rees & Midda 1991).

2.1.1 Histopathogenesis of periodontal disease

According to Rees & Midda (1991), it is now widely accepted that, in most individuals, plaque accumulation leads to gingivitis within a few weeks (Loe *et al.* 1965), but this gingivitis does not inevitably progress to periodontitis. Indeed, why a small proportion of sites with gingivitis progress to periodontitis

is still being researched. Page (1986) divided the development of gingivitis and its progression to periodontitis into four stages:

The initial lesion. This is seen within four days of the beginning of plaque accumulation and is characterized by the presence of a classic acute inflammatory response. There is increased production of gingival fluid and enhanced migration of neutrophils from the tiny blood vessels of the subgingival plexus into the sulcus, in response to plaque. The connective tissue matrix surrounding the blood vessels becomes altered, probably by collagenases and other enzymes released by the neutrophils.

The early lesion. This develops from the initial lesion at about 1 week after plaque accumulation. The acute inflammatory response still persists as is evidenced by the presence of neutrophils, especially in the junctional epithelium. The early lesion is characterized by the presence of lymphocytes, which account for about 75% of the inflammatory cells. Collagen loss in the affected area is about 60-70%.

The established lesion. This develops within a few weeks. It is characterized by a predominance of plasma cells and B-lymphocytes, probably in conjunction with the creation of a small gingival pocket lined with epithelium, but without significant bone loss. Many neutrophils are present in the junctional and pocket epithelium, with macrophages present in the pocket wall.

The advanced lesion. Periodontitis may take years to develop, if at all. This lesion is characterized by the formation of a periodontal pocket, destruction of alveolar bone, soft tissue damage and a predominantly plasma cell infiltrate.

According to Rees & Midda (1991), the advanced lesion marks the progression of gingivitis into periodontitis. These authors also mention that

this is a fairly arbitrary division and there is no clear division between these last two stages. They propose that the actual moment of conversion of gingivitis to periodontitis occurs when the cells of the junctional epithelium leave the amelocemental junction and migrate onto the root surface.

During the initial and early stages of gingivitis, the classic features of acute inflammation predominate, whereas the latter stages are characterized by chronic inflammation with superimposed acute episodes, so that acute and chronic inflammatory features co-exist in the early, established and advanced lesions (Rees & Midda 1991). Furthermore, there appear to be a multitude of cellular and immunological events occurring as a result of the presence of plaque in the gingival sulcus. These interactions are summarized in Figure 2.

The height of alveolar bone is normally maintained by an equilibrium, regulated by local and systemic influences, between bone formation and bone destruction. In periodontal disease the equilibrium is altered so that resorption exceeds formation. There are many factors that are directly or indirectly responsible for this altered equilibrium. This bone loss, which is an indicator of attachment loss, has been estimated to be in the region of 0.1mm per year (Loe *et al.* 1986, Rees & Midda 1991). This equates to 1mm loss in 10 years or about 5mm in a lifetime. Clinically, this means that patients can retain a natural dentition for life and this is what appears to be happening for the vast majority of the population.

The other 15% of the susceptible population, for one reason or another, are in negative balance and their rate of bone loss is well in excess of 0.1mm per year. However, this is an average figure and these patients may loose 1-2 mm of bone in a burst of disease activity, which lasts for a few weeks. The site may remain quiescent for the following five years, only to be followed by another burst of disease activity (Rees & Midda 1991).

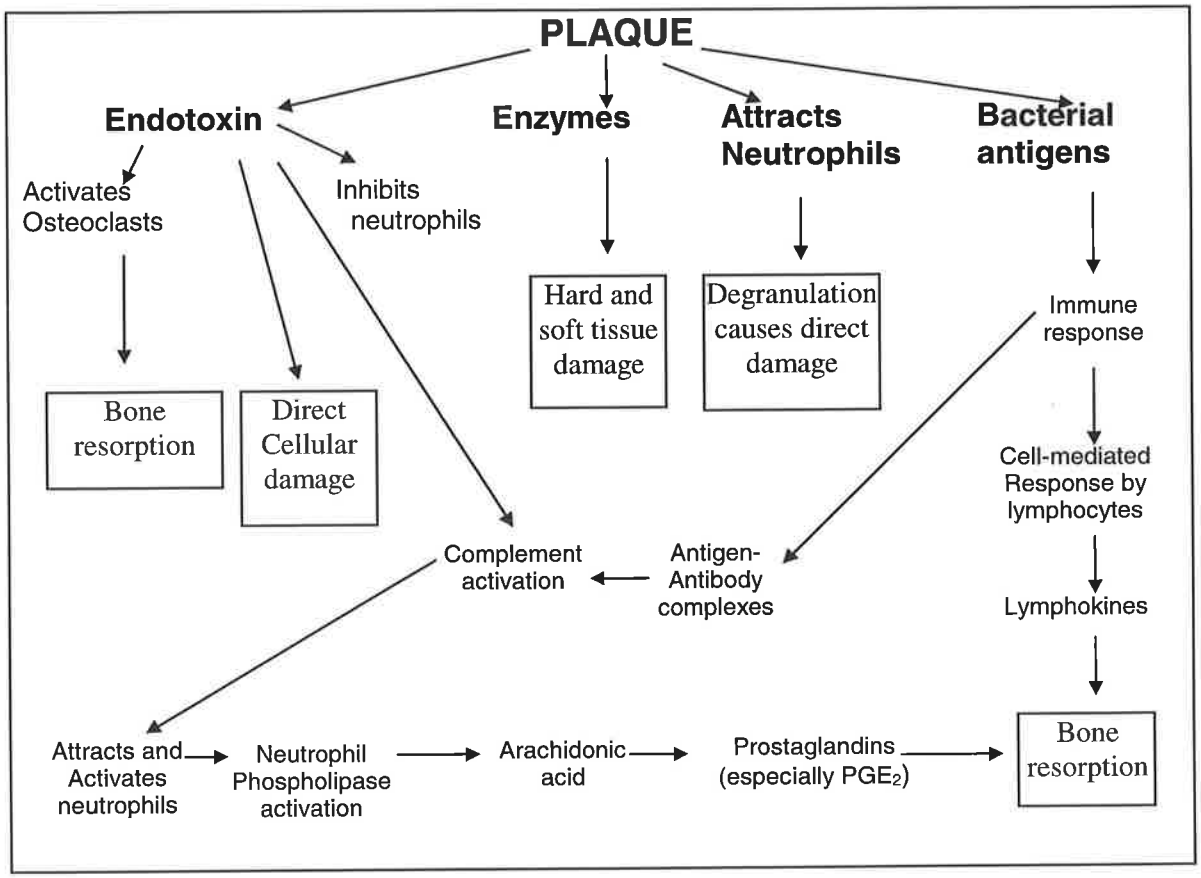

Figure 2: Overview of the interactions in periodontitis (Rees & Midda 1991)

2.1.2 Cellular Events

According to Rees & Midda (1991), damage to the periodontal tissues can occur in two main areas: either through injury by plaque bacterial toxins, or by activation of the host defence mechanisms.

2.1.2.1 Damage by bacterial toxins

The bacteria constituting the oral flora produce many substances which can cause direct damage to the periodontium. Over 300 of these have been identified (Page & Schroeder 1981; Moore 1987), including the following:

- Enzymes, such as collagenase, protease, hyaluronidase and β-glucuronidase. They tend to destroy the connective tissue ground substance thereby facilitating bacterial penetration.

- Cytotoxic factors, such as ammonia, hydrogen sulphide, lactic, acetic and formic acids. These may accumulate and reach toxic levels. Alternatively, they may be used as nutrients by other bacteria.

- Bacterial toxins are of two major types, namely exotoxins and endotoxins. Exotoxins are proteins released from multiplying bacteria (mainly Gram positive) which cause direct tissue damage in sites removed from the focus of bacterial growth. Exotoxins display affinities for specific cell types. For example, the periodontal pathogen *Porphormonas gingivalis* secretes an epitheliotoxin which damages epithelial cells. Endotoxins are lipopolysacharides (LPS) which are major components of the outer membrane of Gram negative bacteria and are only released after the death of the organism. *In vitro* LPS can stimulate immunoglobulin production, activate complement and blood clotting, and initiate prostaglandin production. LPS can also have significant effects on neutrophils and activate OCs (Van Dyke & Zinney 1989, Rees & Midda 1991).

2.1.2.2 Activation of the host defence mechanisms

The gingival tissues rely on several host defence systems in responding to plaque irritants. These defense systems include the phagocytic neutrophils, the complement system, the immune system and various chemical mediators of inflammation.

Neutrophils

These white blood cells have the ability to phagocytose plaque bacteria and other foreign matter. They form the first line of defence against bacterial invasion and respond in a nonspecific manner. The neutrophils possess lysosomes containing a variety of substances which can kill bacteria.

Unfortunately these substances are also released into the gingival tissues, causing localized destruction.

Plaque bacteria have developed a series of mechanisms to avoid or alter polymorph influence. Endotoxins modulate certain neutrophil functions such as chemotaxis, engulfment and killing.

The bacteria which are strongly implicated in periodontitis have developed various strategies against neutrophils.

Complement System

This is a series of at least 20 individual proteins which are present in plasma in an inactive form. They all form part of a complex cascade mechanism which can be activated by bacteria antibody complexes or by exotoxin, endotoxin or proteases discharged from neutrophils. The net result of complement activation is threefold. Firstly, neutrophils are attracted to the site of inflammation; secondly, lysis of Gram negative bacteria takes place; and finally, opsonization occurs. This refers to the coating of the bacteria by a fragment of complement so that they are recognized by phagocytic cells such as neutrophils, and destroyed.

The Immune System

Most of the substances produced by plaque bacteria are antigenic, eliciting both cell and antibody-mediated reactions. Plasma cells produce antibodies to numerous periodontal pathogens, which helps the rest of the cells of the immune system to recognize the bacteria as foreign matter. Activation of the cell-mediated immune system results in the production of a large family of substances known as lymphokines. These include various substances which can attract and activate neutrophils, macrophages and OCs.

Chemical mediators of inflammation

Prostaglandins and leucotrienes are two groups of substances, which are metabolites of arachidonic acid. This acid is formed from cell membrane phospholipid by the action of the enzyme phospholipase A_2 (released from neutrophil lysosomes).

Both prostaglandins and leucotrienes are important in the mediation and regulation of inflammation. In particular, much attention has been focussed on PGE_2, because it activates OCs and is thought to be involved in the resorption of alveolar bone. Levels of PGE_2 seem to be markedly raised during periods of periodontal destruction and have therefore been suggested as a marker of disease activity (Offenbacher *et al.* 1986).

Antigens derived from the subgingival microbiota bring about immune reactions which cause harm to the host. Recent evidence demonstrates a major role for host immune cells, like macrophages, and their soluble products as mediators for these alterations in the host immune response (Meikle *et al.* 1986, Alexander & Damoulis 1994). Some of these soluble products are called cytokines.

Howells (1995), described cytokines as important regulatory proteins, produced by activated immune cells and other cells of the body. These small proteins act as local messengers, usually delivering their signals over short distances by binding high affinity surface receptors. Cytokines are produced by most cells, but usually following activation. In this way cytokines form networks, one cytokine stimulating or inhibiting the production of other cytokines. The key factor in the way cytokines work is their effectiveness at very low concentrations (usually 1-1000 pg/ml). This is primarily due to the high affinity of their receptors. A cytokine may act on the producing cell in an autocrine way, or on an adjacent cell in a paracrine fashion. Cytokines like IL-1 and TNFα are biologically active in membrane-bound forms and relatively

small amounts of these cytokines may be potent in close cell-to-cell contact. Therefore, it does not follow that the most abundant cytokines are the most important (Howells 1995).

Howells (1995) further explained that the term cytokine includes proteins described previously as interleukins, inteferons, tumour necrosis factor, the chemokine family of chemoattractant cytokines and growth factors. These cytokines are important mediators in almost every aspect of cell biology including immunity and inflammation. A single cytokine may have different effects on different cells which express a corresponding receptor, that is "pleiotropy". In addition, several cytokines stimulate common activation pathways and show overlapping activities, known as "redundancy". What has become clear is that cytokines in excess, or when disregulated, may be damaging. A major problem in cytokine research has been the identification of the essential or pivotal cytokines involved in disease processes (Howells 1995).

The pleiotropic actions of cytokines include numerous effects on the cells of the immune system and modulation of inflammatory responses. Several cytokines have been detected in the gingival crevicular fluid (GCF) and gingival tissues in patients with periodontitis (Alexander & Damoulis 1994) (Table 1).

Interleukin-1

Interleukin 1 (IL-1) is a multifunctional cytokine that is one of the key mediators of the body's response to microbial invasion, inflammation, immunologic reactions and tissue injury. The macrophage-monocyte predominantly produces large amounts of IL-1α and IL-1β, which are distinct but structurally related molecules (Tatakis 1993; Alexander & Damoulis 1994). Both forms have similar proinflammatory properties, with IL-1β being more potent (Stashenko *et al.* 1987a,b; Alexander & Damoulis 1994).

Periodontal tissue			
	messenger RNA	Protein	GCF
IL-1α	+	+	+
IL-1β	+	+	+
TNF-α	+	+	+
IL-2	+/-	+/-	?
IL-4	-	-	?
IL-5	+	?	?
IL-6	+	+	+
IL-8	+	+	+
IFN-γ	+	+	+

GCF-gingival crevicular fluid; IFN-interferon; IL-interleukin; TNF-tumor necrosis factor; +, present; -, absent.

Table 1. Presence of cytokines in periodontitis (Alexander & Damoulis 1994)

IL-1 has many diverse biologic activities (Table 2). These biologic activities are consistent with the potential role of IL-1 as a local mediator of tissue destruction in human periodontitis.

IL-1α and IL-1β were predominantly produced by the macrophages in gingival tissues of patients with moderate to severe periodontitis (Matsuki *et al.* 1992). These results suggest that IL-1 is produced locally by cells present in diseased periodontal tissues and that the macrophage is the predominant IL-1 producing cell.

The potential role of IL-1 in periodontal tissue destruction has been reported by several researchers who used *in vitro* models to examine the effects of IL-1 on periodontal ligament cells (Tatakis 1993, Alexander & Damoulis 1994). IL-1 has been shown to induce the production of metalloproteinases, plasminogen activator, IL-6 and PGE_2 in human gingival fibroblasts

(Richards& Rutherford 1988, Bartold & Haynes 1991, Alexander & Damoulis 1994). IL-1 stimulates procollagenase and prostaglandin syntheses in periodontal ligament cells (Richards & Rutherford 1988, Alexander & Damoulis 1994). IL-1β inhibits alkaline phosphatase activity of the periodontal fibroblasts in a way that is similar to effects on bone cells (Piche *et al.* 1989, Nojima *et al.* 1990, Takeshita *et al.* 1992, Alexander & Damoulis 1994). Whether these effects of IL-1β on the human periodontal cells are direct or indirect is not clear.

Activity	Study
Inhibition of bone formation	(Stashenko *et al.* 1987) (Nguyen *et al.* 1991)
Enhancement of bone resorption	(Gowen *et al.* 1986) (Dinarello 1988)
Stimulation of prostaglandins and thromboxane synthesis	(Tatakis 1993)
Stimulation of collagenase and protease production	(Mizel *et al.* 1981)
Potentiation of neutrophil degranulation and superoxide production	(Dinarello 1988)
Enhancement of endothelial cell leukocyte adhesion	(Bevilacqua *et al.* 1985)
Stimulation of fibroblast and kerotiocyte proliferation	(Schmidt *et al.* 1982)

Table 2. Biologic activities of interleukin-1 (Alexander & Damoulis 1994)

A potential indirect effect of IL-1 on periodontal fibroblasts could occur via a Prostaglandin E_2 (PGE_2) dependent pathway. The sequelae of PGE_2 release

include connective tissue destruction as well as bone resorption (Offenbacher *et al.* 1993, Alexander & Damoulis 1994). Human periodontal fibroblasts have been reported to respond to IL-1 with an increase in PGE_2 production, but the mechanism of action is not clearly understood (Offenbacher *et al.* 1993, Alexander & Damoulis 1994).

IL-1 has been shown to have strong stimulatory effects on bone resorption and has been shown to have inhibitory effects on bone formation (Stashenko *et al.* 1987a, Nguyen *et al.* 1991, Tatakis 1993, Alexander & Damoulis 1994). Chaudhary *et al.* (1992), reported that IL-1β and TNF stimulated highly differentiated human osteoblast (OB) cells to produce IL-6, IL-8 and GMCSF but not IL-4 in culture media. The cytokines IL-6, IL-8 and GMCSF may act as autocrine and paracrine factors in modulating bone metabolism (Chaudhary *et al.* 1992, Alexander & Damoulis 1994).

The role of IL-1 in resorption and formation is partially regulated by its interactions with osteotropic hormones. Potent synergistic effects of IL-1 with parathyroid hormone (PTH) on bone resorption in bone organ cultures have been reported by Dewhirst *et al.* (1987). Kanatani *et al.* (1993) strongly suggested that $1,25(OH)_2VitD_3$ modulates the secretion of local regulators of bone remodelling from monocytes, resulting in inhibition of OB proliferation.

Despite all the evidence accumulated on the biologic activities of IL-1, its exact contribution to the pathogenesis of periodontal disease is still unclear. The local production of IL-1 by cells of the periodontium suggests that it can stimulate gingival and periodontal fibroblasts in an autocrine or paracrine fashion to induce the production of other cytokines, matrix degrading enzymes and PGE_2 (Alexander & Damoulis 1994). Studies have clearly demonstrated that IL-1 is a potent stimulator of bone resorption and that this effect is mediated via PGE_2 dependent and independent pathways (Stashenko *et al.* 1987b). These results strongly suggest that IL-1 plays a role

in periodontal tissue destruction. Further assessment of the effects of IL-1 on periodontal tissues is important in improving our understanding of the pathogenesis of periodontal diseases.

Interleukin-6

IL-6 is a multifunctional cytokine that regulates immune responses, acute phase reactions and hematopoiesis (Hirano 1991; Alexander & Damoulis 1994). IL-6 was originally identified as a B-cell differentiation factor and thus one of its major functions is antibody induction (Hirano 1991, Alexander & Damoulis 1994). IL-6 acts on and is constitutively produced by lymphoid and non-lymphoid cell types. IL-1, TNFα and inteferon-γ induce IL-6 production (Shalaby *et al.* 1989, Alexander & Damoulis 1994).

It has biologic effects that overlap those of other cytokines, such as IL-1 and TNFα, which have been reported to mediate inflammatory tissue destruction (Alexander & Damoulis 1994).

Although IL-6 production in periodontal tissues has been identified, its activities have not been well characterized. Kamagata *et al.* (1989) showed that IL-6 levels were higher in culture supernatants of gingival tissues from gingivitis and periodontitis patients than in healthy control patients. Similar results were reported by Bartold & Haynes (1991), who found that stronger staining for IL-6 was present in inflamed gingival tissues than in healthy control tissues. *In situ* hybridization showed that most periodontal ligament cells express IL-6 *m*RNA in response to IL-1 (Matsuki *et al.* 1992, Shimizu *et al.* 1992, Alexander & Damoulis 1994). The results from these studies show that IL-6 is widely distributed among cells of the periodontium.

Because IL-6 has been associated with diseases that are accompanied by bone resorption, such as osteoporosis and multiple myeloma, IL-6 may mediate bone resorption in pathologic states such as periodontal disease or

rheumatoid arthritis (Jilka *et al.* 1992, Fujihashi *et al.* 1993, Alexander & Damoulis 1994). Several studies have reported that OB like cells produce IL-6 and that IL-6 does not have any inhibitory effects on these cells (Ishimi *et al.* 1990, al-Humidan *et al.* 1991, Alexander & Damoulis 1994). Some studies have reported that IL-6 can induce osteoclastic bone resorption and that this activity is exerted via stimulation of OC formation rather than via the activation of mature OCs (Lowik *et al.* 1989, Ishimi *et al.* 1990, Kurihara *et al.* 1990, Alexander & Damoulis 1994). Other studies report that IL-6 does not exert any effect on bone resorption (al-Humidan *et al.* 1991, Fang & Hahn 1991, Alexander & Damoulis 1994). Hughes & Howells (1993) showed that IL-6 inhibits bone formation. These controversial results may reflect differences in the assay systems used.

Findings suggest that IL-6 may act as an autocrine factor, a paracrine factor, or both in pathologic states such as periodontal disease. This is by stimulating the formation of OCs, activation of osteoclastic bone resorption and the inhibition of osteoblastic bone formation (Alexander & Damoulis 1994).

Interleukin-6 Receptor (IL-6R)

IL-6, suggested to be an osteotropic factor, exerts its activity via a cell surface receptor that consists of two components; a ligand-binding protein gp80 (IL-6R) and a non ligand-binding but signal-transducing protein gp130 (Taga *et al.* 1989). Recently, the genetically engineered human and mouse soluble IL-6R (sIL-6R), which lacks transmembrane and cytoplasmic domains, has been reported to mediate the IL-6 signals through gp130 in response to IL-6 (Taga *et al.* 1989, Saito *et al.* 1991).

The role of IL-6 and/or sIL-6R has been examined in OC formation using a co-culture of osteoblastic cells and bone marrow cells (Tamura *et al.* 1993). Neither recombinant mouse IL-6, nor mouse sIL-6R, induced osteoclast-like cell (OCL) formation when they were added separately. In contrast,

simultaneous treatment of the co-cultures with IL-6 and sIL-6R strikingly induced TRAP-positive-multinucleated cell formation. The multinucleated cells possessed abundant calcitonin (CT) receptors and the ability to form resorption pits on dentine slices. The OCL formation induced by IL-6 and sIL-6r was dose-dependently inhibited by the addition of monoclonal anti-mouse sIL-6R antibody, MR16-1. However, this antibody showed no inhibitory effect on OCL formation induced by $1\alpha,25(OH)_2VitD_3$ and IL-1 (Tamura *et al.* 1993). These findings indicate that a complex of IL-6 and sIL-6R mediates the signals for inducing OC recruitment, but that IL-6 and sIL-6R alone do not.

sIL-6R has been detected in the urine and serum of healthy subjects and its level is increased in patients with multiple myeloma (Gaillard *et al.* 1993) and rheumatoid arthritis (Kotake *et al.* 1996). Furthermore, excess IL-6 is produced systemically and locally in patients with multiple myeloma (Kawano *et al.* 1988) and rheumatoid arthritis (Hirano *et al.* 1988). These findings suggest that the elevated levels of both IL-6 and sIL-6R form a complex, which is involved in bone destruction in these patients. Hence, it would appear that excess production of IL-6 is not sufficient to induce Osteoclastic bone resorption, and that the production of sIL-6R is indispensable for IL-6-induced Osteoclastic bone resorption in patients with multiple myeloma and rheumatoid arthritis.

Tumour Necrosis Factor (TNF)

TNFα and β are proinflammatory cytokines that are produced primarily by macrophages and lymphocytes, respectively (Manogue *et al.* 1991, Alexander & Damoulis 1994). TNF has been reported to have similar biologic effects as IL-1 and IL-6 (Manogue *et al.* 1991, Alexander & Damoulis 1994).

TNFα has been shown to induce the secretion of collagenase, PGE_2 and IL-6 by human fibroblasts and bone culture cells (Elias *et al.* 1987, Meikle *et al.*

1989, Chaudhary *et al.* 1992, Alexander & Damoulis 1994). It has also been reported to induce bone resorption *in vitro* and *in vivo* (Bertolini *et al.* 1986, Johnson *et al.* 1989, Van Der Pluijm *et al.* 1991, Alexander & Damoulis 1994). However TNFα is 100 to 1000 fold less potent a stimulator of bone resorption than IL-1. TNF has been detected in gingival crevicular fluid of patients with gingivitis and periodontitis (Rossomando *et al.* 1990, Alexander & Damoulis 1994).

The role of TNFα and TNFβ in the pathogenesis of periodontal disease is still unclear. However the ability of TNF to induce the production of matrix degrading enzymes and its bone resorbing activity strongly suggest that it could play an important role in connective tissue destruction and bone resorption in periodontal disease (Alexander & Damoulis 1994).

Cytokine Networks

A classic example of a cytokine network is the systemic shock induced by Gram-negative sepsis or following inoculation with LPS (Beutler & Cerami 1988, Tracey & Cerami 1993). In all species the response is similar. TNFα is produced first, followed shortly afterwards by IL-1, which in turn is followed by IL-6 and IL-8 (Waage *et al.* 1989). This cascade shows significant amplification with relatively low levels of TNFα inducing greater levels of the cytokines further downstream. This sequence of TNFα, IL-1 and IL-6 has also been shown in vitro (Dinarello *et al.* 1986). TNFα and IL-1 are profoundly pro-inflammatory and have a number of overlapping functions. Both have a potential to initiate tissue destruction and bone loss in periodontal disease. They are regarded as pivotal cytokines in periodontal disease (McFarlane *et al.* 1990).

2.1.3 Microbiology of periodontal disease

Johnson *et al.* (1992) described dental plaque as a complex and variable mixture of a wide variety of different types of bacteria. The mouth is the major (or sometimes only) habitat for these organisms, which are characterized as 'normal oral flora'. Even those organisms that appear to be quantitatively associated with disease are usually present as members of the normal oral flora. This suggests periodontal disease results from a disturbance of the normal balance between the host and the usually benign commensal flora. Also it is apparent that there is no single organism which is the causative agent of any of the periodontal diseases. Nevertheless, a number of organisms are involved with an ecological shift in the proportion of different bacteria, and a shift in the types and concentration of bacterial products (Johnson *et al.* 1992). In this respect, it is becoming increasingly apparent that there is no single etiology for the various periodontal diseases. Destructive periodontal diseases are the result of environmental, host and bacterial factors. Microorganisms, however, are essential components of any model for progressive periodontitis (Wolff *et al.* 1994).

Lindhe (1989) proposed three phases in the changes of the microbiota over a two-week period. During the first phase, the microbiota is dominated by Gram positive cocci, Gram positive rods, and Gram negative cocci. During the second phase, filamentous organisms appear and during the third phase, spirille and spirochetes (Theilade *et al.* 1966). When gingivitis has become established, cultivation of the bacteria from the infected sites discloses an increase in the number of anaerobic bacteria in relation to the facultatively anaerobic ones (Slots *et al.* 1978).

Lindhe (1989) further explained that, in the early stages of periodontitis, the bacterial flora of the gingival pocket is similar to that accompanying gingivitis

(Moore *et al.* 1987). Cultivation of samples from advanced cases of periodontal disease demonstrates a predominance of Gram negative anaerobic rods (Slots 1977). Microscopically, it can also be observed that samples from advanced cases of periodontal disease usually contain high numbers of spirochetes (Listgarten & Hellden 1978).

Plaque from individuals with periodontitis has been associated with more than 350 microbial species. Furthermore, a strong case can be made for implicating a number of specific bacteria as risk indicators for destructive periodontal disease (Table 3).

Microorganism	Selected references associating microorganisms with periodontitis
Porphyromonas gingivalis	(Haffajee *et al.* 1991; Beck *et al.* 1992; Dahlen *et al.* 1992; Wolff *et al.* 1993)
Prevotella intermedia	(Bragd *et al.* 1987; Wolff *et al.* 1988; Dahlen *et al.* 1990; Beck *et al.* 1992; Wolff *et al.* 1993)
Actinobacillus actinomycetemcomitans	(Wolff *et al.* 1985; Beck *et al.* 1992; Skaar *et al.* 1992; Wolff *et al.* 1993)
Eikenella corrodens	(Mandell 1984; Dzink *et al.* 1985; Wolff *et al.* 1985; Wolff *et al.* 1993)
Fusobacterium nucleatum	(Wolff *et al.* 1985; Wolff *et al.* 1993)
Bacteroides forsythus	(Tanner *et al.* 1984; Lai *et al.* 1987)
Camphylobacter rectus	(Tanner & Bouldin 1989; Moore *et al.* 1991; Lai *et al.* 1992)
Spirochetes or *Treponema* species	(Listgarten & Levin 1981; Loesche *et al.* 1985; Pihlstrom *et al.* 1985; Wolff *et al.* 1985)

Table 3. Microorganisms as risk indicators for periodontitis (Wolff *et al.* 1994)

Wolff *et al.* (1993) immunologically screened the natural distribution of 5 bacterial pathogens, *P. gingivalis, A. actinomycetemcomitans, P. intermedia, F. nucleatum* and *E. corrodens*, in subgingival plaques taken from 3,052

molar sites in 886 subjects with early periodontitis. Sites negative ($<10^3$ bacteria) for a bacterial pathogen, to low ($>10^3$ $<10^5$ bacteria) and then high ($>10^5$ bacteria) positive levels, exhibited a progressively higher mean probing depth ($P<0.001$). It is important to emphasize that although these bacterial pathogens are more likely found at sites with greater probing depth, greater attachment loss, or greater bone loss, they are also found at sites with little or no evidence of disease (Wolff *et al.* 1993).

Wolff *et al.* (1993) examined the site distribution and prevalence of the 5 periodontal bacterial pathogens in a population with early periodontitis and showed that *prevalence* in subjects was lowest for *P. gingivalis* (32%) and highest for *E. corrodens* (49%). Site-based frequency distribution of these bacterial species ranged from 10.3% for *P. gingivalis* to 18.7% for *E. corrodens.*

Chen & Wilson (1992) described *E. corrodens* as a facultative Gram-negative bacillus, which is a common inhabitant of the oral cavity and the intestinal and genital tracts. Its primary ecologic niche within the oral cavity appears to be dental plaque, both in periodontally healthy individuals and in periodontitis patients. However, *E. corrodens* is recognized as an infrequent human pathogen capable of causing extraoral infections, either as the sole infectious agent or as part of a mixed infection. Its potental role in the etiology of periodontal disease is not well understood. *E. corrodens* is often present in the supra- and subgingival plaque of periodontally healthy subjects. On the basis of cross-sectional and longitudinal studies, *E. corrodens* appears to be somewhat more prevalent in subgingival plaque samples of periodontitis subjects compared with periodontally healthy individuals (Chen & Wilson 1992).

E. corrodens typically exhibits corroding colony morphology which is characterized by a circular and irregular margin with a diameter typically <

3mm. Such colonies contain a rough, nonrefractile, opaque perimeter; a moist umbonate center; and appear to pit the blood agar surface. Strains with the corroding colony morphology may yield non-corroding colony variants, which are usually smaller, exhibit a circular margin and a smooth colony surface, and lack the granular perimeter. *E. corrodens* is capable of both aerobic and anaerobic growth (Chen & Wilson 1992).

Chen & Wilson (1992) further explained that the virulence factors which may influence the capacity of *E. corrodens* to cause extraoral or periodontal infections were sparse. There has been great interest in the various components of bacterial surface structures because of their potential role in the pathogenesis of bacterial infection and their accessibility to host defences. Kato *et al.* (1987) examined lipopolysaccharides (LPS) of 7 *E. corrodens* isolates by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and showed that the LPS consisted of low molecular weight bands and a few high molecular weight bands. There is considerable strain heterogeneity regarding the mobility and number of low molecular weight LPS bands among different strains. This indicates that there is appreciable structural heterogeneity in LPS of *E. corrodens* (Chen & Wilson 1992).

Although *E. corrodens* LPS differs from enterobacterial LPS in biochemical composition, it nonetheless exhibits similar biological activity. Thus, *E. corrodens* LPS has shown to be mitogenic for mouse spleen cells, to induce bone resorption in organ culture, and to exhibit Limulus amebocyte lysate clotting activity (Progulske *et al.* 1984). In addition, *E. corrodens* LPS has been shown to stimulate the release of TNF and IL-1 from human monocytes (Lindemann *et al.* 1988).

SDS-PAGE analysis of the outer membrane fractions of *E. corrodens* indicated that this species contains a principal outer membrane protein (POMP) (with an apparent mass of 33 to 42 kD) as well as several proteins of

lesser staining intensity (Maliszewski *et al.* 1983, Progulske & Holt 1984, Chen & Wilson 1990). The function of the POMP is unknown, although it has been suggested to be a porin. The outer membrane protein patterns (including POMP and minor membrane proteins) as assessed by SDS-PAGE appear to be diverse among *E. corrodens* strains (Chen & Wilson 1990).

Whether *E. corrodens* outer membrane proteins represent potential virulence factors for this species is unresolved. Tufano *et al.* (1986) reported that the POMP induced the release of lysosomal enzymes from mouse macrophages at low doses and exhibited cytotoxic effect at higher doses. The POMP also exhibited dose-dependent stimulation or depression of macrophage phagocytosis, and was capable of consuming serum complement activity and of inducing platelet aggregation (Tufano *et al.* 1986). These observations suggest that the predominant outer membrane proteins may contribute to the pathogenic potential of *E. corrodens* infection.

Reddi *et al.* (1995) extracted the surface associated material (SAM) from the surface and lipid A-associated proteins (LAPs) of the cell walls in a range of periodontopathic bacteria including *E. corrodens.* These bacterial fractions were assayed to determine their composition and their capacity to induce bone resorption was determined by use of the neonatal murine calvarial bone resorption assay, by way of calcium release. The SAM from *E. corrodens* demonstrated bone resorbing capacity at concentrations as low as 1ng/ml which, given the molecular weights of the active components, is in the picomolar range of activity. In contrast, the SAM from *P. gingivalis, P. intermedia* and *C. rectus* were significantly less potent and showed a lower efficacy. The LAPs all showed significant and similar capacities to induce bone breakdown (Reddi *et al.* 1995).

SAM is a complex mixture of proteins and carbohydrates with potent biological actions on isolated bone and on various mammalian cell

populations (Reddi *et al.* 1996). These investigators measured the composition of the SAMs of five major periodontopathogens. When fractionated by SDS-PAGE the SAMs from all the bacteria contained Coomassie blue-stainable bands ranging in molecular weight from <14kDa to >66kDa. The composition of the various SAM preparations in terms of protein, carbohydrate and lipopolysaccharide is shown in Table 4. The lipopolysaccharide content of the SAMs was low (Table 5).

SAM	Lowry	Brad-ford	Dc Kit	Lipid	CHO	Nucleic acids	% Total
A. actinomyce-temcomitans	70	35	60	12	13	8	93*
E. corrodens	12	44	64	14	6	14	98
P. gingivalis	42	7	49	8	18	3	78
Pr. Intermedia	14	5	21	22	17	2	62
C. rectus	3	9	11	5	1	1	18

*The percentage total has been calculated using the maximum protein values.

Table 4. Composition of SAM (Reddi *et al.* 1996)

SAM	Endotoxin (IU*/ng)
A. actinomycetemcomitans	0.009
P. gingivalis	2×10^{-5}
Pr. intermedia	3×10^{-6}
E. corrodens	6×10^{-4}
C. rectus	3×10^{-4}

*1 µg of *E. coli* LPS contains 7.000 IUs.

Table 5. Endotoxin content of SAM (Reddi *et al.* 1996)

2.2 BONE RESORPTION AND OSTEOCLASTS

The OC, first named by Kolliker (1873), is the primary cell responsible for bone resorption. OCs are multinucleated giant cells with elaborate membrane specializations which engineer the resorption process. In concert with OBs, hormones, and mediators produced by immune system cells, they play a role in normal skeleton remodelling, maintenance of the skeleton as a structural support system, pathological bone resorption, and mineral homeostasis.

2.2.1 General structure of osteoclasts

OCs are motile cells, usually between 10-100μm in diameter and capable of moving along the bone surface between resorption sites. They are multinucleated and may contain up to 100 nuclei. The majority have between 10 and 20 nuclei, although there is some species variation. OCs are highly vacuolated in their active state and have many mitochondria, indicative of their high metabolic rate. Actively resorbing cells are found in concavities called *Howship's lacunae* along the bone surface. Active OCs show the presence of specialised resorbing structures, namely the ruffled border and the clear zone. The ruffled border is a series of interdigitating membraneous folds directly under which the resorption process occurs. It is surrounded by the clear zone, which is a circular area of filament rich and organelle free cytoplasm anchoring the cell to the underlying bone. The expression and size of these resorbing structures has been shown to be proportional to resorptive activity. The actively resorbing OC is, therefore, a polarized cell. The ruffled border/clear zone in apposition to the bone surface may be referred to as the ventral or apical area of cell membrane, in contrast to the dorsal or basal area of the cell which juxtaposes the extracellular matrix.

2.2.2 How osteoclasts resorb bone

2.2.2.1 Removal of osteoid

Bone surfaces are normally covered by a thin layer of OB-covered osteoid which is removed prior to resorption of the underlying bone. There are several theories of osteoid removal and it appears that the OB may participate in the removal of this particular organic matrix component (Chambers & Fuller 1985, Sasaki *et al.* 1985). For example, *in vitro* studies have shown that the OB can produce a latent collagenase and tissue plasminogen activator and that the production of both of these is increased by PTH and other agents which stimulate bone resorption. Tissue plasminogen activator converts plasminogen to plasmin, which can then activate latent collagenase. Subsequent detachment of the covering OB layer and exposure of the mineral facilitates attachment of OCs (Jones & Boyde 1976). Several studies have revealed that OCs are attracted by the hydroxyapatite component of the bone (Chambers 1981, Chambers *et al.* 1984).

Chambers (1988) suggested that osteoblastic cells are necessary for the initiation of bone resorption. His group found that the OC are not induced to resorptive activity by incubation on native, uncut bone surfaces unless the unmineralized surface layer was first removed by mammalian or bacterial collagenase (Chambers *et al.* 1985). Although OCs are capable of destroying both the organic and mineral components of bone, contact with bone mineral, and not the organic material, induces OCs to resorptive activity.

2.2.2.2 Attachment

An OC needs to attach itself to the mineralized substratum before resorption can occur, a specialized cell-matrix attachment must be accomplished to create an isolated compartment at the OC-bone interface. If this specialized compartment were not present, vectorial transport of degradative acid and

enzymes would be dissipated into the general extracellular space. This would damage adjacent cellular material and result in horribly inefficient bone degradation (Blair *et al.* 1993).

In 1984, Marchisio *et al.* described a form of cell substratum contact, which they suggested represented an OC specific adhesion device. This mechanism consists of short protrusions extending from the thick network of intermediate filaments (vimentin type) found in the clear zone. These protrusions contacted the substratum and showed a core of F-actin, fimbrin and α-actinin containing material, presumably organized as a network of microfilaments, and surrounded by a rosette-like structure in which vinculin and α-actinin were found. The authors interpreted the rosettes as representing circular forms of close cell-substratum contact.

In 1993, Blair *et al.* described that OC attachment is directed by specific receptor-substrate binding. Cell membrane integrins are critical to this process, but the process has not been fully characterized. Attachment also involves, and may be modified by, changes in intracellular [Ca^{2+}] (Teti *et al.* 1990). This, in turn, may regulate OC detachment from mature resorption pits to allow escape of degraded collagen fragments, phosphates, and calcium itself. There is direct experimental data for retraction of OC resulting from a high extracellular [Ca^{2+}] (Zaidi *et al.* 1989). Supporting this possibility, direct microelectrode measurement has recently shown that extracellular calcium at the cell attachment site is ~20mM (Silver *et al.* 1988).

Integrins are a family of heterodimeric membrane proteins that recognize and bind to specific proteins in the extracellular matrix, characteristically at arginine-glycine-aspartic acid (RGD) sequences (Albelda & Buck 1990, Horton & Davies 1989). OCs have been shown to contain several integrin subunits (Zambonin-Zallone *et al.* 1989), and one integrin has been ascribed a specific function. A monoclonal antibody specific for the vitronectin receptor

$\alpha_v\beta_3$ (Davies *et al.* 1989), an integrin that binds to a large number of matrix proteins (Ruoslahti 1991), recognizes OCs and inhibits bone resorption (Chambers *et al.* 1986, Horton *et al.* 1985b). This has been shown to interact with osteopontin and bone sialoprotein (Reinholt *et al.* 1990), suggesting a function for these minor bone matrix proteins (Heinegard & Oldberg 1989). Integrin-matrix interaction appears to regulate, and may be modified by intracellular [Ca^{2+}] (Teti *et al.* 1990). This suggests that integrins may be critical elements in the OC intracellular calcium regulation as well as in matrix attachment (Blair *et al.* 1993).

In 1993, Blair *et al.* wrote that the understanding of matrix attachment is too incomplete to allow additional specific elements to be discussed with reasonable hope that they will hold up. However, diverse matrix and cell-surface attachment proteins are possibilities, as is signal-transduction with OC cytoskeletal changes. On the other hand, calcium and integrins do not represent a functionally complete attachment system, so further elements of attachment certainly remain to be defined. It is of pressing interest to define more fully the biochemistry of cell attachments, because this may resolve some causes of abnormal bone, such as osteopetrosis. This assertion is not hypothetical; note, for example, the study of Key (1987) showing osteopetrotic cells that are capable of acidifying cellular vacuoles, but not their bone attachment site. Furthermore, if the process of OC attachment involves unique bone proteins, the possibility of therapeutic modification of the process could be pursued, with effects on other organ systems, under this possible scenario, expected to be minimal.

2.2.2.3 Phagocytosis

Recent studies aimed at elucidating the mechanisms of acidification of the extracellular resorbing zone and the release of enzymes into this space favour the view that degradation of both the inorganic and the organic

components of bone is an extracellular event (Vaes 1988, Baron 1989, Pierce 1991). There is also a body of evidence based primarily on TEM studies, which indicates that phagocytosis also plays a role in bone resorption, particularly with respect to disposal of the mineral component. The ability of normal OCs to take up macromolecules by endocytosis has been demonstrated using TEM and the markers, peroxidase and thorium dioxide.

Partial decalcification of the bone surface under the ruffled border of OCs has been recognized as the first extracellular phase in bone resorption and results in the "release" of apatite crystals from the matrix. These crystals are distributed in the extracellular channels of the ruffled border and in endocytic vacuoles. Vacuolar structures containing mineral crystals have been observed (Pierce 1991), but collagen has not been demonstrated within these vacuoles or in the channels of the ruffled border.

Perhaps the simplest explanation for the presence of intracellular crystals is that they are dislodged from the collagen framework and partly solubilised in the extracellular acid milieux. The complete dissolution occurs either extracellularly or intracellularly, the proportions of which depend on such factors as variation in activity between cells, type of bone, stage of resorption, species and age of the animal. At the same time collagen is being degraded, thus allowing further opportunity for release of intact mineral crystals and their subsequent phagocytosis (Pierce 1991).

2.2.2.4 Exocytosis

Lucht & Norgaard (1976), using the TEM tracer tritiated leucine, performed one of the first studies to conclusively show that a newly synthesized protein, which they suggested represented a lysosomal enzyme, was transported from the OC to the extracellular resorption zone. The OC produces large amounts of lysosomal enzymes, in particular acid hydrolases, which are produced in

the Golgi apparatus. The lysosomal enzymes are thought to participate in lysosomal degradation within the cell and enzyme secretion after fusion of the primary lysosome with the ruffled border membrane.

Tartrate-resistant acid phosphatase (TRAP) is a lysosomal hydrolase, which has been used as a marker enzyme for OC function (Hammarström *et al.* 1971, Minkin 1982). The exact functional significance of TRAP remains to be determined. Clark *et al.* (1989) confirmed that TRAP is specifically localized in OCs, but not in other cells of bone or epiphyseal cartilage. However, the presence of TRAP in cells of the mononuclear phagocyte system (MPS) has been observed. Clark *et al.* concluded that the extent of acid phosphatase release and its regulation by calciotropic hormones implies a central role for acid hydrolase secretion in OC bone resorption.

OCs contain other membrane bound enzymes, including a (Na^+,K^+) adenosine triphosphatase (ATPase) (Baron *et al.* 1986) and carbonic anhydrase isoenzyme II (CA II) (Anderson *et al.* 1982). Sundquist *et al.* (1987) stated that the specific activity of CA II in OCs was so high that it probably represents one of OCs major soluble proteins.

2.2.3 Regulation of osteoclast function

2.2.3.1 Systemic factors

Systemic regulation of OCs is mediated primarily by the osteotropic hormones, which act by increasing the phenotypic expression or the number of cells (Table 6).

PTH stimulation of bone resorption causes an increase in size of rat OCs and an increase in ruffled border and clear zone areas. The number of cells are also significantly increased on stimulation with PTH, *in vivo* and *in vitro* (King *et al.* 1978; Holtrop *et al.* 1979). Stimulation of OCs in cultured long bones of

fetal rats by 1,25$(OH)_2VitD_3$, similar to PTH, produced a comparable increase in ruffled border, clear zone and total cell size, but no significant changes in cell numbers (Holtrop & Raisz 1979). Hence, these authors concluded that stimulation of bone resorption by PTH and 1,25 $(OH)_2VitD_3$ is mediated primarily by increased activity of existing OCs.

Factor	Formation	Resorption
PTH	(+)	(+)
Calcitriol	(+)	(+)
PGE_2	(+) (murine)	(+) (murine)
PGE_2	(-) (human)	(-) (human)
IL-1	(+)	(+)
M-CSF	(+)	(-)
TGFα	(+)	(+)
TNFα and TNFβ	(+)	(+)
IL-6	(+)	(+)
IL-11	(+)	?
CT	(-)	(-)
TGFβ	(-)	(-)
γ-Inteferon	(-)	(-)
IL-4	(-)	(-)
Sex steroids	(-)	(-)
Biphosphonates	?	(-)

Table 6. Factors that regulate OC activity (Roodman 1996). Stimulation of OC formation and/or resorption is denoted by (+); inhibition of formation and/or resorption is denoted by (-).

Another hormone, oestrogen (E_2), is known to inhibit osteoclastic resorption and an E_2 receptor has been identified on the OB cell membrane (Scheetz *et al.* 1987). E_2 deficiency is known to increase IL-1, IL-6, TNF_α, and GM-CSF

levels. Using *in vivo* studies, Wallach *et al.* (1993) suggested that E_2 deficiency leads to increased bone resorption by causing a surge of IL-6 production and augments IL-6 stimulation, by IL-1 and PTH. In addition, E_2 deprivation may lessen the impact of IL receptor antagonists, thereby unblocking IL resorptive activity.

In contrast to E_2, receptors for CT have been characterized on the OC surface membrane (Warshawsky *et al.* 1980, Nicholson *et al.* 1986). CT is a peptide hormone secreted by the parafollicular cells of the thyroid gland and is a potent inhibitor of Osteoclastic bone resorption. CT has a direct suppressive effect on Osteoclastic resorption. Physiologic doses of CT cause inhibition of motility, intense ruffling of lamellipodia (Chambers *et al.* 1984a) and flattening of the ruffled border with detachment from the bone surface (Kallio *et al.* 1972). It has been demonstrated that this hormone exerts its inhibitory effects on OCs by raising intracellular levels of cAMP (for review, see Ransjö 1988).

Continuous exposure to CT has shown to result in acquired resistance by OCs. The mechanisms of this 'escape' phenomenon are not yet established. However, it has been suggested that this phenomenon may result from a prolonged CT induced loss of CT responsiveness due, at least in part, both to reduced synthesis of CT receptor and to the appearance in bone of CT receptor deficient OCs (Ikegame *et al.* 1996).

2.2.3.2 Local factors

Other than the osteotropic hormones there are also locally released factors such as inflammation-associated prostaglandins and cytokines, as well as growth factors which can have powerful effects on both OBs and OCs. Several of these factors have been proposed as intercellular messengers which couple formation to resorption.

Growth factors can have powerful local effects on bone resorption. They are synthesized within a variety of tissues, where they act on their cell of origin (autocrine factors) or on adjacent cells (paracrine factors) (James & Bradshaw 1984, Canalis *et al.* 1989). They may be classified as transforming or non-transforming depending on their ability to induce normal cells to form tumour-like colony growth *in vitro*. TGF(α & β) are produced by both neoplastic and normal cells and stimulate bone resorption *in vitro* (Tashjian *et al.* 1985). Members of the TGF-β superfamily include the bone morphogenetic proteins (BMPs) (Celeste et al. 1990). Both TGFs and parathyroid hormone-related protein (PTHrP) are implicated in the bone resorption associated with the hypercalcaemia of malignancy (Ibbotson et al. 1983, Cornish et al. 1997).

There are many non-transforming growth factors that can be isolated from bone matrix. These are known or suspected to originate in cells present in bone tissue (Canalis *et al.* 1988, Mohan *et al.* 1988, Urist *et al.* 1983, Canalis *et al.* 1989). However, it is possible that factors secreted by extraskeletal tissues are trapped by the bone matrix from which they may be released and subsequently act as local growth factors.

Colony stimulating factors (CSFs) are hematopoietic growth factors that are produced by macrophages, stromal cells, endothelial cells, and T lymphocytes to induce the clonal growth of hematopoietic progenitors *in vitro* and *in vivo*. Since the OC is hematopoietic in origin, it is not surprising that these factors may act as stimulatory factors for OC formation. Macrophage colony-stimulating factor (M-CSF), also called CSF1, appears to play an important role in OC development (Yoshida *et al.* 1990). It stimulates both the proliferation and differentiation of OC precursors (Tanaka *et al.* 1993), and may also affect mature OCs (Weir *et al.* 1993). The effects of other CSFs are not as clear as those for M-CSF. Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates the growth of OC precursors and induces OC formation when human bone marrow cultures are treated sequentially with GM-CSF followed by $1,25(OH)_2VitD_3$ (MacDonald *et al.* 1986).

Whether growth factors are themselves sufficient to mediate the required communication between OCs and OBs is unclear. Other mechanisms and short acting mediators may be operative, such as direct cell to cell contact, and the secretion of oxygen radicals, peroxide, nitrous oxide, prostaglandins, prostacyclins, and other soluble mediators. Many activators of bone resorption do not act on the OC directly. Rather they act indirectly via activation of the OB. It is likely that osteoblastic cells produce a factor(s) responsible for OC differentiation in response to osteotropic hormones and cytokines. Such factors must be expressed on the plasma membrane of osteoblastic cells and play a critical role in its recognition system through a cell to cell contact mechanism. Cell-cell communication is thus an essential prerequisite for normal bone turnover (Wallach *et al.* 1993).

2.2.3.3 Osteoblastic control

The concept of osteoblastic involvement in OC development was first proposed by Rodan & Martin (1982). The histological observations by Ejiri (1983) strengthened this hypothesis of a cellular interaction between mononuclear pre-OCs and osteoblastic stromal cells. Using a coculture system with fetal bone rudiments, Burger *et al.* (1984) demonstrated that living bone cells are required for OC development. The mechanisms responsible for the activation and deactivation of the OC and the OB are closely related. This link of osteoclastic and osteoblastic activity in a bone remodelling unit is referred to as *coupling,* i.e. where a team of OCs remove bone (pit depth of approximately 50 μm), that is filled in with new bone by OBs (Parfitt 1982).

Rodan & Martin (1982) first proposed that the OB was the central cell controlling bone resorption, and that it was the OB and not the OC that were the targets for many agents stimulating resorption. These authors suggested that access was provided for the OCs to the bone surface only after these

agents changed the shape of the OBs, causing them to retract from the bone surface. Short range soluble factors released by the OB control the OC recruitment and function following PTH (McSheehy & Chambers 1986) or $1\alpha,25(OH)_2VitD_3$ (McSheehy & Chambers 1987) administration. Hence, OBs are attributed with a central role in the deposition and resorption of bone. PTH, $1,25(OH)_2VitD_3$ and IL-1 act first on the OB via receptors (not found in the OC) to initiate resorptive activity (Luben *et al.* 1976,Partridge *et al.* 1980, Silve *et al.* 1982, Narbaitzf *et al.* 1983, Bird & Saklatvala 1986). Udagawa *et al.* (1995) reported that the ability of IL-6 to induce OC differentiation depends on signal transduction mediated by IL-6 receptors expressed on osteoblastic cells and not on OC progenitors.

Isolated OCs are incapable of resorption after exposure to leukotrienes B_4, C_4 and D_4 (Chambers 1988), or IL-1, TNF and Lymphotoxin which stimulate resorption in cocultures (Thomson *et al.* 1986).

2.2.3.4 Role of stromal cells in osteoclast development

Burger *et al.* (1982) developed a co-culture system in which mouse marrow cells were cultured with stripped fetal bone rudiments devoid of OC progenitors. These authors clearly showed that living bone rudiments were required for OC development. Observations of mouse bone marrow cultures also suggested that bone marrow-derived stromal cells play an important role in OC development (Takahashi *et al.* 1988a). In mouse marrow cultures, OC-like multinucleated cells were formed in response to osteotropic hormones and cytokines such as $1\alpha,25(OH)_2VitD_3$, PTH, and IL-1 (Takahashi *et al.* 1988a, Akatsu *et al.* 1989, 1991). The multinucleated cells formed in mouse marrow cultures showed typical characteristics of OCs including intense tartrate-resistant acid phosphatase (TRAP) activity, abundant CT receptors, and bone resorbing activity (Takahashi *et al.* 1988). In the course of investigating OCL formation in mouse marrow cultures, OCLs were formed

mainly near the colonies of alkaline phosphatase (ALP)-positive stromal cells (possibly osteoblastic cells) (Suda *et al.* 1996). To examine whether or not osteoblastic cells are involved in OC development, a co-culture system was established of spleen cells and osteoblastic cells obtained from newborn mouse calvaria (Takahashi *et al.* 1988). Many OCLs were formed in co-cultures treated with $1\alpha,25(OH)_2VitD_3$. No OCLs were formed in separate cultures of osteoblastic cells and spleen cells even in the presence of $1\alpha,25(OH)_2VitD_3$.These findings indicated that osteoblastic stromal cells were somehow involved in OC development.

Takahashi *et al.* (1988a) demonstrated that bone marrow derived stromal cell lines, MC3T3-G2/PA6 and ST2, could be substituted for primary osteoblastic stromal cells in inducing OC development in cocultures with spleen cells. At present it is difficult to identify the common phenotype in MC3T3-G2/PA6, ST2, KS-4, ts8, and C3H-10T1/2 cells that could support OC differentiation. MC3T3-G2/PA6 cells and ST2 cells are classified as preadipocytes. It may be important to point out that most of the stromal cell lines that can support OC differentiation are endowed with the ability to support hemopoiesis in cocultures with bone marrow cells. This may also indicate that there is a common mechanism between hemopoiesis and OC formation.

2.2.4 Origin of osteoclasts

The formation of OCs differs from other bone cells, OBs and osteocytes, which derive from osteoprogenitor cells in the skeleton. OCs are thought to be derived from extraskeletal mononuclear haemopoietic precursor cells in the bone marrow or spleen and transported to the site of resorption via the bloodstream (Marks 1983). It is now well established that OCs are of hematopoietic origin. According to Suda (1992), the decisive evidence for this has come from three different types of investigations: parabiosis experiments,

chick-quail chimera experiments, and the restoration of bone resorption in osteopetrosis by transplanting normal marrow cells or spleen cells.

It was implicated in earlier studies that the monocytes or macrophages were the mononuclear precursor cells, but Marks & Popoff (1988) suggested that if the cells of the MPS are related to the OC, it is via a common progenitor cell.

The key functional feature which defines a cell as an OC is now generally accepted as its ability to form lacunar resorption pits on a bone substrate (Andersson *et al.* 1992, Chambers & Horton 1984). Original studies which attempted to investigate whether monocytes and macrophages possess this functional ability failed to demonstrate any evidence of resorption pit formation when various mononuclear phagocyte populations were cultured on bone slices in an SEM bone resorption assay (Ali *et al.* 1984, Chambers & Horton 1984). Quinn *et al.* (1996) duplicated a number of these earlier experiments in mice and found that an essential element required for mononuclear phagocyte differentiation into bone-resorbing cell was the presence of a bone stromal cell population. Various mononuclear phagocyte cell populations were used and only when mononuclear phagocytes were cocultured with UMR106 osteoblast-like cells in the presence of $1,25(OH)_2VitD_3$ was there evidence of OC differentiation, such as TRAP positivity and lacunar bone resorption. The formation of osteoclastic bone-resorbing cells by mononuclear phagocytes would appear to argue in favour of OCs being derived from this population of mononuclear cells.

2.2.5 Relationship between osteoclasts and the mononuclear phagocyte system

Mononuclear phagocytes were initially considered to be likely precursor cells for OCs on the basis of the numerous cytochemical, morphological, and

functional features which they were known to have in common with OCs (Ali *et al.* 1984, Chambers 1985, Quinn *et al.* 1996). These features include:

- OC and macrophage polykaryons are formed by fusion of mononuclear precursors. Nuclear division is never detected in OCs even after administration of PTH. Macrophages also tend to fuse to each other to form macrophage polykaryons.

- There are several ultrastructural similarities between OCs and macrophages. Like OCs, macrophages possess relatively high cytoplasmic concentrations of lysosomes and mitochondria.

- Some monoclonal antibodies that react with macrophages have been shown to recognize OCs as well (Sminia & Dijksira 1986, Athanasou & Quinn 1990). In addition Nijweide *et al.* (1985) reported that some monoclonal antibodies for OCs recognise Kupffer cells and intestinal macrophages.

- Both OCs and mononuclear phagocytes adhere strongly to the surface of culture dishes, and the adhesion is resistant to trypsin (Chambers 1979).

- Monocytes are attracted to bone (Mundy *et al.*1978, Quinn *et al.* 1996), and both monocytes and macrophages like OCs can resorb bone as measured by ^{45}Ca release from devitalized bone fragments (Mundy *et al.* 1977, Teitelbaum *et al.* 1979).

It has been hypothesised that mononuclear phagocytes are precursors of OCs. However, OCs and macrophages are different in several significant aspects.

- Studies with enzyme histochemistry have revealed that TRAP activity is localised only in OCs, and not in macrophages present in bone (Hammarström *et al.* 1971, Minkin 1982).

- Fcγ and C3 receptors are known to be functional markers for monocytes and macrophages but not for OCs (Chambers 1985).

- OCs also fail to express some leukocyte antigens, which are commonly expressed on mononuclear phagocytes (Horton *et al.* 1985b, Athanasou & Quinn 1990).

- One of the most critical features in mononuclear phagocytes is the lack of CT receptors, a functional hallmark of mammalian OCs.

- In addition, neither macrophages nor macrophage polykaryons are capable of elaborating ruffled borders adjacent to the bone surface (Chambers 1985).

Thus OCs and macrophages share similar ultrastructural features and functions, but they appear to be functionally distinct cell populations (Suda *et al.* 1992).

Demonstration of the leucocyte common antigen (LCA) on the OC cell membrane favours the derivation of OCs and peripheral blood leukocytes from a common multipotential stem cell and not from separate stem cells (Athanasou 1996; Suda *et al.* 1992).

Horton & Davies (1989) characterised the OC functional antigen (OFA), a molecule recognised by antibodies 13C2 and 23C6. OFA was found to be a member of a family of tissue specific extracellular matrix receptors termed integrins, and shows a homology with the vitronectin receptor (VNR). OCs

motility and bone resorption are inhibited with antibodies to these receptors which suggests that OFA-ligand interaction is required for bone resorption. Osteopontin is a protein and like vitronectin is also recognised by OCs in bone.

Helfrish *et al.* (1989) considered 3 candidates for OC progenitor cells from the circulating blood cells.

1. The peroxidase-negative macrophage; first suggested by Thesingh (1986).
2. The colony forming unit spleen and the granulocyte macrophage colony forming unit.
3. They also suggested that it could be an unidentified cell, which is not part of the macrophage-monocyte lineage but which is able to differentiate into OCs.

Recent studies have concluded that OCs originate from either pluripotent haemopoietic stem cells or from separate OC stem cells (Suda *et al.* 1992).

Hattersly & Chambers (1989a) used bone marrow cultures to conclude that additional characteristics present in bone marrow stroma are specifically required for OC differentiation. They also showed that components of calcified dentine is required for further differentiation into functional OCs after marrow culture (Sasaki *et al.* 1989).

2.2.6 Model systems for studying osteoclast formation and bone resorption

Progress in understanding the molecular events that occur during OC differentiation and osteoclastic bone resorption has been difficult because, OCs are few in number, are fragile when isolated from bone, and are difficult to isolate because they are embedded in a calcified matrix; furthermore, no

OC cell lines are available. To circumvent these problems, a variety of model systems have been developed to study OCs and OCLs *in vitro* and *in vivo*. These model systems have been used to identify OC precursors and to characterize their surface phenotype and for identification and characterization of factors affecting OC activity and formation. Furthermore, these models form the basis for *in vitro* resorption assays.

2.2.6.1 In vivo *models of osteoclast formation*

Baron *et al.* (1986b) developed an *in vivo* model for studying OC formation in rodent alveolar bone. OCs in alveolar bone are responsive to changes in the plasma concentration of PTH or CT and have enzymatic properties similar to those of OCs in other parts of the skeleton. This system has been used to study OC formation from mononuclear precursors. Bone resorption is induced by extracting molars from adult rats and inducing bone remodelling along the periosteum of the mandible. The animals are killed and the mandibles are processed for light microscopy, ultrastructural studies and cytochemical studies. Although these techniques have provided important insights into the sequence of OC differentiation, they are tedious, and it is difficult to isolate cells from these preparations for additional studies.

Recently, Uy *et al.* (1995) reported an *in vivo* model to examine the mechanisms of action of osteotropic factors on various stages of OC formation. In this model system, mice are either injected with a factor or implanted with Chinese Hamster ovary cells which have been transfected with the cDNA for the factor of interest and constitutively express the factor at high levels. This *in vivo* model system should help to dissect the site of action in the OC lineage of various cytokines involved in normal bone remodelling, as well as in states of increased bone turnover, such as osteoporosis.

2.2.6.2 Isolation of mature osteoclasts from bone

Several techniques have been developed for isolating mature OCs from long bones. One of the most commonly used sources for OCs has been the endosteal surface of chick long bones. In one approach, Osdoby & co-workers (1982) prepared OCs from embryonic chick tibia by removing the marrow and releasing the OCs using calcium-magnesium free buffers. The cell suspension was sieved through nylon mesh to trap the large multinucleated OCs. However, only a small percentage of these cells were viable and resorbed bone. A similar approach was used by Zambonin-Zallone *et al.* (1982), using hypocalcemic egg-laying chickens. With their methods, large numbers of OCs can be isolated, although the viability of these cells is still in question. Chambers *et al.* (1984b) and Tezuka *et al.* (1992) isolated OCs from the long bones of rats or rabbits using mechanical disaggregation. However, these systems suffer from two shortcomings: 1) The majority of the isolated cells may not be viable, 2) they may contain mixed populations, and 3) yields of OCs may be relatively low.

Chambers (1985) used isolated OCs deposited on bone slices as an assay for osteoclastic bone resorption. Factors are applied to the cells, and the number of resorption pits and the area of the calcified matrices resorbed are determined microscopically. However, such techniques can give only limited information on the effects of these factors on OC differentiation and on the regulation of osteoclastic bone resorption, since no new OCs form, and the cell populations tested are not homogeneous. The majority of studies using isolated OCs have suggested that most osteotropic factors act indirectly on OCs via the OB or stromal cell. Furthermore, there is a large variability in the capacity of these cells to resorb bone, so that large numbers of bone slices are required to obtain reproducible results (Roodman 1996).

2.2.6.3 Giant cell tumours as a model of osteoclasts

Several groups have used human giant cell tumours of bone as a source of human OCs. The giant cells from these tumours express an OC phenotype (Horton *et al.* 1985). They are extremely large, multinucleated, contain high levels of TRAP (a marker enzyme for OCs) have CT receptors (contract in response to CT) and form resorption lacunae on calcified matrices. These cells most likely represent very highly activated OCs and their study has revealed information on the OC phenotype and the effects of different factors on osteoclastic bone resorption. In addition it has allowed the preparation of human OC cDNA libraries (Bartkiewicz *et al.* 1995).

2.2.6.4 Bone organ culture systems

Bone organ culture systems have provided to be useful bioassays for studying factors controlling osteoclastic bone resorption and OC formation. These organ culture systems have identified new agents that stimulate, as well as inhibit, bone resorption. Among the most frequently used systems are the fetal rat or mouse calvarial assay or fetal rat long bone system, in which pregnant rats or mice are injected with calcium-45, and the radius and ulna or the calvaria are dissected from the fetuses or neonatal rodents. These bones are placed on membranes suspended on wire mesh and floated over a chemically defined media, and various osteotropic factors are added to the media. After an appropriate time period calcium-45 released from the bone relative to the total amount of calcium-45 in the bone fragment is determined. However, since these organ cultures represent mixed cell populations, determination of whether a factor(s) acts directly on OCs or indirectly through marrow stromal cells or other cells present in the bone organ culture is problematic. Furthermore, since most of these organ culture systems use fetal or neonatal tissues, these organ culture systems may not reflect the behaviour of OCs in adult animals.

2.2.6.5 Bone marrow culture systems

Since the OC is hematopoietic in origin, bone marrow culture techniques have been applied to studies of OC development. In these cultures OC-like multinucleated cells form. Burger & co-workers (1982) were the first to describe a marrow culture system that formed OCs. These authors used fetal bone rudiments that were devoid of OCs and cocultured them with marrow as a source of OC precursors in the presence of M-CSF. The effects of different factor(s) in this system were determined using histomorphometry, which is time consuming and labor intensive. Lowik *et al.* (1989) used fetal mouse metacarpals and metatarsals to distinguish whether factors stimulate OC formation or act on OCs once they are formed.

Testa *et al.* (1981) modified the long-term marrow culture systems to form OCLs. In their original work, multinucleated cells formed in feline bone marrow cultured in α-MEM with horse serum in the absence of any osteotropic factors. These multinucleated cells were not well characterized but were thought to be OC-like (OCL) because of their morphology. Ibbotson *et al.* (1984) further characterized this culture system, demonstrating that osteotropic factors could modulate formation of these OCLs appropriately, and that the precursor for these cells was a cell of the monocyte-macrophage lineage.

Since these initial studies, marrow culture systems using mouse marrow, in which TRAP-positive multinucleated cells that extensively resorb bone form in 5 to 6 days are observed (Takahashi *et al.* 1988). Baboon marrow cultures (Roodman *et al.* 1985), and human marrow cultures (MacDonald *et al.* 1987, Kassem *et al.* 1991, Thavarajah *et al.* 1991) in which OCLs form in 3 weeks, have also been developed. Multinucleated cells from these cultures fulfill the functional criteria of OCs. They are TRAP positive, react with the 23c6 monoclonal antibody, express CT receptors, contract in response to CT, and form resorption lacunae on calcified matrices such as sperm whale dentine,

although the resorption lacunae are not as prominent or as frequent as with murine marrow cultures. Both murine (Udagawa et al. 1989) and human (Fujikawa et al. 1996) marrow culture systems have been used to identify the mechanisms of action of a variety of factors on osteoclast formation. The murine coculture model was used in the current study, and is described further in Section 5.2.2 of the Discussion (p 98).

2.2.6.6 Osteoclast-like cell lines

Since an OC precursor cell line is currently not available, investigators have used hematopoietic cell lines to form OCL lines. Hattersly *et al.* (1989) reported the generation of OCLs from FDCPA4 cells, murine hematopoietic multipotential cell lines *in vitro*. These authors demonstrated that these pluripotent hematopoietic cells can form low numbers of multinucleated cells that express an OC phenotype and form resorption lacunae on calcified matrices. Similarly, Hagenaars *et al.* (1989) used FDCP-C2-GM cells, another multipotent hematopoietic murine cell line, to form some OCLs. Yoneda *et al.* (1991) have also used HL-60 cells, a human promyelocytic cell line, to form OC-like multinucleated cells. However, the number of OCLs formed was extremely low. Gattei *et al.* (1992) used a human myelo-monocytic leukemia cell line as a model for OC precursors, but found that, although the cells have some characteristics of OCs, they do not resorb bone (Gattei *et al.* 1992). Chambers *et al.* (1993) recently reported that cells from transgenic mice, which contain a transgene composed of the SV40 large T antigen driven by an MHC promoter, can be used to produce OCs *in vitro*. Several marrow stromal cell lines that supported OC differentiation from spleen cells or marrow cells were also derived from these mice. Thus, cells derived from these transgenic mice do not appear to be committed to the OC lineage but, rather, to be earlier precursors that have the potential to form small numbers of OCs *in vitro*. None of these cell lines described above form large numbers of OCs that are suitable for biochemical or molecular biological studies.

Recently, Shin *et al.* (1995), reported that the murine macrophage cell line BDM-1 could form OCLs when cocultured with primary OBs and 1,25-$(OH)_2VitD_3$ for 14 days. Subclones of this cell line that did not express the F4/80 macrophage antigen were the only cells that could form OCLs and only 5-9% of the cells could form OCs. As noted above, development of an OC precursor cell line would be a major advance in studies of the cell biology of the OC.

2.3 HYPOTHESIS AND AIMS OF THE STUDY

With increasing evidence for the local production of cytokines in the inflamed periodontium (Kjeldsen *et al.* 1993), chronic destructive inflammation, such as that seen in periodontal disease, is now thought to be due to the overproduction of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα (Birkedal-Hansen 1993).

In the current study, it is **hypothesised** that:

> *Bacterial products such as SAM are released from the subgingival plaque and activate macrophages and stromal osteoblastic cells in the gingiva and periodontium to synthesise and release pro-inflammatory cytokines, some of which may be responsible for the pathologic bone resorption seen in periodontal disease. Alternatively, bacterial products like SAM could directly stimulate formation of OCs from precursor cells and/or activate mature OCs to further stimulate bone resorption (Fig. 2).*

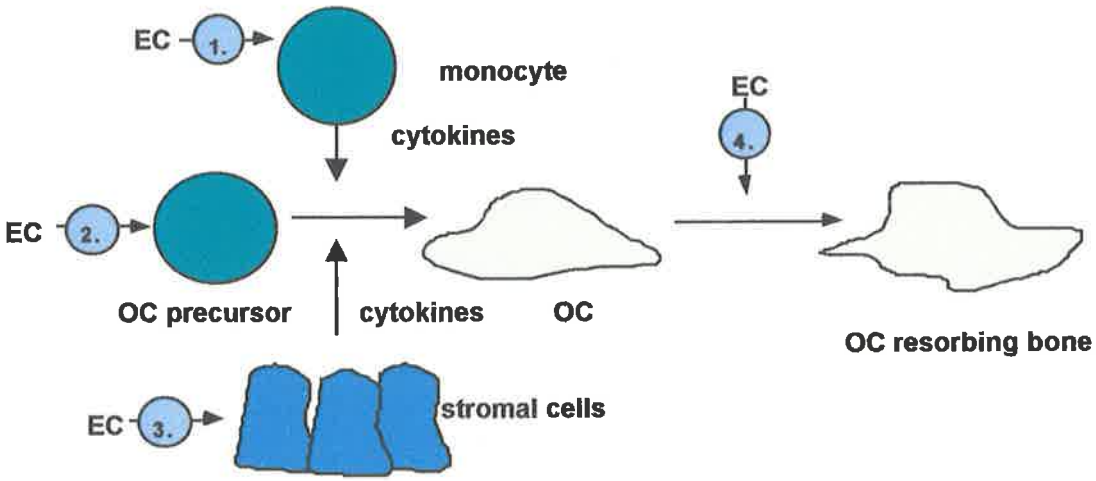

Figure 3. Diagrammatic representation of the four possible stages at which EC SAM may induce bone resorption.
1. *Stimulation of osteoclastogenic cytokine release from monocytes.*
2. *Stimulation of OC precursors to become OCs directly.*
3. *Stimulation of osteoclastogenic cytokine release from osteoblastic stromal cells.*
4. *Stimulation of bone resorbing activity of mature OCs.*

Hence, the specific **aims** of this study were:

Aim 1: To determine whether EC SAM stimulates human monocytes to release cytokines that induce osteoclastogenesis.

Aim 2: To establish if there are any direct effects of EC SAM on the activation of mature OCs or their formation from precursors.

Aim 3: To determine whether EC SAM induces stromal osteoblastic cells to release osteoclastogenic cytokines.

CHAPTER 3 MATERIALS AND METHODS

3.1 STIMULATION OF OSTEOLYTIC CYTOKINES BY *E. CORRODENS* SAM

In the current study, SAM from *Eikenella corrodens & Porphyromonas gingivalis* were extracted and their effects in stimulating monocytes/macrophages to release osteolytic cytokines (IL-1β, TNFα, IL-6, IL-6R and GM-CSF), was determined.

3.1.1 Bacterial Cultivation

Eikenella corrodens (33EKL) was grown in media maintained at a pH of 7.2, containing 20 gm/l bacteriological peptone (Oxoid, Australia), 10 gm/l yeast extract (Oxoid, Australia), 2 gm/l potassium nitrate and 0.5 gm/l cysteine (Sigma Chemical Company, Castle hill, Australia). While *Porphyromonas gingivalis* (W50) was grown in media maitained at a pH of 7.4, containing 37 gm/l brain heart infusion broth (Oxoid, Australia) and 5 mg/l haemin. Both organisms were separately cultured in chemostats (New Brunswick Scientific, Bioflo, model C30) with a working volume of 360 ml and a dilution rate of 0.1 h^{-1} at 37°C. *P. gingivalis* cultivation was done at specific atmospheric conditions of 10% CO_2 to 90% nitrogen. Once steady state conditions were established, the bacteria were collected, centrifuged at 4000rpm at 4°C for 40 min to isolate the pellet of bacterial cells. As both these organisms are gram negative, gram staining was performed to rule out any contamination. The pellets of bacterial cells were collected and stored separately at -20°C, until a volume of approximately 100ml of each bacteria was collected.

3.1.2 Extraction of surface associated material

The bacteria were suspended in 0.85% NaCl and their surface associated

material (SAM) was removed by gentle stirring at 4°C for 1 h. The resulting suspension was centrifuged at 4000rpm at 4°C for 1 h and the supernatant was collected. The supernatant was exhaustively dialysed against distilled water and concentrated using the Amicon ultrafiltration cell apparatus, model 8400 (Amicon Div., W.R. Grace & Co., Danvers, MA 01923 U.S.A.) using a filter with a 30,000 molecular weight cut off (Diaflo ultrafiltration membranes, YM30). The concentrate was then lyophilised and stored in a dessicator at 4°C until later use.

3.1.3 LPS extraction

The smooth and rough form of lipopolysaccharide (LPS) of *E. corrodens* was extracted according to the *Darveau-Hancock* procedure (Darveau & Hancock 1983)

Day 1

500 mg of lyophilized *E. corrodens* bacterial cells were resuspended in 15 ml of 10 mM Tris HCl buffer (pH 8.0) containing 2 mM $MgCl_2$, 100 µg of pancreatic DNase I per ml (1.5 mg added directly), and 25 µg of pancreatic RNase per ml (750 µl of 500 µg/ml stock added). In this and all subsequent steps, "Tris HCl buffer" refers to 10 mM Tris HCl buffer (pH 8.0). This suspension was passed through a french press at 16,000 lb/in^2 and sonicated for two 30 sec intervals at maximum output. An additional 100 µg of DNase per ml and 25 µg of RNase per ml was added. The cell lysate was incubated at 37°C for 2 h with gentle shaking. To this cell lysate the following was added: 5ml of 0.5 M tetrasodium EDTA dissolved in tris HCl buffer, 2.5 ml of 20% (wt/vol) SDS in Tris HCl buffer, and 2.5 ml of Tris HCl buffer. The pH was adjusted to 9.5. This lysate was vortexed carefully, and incubated for 1 h at 37°C with gentle shaking. This suspension was ultracentrifuged at 50,000 x *g* for 30 min at 15°C to sediment intact murein (23,500 rpm; Beckman type 40 rotor). The supernatant was removed and pronase (protease from

Streptomyces griseus [Sigma]) at 5mg/25ml was added to give a final concentration of 200 μg/ml. This was incubated overnight at 37°C with gentle shaking.

Day 2

The LPS was precipitated by adding 2 volumes of 0.375 M $MgCl_2$ in 95% ethanol and cooled to 0°C in a -20°C freezer (Care was taken not to let the temperature fall below -5°C). The mixture was centrifuged at 12,000 x *g* for 15 min at 0°C. (The rotor and centrifuge tubes were precooled). The pellet was resuspended in 17.5 ml of Tris HCl buffer, to which 2.5 ml of 20% SDS and 5ml of 0.5 M tetrasodium EDTA (both in 10 mM Tris HCl) was added, and sonicated for two 30 sec intervals at maximum output. The pH was adjusted to 8 by dropwise addition of 2 N HCl and the sample was incubated at 85°C for 30 min in a water bath, and cooled to room temperature. The pH was adjusted to 9.5 with 2 N NaOH. To this sample pronase at 25μg/ml (625μg/25ml) was added, and it was incubated overnight at 37°C with gentle shaking.

Day 3

LPS was precipitated as before with two volumes of 0.375 M $MgCl_2$ in ethanol. The sample was centrifuged at 12,000 x *g* for 15 min at 0°C, and the pellet resuspended in 15ml of Tris HCl buffer, sonicated as described above, and centrifuged at 1000 rpm (150 x *g*) for 5 min in a clinical centrifuge. The supernatant from the low speed centrifugation was made up to 25 ml with Tris HCl buffer, and $MgCl_2$ was added to 25 mM (125mg/25ml). This was ultracentrifuged at 200,000 x *g* for 4 h at 15°C (50,000 rpm; Beckman type 70.1 Ti rotor). The pellet was resuspended in distilled water, and lyophilized. This was stored in a dessicator at 4°C until later use.

3.1.4 Heat or trypsin treatment of SAM

To determine if the active components in the SAM were proteins, SAM from

E. corrodens was diluted to a concentration of 1mg/ml and either heat treated to 100°C for 1 h or exposed to trypsin (0.25%) for 1 h at 37°C. After trypsin treatment, an excess of soya bean trypsin inhibitor (Sigma) was added prior to addition to cells. As a control, *E. corrodens* SAM was treated with only soya bean trypsin inhibitor alone prior to addition to cells.

3.1.5 Activation of peripheral blood mononuclear cells (PBMC) to release cytokines

Human blood buffy coats were obtained from Red Cross Transfusion Service (Adelaide, South Australia). Buffy coats were diluted 1:3 in Hank's balanced salt solution (HBSS) (Gibco BRL, Life Technologies, Melbourne, Australia) and layered over a Ficoll-Paque separation gradient (Pharmacia Biotech, Uppsala, Sweden). Following centrifugation at 300 x *g* for 40 min, the mononuclear cell layer was removed, washed three times in HBS and a cell count was performed using a haemocytometer. Two x 10^6 cells were suspended in 1ml of RPMI-1640 medium (Gibco BRL, Life Technologies, Melbourne, Australia) including 25mM HEPES buffer supplemented with 10% fetal calf serum (Gibco BRL, Life Technologies, Melbourne, Australia), 5µg/ml penicillin and 50U/ml streptomycin (Gibco BRL, Life Technologies, Melbourne, Australia) and 50µM 2-Mercaptoethanol (Sigma M6250, St. Louise, MO., USA) and 200µl was added to each well of a 96-well flat bottomed microtiter plate (Nunc, Nalge Nunc International, Denmark). Cells were left to adhere for 1 h at 37°C in 5% CO_2, after which non-adherent cells were removed by rinsing with HBSS. Remaining adherent cells were incubated for 24 h in 1ml of the complete RPMI medium containing various concentrations of *E. corrodens* or *P. gingivalis* SAM or *E. corrodens* lipopolysaccharide (LPS) or *E. corrodens* SAM heat or trypsin treated. Concentrations of 5, 0.5, 0.05, 0.005 and 0.0005 µg/ml were used. Unstimulated monocytes were used as negative controls, and *Escherichia Coli* LPS (Sigma Chemical Company, Castle Hill, Australia), a strong stimulator of macrophage activation, was used as a positive control.

Monocytes from six donors were incubated with the SAMs for 24 h, their supernatants were removed and stored at -20°C prior to testing with enzyme-linked immunosorbent assays (ELISA).

3.1.6 Immunoassay of supernatant cytokine levels

Cytokines were measured using commercial kits and assays developed in our laboratories using commercial antibodies to measure cytokines.

3.1.6.1 *hIL-1β ELISA*

Levels of human IL-1β were measured using paired antibodies in a sandwich ELISA configuration. Briefly, 96-well plates (Nunc ImmunoPlate, Nalge Nunc International, Rochester, New York, USA) were coated with 100µl of 4µg/ml monoclonal anti-human IL-1β (R&D Systems, Minneapolis, MN, USA) in PBS. Plates were allowed to incubate at room temperature overnight. After incubation, fluid was decanted and wells washed four times with wash buffer (PBS, pH 7.4, 0.05% Tween 20). Nonspecific binding sites were blocked using PBS (pH 7.4) containing 1% bovine serum albumin (BSA), and wells incubated at room temperature for 60 min. Plates were washed four times before 100µl/well of standards (rhIL-1β (Cayman Chemical Company, Ann Arbor, Michigan, USA)) or samples were added. A 2 h, room temperature incubation followed. Plates were washed three times and 100µl of detection antibody (100ng/ml biotinylated anti-human IL-1β (R&D Systems, Minneapolis, MN, USA), diluted in Tris buffered saline, pH 7.3 with 0.01% bovine serum albumin (BSA) (Sigma Chemical Company, Castle Hill, Australia)) added to each well. Plates were incubated for 2 h at room temperature before being washed four times with wash buffer. Streptavidin horseradish peroxidase (Genzyme corporation, Cambridge, Massachusetts, USA) was added (100µl/well) and plates incubated for 20 min at room

temperature. Wells were given four final washes and 100µl tetramethylbenzidine (TMB) liquid substrate (Sigma Chemical Company, Castle Hill, Australia) was added to each well. The optical density of each well at 620nm (with a 700nm filter to account for optical density background) was determined using a Titertek Multiskan plate reader after 10 and 20 min. The reaction was terminated by addition of 50µl 0.5M H_2SO_4 to each well. Plates were read at 450nm with optical correction at 700nm.

A standard curve was constructed using dilutions of 0, 4, 8, 16, 32, 64, 128, and 256 pg/ml. The levels of IL-1β released were then determined comparing the optical densities of sample wells against the standard curve.

3.1.6.2 *hIL-6 ELISA*

The concentration of IL-6 in culture supernatants was determined using a similar protocol to hIL-1β. The only differences were: (a) monoclonal anti-human IL-6 antibody (R&D Systems, Minneapolis, MN, USA) was used as the primary antibody at a concentration of 2µg/ml, (b) biotinylated anti-human IL-6 (R&D Systems, Minneapolis, MN, USA) was used as the secondary antibody, and (c) rhIL-6 (Boehringer-Mannheim GmbH, Mannheim, Germany) standards were used.

A standard curve was constructed using dilutions of 0, 4.1, 12.3, 37, 111, 333, 1000 and 3000 pg/ml. The levels of IL-6 released were determined by comparing the optical densities of sample wells against the standard curve.

3.1.6.3 *hTNFα ELISA*

Human TNFα concentrations were measured in supernatants using paired antibodies (Genzyme DuoSet, Genzyme Corporation, Cambridge, Massachusetts, USA). Briefly, 96-well plates (Nunc ImmunoPlate, NalgeNunc

International, Rochester, New York, USA) were coated with 100µl of 2µg/ml mouse anti-human TNFα in 0.1M $NaHCO_3$ (pH 9.6). Plates were incubated overnight at 4°C. Fluid was decanted and wells washed four times with wash buffer (PBS, pH 7.4, 0.05% Tween 20). Nonspecific binding sites were blocked using PBS (pH 7.4) containing 1% bovine serum albumin (BSA), and wells incubated at 37°C for 2 h. Plates were washed four times before 100µl/well of standards [rhTNFα (Genzyme Corporation, Cambridge, Massachusetts, USA) diluted to concentrations between 16 and 100 pg/ml in RPMI medium as used for monocyte culture] or neat samples were added. A 1 h incubation at 37°C followed. Plates were again washed and 100µl of detection antibody (biotinylated rabbit anti-human TNFα, diluted in wash buffer with 1% BSA) added. Plates were incubated for 1 h at 37°C before being washed four times with wash buffer. Streptavidin horseradish peroxidase was added (100µl/well) and allowed to incubate for 15 min at 37°C. Wells were given four final washes and 100µl tetramethylbenzidine (TMB) liquid substrate (Sigma Chemical Company, Castle Hill, Australia) was added to each well. Plates were read at 620nm (with a 700nm filter to reduce background) using a Titertek Multiskan plate reader after 10 and 20 min. The reaction was terminated by addition of 50µl 0.5M H_2SO_4 to each well. Plates were read at 450nm with optical correction at 700nm.

A standard curve was constructed using dilutions of 0, 16, 32, 64, 125, 250, 500 and 1000 pg/ml. The levels of TNFα released were determined by comparing the optical densities of sample wells against the standard curve.

3.1.6.4 *GM-CSF and sIL-6R ELISA assays*

Human GM-CSF (BioSource International, Camarillo, California, USA) and human soluble IL-6 receptor (BioSource International, Camarillo, California, USA) were measured using commercially available ELISA kits following manufacturers' instructions. Plates were read 10 and 20 min after addition of

tetramethylbenzidine (Sigma Chemical Company, Castle Hill, Australia), and again after addition of 0.5M sulphuric acid solution, using a Titertek Multiskan plate reader. Standard curves were constructed using dilutions of 0, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg/ml for GM-CSF and 0, 62.5, 125, 250, 500, 1000, 2000 and 4000 pg/ml for IL-6 receptor. Cytokine concentrations were determined by comparing optical densities of sample wells against those of known standards.

3.2 MOUSE CO-CULTURE SYSTEM FOR RESORPTION

The direct effects of SAM from *E. corrodens* and *P. gingivalis* were examined, using the mouse coculture model by Tamura *et al.* (1993), on the formation and function of OC.

3.2.1 Seeding of dentine slices and coverslips with ST2 cells

Sperm whale dentine slices (whale tooth, courtesy of Environment Australia), were prepared with the help of a diamond saw, punched out as circles 6mm in diameter and 0.1mm to 0.2mm in thickness, sonicated, and sterilized in 75% alcohol. Sterile 13mm thickened glass coverslips (Lomb Scientific, Taren Point, NSW, Australia) were placed into individual wells of a 16mm flat-bottomed 24 well tray (Falcon, Becton Dickinson Labware, New Jersey, USA), and sperm whale dentine slices were placed into individual wells of a 96-well plate (Nunc ImmunoPlate, NalgeNunc International, Rochester, New York, USA), using a pair of flamed forceps dipped in 100% ethanol.

Bone marrow derived stromal cell line ST2 was established from long term marrow cultures of BC8 mice. A 250ml tissue culture flask (Falcon, Becton Dickinson Labware, Franklin Lakes, New Jersey, USA) containing confluent ST2 cells (from St. Vincent's Hospital, Melbourne) was washed twice with endotoxin-free Dulbecco's phosphate buffered saline (PBS) (Gibco BRL, Life Technologies, Melbourne, Australia). Two ml of 0.2g/l EDTA and 0.5g/l trypsin

(Type XI, Sigma Aldrich, 14 Anella Avenue, Castle Hill, NSW, Australia) was added to dislodge the confluent ST2 cells. The ST2 cells were suspended in α-Minimum Essential Medium (α-MEM) (ICN Biomedicals, Seven Hill, NSW, Australia) containing 1% L-Glutamine (Gibco BRL, Life Technologies, Mulgrave, VIC, Australia), 1% L-Ascorbic acid Phosphate (AS2P) (Novachem, South Yarra, VIC, Australia) and 10% endotoxin free foetal calf serum (FCS) (Gibco BRL, Life Technologies, Melbourne, Australia). Following centrifugation at 14,000-16,000 rpm for 10 min, a cell count using a haemocytometer was performed on the pellet of cells, and cells were resuspended at a concentration of $2x10^4$ cells/ml. One ml of the cell suspension in α-MEM, containing 1% L-Glutamine, 1% AS2P and 10% FCS was placed into each well of a 16mm flat-bottomed 24 well tray containing the sterile coverslips and 250µl into each well of a 96 well plate containing the sperm whale dentine slices. Cells were left to adhere for 1 h at 37°C in 5% CO_2, after which the non-adherent cells were removed by washing with α-MEM. The remaining adherent cells on the coverslips and dentine slices were incubated overnight with 1ml and 250µl of complete α-MEM respectively.

3.2.2 Preparation of mononuclear hemopoietic bone marrow cells

Under sterile conditions, two 6-9 week old C57/Bl6J mice, from The University of Adelaide Animal House, were sacrificed by cervical dislocation under the effect of diethyl ether (AnalaR, BDH Chemicals Australia Pty. Ltd., Kilsyth, VIC, Australia). The tibiae, femora and humeri bones were dissected out. With a pair of sharp sterile scissors the ends of these bones are cut off to expose the marrow canals. A 21 gauge needle was inserted into one end of the bone marrow canal, and using a 10ml syringe filled with PBS, the marrow was flushed into a sterile 50ml conical tube (Falcon, Becton Dickinson Labware, New Jersey, USA). The pellet of marrow cells was isolated by centrifugation for 10 min at 14,000-16,000 rpm and $1x10^6$ marrow cells were suspended in 1ml of complete α-MEM and used for the co-culture system.

3.2.3 Coculture system

400µl and 200µl of the bone marrow cells at $1x10^6$ cells/ml concentration were added to each coverslip and dentine slice pre-cultured with ST2 cells the previous day. Cells were allowed to adhere for 90 min at 37°C in 5% CO_2 before removing nonadherent cells by washing with α-MEM. The dentine slices with the adherent ST2 and marrow cells were transferred to wells of a 24-well plate, placing 2 dentine slices in each well. To each well containing coverslips or dentine slices, 1ml of complete α-MEM medium containing 1% of 10^{-8} M $1\alpha,25(OH)_2VitD_3$ (Calcitriol, Novachem, South Yarra, VIC, Australia), 10% of 10^{-6} M dexamethasone (Royal Adelaide Pharmacy, North Terrace, Adelaide, Australia), and 10% of the SAM of *E. corrodens* or *P. gingivalis* at a concentration of 5, 0.5, 0.05, 0.005 or 0.0005 µg/ml, were added. For the negative controls, 1ml of complete α-MEM (containing 1% of 10^{-8} M $1\alpha,25(OH)_2VitD_3$ and 10% of 10^{-6} M dexamethasone without the SAM) was added. The co-culture plates were incubated at 37°C in 5% CO_2. Every third day the media was replaced with fresh media.

3.2.3.1 *TRAP staining of coverslips*

At 5 and 14 days, the coverslips were fixed and stained for tartrate-resistant acid phosphatase (TRAP), using the Leukocyte Acid Phosphatase kit (Sigma Aldrich, Castle Hill, NSW, Australia).

TRAP stain was prepared using reagents from the Leukocyte Acid phosphatase kit. Forty four ml of distilled water at 37°C, 2ml of acetate solution, 2ml of naphthol AS-B1 phosphoric acid, 2ml of tartrate solution and contents of 1 capsule of Fast Garnet GBC salt were mixed together in a beaker on a magnetic stirrer for 30-60 sec and filtered through a Whatman

No. 1 filter paper (Whatman Ltd., England). The coverslips were first treated with 4% gluteraldehyde (fixative) for 10 min, rinsed with deionised water and covered with stain, then incubated at 37°C for 60 min, rinsed with deionised water and counterstained with methyl green (Sigma Aldrich, Castle Hill, NSW, Australia) for 8 sec. Coverslips were carefully rinsed, allowed to air dry and mounted onto glass slides. The number of large, multinucleated, TRAP positive cells were counted under the light microscope.

3.2.3.2 *Processing of dentine slices for scanning electron microscopy*

At similar intervals, the dentine slices were processed and examined under the scanning electron microscope (SEM). Dentine slices were treated with ammonia for 30 min, then trypsinised for 15 min and sonicated for 5 min. They were subsequently dehydrated, in a graded series of alcohol (5 min in solutions of 70%, 90%, 95%, and 100% ethanol). Slices were finally placed in 100% acetone and vacuum treated overnight using a Vacuum dessicator (Kartell, Italy). The next day, the dehydrated slices were mounted onto stubbs and were standard coated with carbon. The surface of the slices was then examined using the scanning electron microscope and the number of pits on each slice were counted.

3.3 Stimulation of human osteoblasts by SAM

Release of certain osteolytic cytokines (IL-1β & IL-6) from human osteoblasts was examined under the direct effect of *E. corrodens* and *P. gingivalis* SAM.

3.3.1 Seeding of tray

Human OB were obtained by outgrowth of cells from explants of healthy bone removed at the time of primary hip replacement from 3 patients with a mean age of 69.3 years. All were diagnosed as osteoarthritic. Trabecular bone chips

were cultured in 75 cm^2 flasks (Falcon, Becton Dickinson Labware, New Jersey) in α-MEM supplemented with 10% fetal calf serum (FCS), 5 μg/ml of penicillin, 50 U/ml of streptomycin and 1% ascorbate-2-phosphate. When the cells reached confluence they were washed twice with endotoxin-free Dulbecco's phosphate buffered saline (PBS) and trypsin was added to detach the cells from the bottom of the flask. OB were then resuspended in α-MEM containing 1% L-glutamine, 1% L-ascorbate-2-phosphate (AS2P) and 10% endotoxin free FCS. Following centrifugation at 14,000-16,000 rpm for 10 min, pellets of cells were resuspended to a concentration of $2x10^5$ cells/ml in α-MEM, containing 1% L-glutamine, 1% AS2P and 10% FCS. One ml of the cell suspension was placed into each well of a 16mm flat-bottomed 24 well tray, cells were left to adhere for 1 h at 37°C in 5% CO_2, after which the non-adherent cells were removed by washing with α-MEM. One ml of α-MEM, (containing 1% L-Glutamine, 1% AS2P, 10% FCS) and 10% of SAM of *E. corrodens* or *P. gingivalis* at concentrations of 0, 0.5, 0.05, or 0.005 μg/ml was added to each well containing adherent cells and incubated for 48 h at 37°C in 5% CO_2.

3.3.2 Immunoassay measurement of supernatant cytokine levels

The supernatants were collected after 48 h and IL-1β and IL-6 cytokine levels were measured using enzyme-linked immunosorbent assays (ELISA), as described earlier.

CHAPTER 4 RESULTS

4.1 STIMULATION OF CYTOKINE RELEASE FROM HUMAN MACROPHAGES

4.1.1 IL-1β release

Results of IL-1β release by human macrophages are illustrated graphically in Figure 4 (raw data is presented in Table 7). Figure 4 shows that SAM from *E. corrodens* was the strongest stimulator of IL-1β release from macrophages. SAM of *P. gingivalis* at 5 μg/ml concentration, stimulated 30% of IL-1β produced by the SAM of *E. corrodens* at 5 μg/ml. Although *E. coli* LPS was also a strong stimulator of IL-1β at 5μg/ml, it produced less than 60% of IL-1β release as compared to the SAM of *E. corrodens*. *E. corrodens* LPS only weakly stimulated IL-1β at all concentrations.

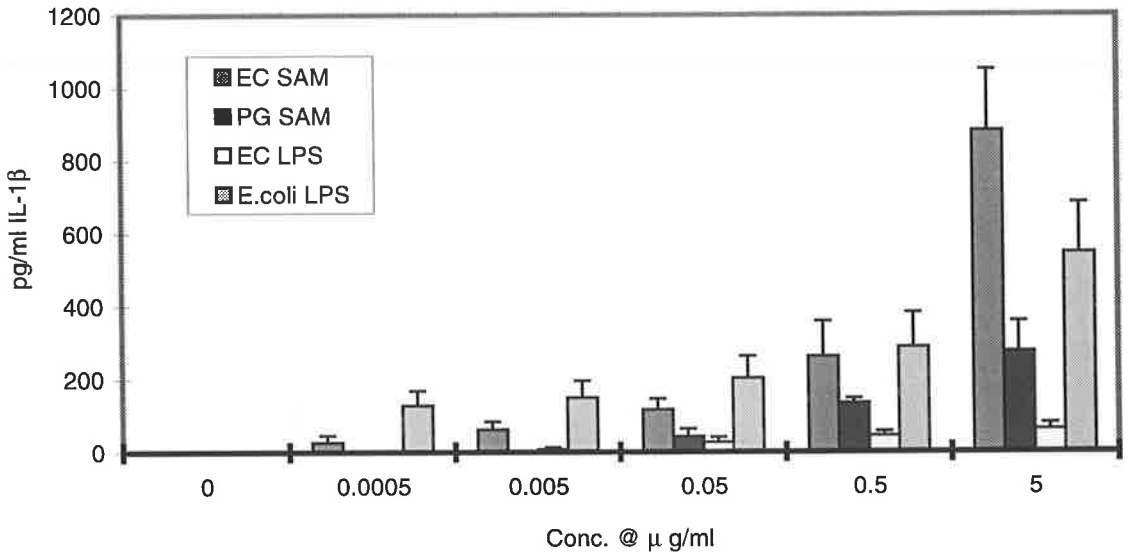

Figure 4: IL-1β release following macrophage stimulation by EC & PG SAMs and *E. corrodens* and *E. coli* LPS. Results are expressed as the mean and standard deviation of five different human macrophage donors.

At concentrations of 0.0005 μg/ml to 0.05 μg/ml *E. coli* LPS released higher levels of IL-1β as compared to the SAM of *E. corrodens.* However, at higher concentrations, the SAM of *E. corrodens* was equal to, or more potent than, *E. coli* LPS at stimulating IL-1β release

E. corrodens **SAM @ μg/ml**	**pg/ml IL-1β Mean**	**Standard deviation**	**Standard error**
5	880.8	377.999	169.046
0.5	261.6	218.043	97.512
0.05	116	68.501	30.635
0.005	61.6	49.772	22.259
0.0005	26.4	43.414	19.415
0	0	0	0
P. gingivalis **SAM @ μg/ml**	**pg/ml IL-1β Mean**	**Standard deviation**	**Standard error**
5	275	189.010	84.528
0.5	133.6	30.900	13.819
0.05	42.2	48.778	21.814
0.005	0.8	1.789	0.8
0.0005	0	0	0
0	0	0	0
E. corrodens **LPS @ μg/ml**	**pg/ml IL-1β Mean**	**Standard deviation**	**Standard error**
5	61.4	41.968	18.769
0.5	44	28.914	12.931
0.05	26.2	32.299	14.444
0.005	8	11.511	5.148
0.0005	0	0	0
0	0	0	0
E. coli **LPS @ μg/ml**	**pg/ml IL-1β Mean**	**Standard deviation**	**Standard error**
5	547	306.504	137.072
0.5	287.2	213.376	95.425
0.05	202	137.505	61.494
0.005	148.6	107.073	47.884
0.0005	127.6	90.207	40.342
0	0	0	0

Table 7. Levels of IL-1β released following macrophage stimulation.

4.1.2 TNFα release

Results of TNFα release by human macrophages are illustrated graphically in Figure 5 (raw data is presented in Table 8). The SAM of *E. corrodens* proved to be the strongest stimulator of TNFα. PG SAM and *E.coli* LPS stimulated similar levels of TNFα which was 86% and 50% of the SAM of *E. corrodens* respectively at concentrations of 0.5 and 5 μg/ml. *E. corrodens* LPS at 5 μg/ml concentration stimulated only 24% of TNFα produced by the SAM of *E. corrodens.*

Even at a low concentration of 0.0005 μg/ml, EC SAM and *E. coli* LPS stimulated the release of elevated levels of TNFα. *E. coli* LPS stimulated slightly higher levels of TNFα compared to EC SAM.

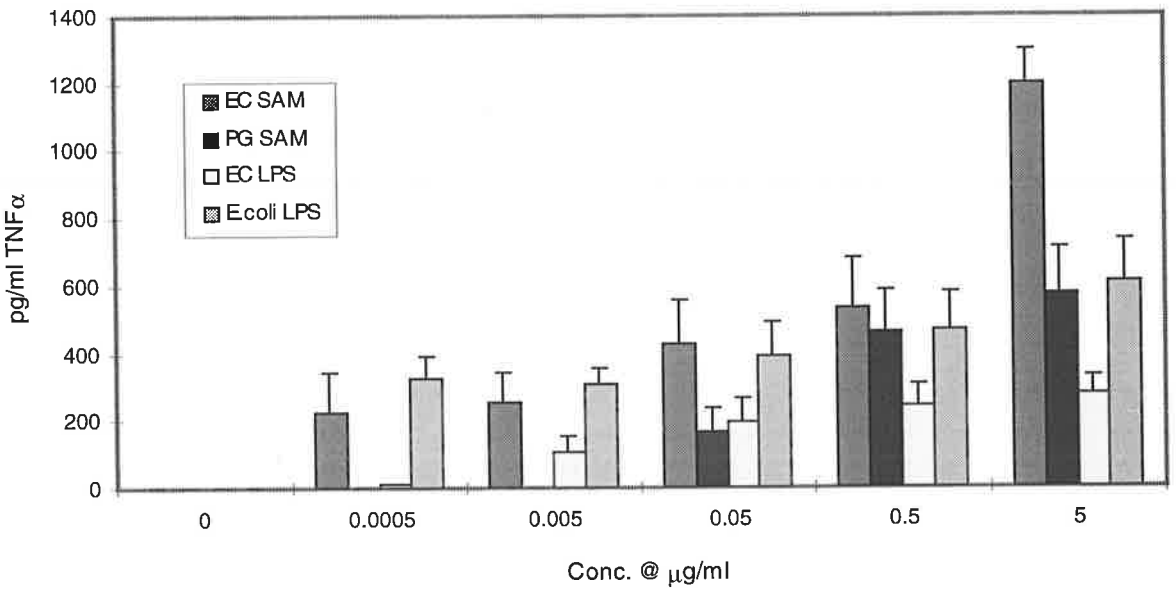

Figure 5: TNFα release following macrophage stimulation by EC & PG SAMs and *E. corrodens* and *E. coli* LPS. Results are expressed as the mean and standard deviation of six different human macrophage donors.

E. corrodens SAM @ μg/ml.	pg/ml TNFα Mean	Standard deviation	Standard error
5	1200	113.62	42.76
0.5	535	361.59	147.62
0.05	427.50	319.50	130.43
0.005	255	220.57	90.05
0.0005	226.67	293.37	119.77
0	0	0	0
P. gingivalis **SAM @ μg/ml**	**pg/ml TNFα Mean**	**Standard deviation**	**Standard error**
5	578.33	334.46	136.54
0.5	465.50	294.55	120.25
0.05	163.33	177.64	72.52
0.005	0	0	0
0.0005	0	0	0
0	0	0	0
E. corrodens **LPS @ μg/ml**	**pg/ml TNFα Mean**	**Standard deviation**	**Standard error**
5	280.83	121.75	49.71
0.5	245.83	152.07	62.08
0.05	194.17	177.21	72.35
0.005	106.67	123.88	50.57
0.0005	9.67	11.04	4.51
0	0	0	0
E. coli **LPS @ μg/ml**	**pg/ml TNFα Mean**	**Standard deviation**	**Standard error**
5	612.50	309.87	126.50
0.5	470.83	272.62	111.30
0.05	393.33	242.62	99.05
0.005	308.67	110.81	45.24
0.0005	327.17	153.96	62.85
0	0	0	0

Table 8. Levels of TNFα released following macrophage stimulation.

4.1.3 IL-6 release

Results of IL-6 release by human macrophages are illustrated graphically in Figure 6 (raw data is presented in Table 9). Macrophages released higher levels of IL-6 in response to the SAM and LPS preparations, than other cytokines. Again, SAM from *E. corrodens* was the strongest stimulator of cytokine release. The SAM from *P. gingivalis* was a strong stimulator, but at 5µg/ml concentration stimulated 63% of IL-6 produced by the SAM from *E. corrodens*. *E. corrodens* LPS was the weakest stimulator of IL-6.

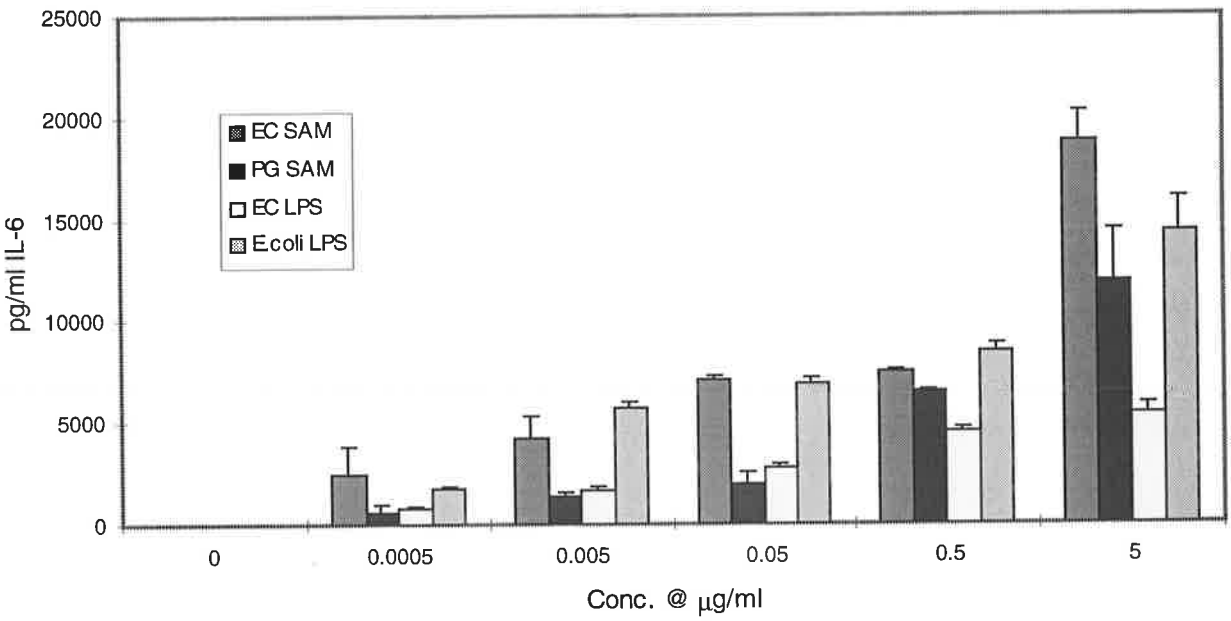

Figure 6: IL-6 release following macrophage stimulation by EC & PG SAMs and *E. corrodens* and *E. coli* LPS. Results are expressed as the mean and standard deviation of three different human macrophage donors.

E. corrodens SAM @ µg/ml.	pg/ml IL-6 Mean	Standard deviation	Standard error
5	18833.33	2466.441	1424.001
0.5	7500	216.506	125
0.05	7041.67	505.181	291.666
0.005	4208.33	1880.547	1085.735
0.0005	2458.33	2309.401	1333.333
0	0	0	0
***P. gingivalis* SAM @ µg/ml**	**pg/ml IL-6 Mean**	**Standard deviation**	**Standard error**
5	11875	4470.388	2580.981
0.5	6533.33	80.363	46.398
0.05	2000	1000	577.350
0.005	1333.33	505.181	291.666
0.0005	625	572.821	330.719
0	0	0	0
***E. corrodens* LPS @ µg/ml**	**pg/ml IL-6 Mean**	**Standard deviation**	**Standard error**
5	5458.33	803.637	463.980
0.5	4541.666	314.576	181.620
0.05	2750	433.012	250
0.005	1666.66	381.881	220.479
0.0005	833.33	144.337	83.333
0	0	0	0
***E. coli* LPS @ µg/ml**	**pg/ml IL-6 Mean**	**Standard deviation**	**Standard error**
5	14416.67	2809.952	1622.327
0.5	8416.67	688.446	397.474
0.05	6875	572.821	330.719
0.005	5741.67	536.384	309.681
0.0005	1750	216.506	125
0	0	0	0

Table 9. Levels of IL-6 released following macrophage stimulation.

4.1.4 sIL-6R (soluble IL-6 receptor)

Results of IL-6R release by human macrophages are illustrated graphically in Figure 7 (raw data is presented in Table 10). No significant stimulation of macrophages for IL-6R release by any of the SAMs or LPS was noted.

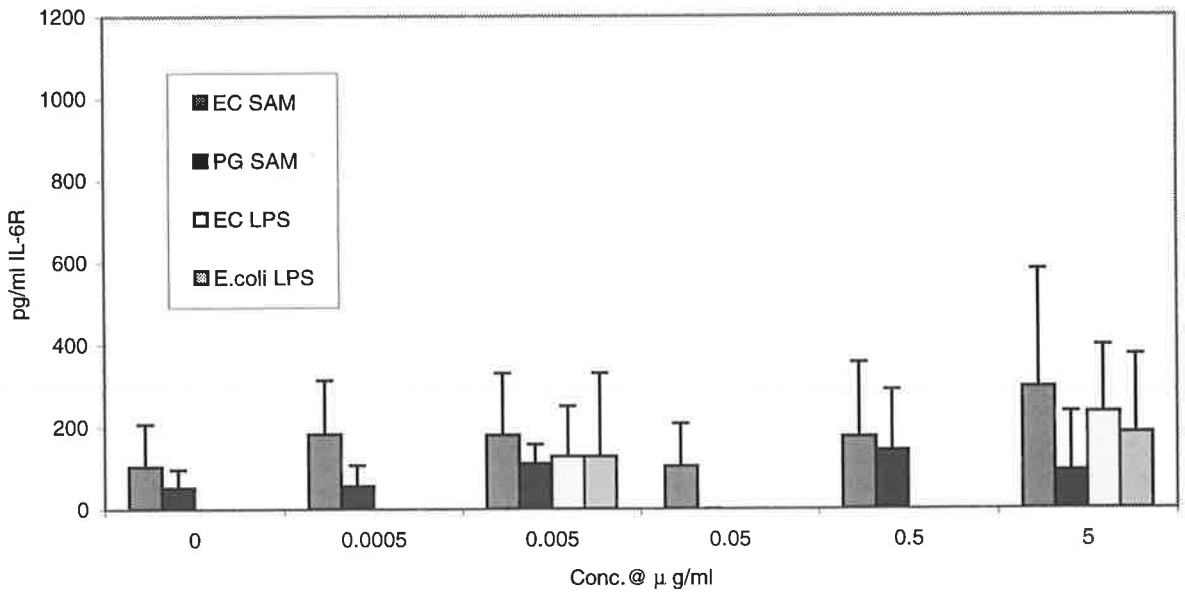

Figure 7: IL-6R release following macrophage stimulation by EC & PG SAMs and *E. corrodens* and *E. coli* LPS. Results are expressed as the mean and standard deviation of two different human macrophage donors.

E. corrodens SAM @ µg/ml.	pg/ml IL-6R Mean	Standard deviation	Standard error
5	296	405.56	181.38
0.5	176	255.50	114.27
0.05	104	148.59	66.45
0.005	180	213.57	95.51
0.0005	183	186.20	83.27
0	104	148.59	66.45
***P. gingivalis* SAM @ µg/ml**	**pg/ml IL-6R Mean**	**Standard deviation**	**Standard error**
5	92	205.72	92
0.5	142	208.39	93.20
0.05	0	0	0
0.005	111	67.12	30
0.0005	56	73.69	32.96
0	52	65.34	29.22
***E. corrodens* LPS @ µg/ml**	**pg/ml IL-6R Mean**	**Standard deviation**	**Standard error**
5	234	230.21	102.96
0.005	128	175.27	78.38
***E. coli* LPS @ µg/ml**	**pg/ml IL-6R Mean**	**Standard deviation**	**Standard error**
5	184	270.70	121.06
0.005	128	286.21	128

Table 10. Levels of IL-6R released following macrophage stimulation.

4.1.5 GM-CSF (Granulocyte macrophage-colony stimulating factor)

Results of GM-CSF release by human macrophages are illustrated graphically in Figure 8 (raw data is presented in Table 11). GM-CSF release was stimulated by SAMs of *E. corrodens* and *P. gingivalis* and *E. coli LPS* but only at concentrations above 0.05µg/ml. At 5µg/ml concentration, EC SAM showed maximum stimulation, followed by PG SAM and *E.* coli LPS, with *E. corrodens* LPS showing no stimulation.

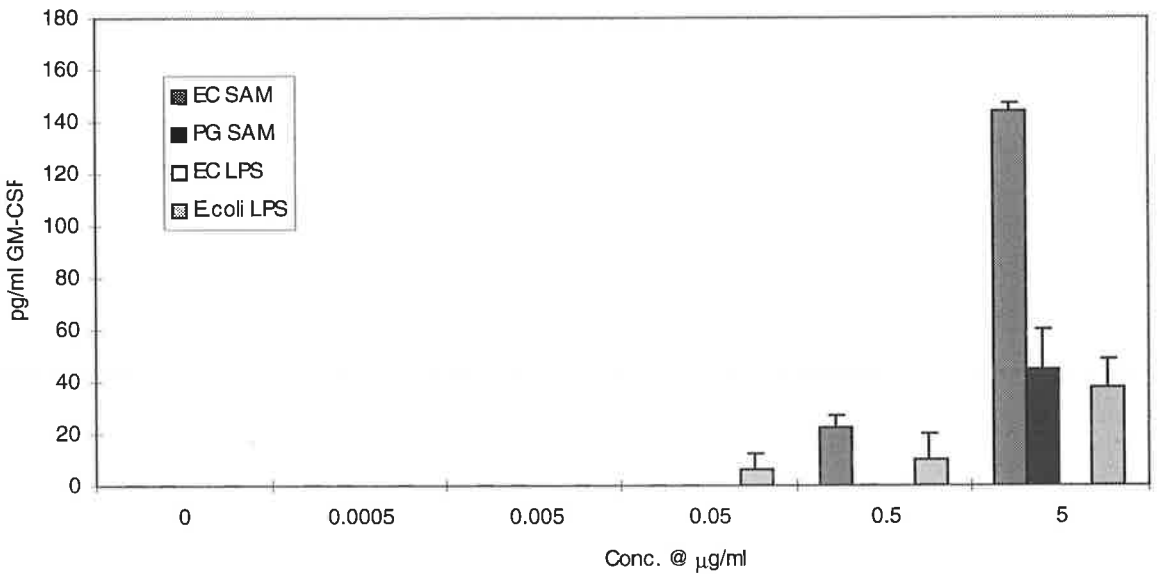

Figure 8: GM-CSF release following macrophage stimulation by EC & PG SAMs and *E. corrodens* and *E. coli* LPS. Results expressed as the mean and standard deviation of three different human macrophage donors.

E. corrodens SAM @ μg/ml.	pg/ml GM-CSF Mean	Standard deviation	Standard error
5	144	5.291	3.055
0.5	22	8.717	5.033
0.05	0	0	0
0.005	0	0	0
0.0005	0	0	0
0	0	0	0
P. gingivalis SAM @ μg/ml	pg/ml GM-CSF Mean	Standard deviation	Standard error
5	44.667	26.407	15.246
0.5	0	0	0
0.05	0	0	0
0.005	0	0	0
0.0005	0	0	0
0	0	0	0
E. corrodens LPS @ μg/ml	pg/ml GM-CSF Mean	Standard deviation	Standard error
5	0	0	0
0.5	0	0	0
0.05	0	0	0
0.005	0	0	0
0.0005	0	0	0
0	0	0	0
E. coli LPS @ μg/ml	pg/ml GM-CSF Mean	Standard deviation	Standard error
5	38	18.357	10.598
0.5	10	17.320	10
0.05	6	10.392	5.999
0.005	0	0	0
0.0005	0	0	0
0	0	0	0

Table 11. Levels of GM-CSF released following macrophage stimulation.

4.1.6 Heat and trypsin treatment of the SAM of *E. corrodens*

Results of stimulation of human macrophages by heat and trypsin treated EC SAM are illustrated graphically in Figure 9 (raw data is presented in Table 12). Heating the SAM of *E. corrodens* to 100°C for 1 h inhibited the IL-1β stimulating activity of EC SAM (Table 12). Exposure of EC SAM to trypsin for 1 h also inhibited its ability to stimulate IL-1β (Figure 9). Soya bean trypsin inhibitor is used to stop the reaction. However, it also stimulates IL-1β production, possibly resulting in falsely high IL-1β levels. This may account for the minimal stimulatory activity observed by the trypsin-treated SAM.

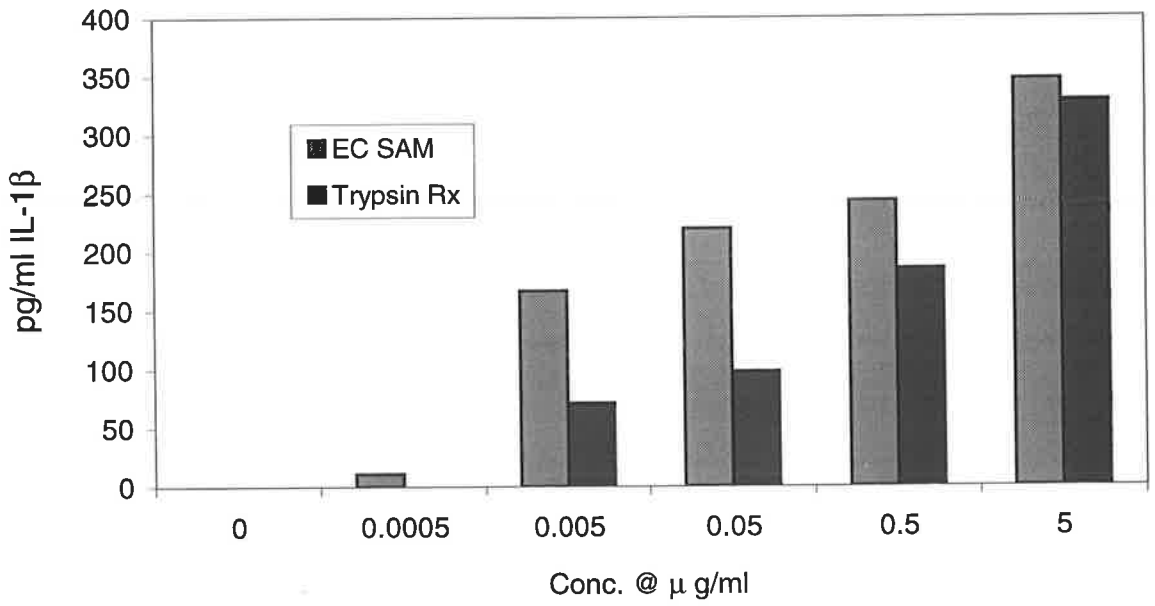

Figure 9: IL-1β release following macrophage stimulation by trypsin treated and un-treated EC SAM. Results expressed as the mean and standard deviation of three human macrophage donors.

E. corrodens SAM denatured @ µg/ml	% of *E. coli* LPS @ 5µg/ml
5	35
0.5	31.8
0.05	30.4
0.005	19.1
0.0005	9.5
E. corrodens SAM @ µg/ml	% of *E. coli* LPS @ 5µg/ml
5	127.5
0.5	44.1
0.05	40.4
0.005	38.9
0.0005	18.4

Table 12. Levels of IL-1β released following macrophage stimulation by heat treated and untreated SAM of *E. corrodens* as a% of IL-1β release by *E. coli* LPS at 5µg/ml.

4.2 EFFECT OF *E. CORRODENS* & *P. GINGIVALIS* SAM ON A 5 DAY COCULTURE SYSTEM

4.2.1 Resorption pits at 5 days

Dentine slices cocultured with ST2 and marrow cells, after five days of treatment with EC & PG SAMs had an average of 100 - 300 resorption pits per slice (Figure 10 & 11 and Table 13). Slices not treated with SAM (Control slices), had an average of 150 pits per slice.

EC SAM at 0.0005 and 0.05 µg/ml concentrations stimulated more resorption pits then PG SAM, but less at 0.005 µg/ml. More pits were there on the EC & PG SAM treated slices than on the control slices except for EC SAM at 0.005

µg/ml concentration. EC SAM at 0.05 µg/ml concentration stimulated resorption to produce an average of 285 pits per slice, and PG SAM 270 pits per slice. Generally, no major differences in the ability to stimulate formation of resorption pits was seen between EC & PG SAMs. Both SAMs produced the highest number of resorption pits at 0.05 µg/ml concentration.

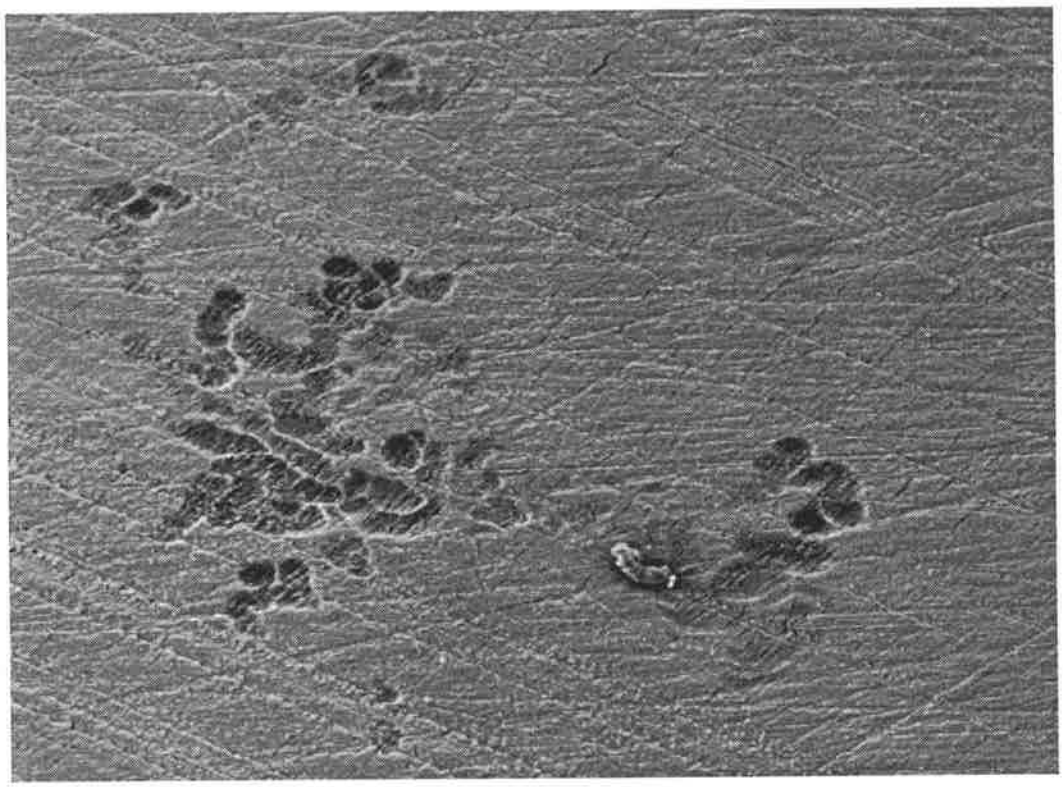

Figure 10: Resorption pits on dentine slices observed under the scanning electron microscope.

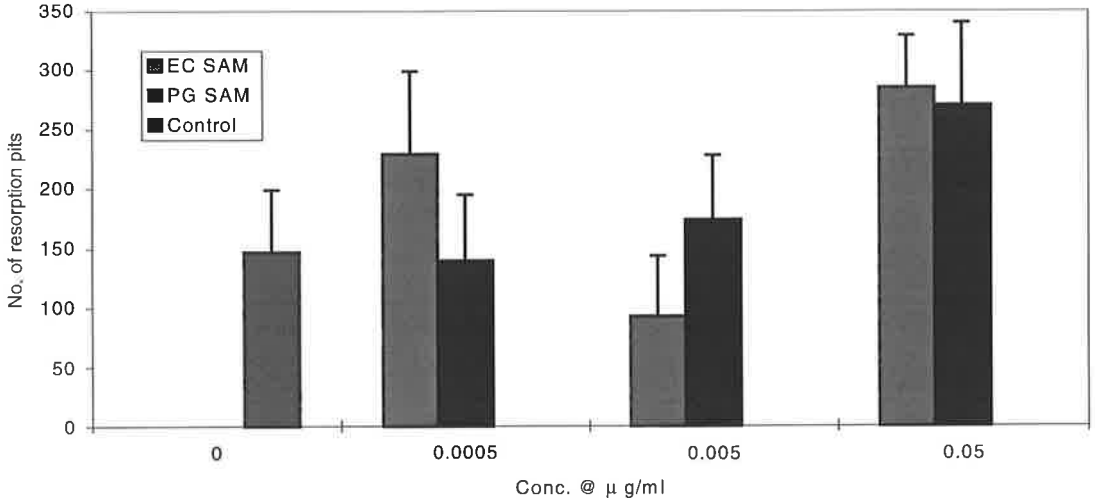

Figure 11: Effect of EC & PG SAMs on a 5 day coculture system of ST2 and mouse marrow cells in causing resorption pits. Experiments were performed in triplicate and results are expressed as the mean and the standard deviation.

Control	Mean (Pits)	SD	SE
0 µg/ml	147	91	51
EC @ µg/ml	**Mean (Pits)**	**SD**	**SE**
0.05	285	77	44
0.005	93	88	51
0.0005	229	191	70
PG @ µg/ml	**Mean (Pits)**	**SD**	**SE**
0.05	270	191	70
0.005	174	94	54
0.0005	140	95	55

Table 13. Effect of EC & PG SAMs in causing resorption in a 5 day coculture system of ST2 & mouse marrow cells

4.2.2 TRAP positive multinucleated cells at 5 days

EC & PG SAMs appeared to stimulate the formation of large TRAP positive multinucleated cells (Figures 12 & 13). 250 TRAP positive multinucleated

cells were seen on the control coverslip, while EC & PG SAMs stimulated the formation of a minimum of 375 cells (Table 14).

PG SAM was a stronger stimulator then EC SAM in the formation of TRAP positive multinucleated cells at concentrations of 0.005 μg/ml and above. EC SAM formed only 29% of the cells formed by PG SAM at 0.05μg/ml concentration. PG SAM was a strong stimulator of TRAP positive cell formation at a concentration of 0.05 μg/ml with a more than five fold increase in numbers of TRAP positive cells when compared to the control. It also showed a more than three fold increase when compared to EC SAM at the same concentration.

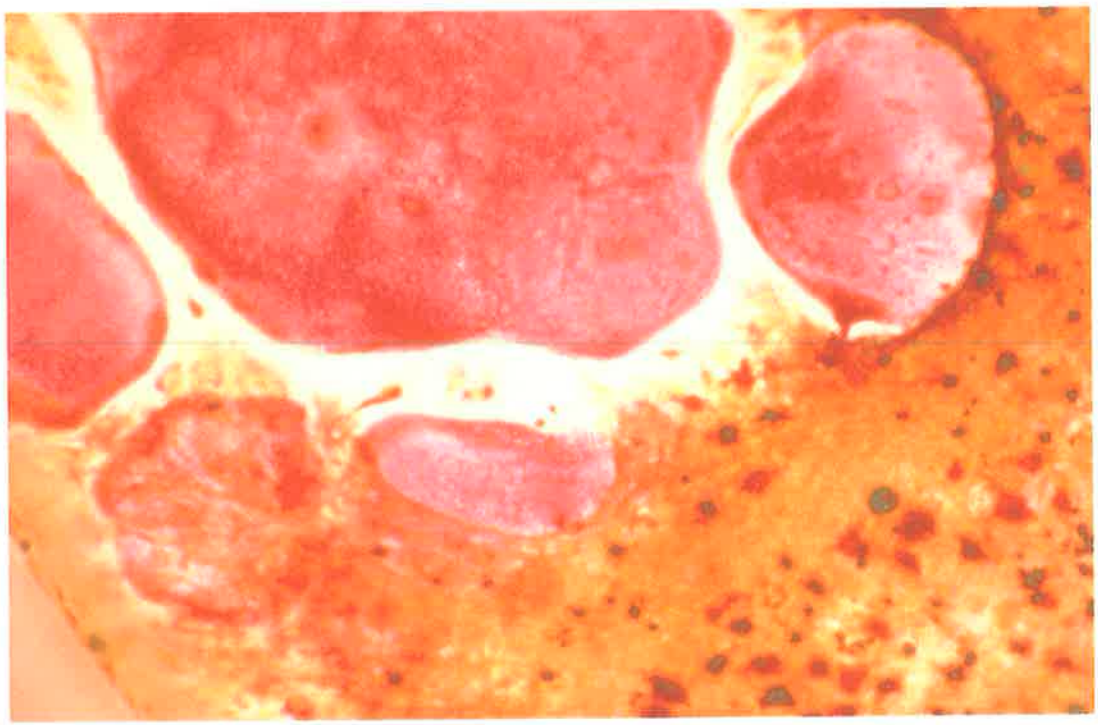

Figure 12: Light microscopic observation of red TRAP positive multinucleated cells on coverslips.

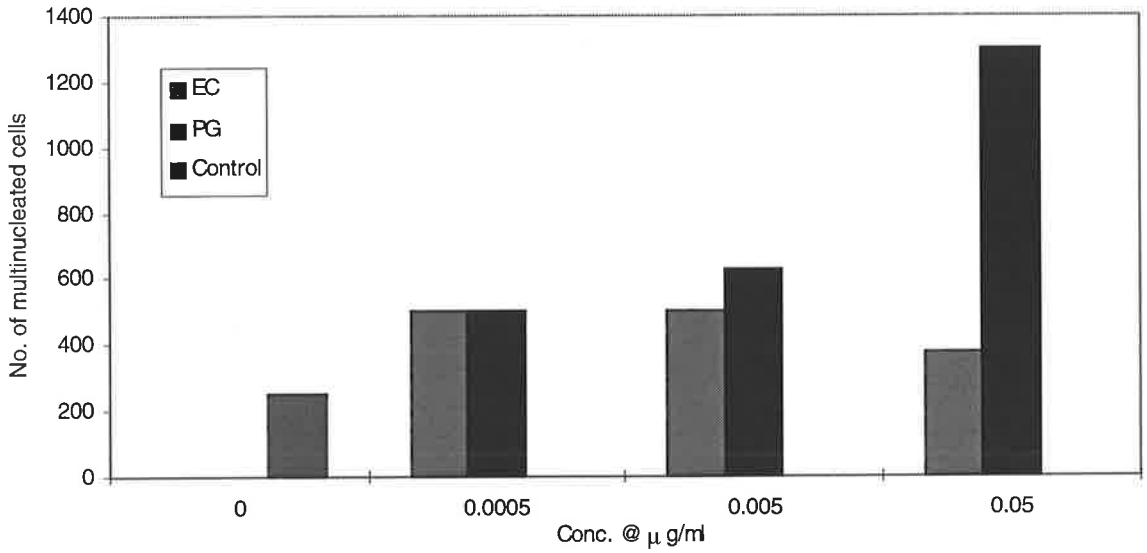

Figure 13: Effect of EC & PG SAMs in the formation of TRAP positive multinucleated cells in a 5 day coculture system of ST2 & mouse marrow cells.

EC @ µg/ml	No. of multinucleated cells
0.05	375
0.005	500
0.0005	500
PG @ µg/ml	
0.05	1300
0.005	625
0.0005	500
Control @ 0 µg/ml	250

Table 14. Effect of EC & PG SAMs on the formation of TRAP positive multinucleated cells in a 5 day coculture system of ST2 & mouse marrow cells.

4.3 EFFECT OF *E. CORRODENS* & *P. GINGIVALIS* SAMS ON A 14 DAY COCULTURE SYSTEM

4.3.1 Resorption pits at 14 days

Figure 14 shows resorption pits on a dentine slice cultured with EC SAM for 14 days. Control dentine slices untreated with SAMs had an average of 162 resorption pits per slice (Figure 15 and Table 15). The number of resorption pits for EC & PG SAMs treated slices for all time periods and concentrations showed averages below the control level.

Slices treated for 14 days with EC SAM, produced only 73 & 59 pits at 0.005 and 0.05 μg/ml concentrations respectively. Cultures treated with EC SAM from 0-7 days produced 96 and 88 pits, and 7-14 day treated cultures produced 156 and 154 pits at 0.005 and 0.05 μg/ml concentrations respectively. Treatment of the cocultured slices from 7-14 days with EC SAM resulted in formation of more pits than slices treated from 0-7 or 14 days.

PG SAM at 0.005 and 0.05 μg/ml concentrations stimulated the formation of 143 and 132 pits when exposed to the coculture slices from 7-14 days. Like EC SAM, PG SAM stimulated formation of more pits on treatment of cultures from 7-14 days (Figure 15 and Table 15).

Overall both EC & PG SAMs produced an inhibitory effect on the coculture system, producing less resorption pits as compared to the pits seen on the control slices.

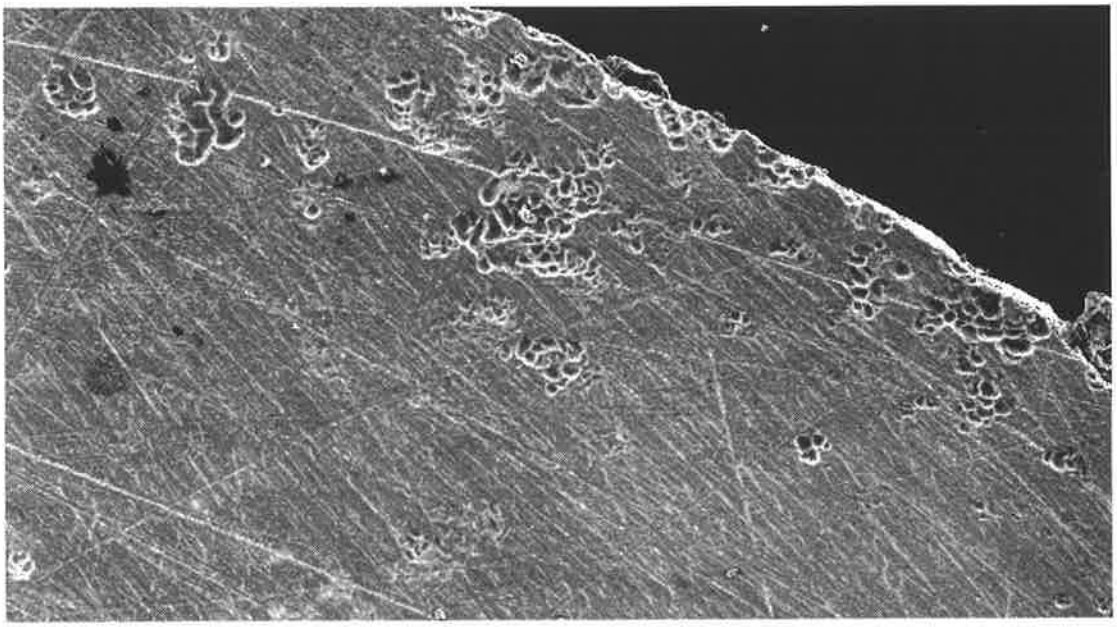

Figure 14: Resorption pits on an experimental dentine slice observed under the scanning electron microscope at 14 days.

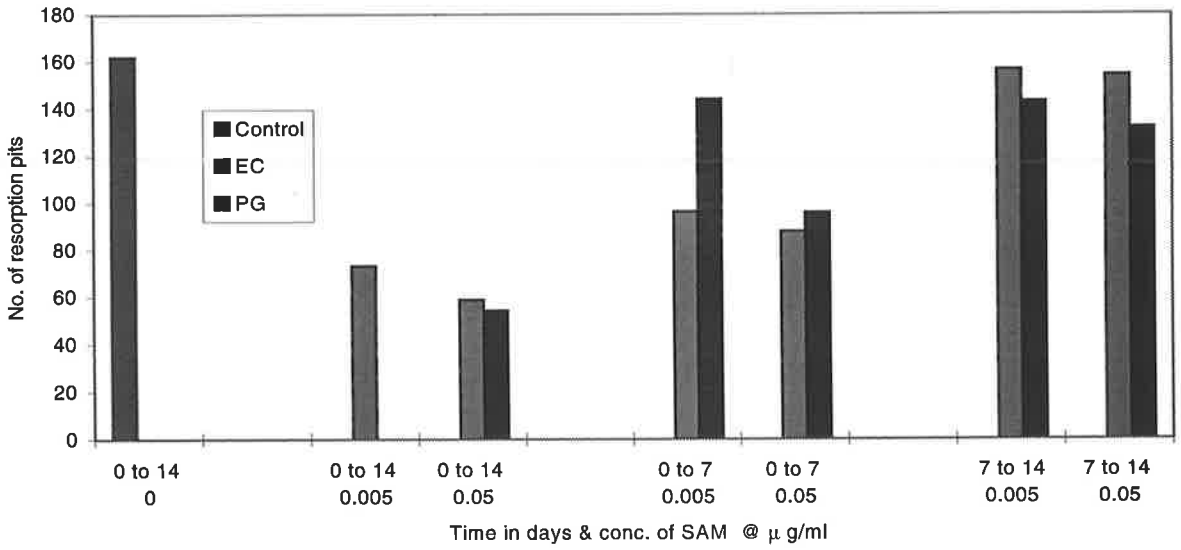

Figure 15: Effect of EC & PG SAMs on a 14 day coculture system of ST2 and mouse marrow cells in causing resorption pits. The time and period of exposure to the SAMs variable. Done in duplicate, results are expressed as the mean of the two values.

Control	Mean (Pits)	SD	SE
0 μg/ml	162	71.01	28.995
EC @ μg/ml for 14 days	**Mean (Pits)**	**SD**	**SE**
0.005	73.5	57.27	40.50
0.05	59	5.65	3.99
PG @ μg/ml for 14 days	**Mean (Pits)**	**SD**	**SE**
0.05	110.5	54.44	38.50
EC @ μg/ml from 0-7 days	**Mean (Pits)**	**SD**	**SE**
0.005	96.5	54.44	38.50
0.05	88	18.38	12.99
PG @ μg/ml from 0-7 days	**Mean (Pits)**	**SD**	**SE**
0.005	144	0	0
0.05	96	8.48	5.997
EC @ μg/ml from 7-14 days	**Mean (Pits)**	**SD**	**SE**
0.005	156.5	27.57	19.497
0.05	154	72.12	51.004
PG @ μg/ml from 7-14 days	**Mean (Pits)**	**SD**	**SE**
0.005	143	121.62	86.011
0.05	132	107.48	76.011

Table 15. Effect of EC & PG SAMs in causing resorption in a 14 day coculture system of ST2 & mouse marrow cells.

4.3.2 TRAP positive multinucleated cells at 14 days

Both SAMs strongly stimulated the formation of TRAP positive cells at all times (0-14 days, 0-7 days, or 7-14 days). Figure 15 shows TRAP positive multinucleated giant cells on coverslips after 14 days of coculture with EC SAM. At all time periods, EC SAM stimulated the formation of more TRAP positive multinucleated cells than PG SAM, and both SAMs produced more cells than the control slices (Figure 17, Table 16). Formation of an average of 50 TRAP positive multinucleated cells was seen on the control coverslips.

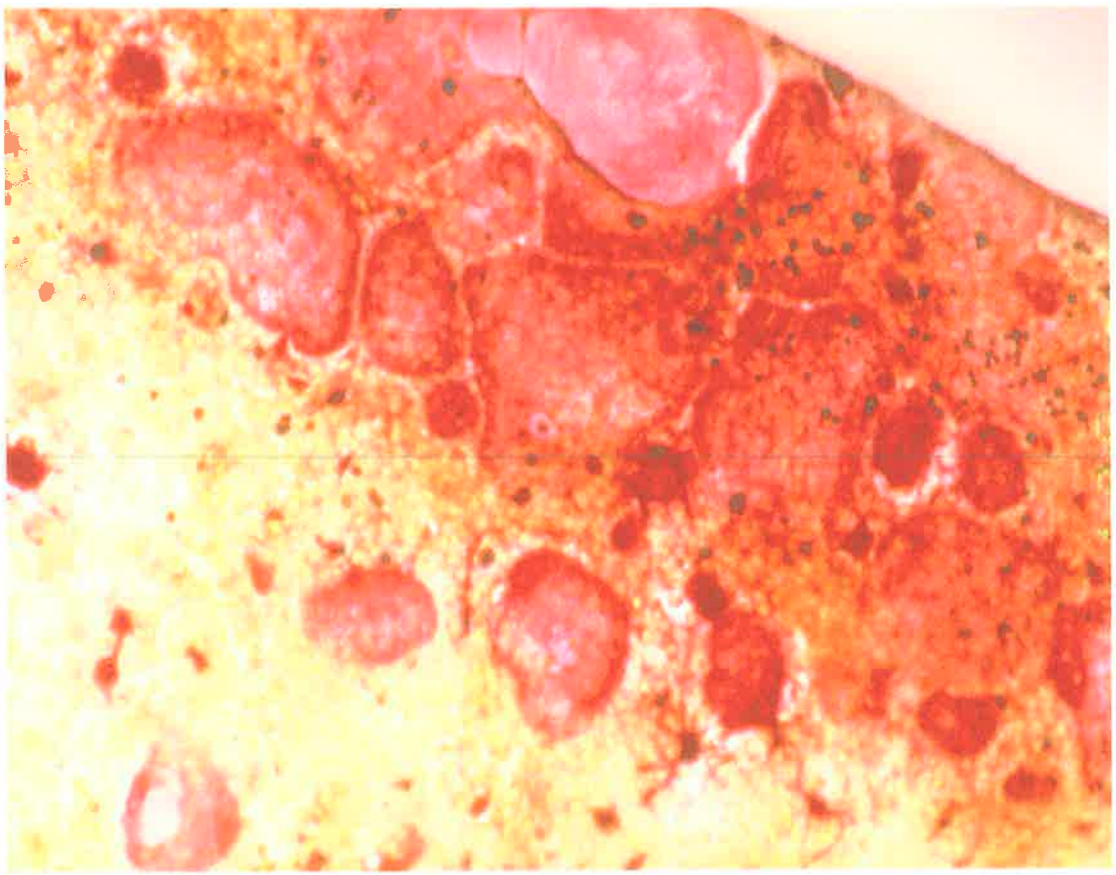

Figure 16: Light microscopic observation of red TRAP positive multinucleated cells on coverslips at 14 days.

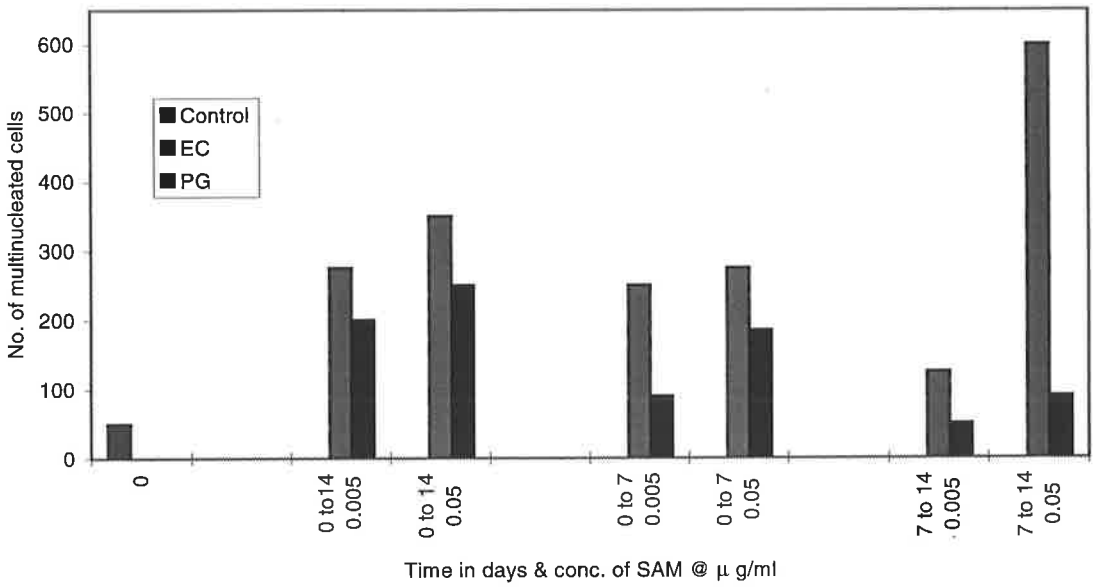

Figure 17: Effect of EC & PG SAMs in the formation of TRAP positive multinucleated cells in a 14 day coculture system of ST2 & mouse marrow cells. Done in duplicate, results are expressed as the mean of the two values.

	No. of multinucleated cells		
EC @ μg/ml	**Day 0-14**	**Day 0-7**	**Day 7-14**
0.05	350	275	600
0.005	275	250	125
PG @ μg/ml			
0.05	250	185	90
0.005	200	90	50
Control @ 0μg/ml	50		

Table 16. Effect of EC & PG SAMs on the formation of TRAP positive multinucleated cells in a 14 day coculture system of ST2 & mouse marrow cells.

4.4 EFFECT OF *E. CORRODENS* & *P. GINGIVALIS* SAMS ON CYTOKINE RELEASE BY HUMAN OSTEOBLASTS

No detectable (<2pg/ml) levels of IL-1β were found using ELISA assay. EC & PG SAMs strongly stimulated human osteoblastic cells to release IL-6 (Figure 18). EC SAM stimulated a release of 3000-3700 pg/ml IL-6, while PG SAM stimulated 6200-9500 pg/ml IL-6 both at concentrations of 0.005-0.5 mg/ml. PG SAM was a stronger stimulator of the release of IL-6 by human osteoblastic cells as compared with EC SAM.

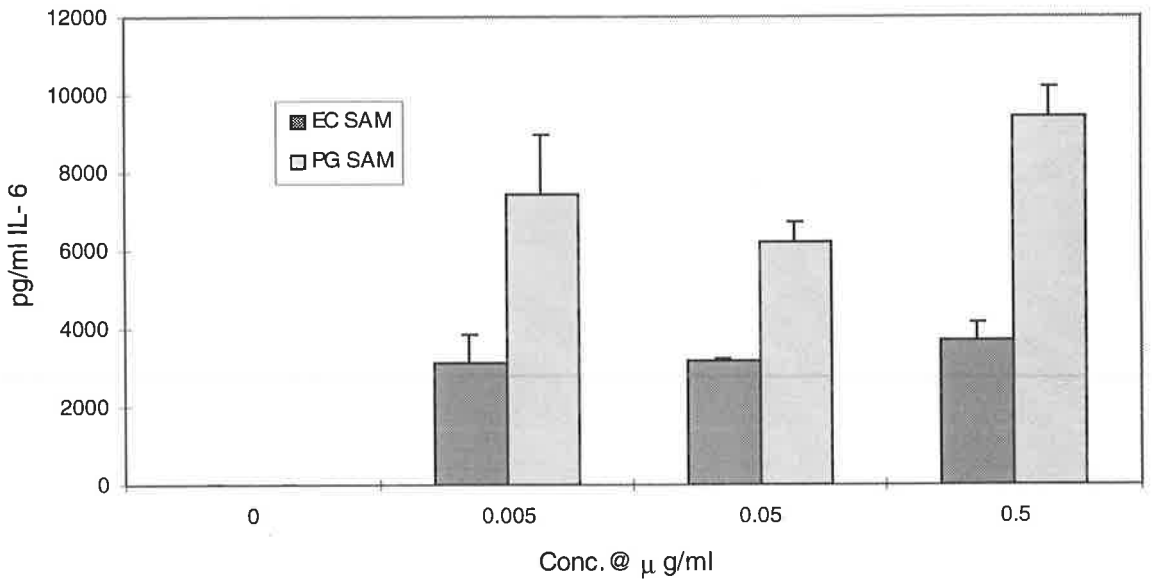

Figure 18: IL-6 release following overnight incubation of human osteoblast-like cells with EC & PG SAMs. Results are expressed as the mean and standard deviation of three different human osteoblastic donors.

EC & PG SAMs did not stimulate release of detectable levels of IL-1β (i.e. <4 pg/ml) from human OB-like cells.

When compared with the results from section 4.1, EC SAM stimulated more release of IL-6 from macrophages than osteoblasts at similar concentrations. The OB-like cells released approximately 50% of that produced by macrophages at 0.5 μg/ml concentration (Figure 19).

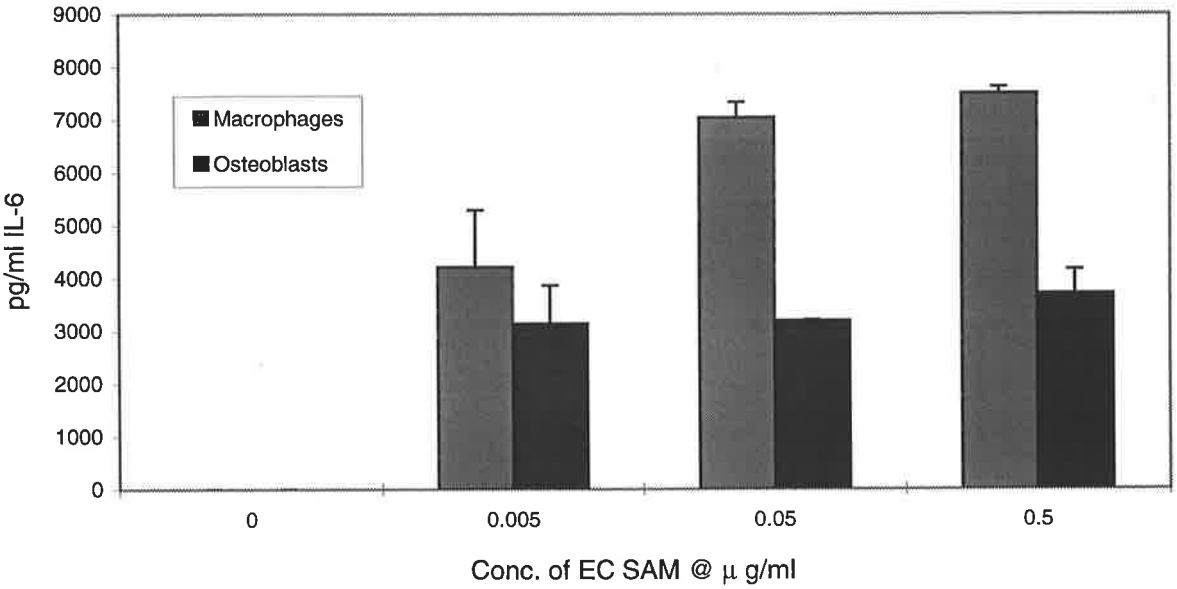

Figure 19: Comparison of macrophage and osteoblast-like cell IL-6 release after stimulation by EC SAM. Results are expressed as the mean and standard deviation of three different human osteoblastic cell donors combined and macrophages.

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CHAPTER 5 DISCUSSION

5.1 CYTOKINE RELEASE IN RESPONSE TO PERIODONTO-PATHOGENIC BACTERIA

5.1.1 Methods

The main organism under investigation in this study was *E. corrodens*. *P. gingivalis* was chosen as a second organism with which to compare the biological effects of *E. corrodens*. Although the prevalence of *P. gingivalis* in the subgingival plaque of a population of patients with early periodontitis has been found to be as low as 32%, many studies have implicated this organism as a high risk factor in periodontal disease (Wolff 1993)

In the current study, the SAMs from *E. corrodens* and *P. gingivalis* were extracted using the procedure described by Wilson *et al.* (1985, 1993) and Reddi *et al.* (1995). This is a simple saline extraction technique specific for the extraction of SAM.

E. corrodens LPS was extracted using the Darveau-Hancock procedure. According to Gerhardt *et al.* (1994), this method of LPS extraction removes both the rough and smooth forms of LPS present on the organism, as distinct from other methods that are specific for either the smooth or rough forms. LPS of periodontopathogenic bacteria has received considerable attention as a possible mediator of the tissue destruction accompanying chronic periodontitis. Furthermore, LPS was one of the first recognised and long established pathogenic entities in bone resorption. A highly purified preparation of *E. coli* LPS, used as an international standard in pyrogen assays, is one of the most potent cytokine-stimulating agonists known. Hence *E. coli* and *E. corrodens* LPS were used in this study as positive controls. Furthermore, to rule out the presence of LPS activity in SAM, both SAM and

LPS of *E. corrodens* were used, and their *in vitro* biological activities relevant to the pathogenesis of periodontal disease were studied.

Immunoassays used to measure cytokine levels are either developed or purchased as commercial ELISA kits. IL-1β, TNFα, IL-6, sIL-6R and GM-CSF commercial ELISA kits were purchased and used in this study as they are accurate, less time consuming, and have standardized values used universally.

5.1.2 Release of cytokines by *E. corrodens* and *P. gingivalis*

In this study, the SAMs extracted from *E. corrodens* and *P. gingivalis* were compared with respect to their capacity to stimulate human peripheral blood mononuclear cells to release IL-1β, TNFα, IL-6, sIL-6R and GM-CSF. The results were compared with those of the international *E. coli* LPS standard (NIBSC 84/650) and LPS extracted from *E. corrodens*. This permitted examination of the ability of the macrophage, one of the major cells present in the normal and inflamed periodontium, to be stimulated by the above bacteria to produce cytokines. Each SAM and LPS was investigated over a dose range in order to compare potencies and efficacies. To determine if the active component of *EC* SAM contributing to the cytokine-inducing activity was proteinaceous, this fraction was exposed to heat and trypsin in an attempt to denature the protein.

5.1.2.1 IL-1β release

E. corrodens SAM was the strongest stimulator of IL-1β release, followed by *E. coli* LPS, *P. gingivalis* SAM and finally by *E. corrodens* LPS. Nevertheless, at low concentrations, *E. coli* LPS was a stronger stimulator than *E. corrodens* SAM, with *E. corrodens* LPS and *P. gingivalis* SAM showing very little or no stimulation. This indicates that *E. corrodens* SAM was the most

potent stimulator of IL-1β release of the periodontopathogenic bacteria. In contrast, the LPS from *E. corrodens* was a very weak macrophage-stimulating agonist ,suggesting that LPS is not responsible for the biological activity observed in this organism. Even though *P. gingivalis* SAM was capable of stimulating the peripheral blood mononuclear cells examined to release IL-1β, its maximal response was around 30% of that produced by *E. corrodens* SAM. The *E. coli* LPS standard, considered universally as a potent inducer of cytokine activity, was the most potent stimulator of mononuclear cells at low concentrations, but was capable of a maximal response of only 62% of that produced by EC SAM at 5μg/ml. EC SAM can hence be considered as a highly potent agonist in IL-1β cytokine stimulation.

5.1.2.2 TNFα & IL-6 release

The potency of the SAMs and LPS in stimulating TNFα release was lower than their capacity to stimulate IL-6 release but was greater than their ability to stimulate IL-1β release. Again, the most potent in stimulating the release of TNFα & IL-6 was SAM from *E. corrodens.* Following in succession of potency for both cytokines was LPS from *E. coli,* followed by SAM from *P. gingivalis* and finally by LPS from *E. corrodens.* It was concluded that IL-6 may be released more easily by inflammatory cells than other cytokines such as TNFα and IL-1β.

5.1.2.3 sIL-6R release

E. corrodens SAM was the most potent stimulator of sIL-6R release at all concentrations. Nevertheless, these levels were extremely low and followed no discernible pattern. *P. gingivalis* SAM, *E. coli* and *E. corrodens* LPS produced vague and haphazard levels of release of this cytokine. These results appeared inconsistent with those found for IL-6, and further studies

might explain this discrepancy.

5.1.2.4 GM-CSF release

GM-CSF was released only at concentrations of 0.005, 0.5 and 5 μg/ml. *E. corrodens* SAM showed the highest potency, releasing three times the level of GM-CSF compared with *P. gingivalis,* at 5 μg/ml concentration. At the low 0.005 μg/ml concentration, only *E. coli* LPS was capable of stimulating GM-CSF release.

5.1.2.5 Effects of heat or trypsin treatment

Both heat and trypsin treatments reduced the IL-1β stimulating activity of the SAM of *E. corrodens,* indicating that the active cytokine-stimulating components in the SAM are largely proteinaceous in nature. The reduction of activity caused by trypsin treatment was less compared with that caused by heat treatment, which may be attributed to the soya bean trypsin inhibitor added to stop the activity of trypsin. The effects of the inhibitor alone on monocytes were tested. Results showed that the soya bean trypsin inhibitor stimulated IL-1β, indicating that the inhibitor may be responsible for the weak stimulation of IL-1β in the trypsin-treated SAM samples.

5.1.3 Cytokine release: Conclusions

The results of this study showed that the SAMs of *E. corrodens* and *P. gingivalis* had the capacity to stimulate peripheral blood mononuclear cells to produce osteolytic cytokines. *E. corrodens* SAM was clearly the more potent of the two at stimulating the release of mediators of chronic inflammation (IL-1β, TNFα, IL-6 & GM-CSF). As expected, the *E. coli* LPS was a potent stimulator of mononuclear cells, inducing the synthesis of all of the above

cytokines. The fact that the LPS of *E. corrodens* stimulated lower levels of cytokines compared to those of EC SAM may mean that constituents other than LPS are primarily responsible for the biological activity of EC SAM.

The only cytokine that showed low and variable results was sIL-6R. However, levels of IL-6 release were generally the highest amongst the cytokines studied, and one might have expected to find similar high levels of sIL-6R. Tamura *et al.* (1993) found that neither recombinant mouse IL-6 nor mouse sIL-6R induced OCL formation when they were added separately to cultures. Hence the effect of IL-6 on OCL formation might be reduced in the absence of high levels sIL-6R. More studies are required to investigate the role of sIL-6R in the response to periodontopathogenic bacteria.

Heat and trypsinisation both inhibited the IL-1β stimulating activity of EC SAM, indicating that the active cytokine-stimulating components in the SAM were probably proteinaceous in nature. Simple Amicon membrane filtration has demonstrated that the molecular weight of the active component of the *E. corrodens* SAM is >10kDa (Reddi *et al.* 1995).

In this study, concentrations of 0.0005 & 0.005 μg/ml of EC SAM were capable of releasing significant amounts of IL-6, TNFα & IL-1β, with IL-6 showing maximum stimulation at these low concentrations. Hence, extremely small numbers of EC bacteria may be capable of activating macrophages to play a role in producing chronic inflammation, and may also induce the bone destruction associated with the chronic inflammatory periodontal diseases. As these bacteria do not invade the periodontium in any appreciable number, it is likely that the stimulus to cytokine production is their solubilized components, which can diffuse into the gingiva and associated periodontal tissues and bind to different cells or infiltrating leukocytes (Liakoni *et al.* 1985).

PG SAM was also capable of stimulating osteolytic cytokines and, at certain concentrations, was shown to be similar to *E. coli* LPS. However, over the range of concentrations tested, it was clearly less potent than EC SAM in stimulating release from macrophages of all of the cytokines investigated.

In conclusion, the results of this study have indicated that EC SAM is a stronger stimulator of cytokine release than PG SAM. It can also be concluded that, even at very low concentrations (0.005 μg/ml), EC SAM is a stimulator of cytokine release. We also have strong evidence that the cytokine stimulating activity of EC SAM is due to a protein present in this fraction. This may be interpreted to mean that the mere presence of this organism would be capable of inducing a pathological response in the periodontium.

5.2 EFFECTS OF *E. CORRODENS* & *P. GINGIVALIS* SAM ON OCLs IN CULTURE

Reddi *et al.* (1995) examined the capacity of bacterial components loosely associated with the cell wall (such as SAM, LPS and LAPs) of the main periodontopathogens to induce bone resorption *in vitro*. These authors used a neonatal murine calvarial bone resorption assay and found that SAM from *E. corrodens* proved to be the most potent stimulator, releasing 50% of the maximum radiolabelled calcium release from mouse calvaria at a concentration of 1ng/ml. The concentration of SAM required to stimulate a >50% response was found to be less than 100pM which is within the potency range of the most potent known osteolytic cytokine, IL-1 (Gowen & Mundy, 1986). It was suggested that *E. corrodens* has an extremely potent osteolytic protein, or proteins, associated with its SAM which could play a major role in the bone destruction in chronic inflammatory periodontal diseases.

5.2.1 Bone resorption culture systems

To study the direct effects of EC SAM & PG SAM on OC, the selection of an appropriate assay system is important. Progress in understanding the molecular events that occur during OC differentiation and osteoclastic bone resorption has been difficult because OC are few in number, are fragile when isolated from bone, and are difficult to isolate because they are embedded in a calcified matrix. Furthermore, no OC cell lines are available. A variety of model systems have been developed to study OC and OCL *in vitro* and *in vivo*.

Bone organ culture systems have provided and continue to be useful bioassays for studying factors controlling osteoclastic bone resorption and, in some cases, OC formation. Furthermore, these organ culture systems have been useful for identifying new agents that stimulate bone resorption. In their study of EC SAM, Reddi *et al.* (1995) used the calvarial bone resorption assay and measured the release of radiolabelled calcium to determine osteolytic activity. The mouse calvarial assay system has been frequently used in the past. In this system, pregnant mice are injected with calcium-45, and the calvaria are dissected from the neonatal rodents. These bones are placed on membranes suspended on wire mesh and floated over a chemically defined media, to which various osteotropic factors are added. After an appropriate time period, the percent of calcium-45 released from the bone relative to the total amount of calcium-45 in the bone fragment is determined. However, since these organ cultures represent mixed cell populations, determination of whether a factor(s) acts directly on OC or indirectly through other cells present in the bone organ culture is problematic. Therefore, to study the direct effect of SAM on the formation and function of OCs, a culture system having mainly preosteoclastic cells or OCL seems desirable.

5.2.2 Coculture of mouse bone marrow and ST2 cells used in the current study

After the haemopoietic origin of OC was established, many investigators focused their attention on the haemopoietic lineage cells which are capable of differentiating into OC. In the current study, a co-culture system developed by Udagawa *et al.* (1990) was used, incorporating mouse bone marrow derived stromal ST2 (osteoblast-like) cells, and bone marrow mononuclear cells (OC precursors) isolated from tibiae of mice. In this system, ST2 cells are pre-cultured for 24 hours on sperm whale dentine slices and coverslips, to which bone marrow mononuclear cells are seeded and cultured for various periods of time in the presence of 1,25-$(OH)_2$Vit.D_3 and dexamethasone. After culture, the adherent cells on the coverslips are fixed and stained for TRAP, in order to establish OCL differentiation. The dentine slices were examined with SEM in order to count resorption pits, an indicator of OC activity.

In the present study, a 5 and 14 day coculture system was used, in order to give a basic estimation of the stimulatory effects of *E. corrodens* & *P. gingivalis* SAMs on both OC formation from marrow mononuclear cells, and their function. In the 5 day co-culture system, the SAMs and hormones 1,25-$(OH)_2$Vit.D_3 and dexamethasone were present in the media all the time. In the 14 day co-culture system, the SAMs were present in the co-culture media at the following time periods (1) Days 1-7, (2) Days 8-14, or (3) for the duration of the 14-day culture. The hormones 1,25-$(OH)_2$Vit.D_3 and dexamethasone were present in the medium for the entire 14 days. TRAP positive multinucleated cells present on coverslips gave an estimation of the stimulatory effects of SAMs on the formation of OCL, and the number of resorption pits gave an estimation of the effect of SAMs on the function of these OCL.

Analysis of results for the 5 day coculture system indicated that PG SAM was a stronger stimulator of OCL formation compared to EC SAM. It is interesting

to compare this with the results of the cytokine studies reported above, in which EC SAM showed greater stimulation of cytokine release. Nevertheless, in the 5-day system, both SAMs proved to have a stimulatory effect on OCL formation, with control cultures forming an average of 250 TRAP positive cells while both SAMs produced an average of 500 cells at 0.0005 μg/ml concentration.

With respect to the effect of the SAMs on the function of OCs in the 5-day culture, it was found that EC SAM was a stronger stimulator at 0.0005 & 0.05 μg/ml than PG SAM, although the differences between the effects of the SAMs was not large. The control dentine slices produced an average of 147 pits, which was equal to the average number of pits produced by PG SAM at 0.0005 μg/ml concentration and more than the pits produced by EC SAM at 0.005 μg/ml concentration. These results suggest that the SAMs were inducing OC formation, but probably not affecting their resorptive activity.

In the 14 day culture, the SAM of *E. corrodens* was clearly a stronger stimulator of OC formation at all concentrations. The control coverslips produced an average of 50 TRAP positive multinucleated cells, and the SAMs produced levels consistently higher than this.

Interestingly, the control dentine slices produced an average of 162 resorption pits, which was more than the maximum number of pits produced by both the SAMs. Hence, the SAMs did not appear to stimulate OC activity, and only caused an increase in OC numbers, at 14 days in this small sample. Comparison of the effects of SAM added at different time periods showed that maximum stimulation was seen when SAM was added at the 7-14 day samples (for both EC and PG SAMs).

5.2.3 Conclusions from mouse coculture studies

Whereas EC SAM consistently produced more OCL on coverslips, there was no indication of stimulatory effects on OC function in the culture system. This observation is in contrast to that of Reddi *et al.* (1995), who found higher levels of calcium-45 release attributable to EC SAM. It should be pointed out that the results of the current culture studies are preliminary, and more work needs to be done on the quantitation of the mouse coculture system, and the use of suitable sample numbers in order to test for statistical significance.

5.3 ROLE OF SAM-STIMULATED OSTEOBLASTS IN THE REGULATION OF OSTEOCLAST FORMATION AND FUNCTION

The interaction of OBs and OCs is essential for normal bone remodelling (Martin & Ng 1994). Although bone loss is caused by OCs, excess bone resorption associated with pathological bone conditions is thought to be mainly regulated by OBs. Receptors for PTH, $1,25(OH)_2VitD_3$ and many other local and systemic factors are found on OBs, and the stimulatory or inhibitory messages are subsequently passed onto OCs and preosteoclasts, regulating their formation, differentiation and function. Hence OBs have been considered to be an important cell that regulates OCs. In the current study, the stimulation of human OBs by the SAM of *E. corrodens* & *P. gingivalis* to induce the release of osteolytic cytokines was examined.

Human OB-like cells were treated with *E. corrodens* & *P. gingivalis* SAM and the levels of IL-1β and IL-6 release were studied. Results indicated that the OB released no IL-1β, but high levels of IL-6. Nevertheless, the level of IL-6 released by the OB was considerably lower than that released by macrophages at similar concentrations. This may be interpreted to mean that OB have some control in inducing IL-6 related bone resorption, but not IL-1β

induced resorption, while macrophages may play a more important role since they can be stimulated to release IL-1β as well as other osteolytic cytokines (Haynes *et al.* 1997).

In contrast to the results of studies described above using monocytes, it was found that *P. gingivalis* SAM was a more potent stimulator of IL-6 release from human OBs than *E. corrodens* SAM. In fact, *E. corrodens* SAM stimulated the release of less than half the amount of IL-6 released by *P. gingivalis* SAM at all concentrations. This observation may mean that specific bacteria have different effects on different target cells. Further studies need to be done using more samples of human OBs and examining release of the other osteolytic cytokines.

CHAPTER 6 CONCLUSIONS & FUTURE STUDIES

6.1 CONCLUSIONS

- The SAMs of *E. corrodens* and *P. gingivalis* have the capacity to stimulate peripheral blood mononuclear cells to produce osteolytic cytokines. EC SAM was the more potent of the two at stimulating the release of IL-1β, TNFα, IL-6 & GM-CSF.

- The fact that the LPS of *E. corrodens* produced minimal stimulation compared to that of EC SAM may mean that some constituent other than LPS is responsible for the observed biological activity of EC SAM.

- Heat and trypsinisation of SAM also indicated that the active cytokine-stimulating components in the SAM were largely proteinaceous in nature.

- Even at low concentrations of 0.005 μg/ml, EC SAM stimulated cytokine release. This may mean that low numbers of this organism present in subgingival plaque could be capable of inducing a pathological response in the periodontium.

- Although results of preliminary mouse coculture studies were somewhat equivocal, they indicated that the SAMs of both *E. corrodens* & *P. gingivalis* may directly stimulate OC formation and possibly function.

- Whereas bacterial SAMs only stimulated IL-6 release from OB-like cells, monocytes could be stimulated to release most of the osteolytic cytokines.

- Specific periodontopathic bacteria may induce different cytokine responses, and have different effects on the destruction of bone as seen in periodontitis. EC SAM would appear to be a more potent stimulator of

monocytes, while PG SAM may be a more potent stimulator of OB mediated bone resorption.

- In the future, bacterial cytokine-inducing components such as EC and PG SAM could be targets for the prevention and treatment of periodontal diseases. If one could determine which were the key components, then the possibility of immunization to prevent the induction of the periodontal diseases takes a step nearer reality.

6.2 FUTURE STUDIES

At the time of writing up this thesis, certain significant advances in the field of cytokines and osteoclastogenesis were published. Cell-to-cell interaction between OBs/stromal cells and OC progenitors is known to be essential for OCL formation. Recently Tsuda *et al.* (1997) purified and molecularly cloned an osteoclastogenesis-inhibitory factor identical to osteoprotegerin (OPG). OPG is a naturally occurring secreted protein with homology to members of the TNF receptor family (Simonet *et al.* 1997) and inhibits osteoclastogenesis by interrupting the cell-to-cell interaction (Yasuda *et al.* 1998). Administration of OPG *in vivo* inhibits osteoclastogenesis and associated bone resorption and blocks the pathological increase in OC numbers and activity seen in animal models that mimic osteopenic disorders in humans (Simonet *et al.* 1997).

Yasuda *et al.* (1998) further reported the expression cloning of a ligand for OPG from a complimentary DNA library of mouse stromal cells. The protein was found to be a member of the membrane-associated tumour necrosis factor ligand family and induced OCL formation from OC progenitors. Lacey *et al.* (1998) further explained how this TNF-related cytokine replaces the requirement for stromal cells, vitamin D3, and glucocorticoids in the coculture model of *in vitro* osteoclastogenesis. These authors stated that the OPG

ligand (OPGL) binds to a unique hematopoietic progenitor cell that is committed to the OC lineage and stimulates the rapid induction of genes that typify OC development. Yasuda *et al.* (1998) concluded that the membrane-bound protein is the osteoclast differentiation factor, a long-sought ligand mediating an essential signal to OC progenitors. Furthermore, Lacey *et al.* (1998) found that OPGL was also seen to directly activate isolated mature OCs *in vitro.* This suggests that OPGL is an OC differentiation and activation factor. The effects of OPGL are blocked *in vitro* and *in vivo* by OPG, suggesting that OPGL and OPG are key extracellular regulators of OC development (Lacey *et al.* 1998).

Future studies may examine the roles of these newer cytokines in pathological bone loss. Furthermore, new knowledge about OPG and OPGL will help us to broaden our ability to develop better *in vitro* culture systems that are more consistent in producing ideal number of OCs with consistent characteristics and function. Further characterization of OPG, OPGL, and their possible receptors will be beneficial, and the regulation of these factors may help us to find a way to control the excessive or lack of osteoclastic activity.

Although the results of the current study indicated a possible role for the bacterial SAMs in the direct stimulation of OC formation and function, further studies using larger sample numbers are necessary in order to establish statistical validity. Such studies using different concentrations and time variables would help us to better understand the effect of these periodontopathogens in periodontal disease. Furthermore, continuing studies may help to explain the relatively small amounts of sIL-6R detected in monocyte cocultures, especially in relation to IL-6 release.

Previous studies have established that signals promoting OC differentiation and activation act via receptors in OBs/stromal cells, suggesting that localized

activation of OBs/stromal cells leads to directed OC formation. In the current study, SAMs of E. corrodens and P. gingivalis stimulated osteoblast-like cells to release IL-6, but no IL-1β release was noted. It would be of interest to discover if any of the other osteolytic cytokines are released by these cells, and at what levels.

Most of the information we have to date on OC formation, differentiation, and function are from studies done on the mouse system. It is important to perform the same studies done on human OCs, as cells of different species may vary in their behaviour. Previous studies which have attempted to generate human OCs in long-term cultures have proven elusive. Although there have been a number of reports of human OC production in culture from haematopoietic precursors (Flanagan *et al.* 1992), results have been controversial due to doubts about the functional authencity of the OCLs formed. In the absence of a reliable means to generate human OCs *in vitro,* a common approach to the study of mature human OCs has been to source OCLs from giant cell tumours, or osteoclastomas (Horton et al. 1985). Recent work by Fujikawa et al. (1996) has established that human monocytes are capable of differentiating into osteoclastic bone resorbing cells *in vitro*, but only in the presence of rodent-derived stromal cells.

Developing methods for *in vitro* generation of human OCs would be a useful tool to examine various aspects of osteoclastogenesis, including the role of human stromal cells and cells of the osteoblastic lineage in generation of human OCs, identification of the human OC precursor cells, and identification of regulatory cytokines in human osteoclastogenesis. Application of the SAMs of the different periodontopathogens to human OC cultures would also provide more relevant data, and confirm results from the mouse system.

CHAPTER 7 REFERENCES

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