<u>University Of London.</u>

MECHANISMS OF BACTERIALLY INDUCED BONE

DESTRUCTION.

Submitted for the Degree of Doctor of Philosophy

By

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BACTERIALLY INDUCED BONE RESORPTION.

Abstract.

Bacteria implicated in bone diseases contain or produce molecules with potent effects on bone cells. These molecules and their actions on the cells and mediators orchestrating the homeostasis of bone have been the focus of the work presented here.

In the first study saline extraction of *S. aureus* yielded a proteinaceous stimulator of *in vitro* osteolysis and osteoclastogenesis. This osteoclastogenesis could be blocked with calcitonin. Immunoassay for 1,25-dihydroxyvitamin D_3 suggested osteolysis was not operating via upregulation of this hormone's synthesis. Osteoclastogenesis was unaffected by indomethacin and only partially blocked by the IL-1receptor antagonist. Neutralising antibodies to TNF and IL-6 inhibited osteoclastogenesis, as did 5-lipoxygenase (LOX) inhibition.

The finding that 5-LOX activity could modulate osteolysis instigated an investigation into a wider role for 5-LOX products in osteolysis. This revealed the osteolytic activities of several inflammatory agents and hormones to be unaffected by 5-LOX inhibition, however osteolysis and osteoclastogenesis induced by *A. actinomycetemcomitans* LPS was. It was also demonstrated that the *A. actinomycetemcomitans* LPS was able to stimulate *in vitro* production of 5-LOX products. The LPSs from other oral bacteria also proved able to stimulate osteolysis and induce osteoclastogenesis by processes that were inhibited by 5-LOX inhibition. Attention then focused on the effects of *A. actinomycetemcomitans* LPS on the expression of LOXs and cyclooxygenases (COXs) in human osteoblasts. This LPS increased the synthesis of several LOX products, however 5-LOX mRNA expression was constituitive and was not altered by exposure to LPS, except with high doses and long exposure times when it was downregulated. COX-2 and 12-LOX mRNAs were found to be induced on exposure to the LPS, whilst neither 15-LOX nor COX-1 mRNAs were detected.

Finally work moved to *M. tuberculosis* induced osteolysis. Of all the molecules tested only *M. Tuberculosis* cpn10 was found to be a potent stimulator of *in vitro* osteolysis and osteoclastogenesis, and to inhibit osteoblast proliferation. Studies with synthetic peptides of *M. tuberculosis* cpn10 revealed a single conformational unit, the flexible loop, encompassing its osteolytic activity.

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The pleasure and the privilege is mine.

CHAPTER ONE

INTRODUCTION.

1.1. Perspective.

Bacterial infection of bone has had a significant historical impact on society. *Mycobacterium* (*M.*) *leprae*, the causative organism of Leprosy (a condition associated with chronic bone destruction), provided Jesus Christ with an opportunity to demonstrate his divinity.

Figure 1. 1 Pott's' disease with resultant vertebral collapse (red arrow). The onset of tuberculous osteomyelitis is usually insidious. The infection is unrelenting, necrotising, and destructive of bone, cartilage and the surrounding soft tissue. The inflammatory necrosis may extend through the medullary and cortical bone, penetrate the periosteum and progress through the cartilage. Sinuses may tunnel from here to the soft tissue and drain through the skin.

M. tuberculosis, which is the causative organism of tuberculosis, causes horrendous deformity when it infects the spine (Figure 1.1). Vertebral tuberculosis, or Pott's disease (after Percival Pott's of Pott's fracture fame) is a fearsome sight and probably inspired Victor Hugo's characterisation of Quasimodo, the hunchback of Notre Dame.

Another organism *Treponema pallidum*, the causative agent of syphilis, also causes gross skeletal deformation. Staphylococci are another group of

bacteria which leave their mark on bone, causing osteitis and osteomyelitis. A recent biography of Joseph Goebels, the crippled Propaganda Minister of the Third Reich, suggests that his childhood osteomyelitis was the driving force behind his hatred for the Jews (Reuth, 1993).

The most common bacterially-induced bone pathology is found in a group of closely-related conditions known as the periodontal diseases (Figure 1.2). In the developed world these diseases are the most common chronic bacterially induced pathology.
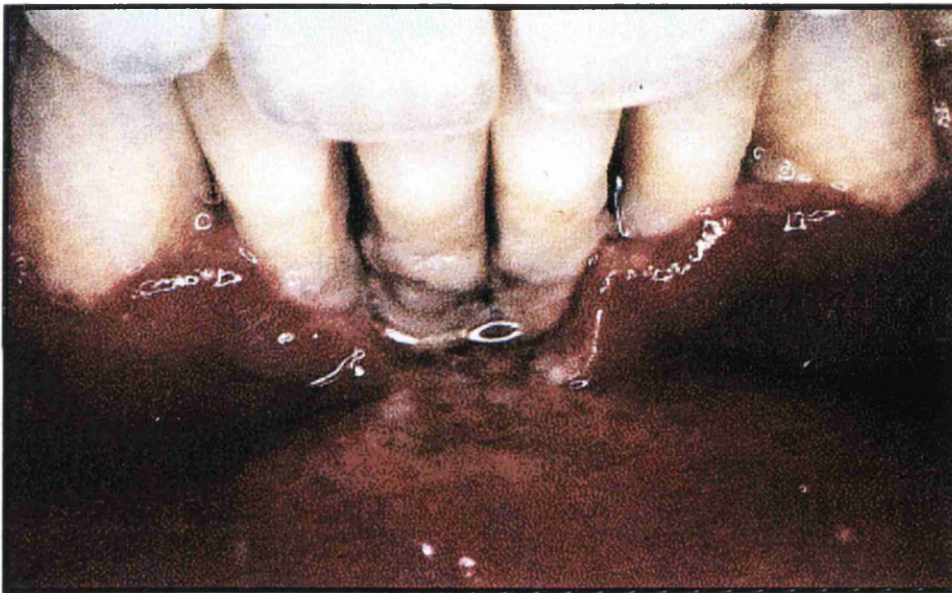
Figure 1. 2 A case of periodontal disease. The gingival inflammation, connective tissue destruction and loss of alveolar bone characteristic of this pathology are clearly visible.

Bacterially induced bone destruction continues to be a major cause of morbidity world-wide and with the rapid increase in the incidence of antibiotic resistant strains of bacteria is likely to become a bigger problem in the future; this is already the case for immuno-compromised individuals.

Very little is known about the mechanisms by which bacteria cause destruction of bone. The studies described in this thesis are part of an ongoing

work programme at the Eastman Dental Institute (EDI), University College London (UCL), aimed at understanding the interactions between bacterial components and bone cells. A number of bacteria and bacterial components have been examined in this study.

A common theme running through this thesis is the interactions between bacterial components and host lipidic mediators, particularly the eicosanoids. Workers at the EDI have long been involved in the study of the role of eicosanoids in bone remodelling and were the first to demonstrate that lipoxygenase (LOX) products are potent inducers of bone remodelling (Meghji *et al*, 1988).

1.2. Bacteria and bone pathology.

Table 1.1 lists some of the bacteria which are known to cause bone pathology. This list is by no means comprehensive, but gives some idea of the variety of organisms involved, and the range of diseases they cause.

The periodontal diseases are a family of related conditions attacking the supporting structures of the teeth (alveolar bone and periodontal ligament), culminating in the loss of these tissues and consequently the teeth. The alveolar process is defined as the parts of the maxilla and the mandible that form and supports the sockets of the teeth. Its loss leads to tooth loss, and therefore alveolar bone resorption is clinically the most important issue in periodontitis. Periodontal diseases are a consequence of the overgrowth by sub-gingival plaque-forming bacteria, which do not seem to invade the periodontal tissues (Freedman *et al*, 1968). Organisms such as *Porphyromonas* (*P.*) *gingivalis*, *Actinobacillus* (*A.*) *actinomycetemcomitans*, *Prevotella* (*Pr.*) *intermedia*, and

Eikenella (*E*) *corrodens* have all been implicated in the initiation and progression of periodontitis (American Association of Periodontology, 1996) (table1.1).

DISEASE	ORGANISM
PERIODONTAL DISEASE	*Actinobacillus actinomycetemcomitans*
	Porphyromonas gingivalis
	Eikenella corrodens
	Fusobacterium nucleatum
	Prevotella intermedia
	Campylobacter rectus
OSTEOMYELITIS	*Staphylococcus aureus*
	Staphylococcus epidermidis
	Salmonella spp.
	Escherichia coli
	Mycobacterium tuberculosis
BACTERIAL ARTHRITIS	*Staphylococcus aureus*
	Mycobacterium tuberculosis
	Neisseria gonorrhoea
	Neisseria meningitidis
	Haemophilus influenzae
	Pasteurella multocida

Table 1. 1 Bacteria involved in pathological bone remodelling and the diseases associated with them.

Osteomyelitis (an inflammation of the bone and bone marrow) is the generic term for bone infections. Unlike the periodontal diseases there is invasion of the tissue by the causative organism. The most serious bone infections are pyogenic osteomyelitis and tubercular osteomyelitis.

Pyogenic (pus forming) osteomyelitis occurs mainly in children at the metaphysis of the long bones, presumably because these regions are highly vascularised and so provide greater access to bacteria. In children the organism most often responsible is *Staphylococcus* (*S.*) *aureus*, which accounts for 60-90% of cases. In adults, osteomyelitis rarely occurs in the long bones, but more often involves the vertebrae, which remain highly vascularised after childhood. *S.*

aureus accounts for about 55% of adult cases, the remainder being due to Gram-negative bacteria and streptococci.

Tuberculous osteomyelitis is usually caused by the blood borne spread of bacteria from active foci of tuberculosis elsewhere in the body, usually the lungs. The bone infection may occur at any age, but is most often seen in children. Infection of the vertebrae (where it is recognised as Pott's disease) and the long bones are most common. The infection may also spread to the contiguous joint, such as the intervertebral joints, in Pott's disease.

The mechanisms by which these organisms initiate and drive the pathogenesis of bone disease are, however, incompletely understood. Nonetheless, several bacteria are known to produce a number of factors capable of inducing bone destruction. Broadly speaking, such bacterial molecules could be referred to as virulence determinants.

1.2.1. Osteoactive virulence determinants.

Since bacteria do not generally invade the periodontal tissues (Wilson, 1995; Wilson *et al*, 1996) the production of diffusible virulence factors is important to the current understanding of periodontal pathogenesis. It is proposed (Wilson, 1995; Wilson *et al*, 1996) that bone destruction is driven by the modulation of host mediator production by bacterial products. These bacterial products stimulate the inflammatory response, releasing cytokines, eicosanoids and other mediators of inflammation. An important element of this thesis is the investigation of how some of these factors affect the host production of eicosanoids, and what relationship this has to bone destruction. It also seems plausible that this mechanism may apply to other diseases, such as tubercular osteomyelitis, where

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invasion of the tissue does occur. Part of this work is concerned with identifying factors produced by the causative organisms of these conditions that may be involved in bone destruction, and again some consideration shall be given to the role of eicosanoids. The following section will consider some virulence determinants known to be osteoactive.

1.2.1.2. Endotoxin and bone resorption.
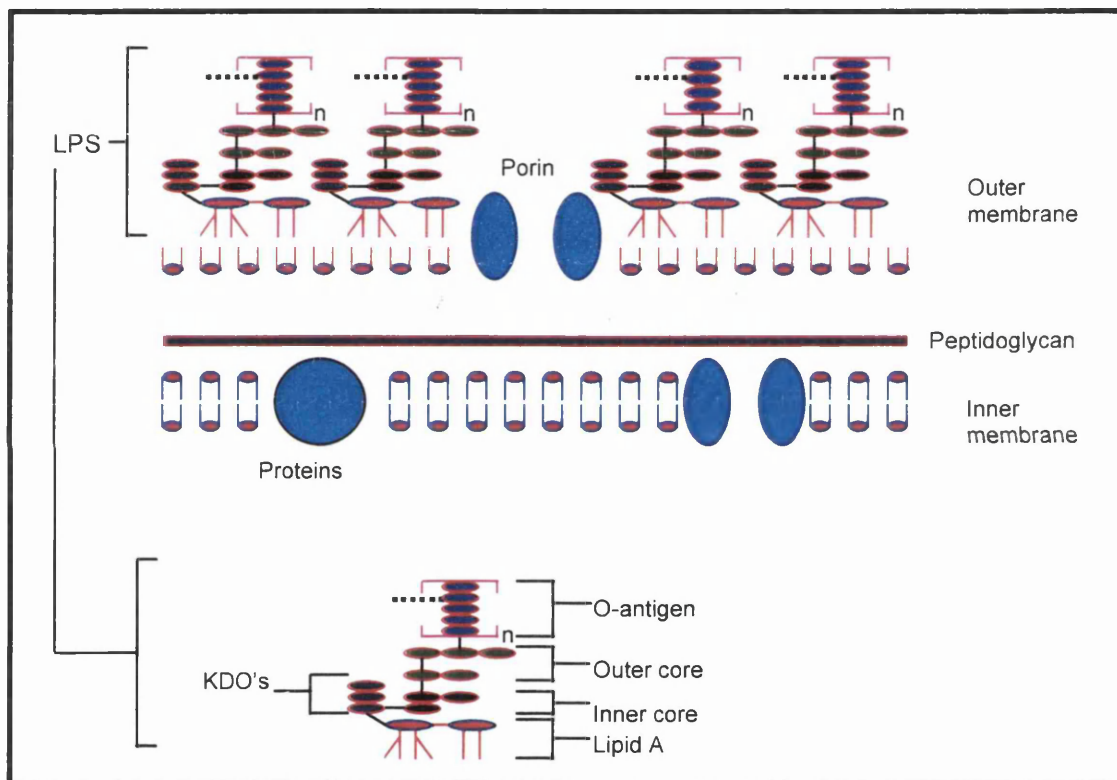
Figure 1. 3 Representation of a gram negative bacterial cell wall. LPS forms the outer leaflet of the outer membrane. Phospholipids make up the other leaflets of the inner and outer membrane. The inner LPS core is composed of two to three 3-deoxy-D-manno-octulosonic acid (KDO) and three heptoses, decorated with phosphate and phosphoethanolamine groups. The outer core usually contains hexoses. The O-antigen contains heptoses as tri to hexa-saccharides (the dashed line represents the bond joining repeating units), the number of repeats (n) is variable within the same culture, and can be up to twenty to thirty repeating units.

The most broadly characterised of these factors is endotoxin. This was first described by Pfeiffer in closing the years of the last century (Westphal *et al*, 1977). Endotoxin is situated on the surface of all Gram negative bacteria

including periodontopathic bacteria (Figure 1.3), and has been found in the sub-gingival region on the root surface of teeth, in a form easily removed by gentle agitation in saline (Wilson *et al*, 1986).

Lipopolysaccharide (LPS) consists mainly of polysaccharide, linked to a bioactive molecule called lipid A. It is generally free of protein and other constituents. Structurally, LPS is formed from three covalently linked distinct molecular structures (Figure 1.4). These are an O-specific chain, a core oligosaccharide (inner core and outer core), and the lipid A component.

Both the LPS and proteins of endotoxin have been well documented as pro-inflammatory agents and are known to have effects on bone. For example it has been known since the early 1970s that highly purified LPS's have the capacity to induce calvarial bone resorption (Hausmann *et al*, 1970, 1972, 1975 and 1978). However, the mechanisms by which LPS induces this bone resorption are unclear, although it seems likely that a direct action on the bone resorbing osteoclast is not involved (Sismey-Durrant *et al*, 1987). This is true for both oral and enteric LPS's. For example, the EDI group have tested *E. coli* LPS and others have tested *P. gingivalis* and *A. actinomycetemcomitans* LPS's in the murine bone marrow cell culture assay for osteoclast recruitment (Ueda *et al*, 1995; Reddi *et al*, 1998) and have failed to find a direct action.

LPS's are well known as being able to modulate the expression of inflammatory and osteolytic mediators by a wide range of cell types (Manthey *et al*, 1994). These mediators include interleukin (IL)-1 (Hanazawa *et al*, 1987; Keeting *et al*, 1991), IL-6 (Hanazawa *et al*, 1988), granulocyte-macrophage colony stimulating factor (GM-CSF) (Hanazawa *et al*, 1995), nitric oxide (Hanazawa *et al*, 1991) and various eicosanoids of the prostanoid family

(Ishihara *et al*, 1990). It has also been demonstrated that *P. gingivalis* LPS can stimulate the production of collagenase by osteoblasts, though not tissue inhibitor of metalloproteinase (TIMP) (Sismey-Durrant *et al*, 1989). It is not surprising then that many authors favour the hypothesis that LPS elicits its osteolytic effects via the osteoblast. Many of the mediators induced by LPS from osteoblasts can increase the proliferation and activity of osteoclasts

Work from the EDI has also established that the protein constituents of the endotoxin from oral pathogens, known as lipid A associated proteins (LAP's), can stimulate bone resorption and induce the release of osteolytic mediators such as IL-6 (Reddi at al, 1995a). Indeed LAP's proved more potent, than the corresponding protein free LPS's, in inducing bone resorption (Reddi *et al*, 1995a).

1.2.1.3. Fimbriae.

Fimbriae are surface structures of bacterial cells that have a role in adherence. Those from *P. gingivalis* have been found able to stimulate fibroblasts and monocytes to produce osteolytic cytokines (Hanazawa *et al*, 1988, 1991 and 1992). Recently, fimbriae have also been shown to cause murine calvarial bone cells to produce resorption pits in bone (characteristic of osteoclast activity). Production of these pits could be inhibited with neutralising antisera to IL-1 or GM-CSF (Kawata *et al*, 1994). It has been reported that fimbriae produce their osteolytic effects via the activation of cellular tyrosine kinase (Hanazawa *et al*, 1992).

There is evidence supporting the involvement of fimbriae in the bone loss associated with periodontal disease. Thus gnotobiotic rats immunised against *P.*

gingivalis fimbriae were protected from tissue destruction induced by *P. gingivalis* (Evans *et al*, 1992). Loss of the fimA gene, coding for the major fimbrial subunit (fimbrillin), blocked *P. gingivalis* induced bone destruction in gnotobiotic rats (Malek *et al*, 1994).

1.2.1.4. Capsular polysaccharide.

A capsular polysaccharide of *A. actinomycetemcomitans* has been shown to stimulate bone resorption by a prostaglandin (PG) dependent mechanism (Nishihara *et al*, 1995; Riancho *et al*, 1994). The induction of osteoclast differentiation induced by this polysaccharide is driven by IL-1α and PGE$_2$ but not by IL-1β or IL-6. Inhibitors of cyclic adenosine monophosphate (cAMP)-dependent kinases could inhibit this osteoclast proliferation.

1.2.1.5. Cell surface associated proteins.

There has been a long standing interest at the EDI in the bone modulating effects of proteins associated with the bacterial surface (Nair *et al*, 1995; Kirby *et al*, 1995). Examination of several osteolytic bacteria revealed most had significant amounts of protein on the external surface and this could be removed by gentle stirring of the bacteria in saline. Oral pathogens such as *A. actinomycetemcomitans* and *E. corrodens* released surface associated proteins (SAP's) which exhibited potent bone resorbing activity in the murine calvarial bone resorption assay (Wilson *et al*, 1985; Meghji *et al*, 1994; Wilson *et al*, 1995), whilst *P. gingivalis* SAP's were found to be one log order less potent (Wilson *et al*, 1993). The SAP's of *P. intermedia* and *Campylobacter* (*C.*) *rectus* were only very weakly active in the same assay (Reddi *et al*, 1995b). SAP's from Gram

positive organisms such as *S. aureus* and *S. epidermidis* have also been studied and these showed substantial osteolytic activity (Nair *et al*, 1995, Meghji *et al*, 1994).

1.2.1.6. Fibroblast activating factor.

P. gingivalis fibroblast activating factor (PGFAF), is a 24kDa protein secreted by *P. gingivalis*, which both promotes fibroblast proliferation (Mihara *et al*, 1993a), and *in vitro* bone resorption, with an accompanying increase in the numbers of cells expressing the widely accepted markers of osteoclast phenotype; positive staining for tartrate resistant alkaline phophatase (TRAP+ve) and multinuclear cells (MNC's). However no resorption pits were associated with these TRAP+ve MNC's (Mihara *et al*, 1993b). This may imply that the PGFAF, whilst able to promote osteoclastogenesis, is unable to activate the cells which it has caused to form.

1.2.1.7. Molecular chaperones.

An osteolytic protein on the surface of *A. actinomycetemcomitans* has been identified, at the EDI, as chaperonin (cpn) 60, a homologue of *E coli* GroEL (Kirby *et al*,1995). This molecule is a molecular chaperone, and such proteins are usually thought of as assisting in the assembly of other proteins (Figure 1.4). It was very surprising to find that it also had potent bone resorbing activity. When other bacterial cpn's were tested it was equally surprising to find the *E. coli* homologue, GroEL also had potent bone resorbing activity. The bone resorbing activity of *E. coli* GroEL could be inhibited with indomethacin, an inhibitor of prostaglandin production. In contrast to these cpn60's, the homologues from *M. tuberculosis* and *leprae*, known as heat shock protein (hsp) 65, could not induce

bone resorption. However the mycobacterial cpn60 co-chaperone, cpn10, could

and some of this work will be presented in this thesis (Meghji et al, 1997).
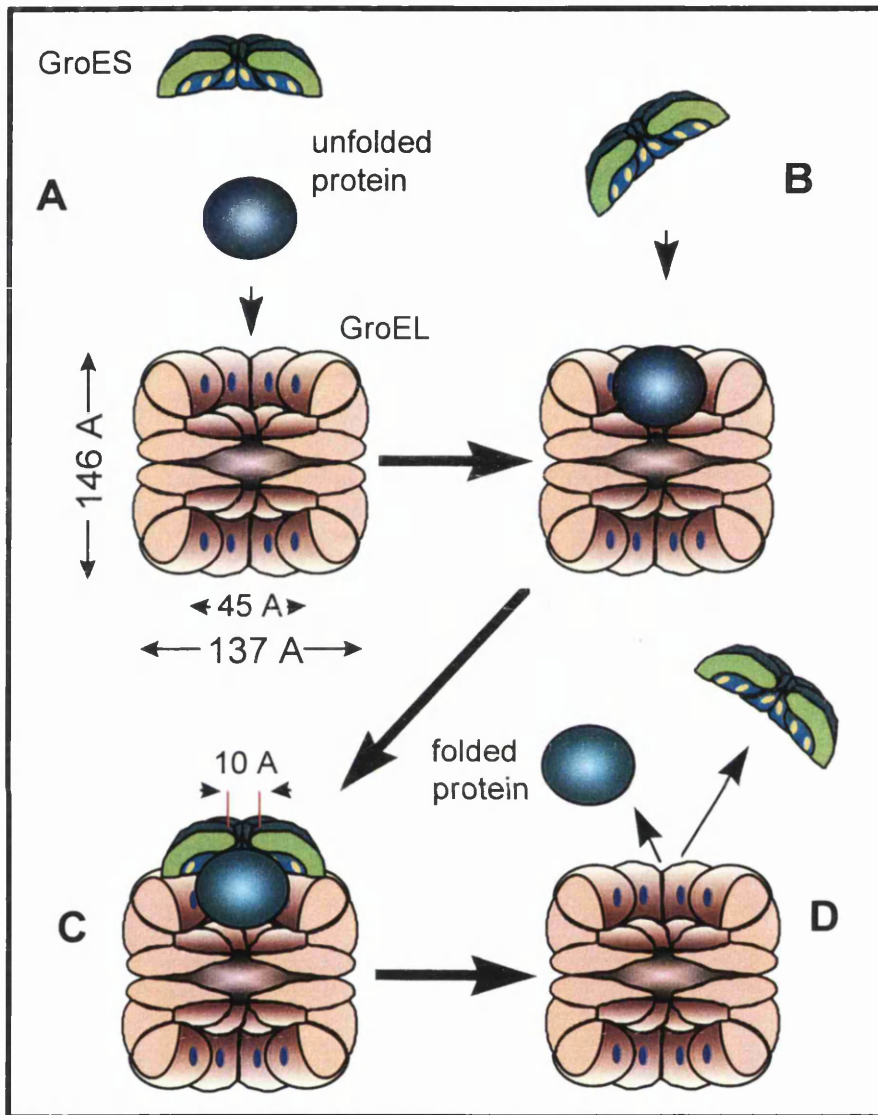
Figure 1. 4 The interaction between GroEL and its co-chaperone GroES during the folding of proteins.

1.3. Bone remodelling.

In order to understand how bacteria can induce bone destruction, the

processes by which bone turnover is normally regulated must be described. This

will include a description of the cellular constitution, structure and normal turnover

of bone.

In general terms, bone can be thought of as a fibre-reinforced matrix containing a sparse population of cells within the matrix and at its marrow cavity surface. This marrow cavity contains a wide variety of cells from which the myeloid and lymphoid cell lineages, found throughout the body in blood, lymph and tissues, are derived.

1.3.1. The osteoblast.

The osteoblast is the mature differentiated cell responsible for the formation and mineralization of bone matrix. Active osteoblasts cover the bone surface in a sheath of closely fitted cells (Figure 1.5).
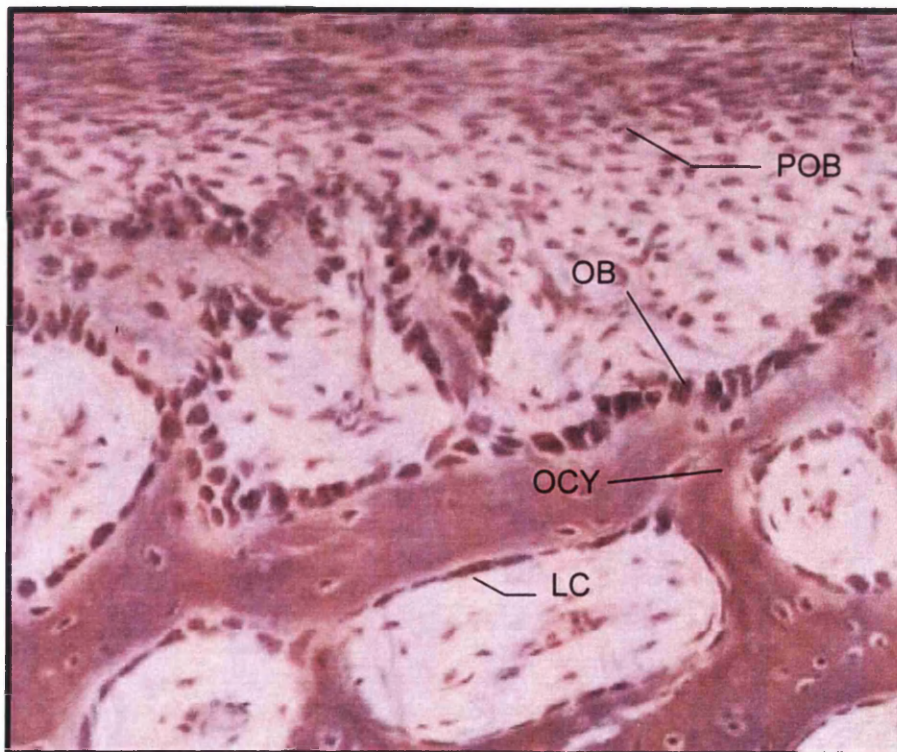
Figure 1. 5 Cuboidal Osteoblasts (OB) lining the bone with layers of preosteoblasts (POB) lying above them, osteocytes (OCY) are dotted through the bone. Elongated lining cells (LC) are also visible.

This sheath consists of a layer of cuboidal basophilic, polarised cells, with a prominent endoplasmic reticulum on the periosteal or endosteal surfaces, at sites of bone deposition.

These cells express high levels of alkaline phosphatase, type 1 collagen and non-collagenous bone matrix proteins. Immediately subjacent to the osteoblasts are additional layers of cuboidal cells, the preosteoblasts, expressing some but not all the above features. Osteoblasts not involved in the synthesis of bone matrix become very long and thin with numerous spaces between them. In this state they are commonly referred to as lining cells. They may become reactivated in fresh rounds of matrix deposition.
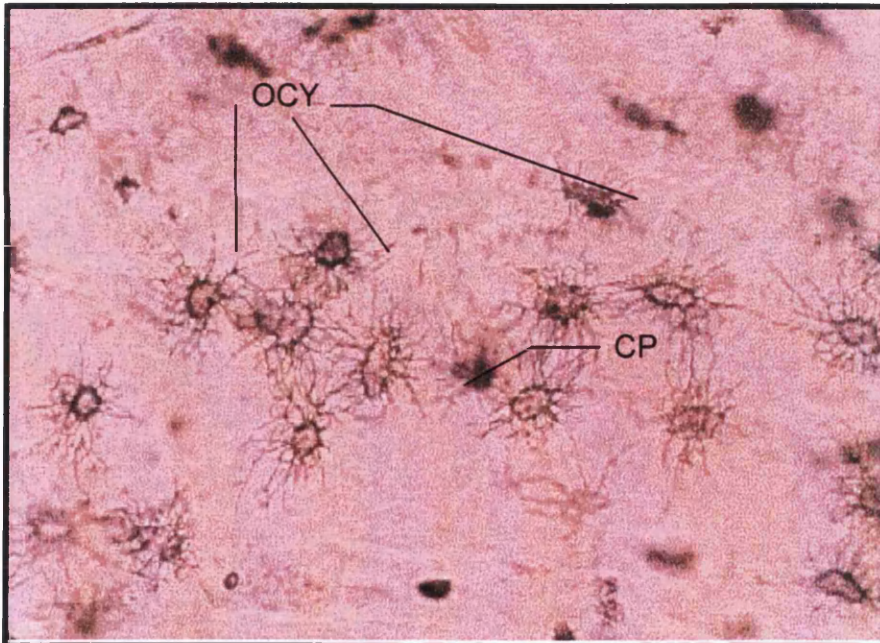
Figure 1. 6 Osteocytes (OCY) embedded within the bone matrix. Cytoplasmic processes (CP) can be seen protruding from the cells into the matrix and communicating with other cells.

The osteoblast exists in a third state, embedded in the mineralised matrix, and termed the osteocyte (Figure 1.6). The name changes from osteoblast to osteocyte when the bone forming cell becomes surrounded by mineralised matrix. Cytoplasmic processes can be seen extending from osteocytes and

communicating with other osteocytes. It is thought osteocytes function in: (i) maintaining bone mass (ii) the regulation of osteoclasts and (iii) the response to mechanical stress.

1.3.1.1. The origins and lineage of the osteoblast.

Osteoblasts represent a heterogeneous family of cells derived from the mesenchymal cells of the marrow stroma. It is from the stromal stem cells that subsequent committed osteogenic precursor cells arise. Osteoblasts share this precursor with adipocytes, reticular cells, fibroblasts, and chondrocytes (Owen *et al*, 1988; Gimble *et al*, 1996; Figure 1.7).
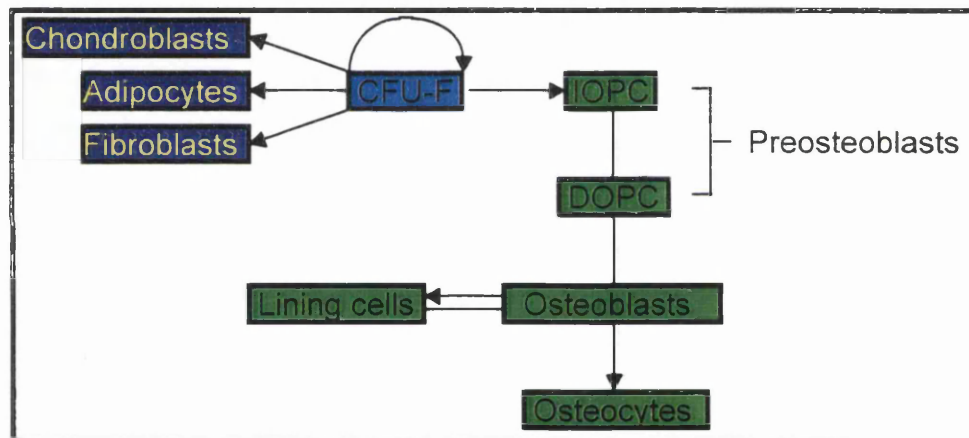
Figure 1. 7 Schema showing the relationship of osteoblasts to other cells in its lineage (Inducible/determined osteoprgenitor cell; I/DOPC, colony forming unit-fibroblast; CFU-F).

Preosteoblasts are recruited from this mesenchymal cell pool (Owen, 1967). These cells do not express any of the above mentioned markers of osteoblasts, but are committed osteoprogenitor cells capable of undergoing osteoblastic differentiation. Some may represent stem cells, though it has not been shown that these cells exhibit stem cell kinetics (an ability to undergo unlimited self-renewal). Some evidence suggests that the majority of the osteoprogenitor cells in bone marrow stromal cell cultures are limited in self-

renewal capacity (McCulloch *et al*, 1991; Kamalia *et al*, 1992; Bellows *et al*, 1990). Mature osteoblasts have a finite lifespan, only a small proportion becoming osteocytes (Owen, 1967). The osteoblast pool is replaced by the further differentiation of preosteoblasts.

1.3.1.2. Characterisation of the osteoblast.

The cellular heterogeneity of bone has been an impediment to the characterisation of the osteoblast. This obstacle has led to the use of isolated matrix free cells, as the major cell population to study (as has been done in this study).

Although the ideal standard for assessment of such cell preparations might be the ability to become osteogenic, measurement of calcification can be non-quantitative. Consequently, indirect assessment of the phenotype has been adopted.

Early attempts to characterise cells *in vitro* utilised histochemical methods on bone slices and the *in vivo* response to parathyroid hormone (PTH). An abundance of alkaline phosphatase in the osteoblast layer has been revealed histochemically, whilst inhibition of bone formation by high levels of PTH was observed. Thus despite its non-tissue specificity, and with little understanding of its role in bone, alkaline phosphatase expression is now generally regarded as a marker of the osteoblastic phenotype (Noda *et al*, 1987; Turksen *et al*, 1991). Similarly, PTH response has also become synonymous with the osteoblast phenotype in bone derived cells. PTH receptors have since been established to be present on osteoblasts (Rao *et al*, 1977; Beresford *et al*, 1984; Modrowski *et al*, 1993). Other molecules secreted by cultured bone explants, and subsequently

shown to be produced by isolated osteoblastic bone cells, have also become established as markers. These include collagenase and collagenase inhibitor, and matrix components such as type 1 collagen, osteonectin and osteocalcin. Amongst all these secreted products only osteocalcin is bone specific. However, together, these products represent a wide array of markers by which osteoblasts may not only be identified but also their position in the developmental pathway established.

1.3.2. The osteoclast.

It was Kolliker, in 1873, who first observed osteoclasts (Figure 1.8) and attributed to them the role of bone resorption. When observing osteoclasts by light microscopy they are recognisable by their size and shape.
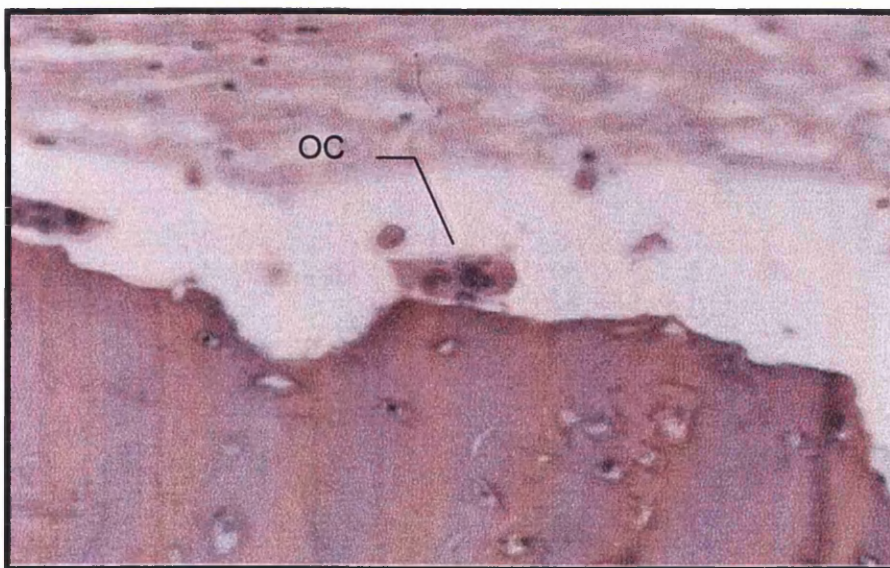
Figure 1. 8 Osteoclasts (OC) lying on a bone surface.

These cells are usually several times the size of osteoblasts, they are polymorphic and are usually found on the bone surfaces in actively remodelling sites, such as at the metaphyses of growing bones or adjacent to tumours. Osteoclasts are large multinucleated cells, 10 nuclei is the norm in healthy tissue.

The nuclei tend to be centrally placed and contain 1 or 2 nucleoli. Osteoclasts have primary lysosomes, numerous and pleomorphic mitochondria, and a specific area of the cell membrane that forms adjacent to the bone surface called the ruffled border.

The ruffled border is only present when the osteoclast is resorbing bone, it forms a system of cytoplasmic projections and represents an extended interface with the extracellular environment, allowing extensive exchange between cell and extracellular space.

1.3.2.1. The origins and lineage of the osteoclast.
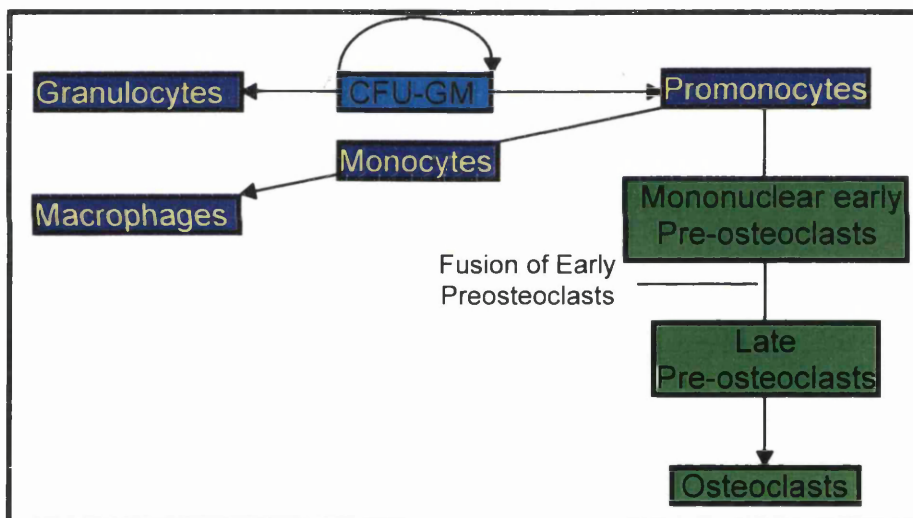
Figure 1. 9 Schema showing the relationship of the osteoclast to other cells in its lineage.

It is now generally agreed that the osteoclast is derived from a multipotent precursor shared with cells of the monocyte/macrophage lineage in the bone marrow (Kahn *et al*, 1975; Jotereau *et al*, 1978; Buring, 1975; Coccia *et al*, 1980) (Figure 1.9). The mononuclear progenitor cells are recruited from the hematopoeitic tissues to bone via the blood stream, these then proliferate and differentiate into multinucleate osteoclasts via a process involving the fusion of

mononuclear early preosteoclasts to form multinucleate, but inactive, late preosteoclasts. The final stage of differentiation results in the formation of a ruffled border and activation.

1.3.2.2. Characterisation of the osteoclast.

Since osteoclasts are difficult to obtain or culture and there is considerable species variation in their behaviour, each new osteoclast model system has been greeted with some degree of scepticism.

Some of the features that aid in identification are an ability to resorb bone, the ability to form a ruffled border, contraction of the cytoplasm on exposure to calcitonin, the presence of calcitonin receptors, cross-reactivity with osteoclast specific antibodi;s (though the absolute specificity of such antibodies has not been shown), appropriate responses to calciotropic hormones and absence of Fc receptors.

The 23c6 antibody to vitronectin has been widely used as a marker for osteoclasts (Horton *et al*,1984,1985a and1985b; Flanagan *et al*, 1988, 1989 and 1992) and can be regarded as having been very useful. However, although it is very heavily expressed on the osteoclast compared to other multinucleated giant cells (such as the macrophage polykaryon) it is not specific to the osteoclast (Flanagan *et al*, 1992). The presence of calcitonin receptors has also elicited much interest, seeming to be unique to the osteoclast in the marrow microenvironment (Owens *et al*, 1996). The presence of tartrate resistant acid phosphatase has been a very helpful marker, but this does not unfailingly distinguish osteoclasts from macrophage polykaryons (Yan *et al*, 1972; Snipes *et al*, 1986; Bianco *et al*, 1987; Hattersley *et al*, 1989). Responsivity to calciotropic

hormones also suffers from not being specific to the osteoclast. Taken alone each of these characteristics are not very useful in the identification of osteoclasts, however in combination they represent reasonable indicators of the osteoclast phenotype.

1.3.3. The bone matrix.

A tissue may be defined as a collection of specialised cells and cell products which mutually organise into well defined structural patterns to fulfil specific functions. By this definition bone falls into the category of a connective tissue, a large part of which is extracellular matrix.

1.3.3.1. Collagen.

Bone is comprised of mainly type 1 collagen which belongs to the fibril forming class 1 collagens (types I, II, III, V, and XI). Class I collagens are the products of eight separate genes, they are similar in size, comprising a single uninterrupted triple helix of three α chains, each about 1015 amino acid residues in length. Type 1 collagen constitutes about 90% of the bone matrix. It is a complex molecule, consisting of a heterodimer of two pro-α1 and one pro-α2 polypeptide chains. Type 1 collagen, like all the fibril-forming collagens, forms an uninterrupted triple helix which is hydroxylated at proline and lysine residues. The hydroxylysine residues are also glycosylated. The formation of the triple helical tertiary structure renders collagen resistant to most proteases at physiological temperatures.

Electron microscopy of collagen fibrils shows bone type 1 collagen molecules are organised in a characteristic staggered arrangement with gaps between the ends of molecules, these gaps are called "end holes" and

mineralization occurs here. Specific interactions occur between collagen and other extracellular molecules such as osteonectin, fibronectin and proteoglycans. Once the alignment of the collagen and other macromolecules occurs, the mineralization process can be initiated.

1.3.3.2. The mineral phase.

Unique among mammalian tissues the formation of bone involves the biological mineralization of the bone proteins to form a mineralised matrix. Following the production of osteoid (formed of non-mineralised matrix proteins) by osteoblasts, the osteoid tissue is mineralised in a controlled manner by the dispersion of mineral within the matrix. Initially, distribution of mineral is within the end holes of collagen as amorphous calcium phosphate. X-ray diffraction reveals this phase to be random and poorly ordered. After this initial deposition a series of solid phase transformations occur that lead to crystalline hydroxyapatite as the final stable solid state.

1.3.3.3. The non-collagenous proteins.

A series of non-collagenous proteins have been isolated from bone, some of which have important biological activities.

1.3.3.3.1. Bone gla protein (osteocalcin).

Osteocalcin is a 6 kDa protein containing three γ-carboxyglutamic acid residues, which enable it to bind calcium (Gallop et al, 1980). It accounts for 20% of the non-collagenous protein content of bone. The γ-carboxylation is a post-translational modification and is vitamin K dependent. Osteocalcin is made exclusively by osteoblasts but is not constitutively released. Osteoblastic cells

produce it in response to 1,25-dihydroxy vitamin D3. Osteocalcin's function is unknown, but it may function to retard mineralization (Poser *et al*, 1979). Osteocalcin is also a chemoattractant for osteoclast progenitors, attracting them towards bone surfaces. Paradoxically, rats treated with warfarin, a vitamin K antagonist, have essentially no osteocalcin in their skeleton yet have no detectable bone abnormalities.

1.3.3.3.2. Osteonectin.

Osteonectin is a 32 kDa acidic glycoprotein, purified from bone matrix. It is the most abundant non-collagenous protein in bone and is synthesised by osteoblasts in bone, odontoblasts in teeth, fibroblasts in skin and in tendon by tendon cells. Its function has not been identified, although it is phosphorylated, glycosylated, highly cross-linked and it binds avidly to both collagen and mineral surfaces and promotes, in vitro, mineral and crystal growth(Termine *et al*, 1981).

1.3.3.3.3. Cell attachment proteins.

The bone matrix contains a number of different cell attachment proteins with the common RGD (arg-gly-asp) motif, which is responsible for mediating attachment to specific integral membrane proteins (integrins) on cell surfaces. These matrix cell attachment proteins include fibronectin, osteopontin, thrombospondin and several sialoproteins.

Osteopontin binds the $\alpha v \beta 3$ integrin receptor on osteoblasts by its RGD sequence. Osteopontin also contains calcium binding sites. It is synthesised by both osteoclasts and osteoblasts. The protein is, itself, highly phosphorylated and exists in multiple forms, due to alternative splicing and post-translational variations in the degree of phosphorylation. Fibronectin is an ubiquitous cell

attachment protein made locally by bone cells and imported to bone by the vasculature. It is not known if it has a special function in bone. Thrombospondin, a trimer of approximately 450Kda, in addition to an RGD motif also contains calcium binding sites, and mediates cell attachment but has no known bone-specific function.

1.4. Remodelling of bone.

Bone turnover takes place throughout life in the process known as normal bone remodelling. The continuous renewal of bone, through the remodelling process, involves both resorption and formation. This requires the concerted activity of both osteoclasts and osteoblasts. The concept that bone formation and resorption are coupled during the remodelling process is over 30 years old (Frost, 1964; Harris et al, 1969). It postulates that resorption, which occurs to meet either the organism's calcium requirements or the tissue's mechanical needs initiates bone formation, which, under balanced conditions, restores the lost bone. This concept is supported by kinetic studies which have shown that changes in the rate of resorption are matched by changes in the rate of formation (Harris et al, 1969; Prestwood et al, 1994). There is also supporting histological evidence, which shows osteoclastic resorption and osteoblastic formation of bone to be contiguous (Frost, 1964). This "coupling" has been amply confirmed as a characteristic of normal bone remodelling (Baron et al, 1984).

1.4.1. Sequence of events in remodeling.

It follows that if remodelling is coupled, then it must also occur in a regulated sequence (Figure 1.10). It has been proposed that remodelling starts at the bone surface, where lining cells in response to initiation signals, e.g. PTH,

release collagenase (Partridge *et al*, 1964) which digests the thin layer of osteoid covering the mineralised matrix. In this way the mineralised matrix becomes accessible to osteoclasts (Chambers *et al*, 1985a and b).
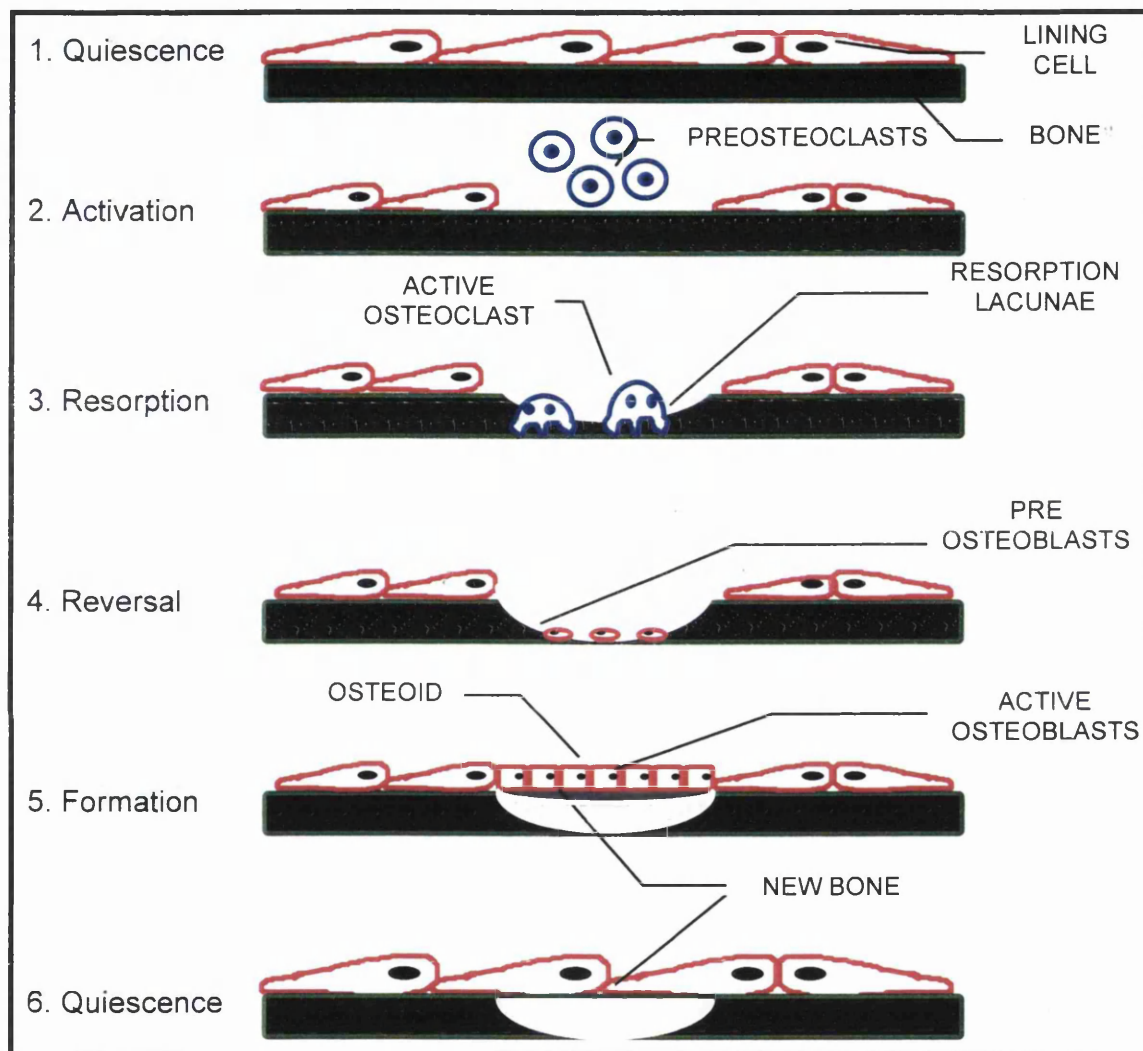
Figure 1. 10 The normal remodelling sequence in adult bone.

Interaction between osteoblast lineage cells and osteoclast lineage cells causes the latter to proliferate and become active osteoclasts (Chambers, 1980; Rodan *et al*, 1981). These newly formed osteoclasts then commence the resorption phase (Eriksen *et al*, 1986).

Towards the end of resorption, pre-osteoblasts appear on the resorption surface. These differentiate, possibly stimulated to do so by the release of

osteogenic stimuli from the matrix by osteoclast activity, and bone formation is initiated. This phase is called reversal -the plane of reversal can be identified microscopically. The osteoblasts form a contiguous layer and, as formation approaches completion, the osteoblasts become flatter and the osteoid surface thinner, until a flat layer of lining cells covers a very thin layer of osteoid on the now quiescent bone surface. Coupling of bone resorption has been taken further with the proposal that stimulation of osteoclastic activity, by most agents, is mediated by osteoblast lineage cells. This is based on observations of hormone action on osteoblasts and osteoclasts (Chambers, 1980; Rodan *et al*, 1981; McSheehy *et al*, 1986; Thomson *et al*, 1986; McSheehy *et al*, 1987). Furthermore, actual contact between osteoblasts and osteoclast precursors appears necessary for osteoclast formation and activation (Suda *et al*, 1992).

It is also possible that once formed, osteoclasts require contact with the mineralised matrix (Suda *et al*, 1992), in a similar way to macrophages being activated by foreign bodies. By analogy to white blood cells, activated osteoclasts may attract additional osteoclasts by releasing chemotactic factors. Interestingly, and of relevance to the work in this thesis, these leukocyte chemotactic factors are LOX products (Samuelson, 1983).

1.4.2. Bacteria And Bone Remodeling.

The coupled nature of bone remodelling makes its control vulnerable to disruption by bacterial factors that can bind to and modulate the behaviour of both mesenchymal (osteoblast) and myeloid (osteoclast) lineage cells. Thus, bacterial factors could affect all of the steps involved in bone remodelling. As has been shown in section 1.3, above, several bacterial components are known to

modulate bone cells and osteoactive host factors. Thus by increasing/decreasing osteoclast recruitment and activity, or decreasing/increasing osteoblast recruitment and activity, bacteria can exert control over the remodelling cycle. Figure 1.11 outlines the points where bacteria may interfere with remodelling. There is much evidence that osteotropic host factors, such as cytokines and eicosanoids are involved in both normal and abnormal bone remodelling.
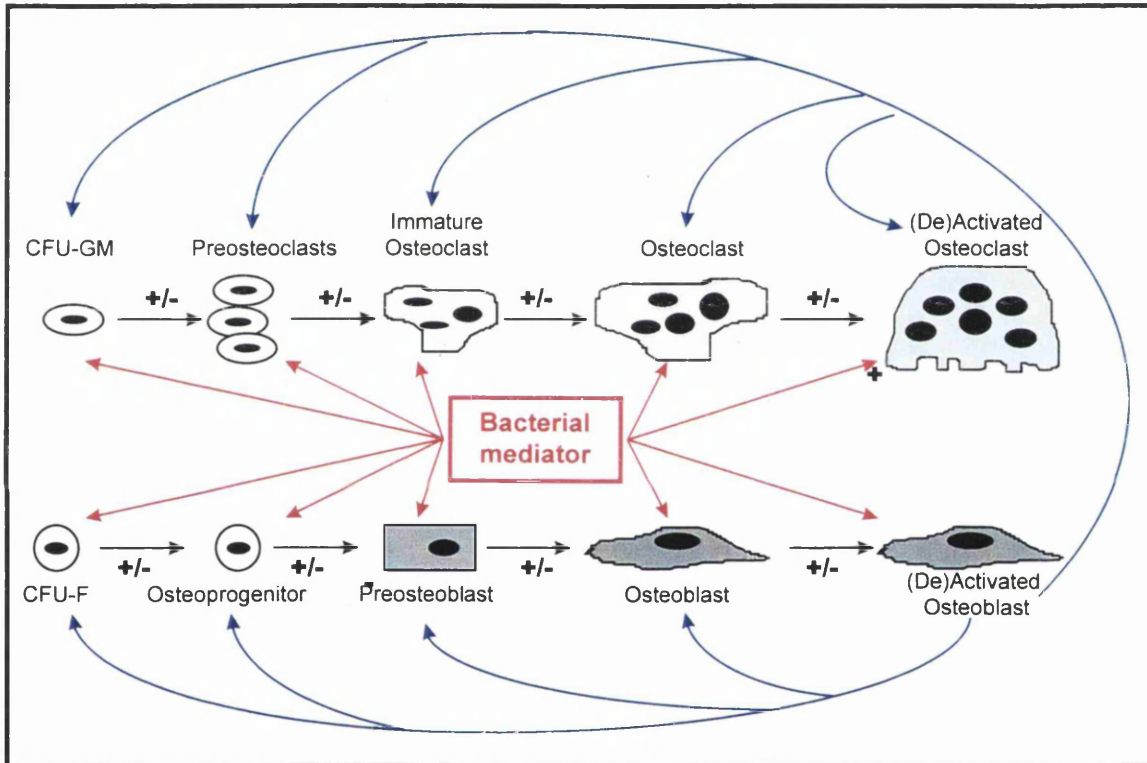
Figure 1. 11 Possible points of interaction between bacterial components and bone cell populations. Bacterial components may directly (-) increase the production and activation of osteoclasts or they may do so indirectly by stimulating the production of host factors (-). They may also act to inhibit the production of bone forming cells either directly or indirectly.

Since few bacterial factors have been shown to act directly on the osteoclast and in view of the osteoblast-osteoclast interaction in the coupling of bone remodelling, the modulation of host factors mediating this interaction is highly relevant to the bone destruction seen in bacterially induced bone lesions. These host factors shall now be considered.

1.4.3. Host factors involved in bone remodeling.

Although remodelling is highly influenced by the structural molecules present in the bone matrix, it is also under endocrine, paracrine and autocrine control. This hormonal control can be regarded as being systemic (endocrine) and local (paracrine and autocrine) (Figure 1.12).
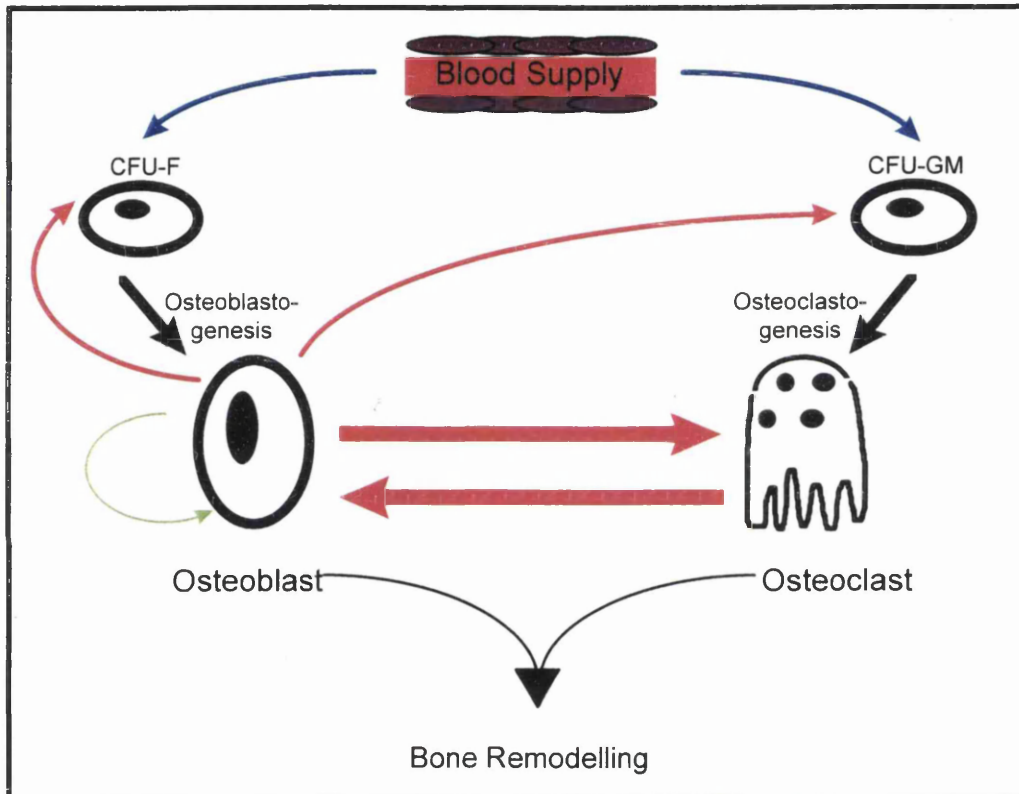
Figure 1. 12 Regulatory relationships involved in bone remodelling. The coloured arrows indicate endocrine (--), paracrine (--) and autocrine (--) regulation.

1.4.3.1. Systemic control of bone remodelling.

Factors acting systemically can be regarded as being responsible for the integration of bone remodelling with the wider metabolism of the organism. The main systemic factors involved in bone remodelling include parathyroid (PTH), Calcitonin, and 1,25-dihydroxy vitamin D3 (VD), all three are under negative feedback control and are regulated by extracellular fluid calcium concentrations.

Other hormones that impact on bone cell function include, oestrogen, androgens, thyroid hormones and growth hormone.

1.4.3.1.1. Parathyroid hormone.

PTH acts on both osteoclasts and osteoblasts, but its effects differ depending on how it is administered. Continuous administration increases osteoclastic bone resorption and suppresses bone formation. Intermittent administration of low doses, however, stimulates bone formation without major effects on resorption. PTH is now known to be secreted in pulsatile bursts (Samuels *et al*, 1993). Some effects of PTH are probably mediated by prostaglandin production by osteoblasts, which have been shown to carry PTH receptors (Rouleau *et al*, 1988).

1.4.3.1.2. Calcitonin.

The peptide hormone calcitonin is secreted by the parafollicular cells of the thyroid gland. Its effects on calcium homeostasis are short lived (24-48 hours). Calcitonin both inhibits the formation of osteoclasts as well as inhibiting the activity of mature osteoclasts (Friedman *et al*, 1986). What separates calcitonin most notably from the other systemic factors is that the osteoclast carries receptors for it (Kurihara *et al*, 1990), and it acts directly on the osteoclast and not via the osteoblast. The effects on osteoclasts are transient (Chambers *et al*, 1983) and are mediated by increasing adenylate cyclase activity to raise intracellular cAMP (Nicholson *et al*, 1987).

1.4.3.1.3. 1,25-dihydroxy vitamin D3.

1,25-dihydroxy vitamin D3 (VD) is a steroid produced mainly in the proximal tubes of the kidney. It probably has its effects in bone through action on osteoblasts (Merke *et al*,1986; McSheehy *et al*, 1987). VD: (i) increases calcium and phosphate absorption by the gut, (ii) increases noncollagenous protein synthesis (Price *et al*, 1980), (iii) inhibits collagen synthesis (Raisz *et al*, 1980) by osteoblasts and (iv) increases the number and activity of osteoclasts (Ibbotson *et al*, 1984; Roodman *et al*, 1985).

1.4.3.2. Local control of bone remodelling.

Bone cell activity is also controlled by regulatory factors produced locally such as cytokines, growth factors, and eicosanoids. These may be stored in the matrix and be released during bone resorption. They do not appear to be regulated by extracellular fluid calcium concentrations, and are thus more vulnerable to modulation by bacterial factors.

1.4.3.2.1. Cytokines.

Cytokines are largely inducible proteins or glycoproteins which can be secreted by any cells in the body. They are able to bind to and activate a wide range of cell types. The major osteoactive cytokines are IL-1, TNF, TGF, IL-6, IFN and the colony stimulating factors. They have their effects at the levels of cellular differentiation activity and cell survivability.

1.4.3.2.2. Eicosanoids.

Eicosanoids, one of the subjects of this work, are derived from the oxidative metabolism of arachidonic acid (Figure 1.13 summarises the relevant

pathways). These derivatives include the prostanoids and thromboxane (TX) products of the cyclooxygenase (COX) pathway; along with the leukotrienes (LT), hydroxyeicosantetraenoic/hydroxyperoxyeicosantetranoic acid (HETE/HPETE), and lipoxin (LX) products of the LOX pathway. Arachidonic acid metabolites have long been known to alter bone cell function.

1.4.3.2.2.1. Prostaglandins.

E-series prostaglandins (PGs) stimulate bone resorption in bone organ cultures (Klein et al, 1970; Tashjian et al, 1972; Goodson et al,1973). However the biological significance of the osteolytic effects of PGs is still unclear. PGs have been shown to have multiple effects on bone cells, and sometimes opposite effects in different species, their role in physiological or diseased remodelling is consequently difficult to discern (Chambers et al, 1983; Akatsu et al, 1989; Chenu et al, 1990). Most evidence indicates PGs are locally active factors and their effects do not appear to be mediated systemically.

The observations by Klein and Raisz (1970) that PGE caused bone resorption in foetal rat long bone assays was confirmed later in mouse calvarial organ cultures by Tashjian (1972) and then by Goodson (1973). However the role of PGs as osteolytic agents was called into doubt when Chambers (1983) observed that isolated osteoclasts responded to PGs with a decrease in motility and contraction of cell size. Calcitonin produces a similar effect. This effect of PGs is transient and Chambers (1983) interpreted this as reflecting inhibition. In human marrow culture systems PGE's could inhibit the formation of cells with osteoclastic characteristics (Chenu et al, 1990). This is in stark contrast to murine marrow culture systems in which PGs stimulate osteoclastic cells to form (Akatsu

et al, 1989). It appears that different species perceive the prostaglandins in different ways in terms of skeletal function.

PGs are certainly involved in mediating the effects of other mediators. Raisz *et al* (1974) found that when complement-sufficient sera was added to bones, PGs were generated. Since then many growth factors and cytokines have been shown to have their effects via the generation of PGs, including the major osteolytic cytokine IL-1 (Boyce *et al*, 1989).
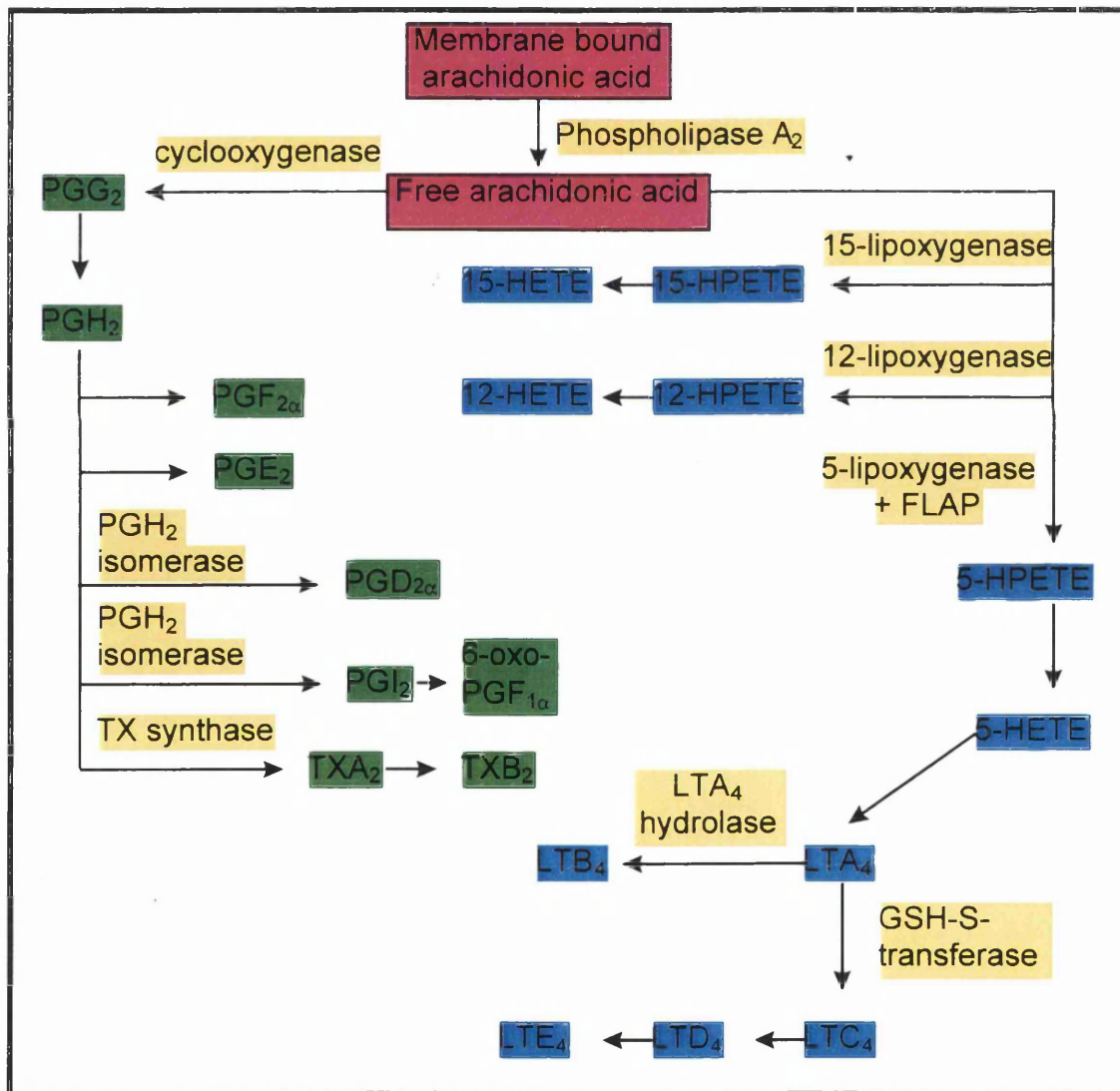
Figure 1. 13 Pathways of arachidonic acid metabolism, showing the production of LOX metabolites(■), COX metabolites(■), and the enzymes involved in their production( ).

PGs also have an effect in bone formation. In short term cultures they inhibit calvarial collagen synthesis (Dietrich *et al*, 1976). However in longer term cultures bone collagen synthesis may be increased (Chyun *et al*, 1984)). In infants receiving PGE pharmacologically there is an increase in bone formation, and in beagle dogs injected with PGs there is also an increase in bone volume (Ueno *et al*, 1985). The relevance of these findings to normal bone remodelling or formation remains unclear. The bone formation response is, however, striking, and it remains possible that the major effects of prostaglandins on bone *in vivo* may be on formation.

1.4.3.2.2. Lipoxygenase products.

In contrast to the prostaglandin's, there has been little interest in lipoxygenase (LOX) products and bone. The first report of LOX metabolites being able to affect bone remodelling came from the EDI in 1988 when Meghji *et al* reported that LT's and HETE's were able to stimulate bone resorption, and later in 1992, from the same group, that 5 and 12 LOX products could stimulate collagen and non-collagenous protein synthesis (Meghji *et al*, 1992). These authors linked the bone resorption observed with clinical situations, such as the periodontal diseases, where there seems to be enhanced production of LOX products.

Later LT's were shown to inhibit osteoblast proliferation (Ren *et al*, 1991) and to act via cAMP (Sandy *et al*, 1991). Gallwitz *et al* (1993) demonstrated that 5-lipoxygenase products could stimulate isolated osteoclasts to resorb calcified matrices. This later group have also confirmed that LTs can stimulate in vitro bone resorption (Garcia *et al*, 1996).

The role of LT's in physiological and pathological bone remodelling has still to be defined. However they are produced in large amounts in inflamed tissues such as chronically infected gingiva (El Attar *et al*, 1983; Davidson *et al*, 1983; El Attar, 1986). Thus the enzymes producing LOX products may be of major importance in bacterial infections of bone and may be modulated by bacterial products.

1.5. Aims of this work.

Normal bone remodelling requires the co-ordinated regulation of osteoblast and osteoclast lineage's. Any interference with these integrated cellular systems can result in dysregulation of remodelling with the consequent loss of bone matrix. Bacteria are important causes of bone pathology in common conditions such as periodontitis, dental cysts, bacterial arthritis and osteomyelitis. It is now established that many bacteria implicated in bone diseases contain or produce molecules with potent effects on bone cells.

The aims of this thesis were to identify mechanisms by which osteolytic bacterial molecules interfere with the bone remodelling sequence. Since bacterially induced disease is distinguished from systemic diseases, such as osteoporosis, by the local nature of the bone destruction, special emphasis has been placed on investigating the role of those most local of local mediators, the eicosanoids, in mediating the effects of the bacteria on bone.

CHAPTER TWO

MATERIALS AND METHODS.

2.1. Preparation and characterisation of bacterial factors.

2.1.1. Sonicate of *Mycobacterium tuberculosis*.

A viable virulent strain of *M. tuberculosis* (strain H37Rv) was sonicated at 4^0C for 1 min intervals, followed by a 1 min rest period, for a total period of 1 h. The sonicated material was then centrifuged at 100,000 g for 1 h, and the supernatant was filtered through a 0.22-mm membrane filter. This was prepared in collaboration with Professor Anthony Coates (Division of Molecular Microbiology, St. George's Hospital Medical School).

2.1.2. *M. tuberculosis* chaperonin 10 and peptides.

Recombinant (r) *M. tuberculosis* cpn10 was expressed in *E. coli* and purified by reversed-phase high performance liquid chromatography (HPLC) to >97% purity as previously described (Fossati *et al*, 1995). The synthetic peptide fragments were prepared by conventional solid-phase synthesis and were purified by isoelectric focusing and by reverse phase HPLC to >95% purity as previously described (Lucietto *et al*, 1997). Both the cpn 10 and the peptides were prepared by Dr. Paolo Mascagni (Italofarmaco, Italy).
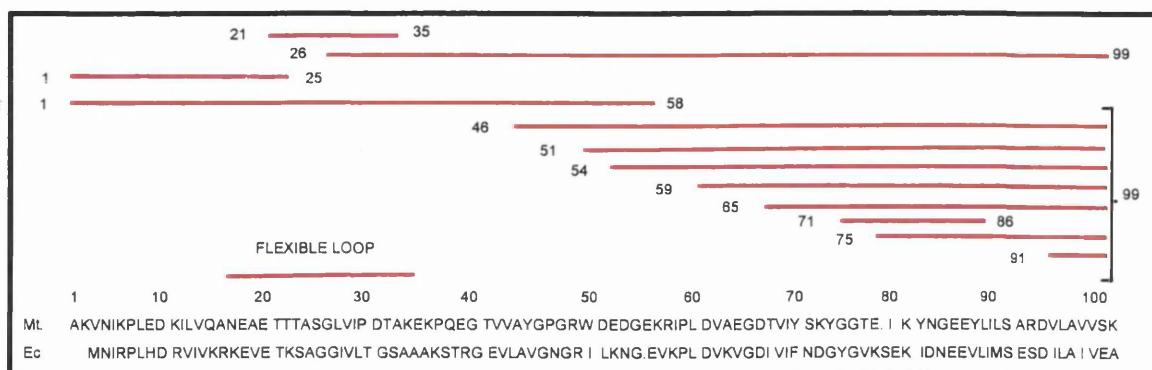
Figure 2. 1 Diagram showing the sequences and relationships of the cpn10 peptides relative to each other and the sequences of *M. tuberculosis* and *E. coli* cpn10.

The relationship of the peptides to *M. tuberculosis* and *E. coli* cpn10 and to each other are illustrated in Figure 2.1. The composition of the peptides was confirmed by amino acid analysis and mass spectroscopy. All peptides were tested for lipopolysaccharide (LPS) using the limulus amoebocyte lysate assay (Whittaker M.A. Bioproducts, Inc., Walkersville, MD). All peptides tested negative, indicating the presence of <0.03 endotoxin U LPS/μg of peptide.

An *M. tuberculosis* cpn10 structural model was generated by Dr. Michael Roberts (St George's Hospital Medical School) using homology modelling of the *M. tuberculosis* cpn10 sequence onto the atomic co-ordinates of the GroES structure of the monomer with the flexible loop assigned. The model was energy minimised with QUANTA/CHARMm (Molecular Simulations, Inc., San Diego, CA) using a non-bonded cut-off of 14 Å and a dielectric constant distance dependence until the root mean squared deviations were 0.001 Kcal/Å. The side chains were minimised first, keeping the backbone fixed. This was followed by minimisation of the whole monomer. The heptamer was generated from the monomer by the symmetry operations relating the GroES subunits. The same energy minimisation procedure was repeated for the final *M. tuberculosis* cpn10 heptamer model, which was displayed with the SYBYL molecular modelling package (Tripos UK, Milton Keynes, UK).

Before addition to the bone explants, r-*M. tuberculosis* cpn10 was passed down a polymyxin B–agarose column (Detoxigel column; Pierce, Rockford, IL) to remove any contaminating LPS.

2.1.3. Bacterial lipopolysaccharides.

Lipopolysaccharides (LPS) were prepared from *Actinobacillus (A) actinomycetemcomitans*, *Eikenella (E) corrodens*, *Prevotella (P) intermedia* and from *Porphyromonas (P) gingivalis* by the method described by Morrison and Leive (1975). Bacteria were grown on agar using established methods (Reddi *et al*, 1996). Briefly, bacteria were washed three times in sterile 0.15M saline over a 2 hour period to remove bacterial surface-associated material, which is itself osteolytic (Reddi *et al*, 1996; Reddi *et al*, 1995). Cells were pelleted by centrifugation and then resuspended in 0.15M NaCl at 4^0C. An equal volume of n-butanol (BDH: Poole, UK) was added, the suspension mixed thoroughly for 10 minutes, and centrifuged for 20 minutes at 35,000g. The aqueous phase was removed and the butanol, together with the insoluble residue, was re-extracted twice with half the volume of saline. The combined aqueous phases were centrifuged at 35,000g for 20 minutes to remove particulate matter and dialysed exhaustively against distilled water. The crude preparation was ultracentrifuged at 100,000g for 1 hour at 4^0C and lyophilised. The lower layer was digested by addition of RNase and DNase (Sigma: 20mg/ml in 0.05M Tris buffer, pH 7.3) overnight at 37^0C and then with 1mg/ml pronase (Sigma) for 6 hours. The suspension was then ultracentrifuged for 1 hour at 100,000g and the lipopolysaccharide, which appears as a clear gel at the bottom of the centrifuge tube, was removed and lyophilised. The LPS content of the LPS preparations was assessed by a commercial chromogenic *Limulus* amoebocyte lysate assay (Pyrogent, Byk-Mallinckrodt, UK) using the *Escherichia coli* international LPS standard (84/691) as a control, this was obtained from Dr. Stephen Poole of the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, UK).

2.1.4. Lipoteichoic acid and muramyl dipeptide.

Lipoteichoic acid (LTA) and muramyl dipeptide were purchased from Sigma (Poole, UK).

2.1.5. *Staphyloccocus aureus* surface associated material.

Staphyloccocus (*S.*) *aureus* NCTC 6571 was cultured aerobically at 37°C on Wilkins-Chalgrens agar (Oxoid, Hampshire, UK), containing 10% horse blood, for 24h. The cultures were routinely Gram-stained to detect contaminating Gram-negative bacteria and were then harvested by washing plates with sterile saline. The cells were then pelleted by centrifugation, washed once with saline and then freeze-dried.

The extraction of surface associated material (SAM) was carried out as described by Wilson *et al* (1986). Briefly, the freeze-dried bacteria were suspended (1g per 100ml) in 0.85% (w/v) saline, and the SAM was removed by gentle stirring at 4°C for 1 hour. The supernatant was collected after centrifugation at 3000g for 1h. The extraction process was repeated twice more, and the SAM was pooled, exhaustively dialysed against distilled water at 4°C, and lyophilised.

In experiments to determine the nature of the active constituents in the SAM, the extract was heat-treated by boiling for 1 hour or was exposed to trypsin (0.5mg/ml trypsin incubated with 5mg/ml SAM) in phosphate buffered saline (PBS: Sigma) for 4 hours at room temperature. After incubation with trypsin, samples were diluted and soya bean trypsin inhibitor was added and activity was compared to equivalent concentrations of SAM which had not been trypsin-treated but to which the soya bean inhibitor had been added.

The protein content of the SAM was determined by the Lowry method (1951) using bovine serum albumin as standard. Carbohydrate was measured by the method of Dubois *et al* (1956) using glucose standards as a control. Lipids were extracted with methanol/chloroform (2:1 v/v), dried and weighed. The DNA content was estimated by UV absorption.

SAM was fractionated into >30kD and <30kD fractions by passage through an Amicon PM30 membrane. The efficiency of fractionation was assessed by protein measurement and SDS-PAGE analysis.

To check on the efficiency of the extraction of the bacterial SAM and the integrity of the resultant cells, bacteria were examined by transmission electron microscopy before and after saline extraction as described by Wilson *et al* (1986). Briefly, a portion of the bacterial suspension was fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer for 1h at room temperature (control cells). A second portion was fixed in the same manner except that the buffer also contained 0.15% ruthenium red. Bacteria were then pelleted by centrifugation at low speed, the fixative decanted and the pellet washed in cacodylate buffer. Control cells were then fixed in 1% osmium tetroxide in cacodylate buffer for 2h at room temperature in the dark. The test cells were treated identically except that the buffer also contained 0.15% ruthenium red. The fixative was decanted after centrifugation and the pellets washed in cacodylate buffer. All specimens were then dehydrated in a graded series of concentrations of ethanol and embedded in araldite. Sections were cut on an LKB ultramicrotome using a diamond knife and these were examined (unstained) in a JEOL 100CXII electron microscope and photographed. The sectioning and electron microscopic examination were performed by Ms Nikki Morden (EM Unit, Eastman Dental Institute)

2.2. Hormones, cytokines and eicosanoids.

All eicosanoids were purchased from Cascade Biochem Ltd (Oxford, England). They were supplied in ethanol stock solutions.

The 1,25-dihydroxycholecalciferol (VitD) was the gift of Roche Ltd (Stevenage, England), it was reconstituted in ethanol and stored under nitrogen.

Bovine parathyroid hormone and all cytokines were the gift of Dr. Stephen Poole (NIBSC).

The role of IL-1 was assessed by adding the IL-1 receptor antagonist (IL-1ra: Synergen, Boulder, Col USA).

Calcium ionophore A23107 was purchased from Sigma and was solubilised in ethanol.

2.3. Antibodies.

2.3.1. Antibodies to *M. tuberculosis* components.

Both the monoclonal antibody (mAb) to *M. tuberculosis* cpn10 (SA12; Minden *et al*, 1984) and the mAb to *M. tuberculosis* cpn60 (TB78; Coates *et al*, 1981) were obtained from murine ascites in a sufficiently high titre to bind to *M. tuberculosis* cpn10 or cpn60 at the dilutions used in this study. Neither of these mAbs are cross-reactive with any other *M. tuberculosis* protein (Coates *et al*, 1989). The mAb to lipoarabinomannan (LAM) (CS-35) of isotype IgG3 was obtained from concentrated tissue culture supernatant with a titer of 1:2,000 by Western blot analysis (Belisle, J.T., personal communication). CS-35 was raised against *M. leprae* LAM and is cross-reactive with *M. tuberculosis* LAM at a dilution of 1:1,000 by Western blot analysis.   CS-35 was used at a 1:1,000 dilution in the bone resorption assay.

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2.3.2. Cytokine neutralising antibodies.

The involvement of tumour necrosis factor (TNF)-α was determined by use of the neutralising monoclonal antibody TN3-19.12 (produced by Prof. Robert Schreiber, Washington University School of Medicine and manufactured by Celltech Ltd, Slough UK).

The role of IL-6 was determined by adding a neutralising rabbit antibody to murine IL-6 (Genzyme).

2.4. Eicosanoid generating enzyme inhibitors.

2.4.1. MK886.

The 5-LOX activating protein (FLAP) ligand: 3-[1-(4-chlorobenzyl)-3-t-butyl-thio-t-isopropylindol-2-yl]-2,2-dimethylpropanoic acid (MK886: Merck Frosst) is an inhibitor of 5-LO which acts by binding to FLAP and preventing the translocation of 5-LO to the cell membranes where it normally exerts its enzymatic actions (Vickers, 1995). This compound is normally active in the concentration range 10nM to 1μM.

2.4.2. BWA70C and BWA4C.

BWA4C and BWA70C (Glaxo Wellcome) are of the hydroxamic acid based iron-ligand class of 5-LOX inhibitors (Tateson *et al*, 1988; Hawthorne *et al*, 1992). These compounds are normally active in the concentration range 10nM to 1μM.

2.4.3. Indomethacin.

Indomethacin (1-[4-Chlorobenzoyl]-5-methoxy-2-methyl-1H-indole-3-acetic

acid) is a non-steroidal anti-inflammatory agent and was obtained from Sigma. It

blocks prostaglandin biosynthesis by inhibiting cyclooxygenase. This compound

is normally active in the concentration range 10nM to $1\mu M$.

2.5. Enzyme and radio immuno-assays.

Peptido-leukotrienes   (pLT)   and   VITD   were   assayed   by   enzyme

immunoassay (EIA) and radio immunoassay (RIA) respectively.

2.5.1. Peptido-leukotriene EIA.
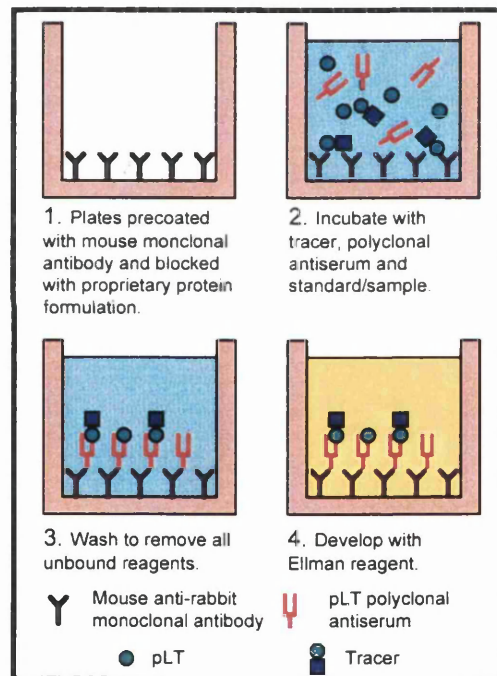
Figure 2. 2 Schematic describing the major steps in the pLT EIA.

The pLT's ($LTC_4/D_4/E_4$) were measured using a commercially available

peptidoleukotriene enzyme immunoassay (EIA) kit (Cayman Chemical, MI, USA)

in which all reagents necessary are provided, and was used according to the

manufacturer's instructions (Figure 2.2). The assay is based on the competition

between peptido-leukotrienes and a pLT-acetylcholinesterase conjugate (the

tracer) for a limited amount of peptido-leukotriene polyclonal antiserum. Since the

concentration of the tracer is constant, while the concentration of the peptido-leukotriene varies, the amount of tracer able to bind to the polyclonal antiserum will be inversely proportional to the concentration of pLT in the well. This antibody pLT/tracer complex binds to a mouse antirabbit immunoglobulin (Ig) G monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to acetylcholinesterase) is added to the well. The product of this reaction has a yellow colour that absorbs strongly at 412nm, this can then be read by absorptiometry, in the studies described in this thesis use was made of a Titertek multiskan spectrophotometer (Flow laboratories).

2.5.2. Dihydroxy vitamin D_3 RIA.
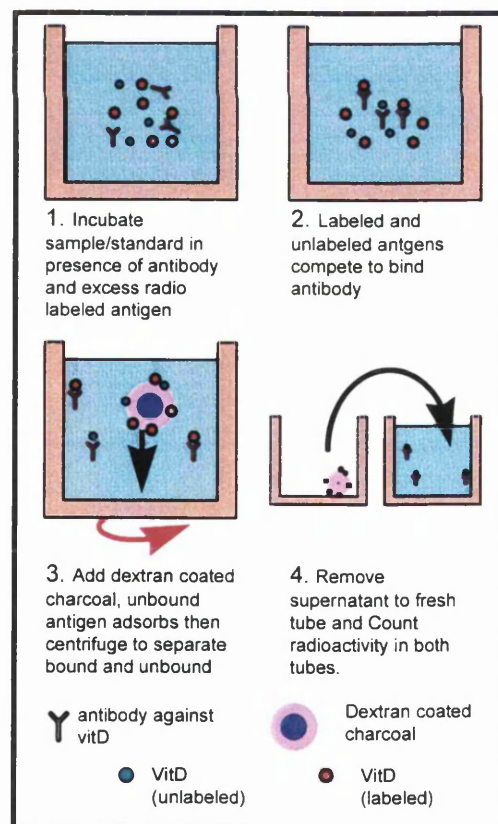
Figure 2. 3 Schematic showing the major steps in the RIA for 1,25-dihydroxycholecalciferol.

The major steps in the 1,25 $(OH)_2$ Vitamin D_3 (VitD) radio-immunoassay (RIA) are represented in Figure 2.3. VitD in the bone marrow cultures incubated with a range of concentrations of SAM over 4, 12 and 48h time periods were assayed by radioimmunoassay (Mawer *et al*, 1990) and this was performed by Dr E. B. Mawer. The principle of RIA is similar to EIA in that a tracer competes with an unlabeled antigen for binding sites on an antibody. In the case of RIA, the tracer is radiolabeled.

2.6. Calvarial bone resorption assay.

Bone resorption was measured by the release of calcium (measured colorimetrically; Zanelli *et al.*, 1969) from explants of neonatal mouse calvaria in culture. This culture technique (Figure 2.4) as described below is based on a method originally described by Reynolds and Dingle (1970) and modified by Meghji *et al* (1988).

2.6.1. Preparation and culture of calvarial bones.

5-day old MF1 mice (Harlan Ltd, Dorset, England) were killed by cervical dislocation. The skin was removed to expose the cranial bones. The dissection was then continued using the lamboidal suture as a guide line, special care being taken not to damage the periosteum. The fronto-parietal bones were trimmed free of any adherent connective tissue and the interparietal bone. Dissected calvaria were pooled in Biggers, Gwatkin, and Jenkins (BGJ) medium (ICN Biomedicals, Inc., Thame, UK) containing 5% heat-inactivated foetal calf serum (GIBCO BRL, Paisley, UK) and 50 mg/ml ascorbic acid (Sigma Chemical Co., Poole, UK) and washed free of blood and brain tissue. The calvaria were then divided along the sagittal suture and placed in a dish of fresh BGJ medium. Each half calvaria was

then placed on a 1cm^2 stainless steel grid (Minimesh FDP quality, Expanded Metal Co. Hartlepool) in a well of a 6-well tissue culture plate containing 1.5ml of BGJ medium. There were five calvaria per 6-well plate, and each plate constituted a single treatment group i.e. five replicates/treatment.
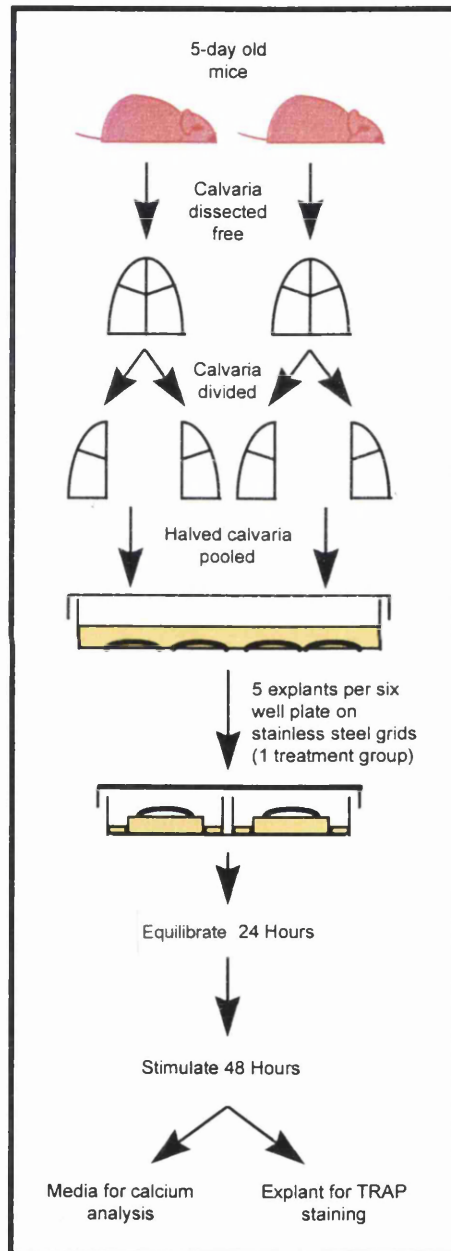
Figure 2. 4 Schematic showing the preparation and culture of neonatal calvaria for use in the bone resorption assay.

The bones were incubated for 24 hours at 37^{0}C in a humidified atmosphere of 5% CO_2/95% air. This 24 hour preincubation period, prior to

stimulation, enabled the adaption of the bones to their new environment, permitting calcium exchange to reach an equilibrium and also to allow prostaglandins release, which in freshly explanted bones is relatively high (Katz *et al.*, 1981), to normalise. This is important since many substances act in bone by the modulation of prostaglandins.

At the end of the preincubation period, the medium is removed and replaced with fresh medium containing the test substances. One treatment group is exposed to PGE_2 ($10^{-6}M$) as a positive control, this group and the experimental groups were compared to a negative control group containing BGJ alone. The cultures were incubated for a further 48 hours and resorption measured as the release of calcium into the culture media.

2.6.2. Assessment of bone resorption.

Calcium concentrations were measured colorimetrically on an autoanalyser (Chem Lab Instruments, Essex) (Figure 2.5), by using the metal complexing dye cresolphthalein complexone (CPC) (Gitelman, 1967). At the end of the incubation period, the culture medium was removed from each well with a disposable transfer pipette into an autoanalyser cup (conical bottomed, 2ml, Chem Lab Instruments, Essex). Calcium was separated from proteins by continuous flow dialysis under acidic conditions. The concentration of dialysed calcium was then determined colorimetrically by complexing with CPC.

Sample cups holding the media and standards were placed in the sample tray in the order shown in table 2.1. The tray could hold 82 samples at once. The first five cups held a series of standards and blanks to calibrate the peak height analysis module. Media and incubated media were also included to account for

evaporation. These were followed by a 10mg/dl standard, a wash with deionised water and then a zero to re-establish the base line. There then followed the 5 replicates of the first treatment group followed by the five replicates of the second treatment group, these ten samples were followed by media, a 10mg/dl calcium standard, a wash with water and a zero. The next two treatment groups were then sampled, finishing with media, 10mg/dl standard, a wash and a zero. This pattern was repeated until sampling was completed.

CUP NO.	SAMPLE	CUP NO.	SAMPLE
1	10mg/dl Ca	15	group1
2	water(zero)	16	group1
3	5 mg/dl Ca	17	group1
4	10mg/dl Ca	18	group1
5	15mg/dl Ca	19	group1
6	BI	20	group2
7	BI	21	group2
8	BI	22	group2
9	BGJ	23	group2
10	BGJ	24	group2
11	BGJ	25	BGJ
12	10mg/dl Ca	26	10mg/dl Ca
13	water(wash)	27	water(wash)
14	water(zero)	28	water(zero)

Table 2. 1 Showing the order in which standards and samples are assayed by automated colorimetric calcium analysis (BGJ; media, BI; media incubated without calvaria, 5,10,15 mg/dl Ca;Calcium standards).

Sampling entailed the removal of a single 100µl aliquot from each cup by the stainless steel sampling probe followed by a 20 second wash with deionised water. The aliquot then mixed with 1M HCl containing 8-hydroxyquinoline (8HQ) at 2.5g/l (to eliminate interference by magnesium), and dialysed against a solution of similar composition containing CPC at 0.7g/l. The dialysate was then mixed with 2-amino-2-methylpropanol (AMP) (90g/l). The absorbance of the

resultant purple-coloured solution was measured in a 15mm flow cell at 570nm.

The flow cell was linked to a peak height analysis module under the control of a

PC.
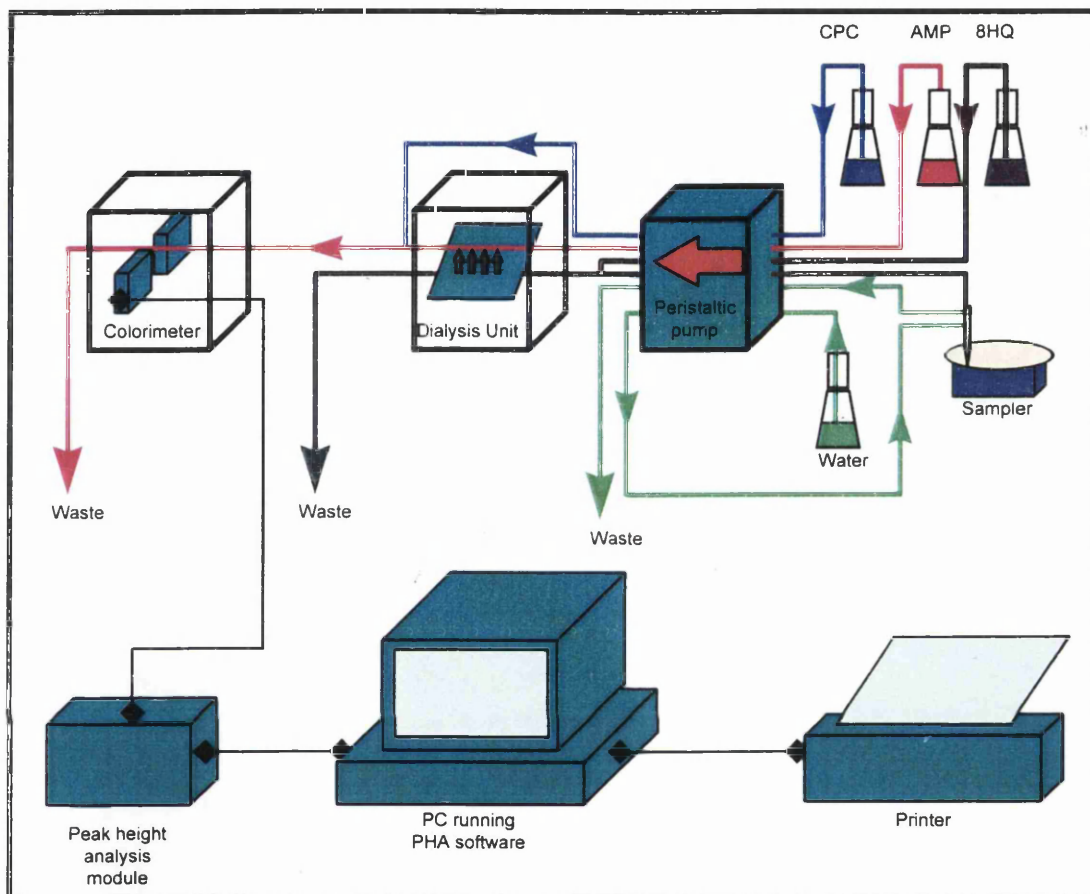
Figure 2. 5 Diagram summarising the equipment used in the automated colorimetric analysis of calcium.

The PHA module calculated a standard curve and then defined the calcium

concentrations in unknowns by comparison to this. The PC displayed this as mg

of calcium per decilitre and provided hard-copy via a printer. The mean calcium

content of media incubated without calvaria (BI) was then subtracted from all the

other readings to give the calcium released by calvaria. Results were expressed

as the mean +/-SE of 5 replicate cultures.

2.6.3. Osteoclast recruitment in calvaria.

After removal of the media supporting the calvarial explants for calcium assay, the explants were then used for the measurement of osteoclast numbers by a modification of the method of Marshall *et al* (1995). The calvaria were fixed in 95% ethanol/5% glacial acetic acid for 24 hour at 4^0C, and were then washed in PBS containing 1 mg/ml bovine serum albumin followed by reaction in the histochemical substrate mixture (obtained from Sigma Chemical Co., and used according to the manufacturer's instructions) for the localisation of tartrate-resistant acid phosphatase (TRAP) activity, a marker enzyme for osteoclasts. Bones were then washed in PBS, fixed and decalcified in 12.5% glutaraldehyde (BDH Chemicals, Ltd., Poole, UK) in 1 M hydrochloric acid (BDH Chemicals, Ltd.) for 5 min. Finally, the bone explants were washed and mounted in Aqua-mount (BDH Chemicals, Ltd.). Each calvarial explant was then scanned by transmitted light microscopy and TRAP-positive cells containing three or more nuclei were counted. The individual counting the cells was unaware of the treatment to which each explant had been exposed.

2.7. Murine bone marrow assay.

The bone marrow cell culture assay for osteoclast recruitment was performed using a modification of the method of Takahashi *et al* (1988) as described below.

2.7.1. Preparation of dentine slices.

Dentine discs were prepared from elephant ivory provided by Dr T. Arnett, who obtained confiscated tusks from Her Majesties Customs and Excise. The tusks were cut into cylindrical sections which were quartered and then shaped

with a hack saw to fit the chuck of a low speed saw (Buelher; figure 2.6).
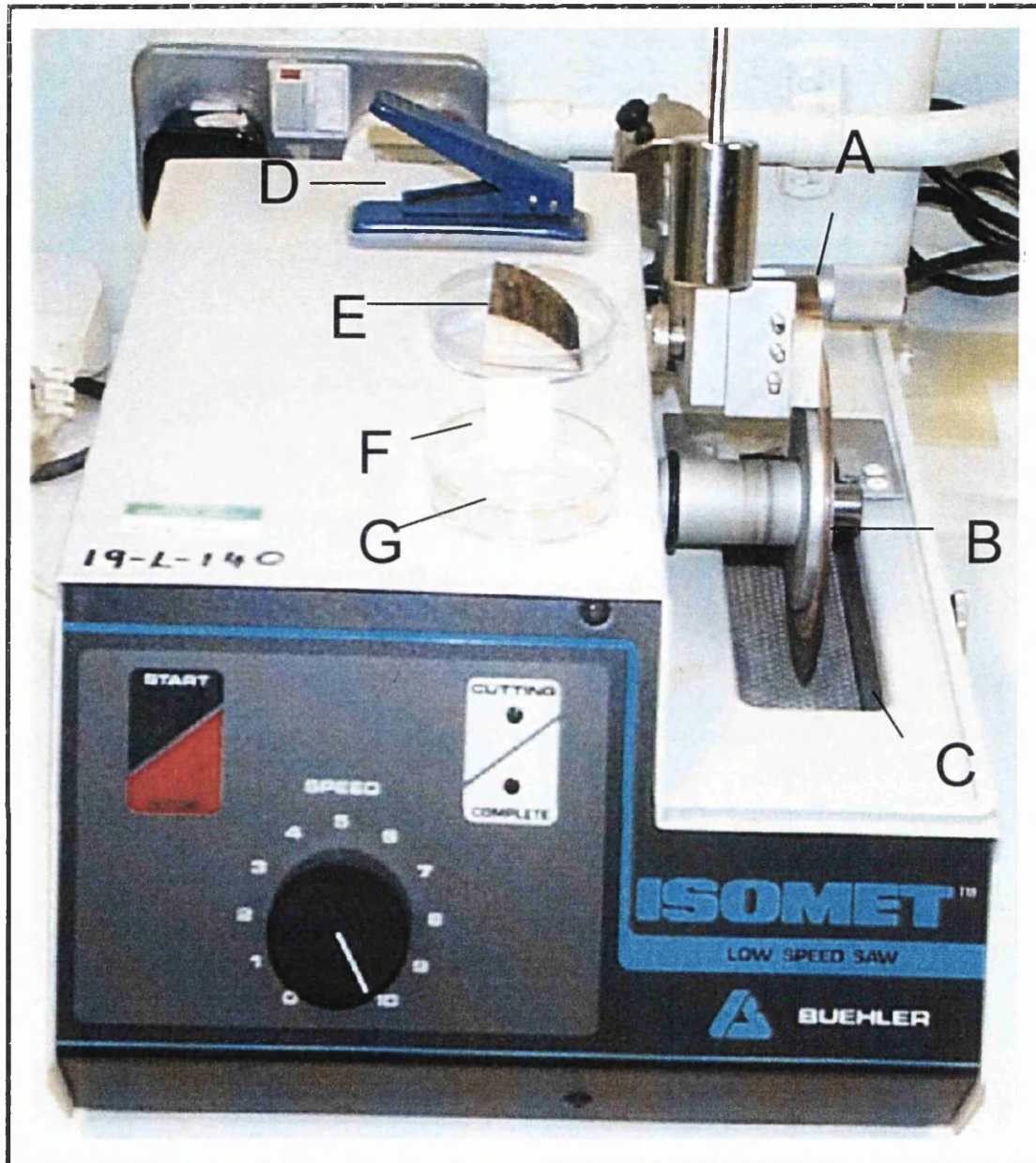
Figure 2. 6 The low speed saw (A; chuck holding tusk segment on blade, B; diamond wafering blade, C; lubricant reservoir), hole punch(D) and ivory in various stages of disc preparation (E; tusk segment, F; wafer cut from segment, G; Discs cut from wafer).

Wafers, 600μm thick, were cut from the ivory sections on the low speed saw, fitted with a diamond wafering blade and lubricated with water. Finally 5mm discs were punched from the wafers with a paper punch (Ryman the stationers PLC). The discs were sonicated for 15 minutes, 3 times, in water to remove

debris and dust from the wafering process. The sonicated discs were then dipped in absolute ethanol to sterilise them and laid out on a sterile Petri dish, in a tissue culture cabinet, to dry. Once dry, immediately prior to use, the discs were placed in a 96 well cell culture plate and covered with 100μl of media, the plate was then put into a cell culture incubator to equilibriate.

2.7.2. Preparation of marrow cells.

Six week old MF-1 mice (Harlan Ltd, Dorset, England) were killed by cervical dislocation. Femorae and tibiae were aseptically removed, placed in a dish of culture medium, and dissected free of adherent tissue. Finally the bone epiphyses were removed. The bone was transferred to a dish of fresh media, split length-wise and the marrow cavity was curetted.

The cells obtained were washed twice and suspended (4×10^6 cells/ml) in Dulbecco's Minimum Essential Medium (DMEM; Gibco BRL, Glasgow, Scotland) supplemented with 10% foetal calf serum (FCS; Gibco BRL), L-glutamine (2mM; Gibco BRL), penicillin/streptomycin (100U/ml & 50μg/ml; Gibco BRL). This suspension was placed (100μl/well) in the 96 well plates in which the dentine slices had equilibriated, and incubated at 37°C with various dilutions of the test substances plus, on occasion, added inhibitors. The cultures were incubated for a total of 10 days, the medium being replaced every 2 days. Figure 2.7 summarises this process.
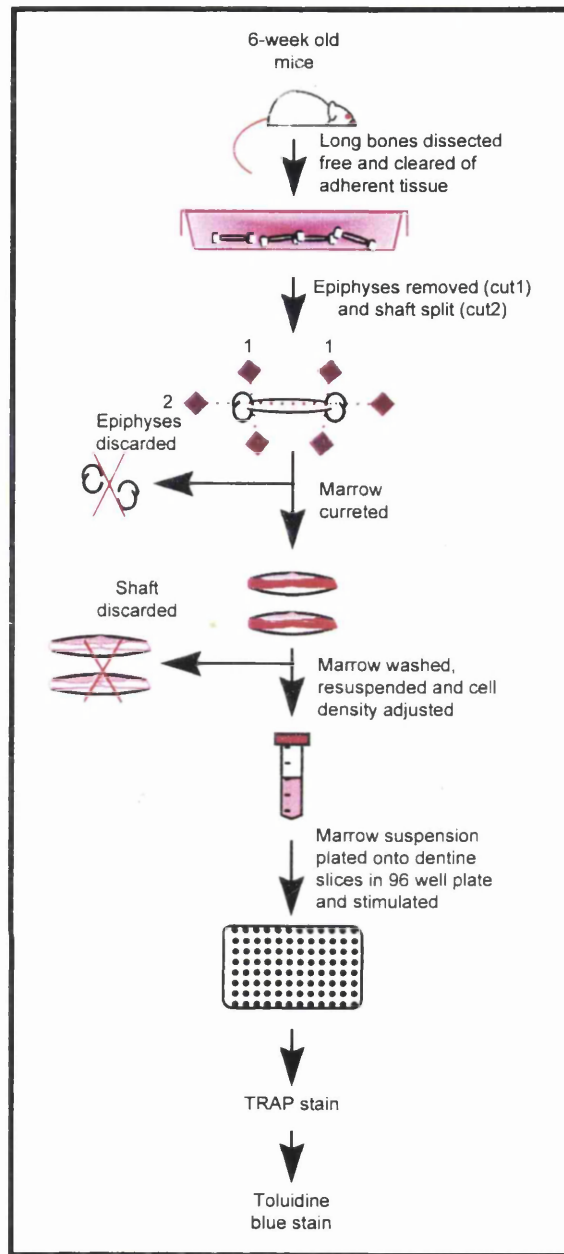
Figure 2. 7 Diagram summarising the derivation and culture of murine bone marrow cells for use in the assay for osteoclast recruitment.

2.7.3. Assessment of osteoclast recruitment.

The cultures were fixed in 1.25% glutaraldehyde (Analar, BDH) and stained for TRAP using a commercial histochemical staining kit (Sigma) according to the manufacturers instructions. All the TRAP-positive MNC with 3 or more nuclei were counted in four replicate wells for each treatment and results were

expressed as the mean and standard deviation. These measurements were carried out in a blinded manner.

2.8. Preparation and culture of osteoblasts.

Primary osteoblasts were obtained by use of the method described in Hughes *et al* (1998) for the preparation of human osteoblasts and this is summarised in Figure 2.8. All tissue culture plastic-ware, media and other reagents were obtained from Gibco BRL (Glasgow, Scotland). Fragments of alveolar bone were obtained from patients undergoing surgery for wisdom tooth extraction. These were dissected free of any adhering soft connective tissue and this was followed by brief treatment with collagenase to remove any vestiges of connective tissue.

The bone fragments were then crushed to give particles of about 2mm in diameter. These particles were cultured in T75 culture flasks in MEM containing 10% FCS, antibiotics and glutamine. Cells were allowed to reach confluence and were used in experiments at early passage. The phenotype of the cells was checked with histochemical staining for alkaline phosphatase.

The osteosarcoma cell line MG63 were also used in this study (section 2.9.). These were maintained in MEM supplemented with 10% FCS and penicillin/streptomycin. Along with primary cells these cell lines were recultured by standard methods using Trypsin/EDTA to lift monolayers for replating. This involved the removal of the culture media from the monolayers, washing the monolayer three times in PBS and the application of 0.5ml of Trypsin/EDTA. The flask was then placed in the tissue culture incubator until cells lifted from the culture surface. Culture medium was then added, the cells washed, and finally resuspended in fresh media and replated.
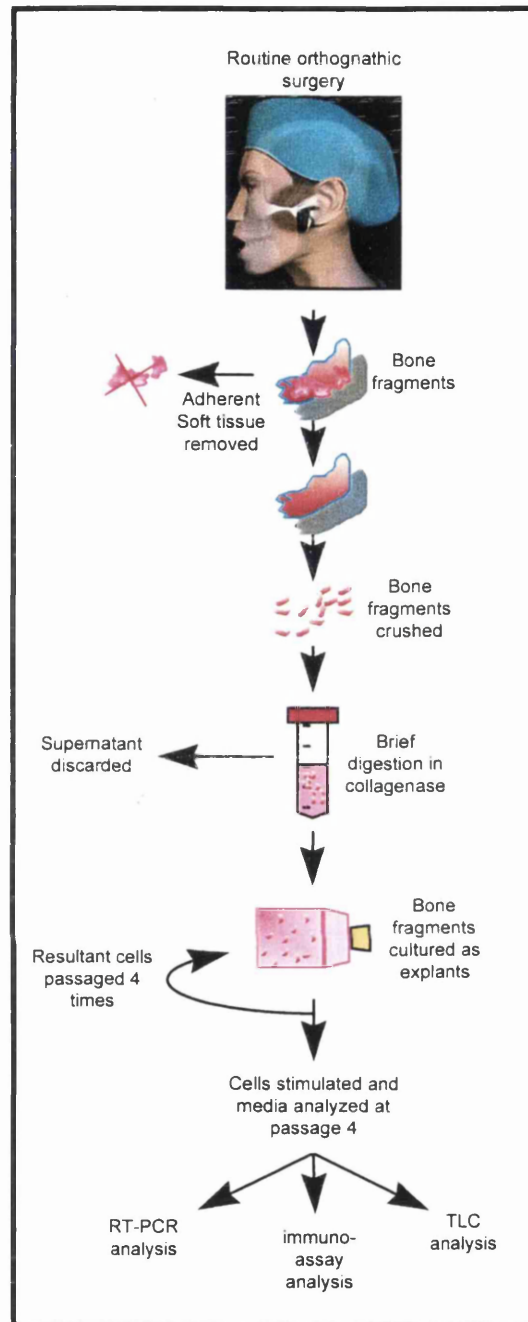
Figure 2. 8 Schematic showing the major steps in the isolation of primary human osteoblasts prior to use in experiments.

Experiments were carried out on osteoblasts as described in Figure 2.9. Cells were lifted from confluent monolayers using Trypsin/EDTA, and were then washed, counted (with cell viability being estimated by the trypan blue exclusion method) and replated onto appropriate cell culture plates in MEM or DMEM supplemented with 10% FCS for 24 hours. The media was then changed to

MEM+2% FCS for a further 24 hours prior to stimulation in MEM+2% FCS.

Monolayer
lifted with
trypsin/EDTA

Cells washed
and pelleted

Cells resuspended,density
adjusted and plated

96-well plates, 10^3
cells/well in 0.2ml
DMEM+10%FCS

24-well plates, 10^5 cells/well
in 1ml DMEM+10%FCS

6-well plates, 3×10^5
cells/well in 3ml
DMEM+10%FCS

Cells allowed to adhere overnight

Media changed to DMEM+2%FCS
and equilibriated overnight

Histochemical staining for
alkaline phosphatase

Labeling with ^{14}C AA,
stimulation extraction of
phospholipids and TLC
analysis

Stimulation, extraction of
total RNA for RTPCR
analysis

Figure 2. 9 Schematic showing the culture of osteoblasts and the analysis of cell responses to exogenous agents (such as LPS).

2.9. Osteoblast proliferation assay to measure activity of cpn10.

The measurement of cell proliferation was carried out as previously described (White *et al*, 1995). In brief, the human osteoblast-like cell line MG63 (CRL 1427; American Type Culture Collection, Rockville, MD) was cultured at a density of 15,000 cells/well in 96-well plates and incubated overnight at 37^0C in DMEM (Gibco) plus 10% FCS (Gibco) in 5% CO_2 /95% air. The media were then removed and cells were washed twice with sterile Hank's solution (Sigma). To measure anti-proliferative activity, various concentrations of r-*M. tuberculosis* cpn10 or truncated peptides were added in DMEM containing 2% FCS, to the MG 63 cells. Cells were incubated for 24 h at 37^0C. During the last 6 h of culture, 0.05 mCi of [^{3}H] thymidine (Amersham International plc, Amersham, UK) was added to cells. The media were then removed and the cells fixed in 5% trichloroacetic acid. 100ml of 0.5 M NaOH was used to lyse cells, this being neutralized by an equal volume of 0.5 M HCl. Radioactivity incorporated into nuclear DNA was measured by scintillation spectrometry. The cytotoxicity of the r-*M. tuberculosis* cpn10 was determined by lactate dehydrogenase release, measured by the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Heidelberg, Germany). Data have been generated from a minimum of three separate experiments.

2.10. Polymerase chain reaction.

The expression of mRNA for 5-LOX and 5-LOX activating protein (FLAP) was determined using the reverse transcription polymerase chain reaction (RT-PCR). This required the preparation of total cellular RNA from appropriately stimulated cells, which were cultured as described in Figure 2.9.

2.10.1. Total RNA preparation from cultured cells.

Total RNA was isolated by the method of Chomzynski and Sacchi (1987). All plastic ware was RNase free as was the water, which was treated with diethylpyrocarbonate (DEPC) to irreversibly inhibit RNase's. All reagents were purchased from BDH (Poole, England) and were of molecular biology grade. Cells were lysed in 600μl of solution D (4M guanidine thiocyanate, 0.5% N-lauryl-sarcosine, 25mM sodium citrate and 36μl 2-mercaptoethanol per 5ml of solution D), the lysate was transferred to 1.8ml tubes and 1200μl of ice cold absolute ethanol added followed by 180μl of 3M sodium acetate, this was vortexed and left at -20^{0}C for 1 hour. The tubes were centrifuged (10000g for 15 minutes), and the supernatant discarded. The pellet was resuspended in 600μl of solution D.

An equal volume of phenol:chloroform: isoamyl alcohol mixture (25:24:1) was added and the tubes vortexed, to form an emulsion. This was left on ice for 15 minutes. The emulsion was then resolved into aqueous and organic phases by centrifugation at 10000g for 15 minutes at 4^{0}C. The top, aqueous, phase was then removed to fresh microfuge tubes taking care not to disturb either the lower phenolic phase or the interface which contained proteins and DNA respectively. 1200μl of ice cold absolute ethanol was then added to the segregated aqueous phase followed by 180μl of 3M sodium acetate, this mixture was vortexed and left at -20^{0}C for 1 hour. The tube was then centrifuged at 10000g for 15 minutes and the supernatant discarded.
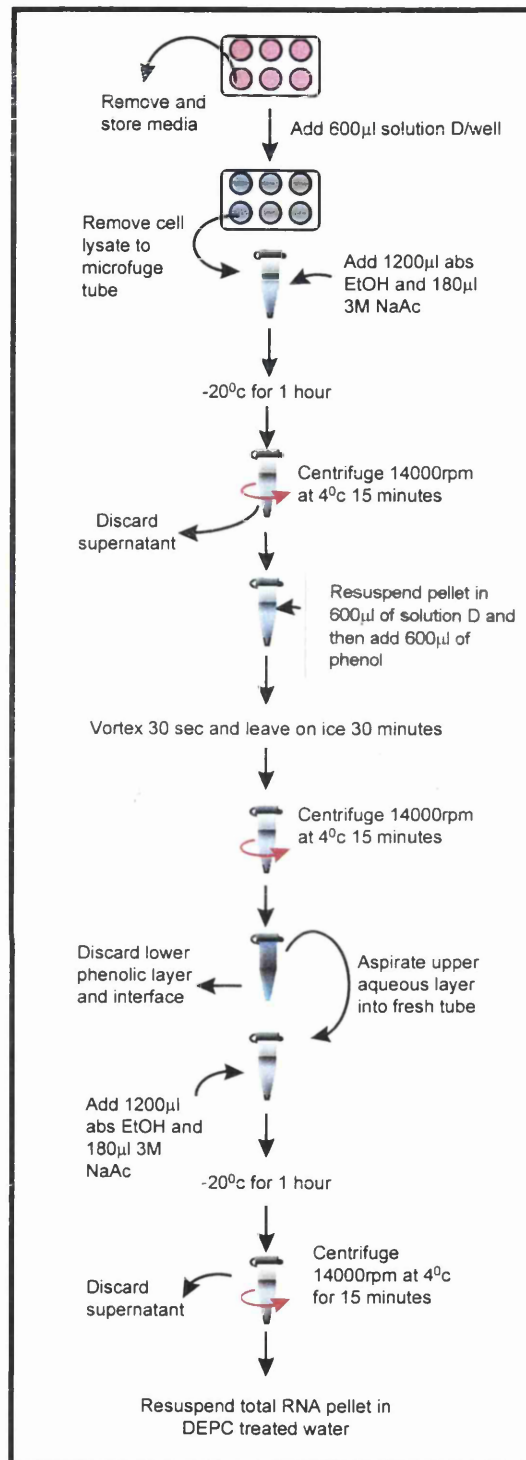
Figure 2. 10 Schematic showing the preparation of total cellular RNA.

The RNA pellet was then washed, first in ice cold 70% ethanol, to remove

salts left over from the isolation, then a second time in absolute ethanol. Each

wash was followed by a brief centrifugation at room temperature in a bench top

centrifuge at 10000g, prior to removal of the washing fluid. The pellet was then allowed to air dry. Once all the ethanol had evaporated the pellet was resuspended in 100μl of RNase free water.

The RNA was quantitated by measuring absorbance at 260nm, purity was assessed by calculating the ratio of absorbances at 260nm and 280nm. RNA was deemed pure enough for PCR if the ratio was 1.8 absorbance units. The procedure is summarised in Fig 2.11.

2.10.2. Preparation of complementary DNA.

Superscript II RNase H⁻ (i.e. with the RNase H activity removed) reverse transcriptase was used to make complementary first DNA strands, using total RNA, primed with an oligo dT primer. All reagents were purchased from GibcoBRL (Glasgow, Scotland) and used as directed by the manufacturer. The protocol is summarised in Figure 2.11. All plastic ware was sterile and RNase free as was the distilled water used. To RNase free tubes 1μl of Oligo $(dT)_{12-18}$ primer (500μg/ml, GibcoBRL) and 1μg of total RNA were added. The volume in the tubes was then made up to 12μl with RNase free sterile distilled water. This mixture was heated in a water bath for 10 minutes to $70^{0}C$ and then quick chilled on ice, the contents were then collected by a brief centrifugation. To this mixture were added the following components; 4μl 5X first strand buffer (250mM Tris HCL at pH 8.3, 375mM KCl, 15mM $MgCl_2$), 2 μl 0.1M dithiothreitol and 1 μl of 10mM dNTP mix (10mM each dATP, dGTP, dCTP, dTTP). To avoid pipetting errors a master mix of these constituents was first made up and 7μl of this added to the RNA/oligo dT mix in the reaction tubes.
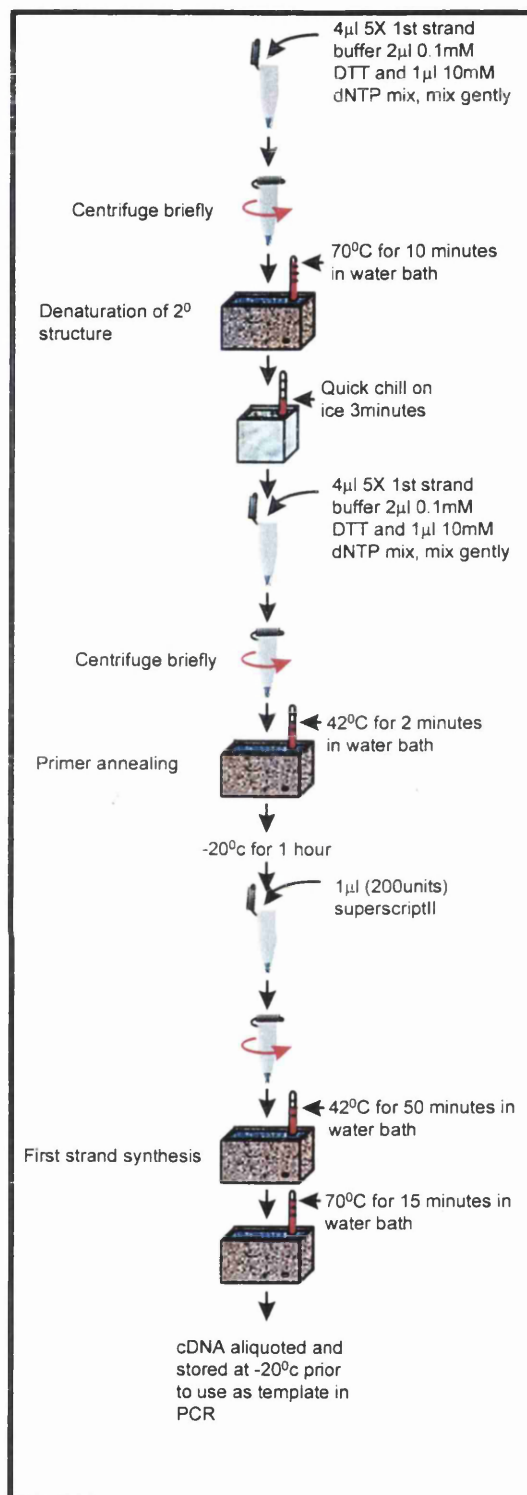
Figure 2. 11 Schematic of the protocol used in cDNA production.

The reaction tube contents were then mixed gently and heated at 42^0C for 2 minutes prior to the addition of 1μl (200U) of the superscript reverse transcriptase to each tube. The tubes were again gently mixed, briefly centrifuged

and the 42^0C incubation then continued for another 50 minutes. The reactions were inactivated by heating to 70^0C for 15 minutes, the cDNA was then aliquoted and stored at -20^0C prior to use as template in the PCR.

2.11.3. Polymerase chain reaction.

PCR was performed using Taq DNA polymerase with the cDNA, produced in the method described above, primed with oligonucleotides described in Table 2.2. The oligonucleotides were synthesised by Genosys (Pampisford, England). All other reagents were purchased from GibcoBRL (Glasgow, Scotland) and were of molecular biology grade. All plastic ware was nuclease free.

Target	Reference
5-LIPOXYGENASE	Brung's *et al* 1995
12- LIPOXYGENASE	Funk *et al* 1991
15- LIPOXYGENASE	Funk *et al* 1991
CYCLOOXYGENASE-1	Funk *et al* 1991
CYCLOOXYGENASE-2	Funk *et al* 1991
GAPD	Maier *et al* 1990.

Table 2. 2 Showing the target mRNA and source reference for the primers used in PCR.

10X PCR buffer (5), 10 mM dNTP mix (1),50mM MgCl$_2$ (1.5), Primer mix 20µMeach (1.25), H$_2$O (40.25)

1µl cDNA

48ml of PCR master mix to 1µl of cDNA

Mix

Centrifuge to collect contents at bottom tube

Tube placed in thermal cycler with heated lid at 104^{0}C

Heated lid closed and block temperature raised to 94^{0}C for 3 min

Lid opened, 1µl taq DNA polymerase added to each tube, lid closed

Begin thermal cycling

duplex denaturation(94^{0}c)   primer annnealing (62^{0}c)

primer extension (72^{0}c)

Stop cycling after 35 cycles

Analyse products by agarose gel electrophoreis

Figure 2. 12 Schematic of the PCR protocol used.

Into each 0.5ml reaction tube was added 1µl of cDNA, these were then kept on ice until the other components of the reaction had been assembled in a master mix and were ready to be added. The master mix contained per reaction;

62

5μl 10X PCR buffer (200mM Tris-HCL at pH8.4 and 500mM KCI), 1μl 10mM dNTP mixture (as described in section 2.5.2), 1.5μl 50mM $MgCl_2$, 2.5μl 3' primer and 5'primer at 10μM each and nuclease free water to 48.75μl. Once the master mix was assembled 48.75μl was added to each reaction. The tubes were gently mixed, spun in a bench top centrifuge and placed in the block of a thermal cycler fitted with a heated lid (Eppendorf 5103, Germany).

The lid temperature was brought up to 104^0C placed over the tubes and the block temperature elevated to 94^0C for 3 minutes, to denature the cDNA. The temperature was then lowered to 80^0C and 0.5μl of Taq polymerase added to each tube and gently mixed. Cycling could then commence, all reactions went through 35 cycles of 94^0C for 1 minute, 62^0C for 1 minute and 72^0C for 1 minute regardless of which primer set was being used. If the PCR was performed overnight the temperature was lowered to 8^0C until the samples were removed for analysis the following morning. The protocol is summarised in Figure 2.13.

2.11.4. Agarose gel electrophoresis.

PCR products were analysed electophoretically on 1.5% agarose gels, and were run against HindIII/EcoRI cut λ-phage DNA molecular weight markers (Figure 2.14).

The gels were made by melting 0.33g of agarose in 30ml of 1XTBE buffer (Made from 10X stock; 0.9M Trizma base, 0.9M Boric acid and 20mM EDTA at pH8.0), in a microwave oven. Once cooled to 50^0C the molten gel was poured into a horizontal casting tray with a comb inserted to form the sample wells. Once set, the comb was removed and the gel, still supported by the tray, was placed in the electrophoresis tank (Minigel, Biometra). The tank was filled with 1XTBE

buffer so the gel was just covered. 10µl aliquots of the PCR reaction mix were mixed with 2µl of 6X sample buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 15% w/v type 400 Ficoll) and loaded into the wells. The same process was repeated with HindIII/EcoRI cut λ-phage DNA molecular weight markers (Figure 2.14).
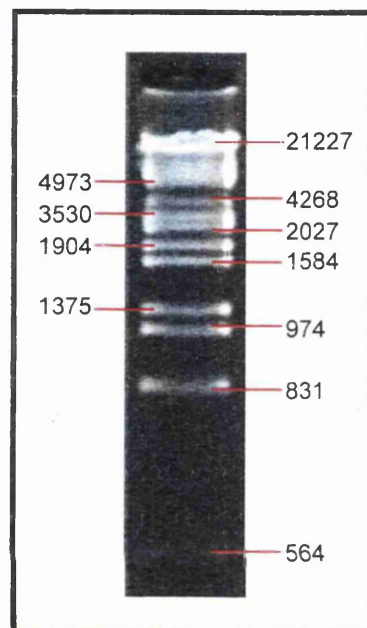
Figure 2. 13 HindIII/EcoRI cut λ-phage DNA molecular weight markers.

Electrophoresis was carried out for 1 hour at 70V. When complete, the gel was removed from the tank and bathed in a solution of ethidium bromide at 1µg/ml for 15 minutes. The gel was destained for a further 5 minutes in distilled water and visualised under UV light. The gel was recorded as a TIFF image with a Sony gel documentation system running UV products digitisation software (Cambridge, UK).

2.12. Thin layer chromatography.

Thin layer chromatography was used to analyse osteoblast production of arachidonic acid metabolites after stimulation with LPS or calcium ionophore.

2.12.1. Labelling and extraction of lipids.

Confluent cultures of primary human osteoblasts were trypsinised from the 75ml flasks in which they were maintained and seeded into 24 well plates at a density of 10^5 cells/ml in 1ml of media (10% FCS). These were then incubated overnight to allow adherence. Once adherent, the media were removed and replaced with 1ml of MEM (10% FCS) containing 0.25μCi/ml of ^{14}C-labelled arachidonic acid (5μl/ml of media; Amersham). The cells were then incubated for 24hr in the presence of radiolabel. After 24hr the media were removed and washed 3 times with PBS to remove unincorporated radiolabel and 1ml of MEM (2%FCS), containing appropriate concentrations of the experimental treatment/control was added and the cells incubated for an appropriate time. The media were harvested and put into 15ml polypropylene tubes on ice and acidified with formic acid to pH 3.5-4. Then 2 volumes of ethyl acetate were added and the tubes were vortexed for 1min, followed by centrifugation for 5 min at 3000rpm. The top organic layer was then removed to a fresh Falcon tube and the bottom aqueous layer was re-extracted with another 2 volumes of ethyl acetate. Then samples were dried down under a stream of dry nitrogen and reconstituted with 100μl of chloroform:methanol (2:1). The protocol is summarised in Figure 2.15.
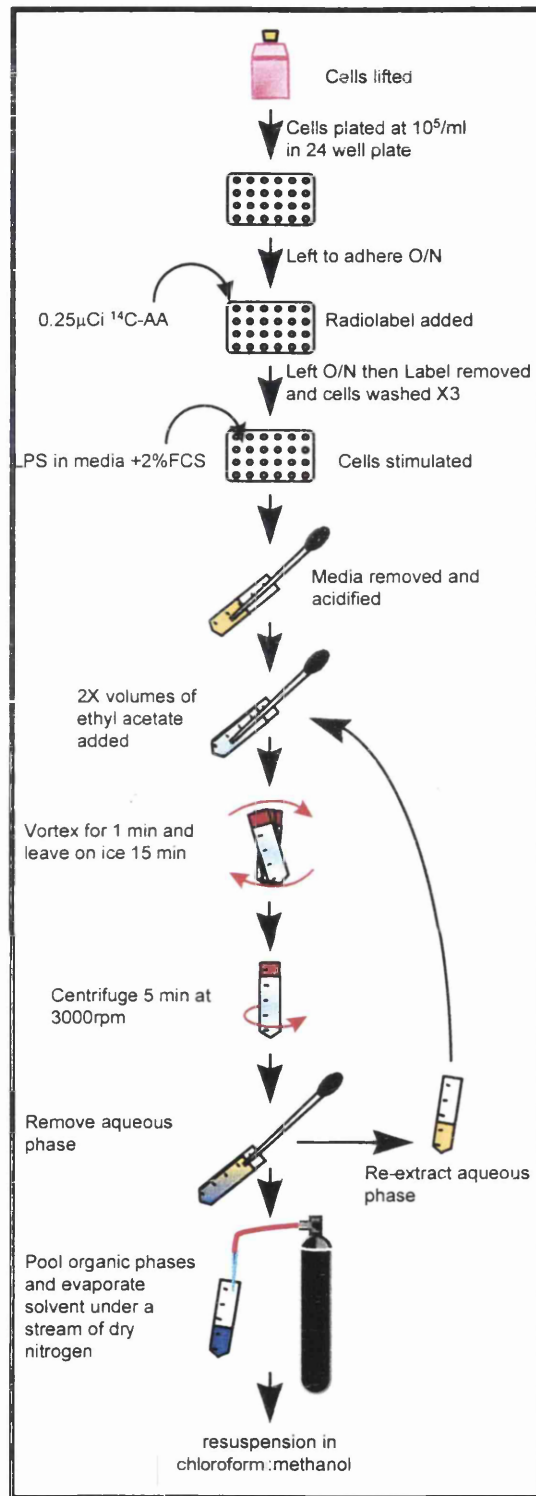
Figure 2. 14 Schematic of the protocol used for the isolation of cellular phospholipids.

2.12.2. Loading, running and analysis of TLC plates.

50μl of each sample was applied to the preabsorbent zone of a TLC plate (L6KD, Whatman) and allowed to dry. This was repeated with the standards. The TLC plate was then placed in a chromatography tank containing the solvent system: petroleum ether (50): diethylether (50): acetic acid (1). The tank was then covered to enable chromatographic separation to occur.
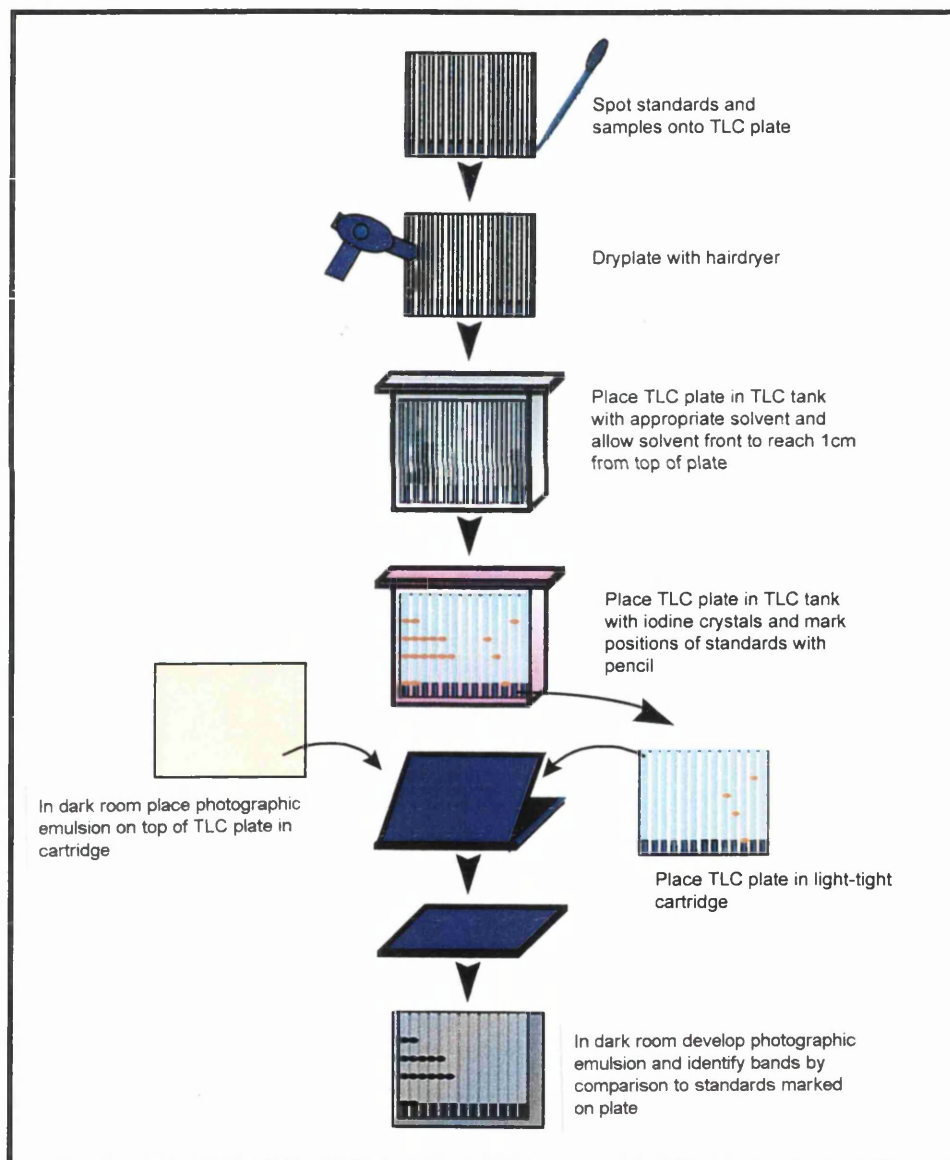
Figure 2. 15 Schematic describing the loading and development of TLC plates.

Once the solvent had reached the top of the plate it was removed from the

tank and allowed to dry in a fume cupboard, and local heating using a hair dryer was used to speed up this process. Once dried, the plate was placed in a tank of iodine vapour for 2 minutes, to visualise the standards, the positions of which were marked on the plate. The plate was then placed in a film cassette with X-ray film and exposed for 8 weeks and then developed. The process is summarised in Figure 2.16.

2.13. Statistical analysis.

The student t-test was used when appropriate to analyse the significance of results throughout this thesis.

CHAPTER THREE

MECHANISMS OF OSTEOLYSIS INDUCED BY

A PROTEINACEOUS SURFACE MATERIAL

ISOLATED FROM *STAPHYLOCOCCUS*

AUREUS.

3.1. Introduction.

Staphylococcus (S.) aureus is a facultatively anaerobic Gram-positive bacterium found on the skin and in the anterior nares of 10 to 30% of healthy individuals and is part of the normal human microflora. It is also a principal cause of bone destruction found in a number of lesions. Haematogenous infection with S. aureus is the major cause of pyogenic osteomyelitis (Jaffe, 1972) and it is the dominant organism associated with infected metal implants (Cioffe, et al; 1988; Ross, 1991). S. aureus is also the causative agent in more than 60% of cases of non-gonococcal arthritis (Goldenberg, 1989). In mice, intra-articular injection of live S. aureus results in the rapid destruction of the subchondral bone of the diarthrodial joints (Bremell, et al, 1992).

In all these conditions associated with S. aureus infections, destruction of the calcified extracellular matrix of bone is rapid and severe. Whether the marked involvement of this particular bacterium in bone destruction is due to its greater propensity, relative to other bacteria, for colonising bone or to more active bone-modulating activity is not clear. Indeed the mechanism by which S. aureus stimulates bone destruction, and particularly that associated with osteomyelitis bone necrosis, is still mysterious.

Workers at the Eastman Dental Institute (EDI) have been investigating the role of various Gram-negative bacteria in the bone destruction which accompanies chronic inflammatory periodontal diseases (CIPD). We have shown that the surface-associated material (SAM) from these bacteria has the capacity to stimulate bone resorption in vitro (Wilson et al 1986, 1993). This very soluble, largely proteinaceous material, removed by a short period of gentle stirring in normal saline (Wilson et al,1986), is also a potent inhibitor of bone collagen

synthesis (Meghji _et al_, 1992a), and has an effect on osteoblasts and other cell populations including fibroblasts, epithelial cells, macrophages and neutrophils (Meghji _et al_, 1992b). Indeed, crude extracts of some of these bacterial SAM's are as much as 2-3 log orders more active in stimulating bone resorption than the corresponding lipopolysaccharides (LPS), which are normally thought to be responsible for bone destruction in the periodontal diseases (Wilson _et al_,1986).

S. aureus is a Gram-positive organism which, by definition, lacks LPS. We have extracted the SAM from this bacterium and have shown that the majority of the material solubilised was protein. The SAM has been shown to be a potent activator of osteolysis in the murine calvarial bone resorption (Nair _et al_, 1995).

In this study the relative potency of SAM compared to other, purified, surface molecules from _S. aureus_ as an inducer of calcium release in the murine calvarial bone explant assay has been examined, revealing it to be much more active in this assay than either muramyl dipeptide or lipoteichoic acid (LTA). The possibility that this material stimulates the maturation of osteoclast precursors has been investigated and has revealed that this surface-associated fraction is a potent inducer of osteoclast maturation and is also an activator of osteoclasts. The role of various mediators (cytokines, prostanoids and lipoxygenase products) in the process of TRAP-positive MNC maturation has been investigated. The possibility that this material acts via the synthesis of $1,25\text{-}(OH)_2$Vitamin D_3 (VitD) has also been investigated.

3.2. Materials and methods.

3.2.1. Origin of SAM, lipoteichoic acid and teichoic acid.

Lipoteichoic acid and teichoic acid were obtained as described in chapter 2 (section 2.1.4). Growth and harvest of S. aureus followed by extraction of SAM was carried out as described by Wilson et al (1986) and in chapter 2 (section 2.1.5.).

3.2.3. Electron microscopy and analysis of SAM.

The protein, carbohydrate, and lipid content of the SAM were determined as described in chapter 2 (section 2.1.5.).Electron microscopy was carried out as described in chapter 2 (section 2.1.5.).

3.2.4. Fractionation, heat and trypsin treatment of SAM.

In experiments to identify the nature of the active principle in SAM fractionation, heat treatment and trypsinisation of SAM were carried out as described in chapter 2 (section 2.1.5.).

3.2.5. Assay of osteolytic capacity.

The ability of the agents tested here to induce calcium release from neonatal murine calvaria was used as a measure of osteolytic capacity. This assay was carried out as described in chapter 2 (section 2.6. and its subsections). The agents to be tested were added to the culture media of the bones at the concentrations described in the relevant results section of this chapter.

3.2.6. Assay of osteoclastogenic capacity.

The ability of the agents tested here to induce recruitment of TRAP+ve MNC's in murine bone marrow cell cultures was used as a measure of osteoclastogenic capacity. This assay was carried out as described in chapter 2 (section 2.7. and its subsections). The agents to be tested were added to the culture media of the bones at the concentrations described in the relevant results section of this chapter. The SAM and its fractions were tested in a number of assays (minimum of three) to ensure reproducibility of results.

3.2.7. Inhibitor studies.

The role of prostaglandins in TRAP-positive MNC formation was tested by adding indomethacin (section 2.4.3.). The role of IL-1 was assessed by add'ng the IL-1 receptor antagonist (section 2.2.). The involvement of tumour necrosis factor (TNF)α was determined by use of the neutralising monoclonal antibody TN3-19.12 (section 2.3.2). The role of IL-6 was determined by adding a neutralising rabbit antibody to murine IL-6 (section 2.3.2). These inhibitors were added to bone cultures at the same time as the SAM, which was used at a concentration of 10μg/ml in all of these studies. Calcitonin (10^{-8} M) was also added to cultures to determine the role of this calciotropic hormone in the SAM-induced formation of osteoclast-like cells.

3.2.8. Dihydroxy vitamin D₃ assay.

The ability of the SAM to induce the production of 1,25-dihydroxy vitamin D$_3$ (VitD) was measured by radio-immunoassay (RIA) was carried out as described in chapter 2 by Dr. B. Mawer (section 2.5.2.).

3.3. Results.

3.3.1. Effect of extraction of SAM on bacterial cell structure.

S. aureus exhibited large amounts of extracellular material when bacteria were stained with ruthenium red and viewed by transmission electron microscopy (Figure 3.1A). When the cells were extracted with saline this material was almost completely removed as can be seen in Figure 3.1B and this extraction was not associated with the lysis of cells or any easily discernible damage to the cell structure.
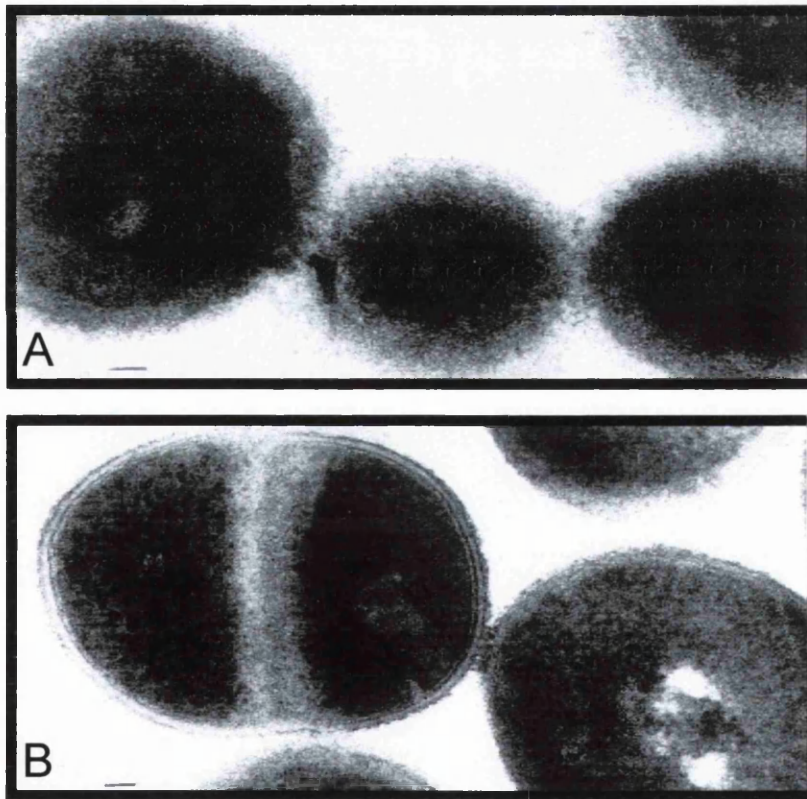
Figure 3. 1 Transmission electron micrographs showing the appearance of *S. aureus* prior to (A) and after (B) extraction of the surface-associated material. The ruthenium-stained surface-associated material can be seen in (A) is virtually completely removed by the extraction with saline without any structural damage (B). (Scale bar 0.1μm.).

3.3.2. Composition of the SAM.

The protein content of the saline extracted SAM was 30% (w/w) and the carbohydrate content was 5%. Lipid and nucleic acids constituted only a few percent of the SAM. The reason that these percentage compositions do not add up to 100% is likely to be due to the assays used underestimating the particular constituents being assayed.

3.3.3. Effect of SAM in the calvarial bone resorption assay.
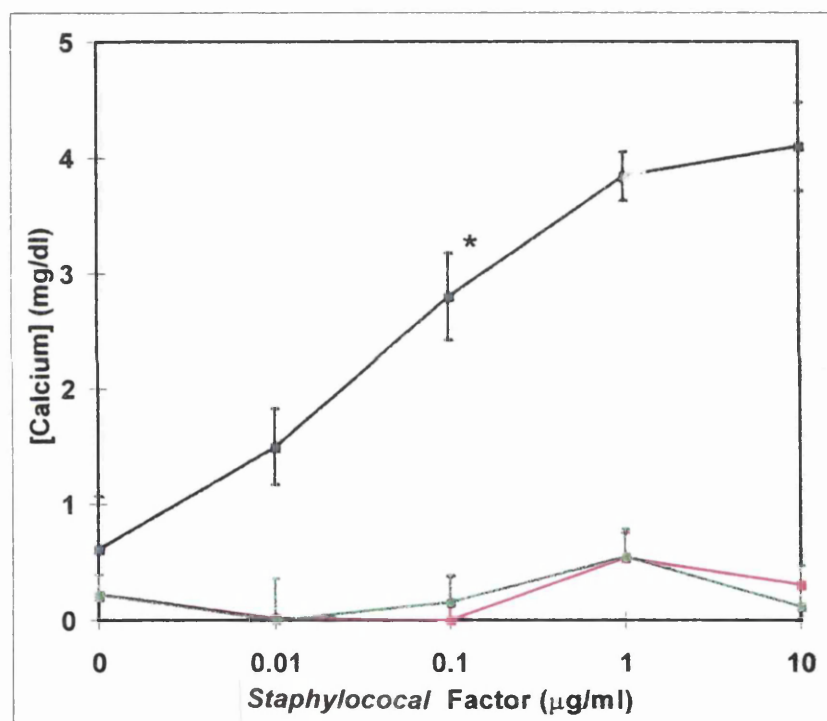
Figure 3. 2 The effects of S.aureus SAM, muramyl dipeptide and lipoteichoic acid on calcium release from neonatal murine calvaria. Neither muramyl dipepetide nor lipoteichoic acid cause any significant calcium release over the concentration range tested. The SAM however is highly potent, causing significant calcium release over the range tested. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

The SAM dose-dependently and reproducibly stimulated calcium release from neonatal murine calvaria. In the data shown in Figure 3.2 there was a 3-fold increase in calcium release at 100ng/ml and at 10μg/ml the calcium release was

eight fold higher than the background release. In contrast, neither the muramyl dipeptide nor the LTA had any significant effect on calcium release.

3.3.4. Effect of heat and proteolysis on SAM induced osteolysis.

Heat treatment or exposure to trypsin totally abolished the ability of the SAM to stimulate calcium release from neonatal murine calvaria (Figure 3.3).
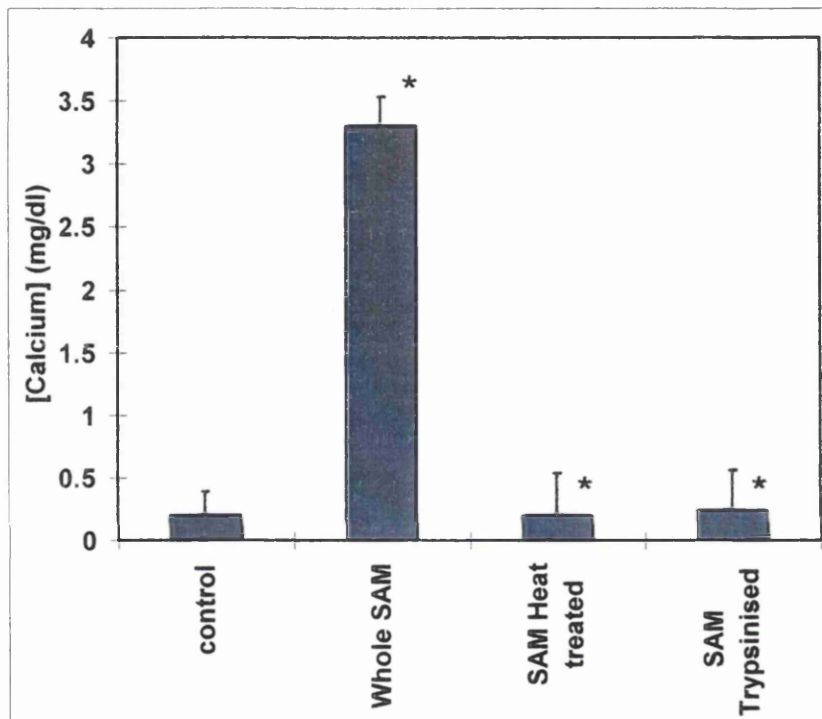
Figure 3. 3 The effect of heating or trypsinisation on the calcium releasing activity of *S.aureus* SAM. Both treatments reduced calcium release to unstimulated control levels. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

3.3.5. Fractionation of SAM and calcium release.

Fractionation of the SAM into two molecular weight populations demonstrated that the osteolytic activity was spread between the two populations (Figure 3.4). The fraction with a molecular weight of >30kDa was 3.5 times as potent as the <30kDa fraction. However in the smaller molecular weight fraction there was still significant osteolytic activity compared to control.
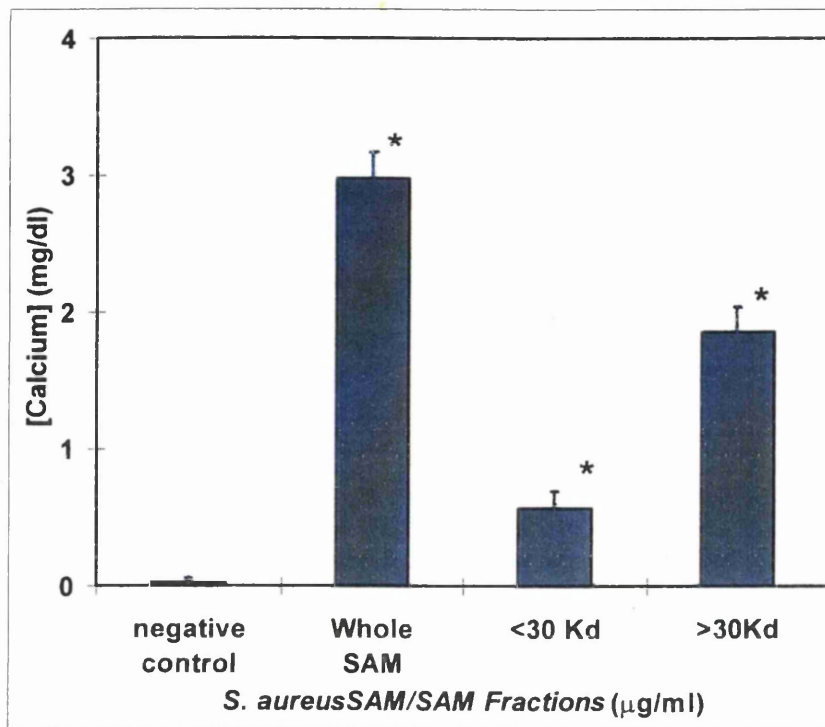
Figure 3. 4 Effects on calcium release from calvaria of <30kDa and > 30kDa SAM fractions compared to whole SAM. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

3.3.6. Effects of *S.aureus* SAM on osteoclastogenesis.

The SAM dose-dependently and reproducibly stimulated TRAP-positive MNC formation in murine bone marrow cultures. In the data shown in Figure 3.5 there was a 3-fold increase in TRAP-positive MNCs at 10ng/ml and at 10μg/ml the osteoclast-like cell numbers had increased 18-fold. Heat treatment or exposure to trypsin totally abolished the ability of the SAM to stimulate TRAP-positive MNC formation (Results not shown). Fractionation of the SAM into two molecular weight populations demonstrated that the TRAP-positive MNC-stimulating activity had a molecular weight of >30kDa. SAM also stimulated pit formation, that is, resorption, on dentine slices in a dose-dependent manner, with significant increase in pit area at a concentration of 100ng/ml and above (Figure 3.6 and 3.7).
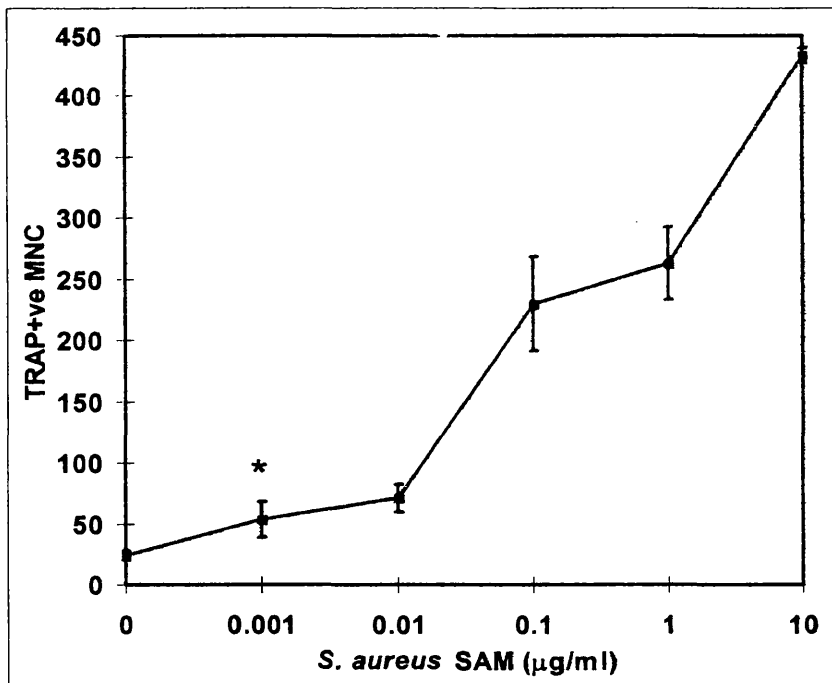
Figure 3. 5 The effect of SAM on the number of TRAP+ve MNC in bone marrow culture. Expressed as mean and SD of quadruplicate cultures (*p<0.01).
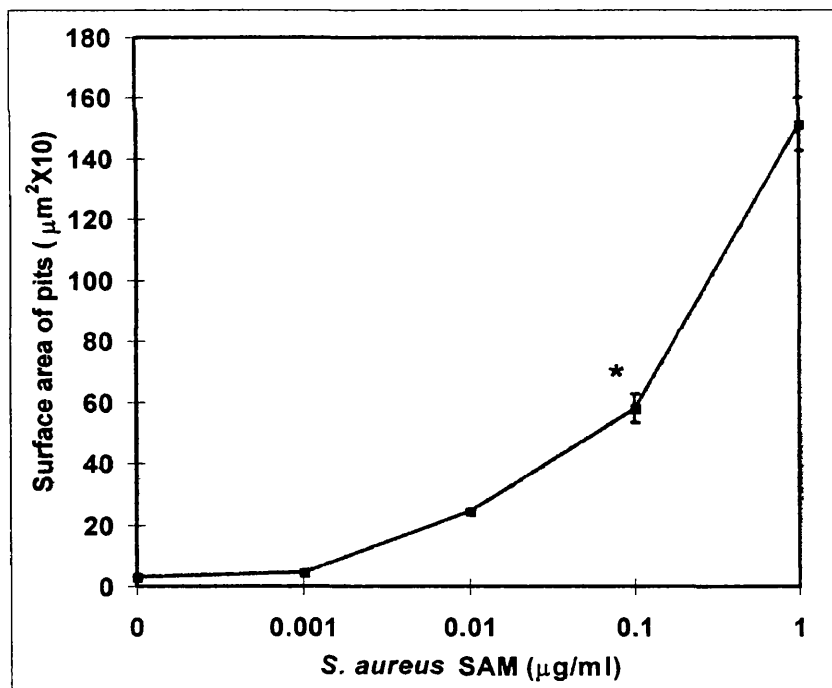
Figure 3. 6 Area of pits excavated by TRAP+ve cells on dentine slices following exposure to SAM from S. aureus. Results expressed as mean and standard deviation of quadruplicate cultures (* p< 0.01).
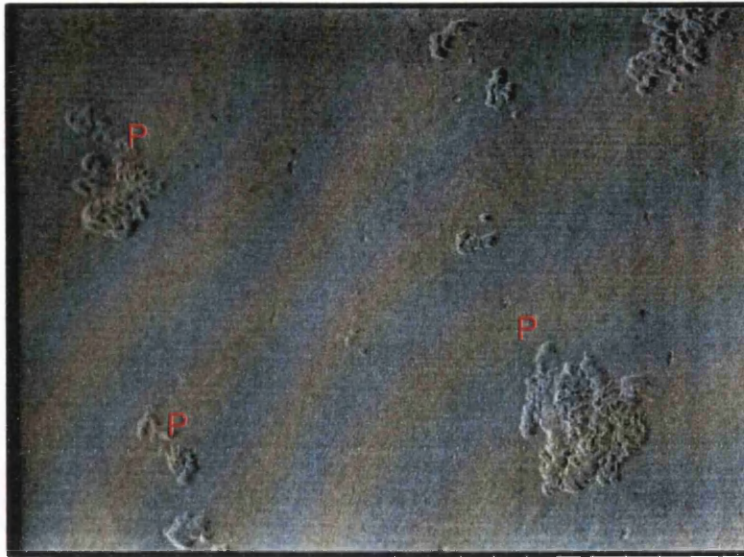
Figure 3. 7 Scanning electron micrograph of a dentine slice on which marrow cells were cultured. Mouse marrow cells were cultured with SAM (1μg/ml) on the dentine slice for 10 days. A number of resorption pits (P) are seen.

3.3.7. Effect of calcitonin on SAM induced osteoclastogenesis.

Calcitonin inhibited *S. aureus* SAM-induced osteoclast formation by >50%, when added at 10^{-9}M (p<0.01) (Figure 3.8).
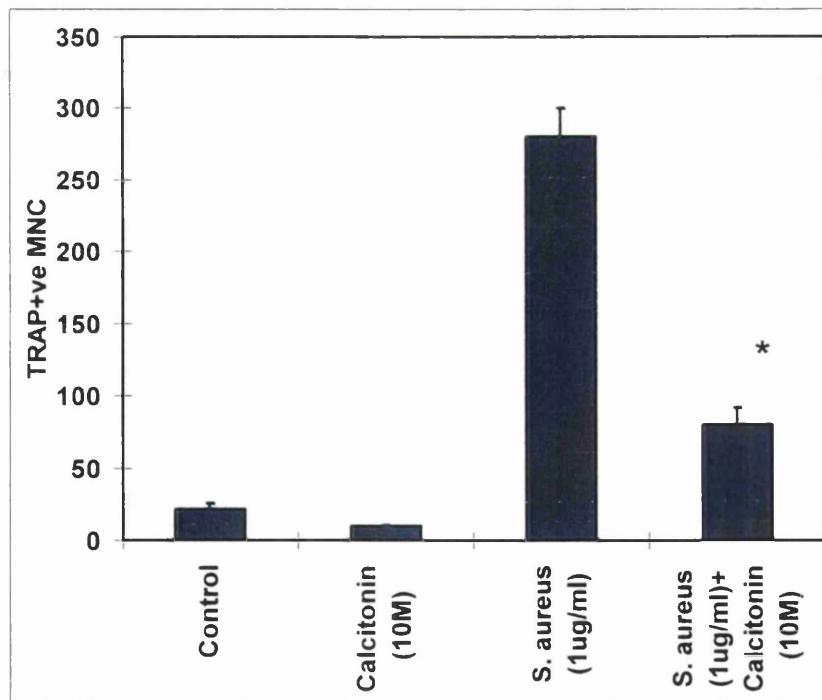
Figure 3. 8 Effect of calcitonin (10-9M) on SAM (1μg/ml) induced osteoclast formation. Expressed as mean and SD of quadruplicate culture (*p<0.01).

There was a minor inhibitory effect observed when calcitonin was added alone, this was expected as calcitonin has a direct inhibitory effect on the activity and formation of osteoclasts (presence of calcitonin receptors are a marker for the osteoclast phenotype).

3.3.8. Effects of anti-IL-6 on SAM induced osteoclastogenesis.

An anti-IL-6 antibody inhibited osteoclastogenesis, with >50% inhibition being seen at 1/500 dilution and complete inhibition at 1/300 dilution (Figure 3.9).
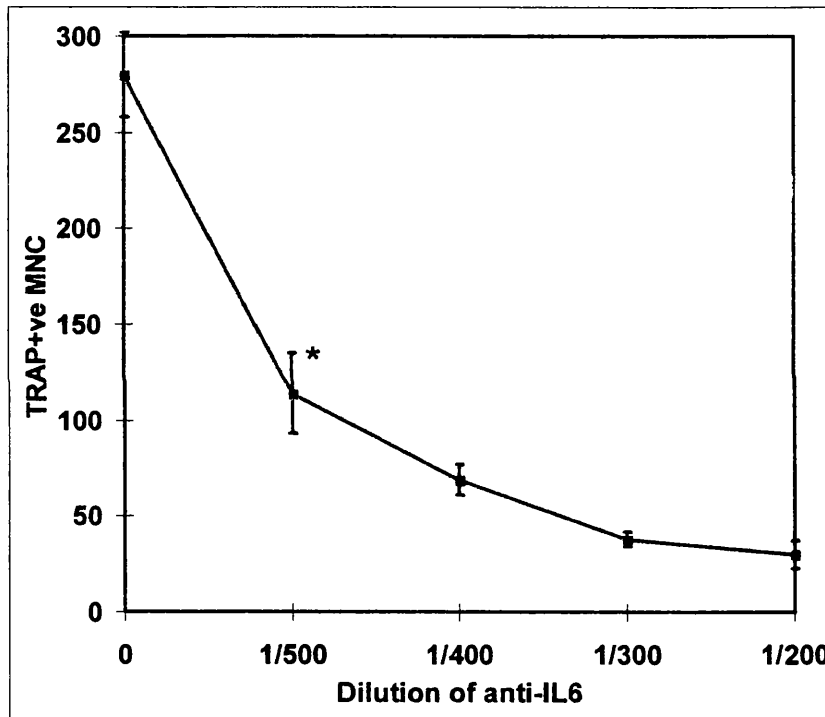
Figure 3. 9 The inhibition of TRAP+ve MNC formation (induced by 1µg/ml _S. aureus_ SAM) by a rabbit antibody to murine IL-6. Results are expressed as mean and standard deviation of quadruplicate cultures. (* p< 0.01).

3.3.9. Effect of anti-TNFα on SAM induced osteoclastogenesis.

A hamster anti-murine TNFα antibody completely inhibited _S. aureus_ SAM stimulated osteoclastogenesis at 10µg/ml with 50% inhibition being seen at 0.1µg/ml (Figure 3.10). This antibody also significantly inhibited the spontaneous generation of TRAP-positive MNC's in unstimulated cultures.
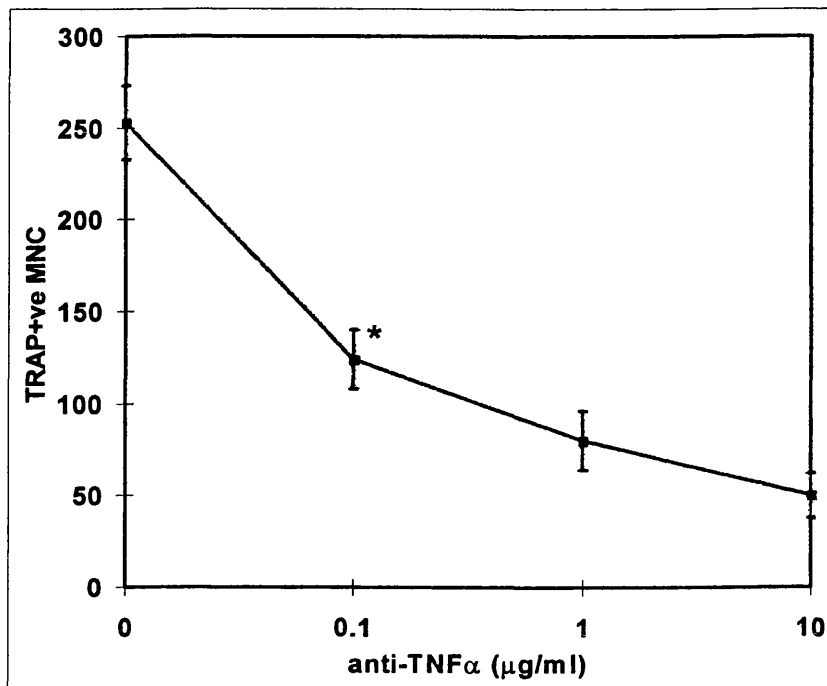
Figure 3. 10 Inhibition of SAM (1μg/ml) induced osteoclastogenesis by anti-TNFα. Expressed as mean (+/-SD) of quadruplicate cultures. (*p< 0.01).

3.3.10. Effects of IL1-ra on SAM induced osteoclastogenesis.

The effects of IL1 were neutralised with the physiological antagonist IL1ra.
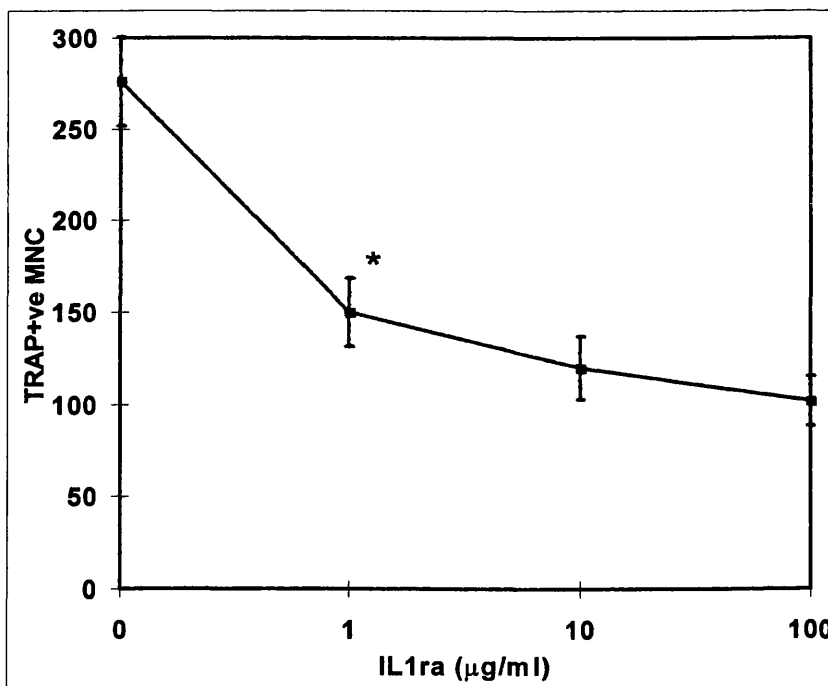
Figure 3. 11 Inhibition of SAM (1μg/ml)-induced osteoclastogenesis by IL-1ra, expressed as mean +/- SD of quadruplicate cultures. (* p< 0.01).

However neutralisation of the cytokine IL-1 by the natural IL-1 receptor antagonist, only inhibited osteoclastogenesis by a maximum of 40% at the highest concentration (100μg/ml) (Figure 3.11).

3.3.11. Effect of indomethacin on SAM induced osteoclastogenesis.

Inhibition of cyclooxygenases (and thus of prostaglandin synthesis), by indomethacin, failed to inhibit the SAM-induced osteoclastogenesis (Figure 3.12).
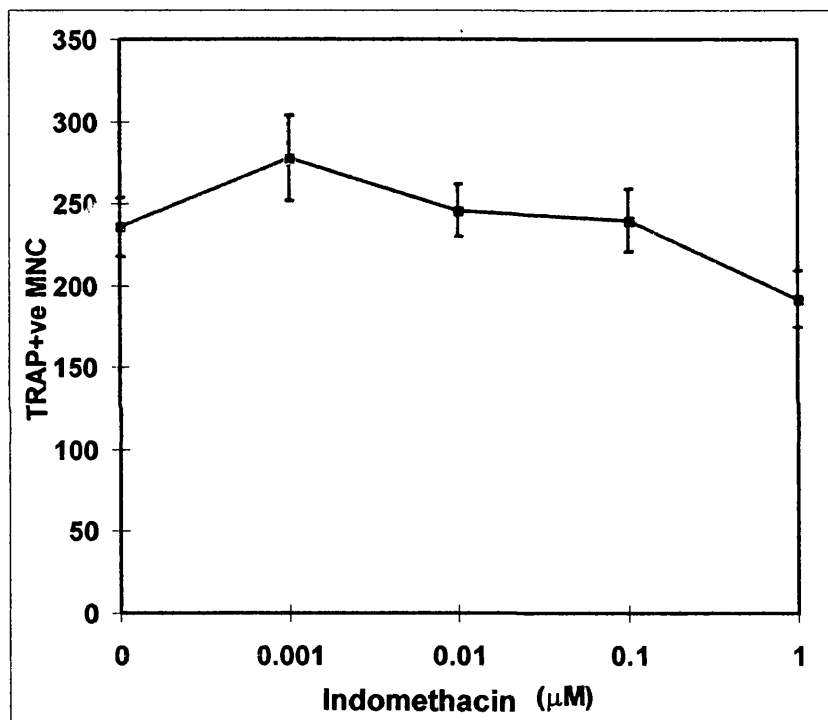
Figure 3. 12 Effect of indomethacin on *S. aureus* SAM (1μg/ml) induced osteoclastogenesis. Indomethacin (10^{-9} to 10^{-6} M) had no significant effect. Results expressed as the mean +/-SD of quadruplicate cultures.

3.3.12. Effect of MK886 on SAM induced osteoclastogenesis.

Inclusion of MK886, a selective inhibitor of FLAP inhibited TRAP-positive MNC formation, with roughly 50% inhibition being seen at concentration of 0.01μM (Figure3.13 on next page).

3.3.13. Effect of BW70C on SAM induced osteoclastogenesis.

A similar, though slightly less potent, inhibitory effect to that seen with MK886 is observed with the 5-lipoxygenase inhibitor BW70C (Figure 3.13), with just under 50% inhibition occurring at a concentration of 0.01µM.
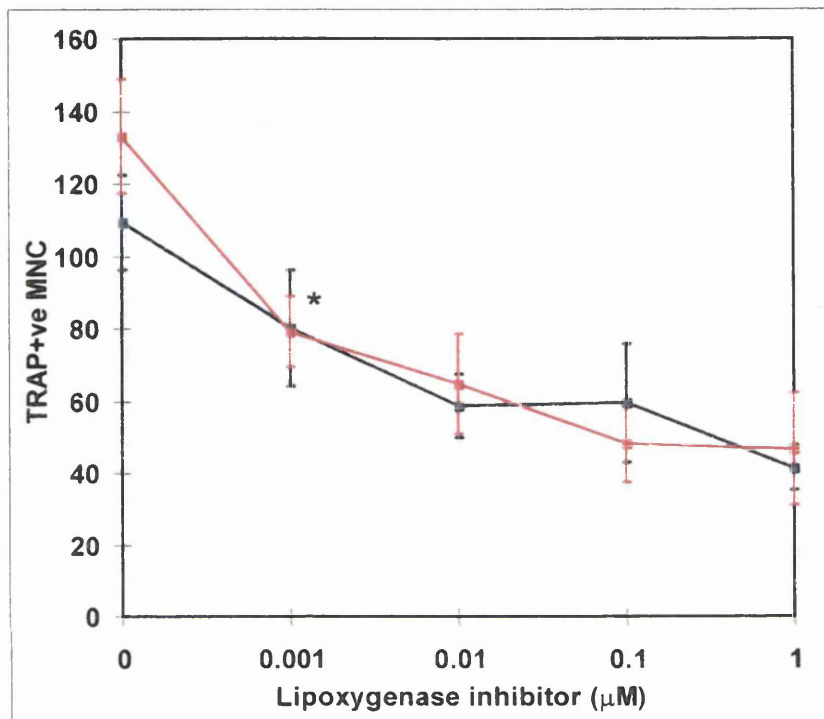
Figure 3. 13 The dose-dependent inhibition of *S. aureus* SAM (1µg/ml)-induced TRAP-positive MNC formation by MK886 and BW70C. Results are expressed as the mean and standard deviation of quadruplicate cultures. (*p< 0.01).

3.3.14. Assay of culture media for 1,25-(OH)$_2$ VITAMIN D$_3$.

The media supporting bone marrow cultures, taken at 4,12, or 48h after initiation of culture, showed insignificant (5-7pg/ml) levels of 1,25 (OH)$_2$ vitamin D$_3$.

3.4. Discussion.

In conditions such as infected orthopaedic prostheses and bacterial arthritis the major pathological change is the destruction of bone. Such osteolysis can be unrelenting and ferocious in progression. *S. aureus* is the major causative organism of acute and chronic osteomyelitis and is also the causative agent in around 60% of cases of non-gonococcal bacterial arthritis (Goldenberg, 1989 & Ho 1993) and of most cases of infected orthopaedic prostheses (Ross, 1991).

The mechanism of bone resorption induced by bacteria may either be due to the direct activity of bacterial constituents on bone cells or to an indirect effect resulting from the stimulation of the synthesis of osteolytic mediators by infiltrating leukocytes or mesenchymal cells. Very little is known about the mechanism of bone destruction induced by *S. aureus*. Previous studies from the EDI have concentrated on the osteolytic activity of surface-associated material from Gram-negative anaerobic and capnophilic bacteria implicated in the pathogenesis of periodontal disease. In this condition there is destruction of the alveolar bone supporting the teeth. The SAM from a number of, but not all, periodontopathic bacteria has been shown to be a potent stimulator of bone breakdown in the murine calvarial bone resorption assay (Wilson *et al*, 1986 & 1993). *S. aureus* is a capsulated bacterium and work from the EDI has demonstrated that the SAM from this organism is an extremely potent bacterial osteolytic mediator capable of stimulating breakdown of neonatal murine calvaria at concentrations as low as 1-10ng/ml (Nair *et al*,1995).

The classic finding of large numbers of osteoclasts at the periphery of the sequestrum in osteomyelitis made it sensible to investigate the effect of the SAM from *S. aureus* on osteoclast generation in bone marrow cultures. Addition of the

SAM to bone marrow cultures produced significant and reproducible increases in TRAP-positive MNC at concentrations as low as 1ng/ml and reproducible dose responses over the range 10ng/ml to 10μg/ml. The bone resorbing activity was completely abolished by either heating the SAM or exposing it to trypsin suggesting that the active components are proteinaceous. Fractionation of the crude mixture of surface components by Amicon filtration revealed that the active constituents in osteolysis were distributed between a <30kDa and >30kDa but that an osteoclast recruiting factor had a molecular weight >30kDa and may be the 32-34kDa protein described in an earlier study (Nair _et al_ 1995). This may indicate the presence of two different activities one inducing the recruitment of osteoclasts and another increasing the activity of extant osteoclasts.

SAM from _S.aureus_ stimulated TRAP-positive MNC formation in the absence of additional external factors and appears to be a competence factor both for the proliferation and for the fusion of osteoclast precursors. This is in contrast to many agents, for example TGFα, that increase MNC formation by stimulating proliferation of the osteoclast precursors (Takahashi _et al_, 1986), but depend on the addition of $1,25-(OH)_2$ vitamin D3 for the fusion of the precursors to form polykaryons (Takahashi _et al_, 1988). We explored the hypothesis that components in the SAM may have been inducing $1,25-(OH)_2$ vitamin D_3 synthesis thus accounting for the effects seen. However assay of the media from the bone marrow cultures revealed negligible quantities of $1,25-(OH)_2$ vitamin D_3.

To determine the mechanism of osteoclast formation, bone marrow cultures were stimulated with a fixed concentration of the SAM and a range of concentrations of "inhibitors" of major osteolytic mediators such as the prostanoids, 5-LOX products, IL-1, TNFα and IL-6 (reviewed by Meghji, 1992).

The activity of the cytokines was inhibited by addition of cytokine neutralising antibodies or the natural IL-1 receptor antagonist (IL-1ra). Cyclooxygenase (COX) activity was inhibited by addition of indomethacin. LOX product generation was inhibited by the addition of the FLAP inhibitor MK886 or the 5-lipoxygenase inhibitor BW70C.

The osteoclastogenic activity of the SAM was significantly inhibited by the inclusion of the antibodies to the cytokines, TNFα and IL-6. Neutralisation of IL-6 and TNF generation in bone marrow cultures totally inhibited the generation of TRAP-positive MNCs. In contrast IL-1ra was a fairly weak inhibitor of osteoclastogenesis. Despite the potent inhibitory activity of indomethacin in the murine calvarial assay (Nair et al, 1995), this compound had no effect on TRAP-positive MNC formation in bone marrow cultures. This may, in part, reflect intrinsic differences in both assay systems. However, it should be noted that TNFα-induced MNC formation is not inhibited by indomethacin (Pfeilschifter et al, 1989). In stark contrast to Indomethacin, the inhibitors of 5-LOX activity had very marked inhibitory effects on TRAP+ve MNC formation induced by SAM.

Inhibitors of LOX, but not COX, are known to interfere with LPS and S. aureus induced production of IL1 (Dinarello et al, 1984). Although this present work does indicate a role for IL1, there certainly seems to be an involvement of LOX products in mediating the effects of bacterial products, including those under investigation here. It has also been reported that LOX inhibitors can suppress the formation of TNF in LPS treated mice (Schade et al, 1989 a & b). It would be worthwhile then to examine, in the systems used here, what effect 5-LOX blockade might have on the evolution of TNF in response to S.aureus SAM.

The preceding disscussion is extremely interesting in the light of previous work emanating from this group which established that products of both 5- and 12-lipoxygenases were potent stimulators of bone resorption in the murine calvarial bone resorption assay (Meghji _et al_, 1988). The 5-LOX product, leukotriene (LT)B_4 was reported to be some 4 log orders more potent than PGE_2 in this assay. It was also found that the LTs were potent, although not terribly efficacious, stimulators of collagen, but not of non-collagenous protein (NCP), synthesis by murine calvaria. In contrast, the 5- and 12- hydroxyeicosatetranoic acids (HETEs) stimulated both collagen and NCP synthesis (Meghji _et al_, 1992). A direct effect of LTs on isolated human osteoclasts has also been reported (Fuller and Chambers, 1989).

The nature of the active moiety in the SAM has not been defined and, given the complexity of the bone marrow assay, this may prove to be a difficult task. It is clear that the active TRAP+ve recruiting agent is a protein of >30kDa. Work is currently in progress to isolate this active constituent.

In conclusion, the surface associated material of the Gram-positive bacterium S _aureus_ is a potent inducer of osteoclast-like cell formation in the murine bone marrow cultures. Activity is seen at concentrations as low as 1ng/ml (w/v). As this active moiety has a molecular mass of >30kDa and if one assumes that activity is due to one component, then the minimum effective concentration would be 25pM. This activity is inhibited by antibodies to IL-6 and TNF, by the 5-LOX inhibitors MK886 and BWA70C but is only partly inhibited by neutralising IL-1 and not at all by the COX inhibitor indomethacin. The ability of the SAM from this bacterium to stimulate osteoclastogenesis and the activity of these osteoclasts at extremely low concentrations must contribute to the pathology of

the various bone lesions associated with infection by *S. aureus*. The active

constituent in this mixture of proteins and carbohydrate represents an important

therapeutic target, as do the host factors that are mediating these effects, in view

of the increasing numbers of isolates of *S. aureus* resistant to antibiotics

(Duckworth, 1993).

CHAPTER FOUR

5-LIPOXYGENASE AND AGONIST INDUCED

OSTEOLYSIS.

CHAPTER 4:   5-LOX AND AGONIST INDUCED OSTEOLYSIS.

4.1. Introduction.

The pioneering work of Raisz and co-workers in the early 1970's (Klein and Raisz,1970) implicated oxidation products of arachidonic acid as mediators of bone resorption. Attention has thus focused on the actions of the products of cyclooxygenase activity - the prostaglandins (PG's) - and it is now accepted that PG's are able to stimulate both the breakdown and the growth of bone (Harvey et al, 1986; Norrdin et al, 1990). However little attention was paid to the other products of arachidonic acid metabolism

Work done at the EDI established that products of the action of both 5- and 12-lipoxygenase (LOX) on eicosatetraenoic acid were potent stimulators of bone resorption in the murine calvarial bone resorption assay (Meghji et al, 1988). The 5-LOX product, leukotriene (LT)B_4 was reported to be some 4 log orders more potent than PGE_2 in this assay. It was also found that the peptidoleukotrienes (pLT's) were as potent as LTB_4 in the promotion of bone resorption. The LT's were potent although not terribly efficacious, stimulators of collagen, but not of non-collagenous protein (NCP), synthesis by murine calvaria. In contrast, the 5- and 12- hydroxyeicosatetranoic acids (HETE's) stimulated both collagen and NCP synthesis (Meghji et al, 1992). In spite of these reports other workers reported that LTs had little effect on bone (Fuller and Chambers, 1989) thus the role of 5-LOX products in bone resorption were neglected for a number of years.   More recent work from Mundy's laboratory has supported the initial findings from the EDI and has demonstrated that LT's can both promote the osteolytic activity of osteoclasts (Gallwitz et al, 1993) and the recruitment of these cells (Garcia et al, 1996). A direct effect of LT's on isolated human osteoclasts

has also been reported (Fuller and Chambers, 1989). It is likely that the failure of other workers to demonstrate the osteolytic activity of LT's is related to the susceptibility of these lipidic mediators to oxidation, a reaction that results in products lacking activity (Gallwitz *et al*, 1993).

The potent actions of LOX products on both calvarial bone resorption and on the activity of isolated osteoclasts suggests that these lipid mediators may play a role in either normal physiological bone turnover and/or in states of bone pathology such as is found in localised inflammatory diseases (e.g. periodontitis or rheumatoid arthritis) or in systemic diseases such as osteoporosis.

To determine the role of 5-LOX in bone resorption, neonatal murine calvarial explants have been stimulated with a range of osteolytically-active molecules in the presence of selective inhibitors of this enzyme. This has revealed that only an oral lipopolysaccharide (LPS)-induced bone resorption was blocked by such inhibitors and the relationship between LPS and osteolysis has been examined. In this study, use has been made of LPS from both *E. coli* and from an oral Gram-negative bacteria (*A. actinomycetemcomitans*), as this oral bacterium and the LPS it produces are implicated in the rapid loss of alveolar bone found in patients with periodontal diseases (Nair *et al*, 1996).

CHAPTER 4: 5-LOX AND AGONIST INDUCED OSTEOLYSIS.

4.2. Materials and methods.

4.2.1. Source and use of osteolytic mediators.

The hormones, eicosanoids and cytokines are all as described in chapter 2 (section 2.2.) and were used at optimal concentrations. Parathyroid hormone (PTH) was used at a concentration of 0.5 units/ml. 1,25 $(OH)_2$ vitamin D_3 (VitD) was used at a concentration of 10^{-8}M. IL-1α and was used at a concentration of 0.5nM. PGE_2 was used at a concentration of 1μM.

The *A. actinomycetemcomitans* LPS preparation was as described in chapter 2 (section 2.1.3.); it was used at concentrations ranging from 0.01 to 10μg/ml as described in the results section of this chapter. The *E. coli* LPS was also that described in the chapter 2 (section2.1.3.), and was used at concentrations ranging from 0.01 to 10 ng/ml.

4.2.2. Assay of osteolytic capacity.

The ability of the osteolytic agents tested here to induce calcium release from neonatal murine calvaria was used as a measure of osteolytic capacity. This assay was carried out as described in chapter 2 (section 2.6. and its subsections). The agents to be tested were added to the culture media of the bones at the concentrations described in the relevant results section of this chapter. Each agonist was tested at least three times to ensure the reproducibility of results.

4.2.3. Assay of osteoclastogenic capacity.

The ability of the agents tested here to induce recruitment of TRAP+ve MNC's in murine calvarial bones was used as a measure of osteoclastogenic

capacity. This assay was carried out as described in chapter 2 (section 2.6.3.).
The agents to be tested were added to the culture media of the bones at the concentrations described in the relevant results section of this chapter. The individual examining the explants was unaware of the treatment to which they had been exposed.

4.2.4. Measurement of peptidoleukotrienes.

The concentrations of pLTs (LTC_4, D_4 and E_4) in the culture media supporting murine calvarial explants was assayed by enzyme immunosorbent assay (EIA) as described in chapter 2 (section 2.5.1).

4.2.5. 5-LOX and COX inhibitors.

The 5-LOX inhibitors MK886, BWA70C and BWA4C were as described in chapter 2 (section 2.4.) as was the COX inhibitor, indomethacin. These were used at concentrations between 0.1 and $10\mu M$, as described in the relevant results sections of this chapter.

4.3. Results.

4.3.1. Effect of 5-LOX inhibitors on VitD$_3$ induced osteolysis.

In calvaria exposed to VitD$_3$, a systemic hormone involved in calcium homeostasis, the selective inhibitor BWA70C had little or no effect over the dose range 100nM to 10mM (Figure 4.1). However, at the very highest concentration BWA4C did have some inhibitory activity (Figure 4.1).
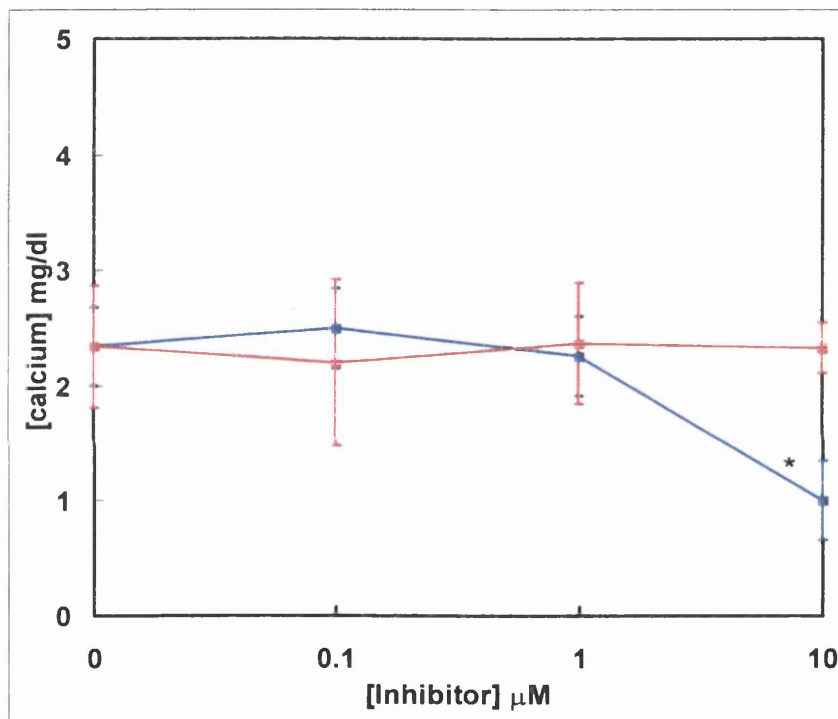
Figure 4. 1 Effects of BWA70C and BWA4C on 1,25-dihydroxycholecalciferol (10nM) induced calcium release from neonatal murine calvaria. Expressed as mean and SD of quintuplicate cultures (* p< 0.1).

4.3.2. Effect of 5-LOX inhibitors on PTH induced osteolysis.

In calvaria exposed to the circulating calciotropic hormone, PTH, the selective inhibitor BWA70C (Figure 4.2), had little or no inhibitory effect over the dose range 100nM to 10mM. However, at the highest concentration BWA4C did have some inhibitory activity (Figure 4.2).
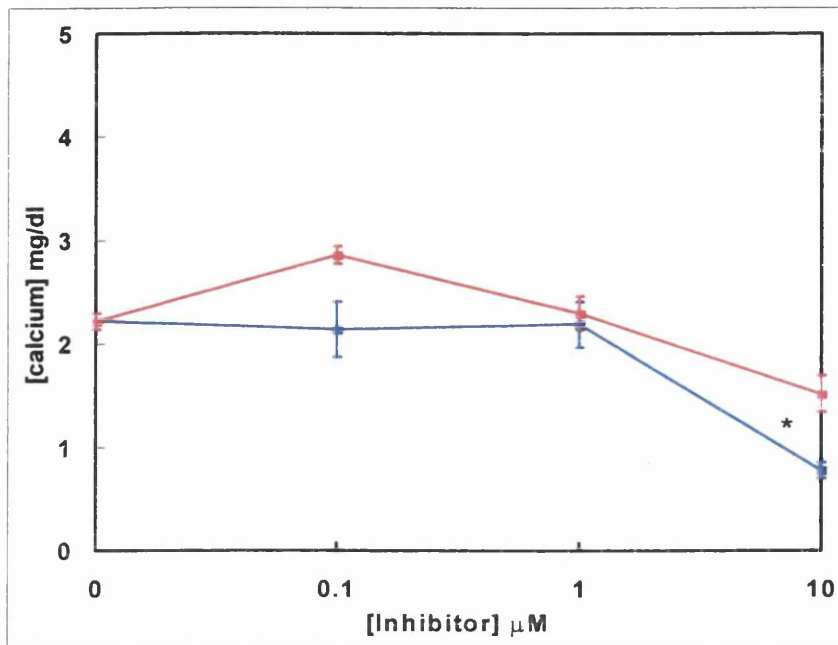
Figure 4. 2 Effects of BWA70C and BWA4C on PTH (0.5 iu/ml) induced calcium release from murine neonatal calvaria. Expressed as mean and SD of quintuplicate cultures (* p< 0.1).

4.3.3. Effect of 5-LOX inhibitors on IL-1α induced osteolysis.

In calvaria exposed to IL1α, BWA70C had little or no inhibitory effect over the dose range 100nM to 10mM (Figure 4.3).
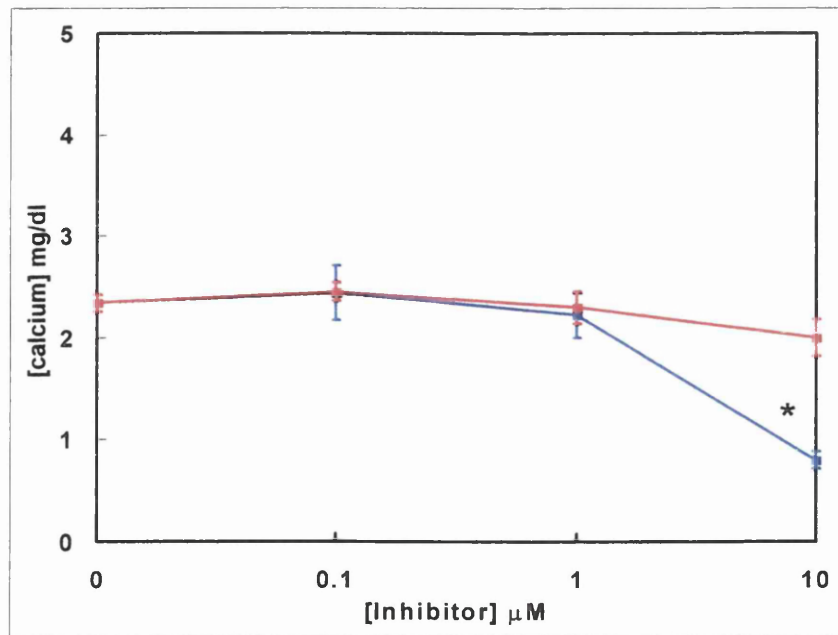
Figure 4. 3 Effects of BWA70C and BWA4C on IL1α (0.5nM) induced calcium release from neonatal murine calvaria. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

However, at the very highest concentration BWA4C did have some inhibitory activity (Figure 4.3).

4.3.4. Effects of 5-LOX inhibitors on PGE$_2$ induced osteolysis.

In calvaria exposed to PGE$_2$, a local mediator known to play a role in the modulation of some effects of the above hormones and cytokines, the selective inhibitor BWA70C, as in the case of the preceding agonists, had little or no effect over the dose range 100nM to 10mM (Figure 4.4). However, at the very highest concentration BWA4C did have some inhibitory activity (Figure 4.4).
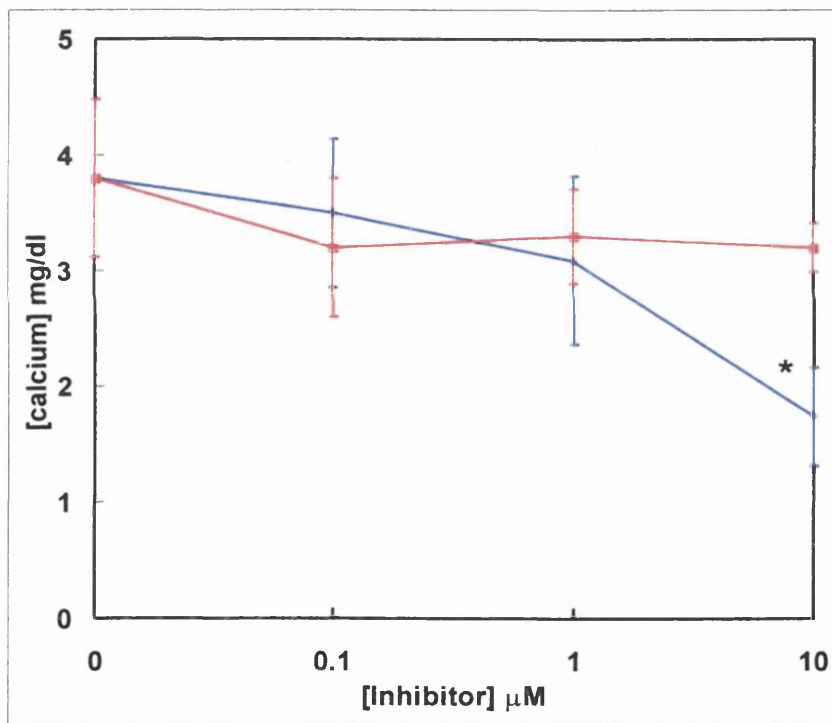
Figure 4. 4 Effects of 5-lipoxygenase Inhibitors (BWA70C and BWA4C) on PGE$_2$ (10^{-6} M) induced calcium release from neonatal murine calvaria. Expressed as mean and SD of quintuplicate cultures (* p< 0.1).

4.3.5. Effect of *E. coli* LPS on osteolysis.

Highly purified *E.coli* LPS proved to be a highly potent and efficacious stimulator of calcium release from murine calvaria (Figure 4.5), causing a significant increase in calcium release at 0.1ng/ml, with the activity increasing up

to a concentration of 1ng/ml. At 10ng/ml the amount of calcium being released

reached a plateau, possibly reflecting the cytotoxic effects of LPS (Figure 4.5).

This high potency and efficacy is not unexpected, considering a similar pattern of

dose dependent activity is observed in the induction of osteolytic cytokines by

peripheral blood monocytes exposed to highly purified *E. coli* LPS.
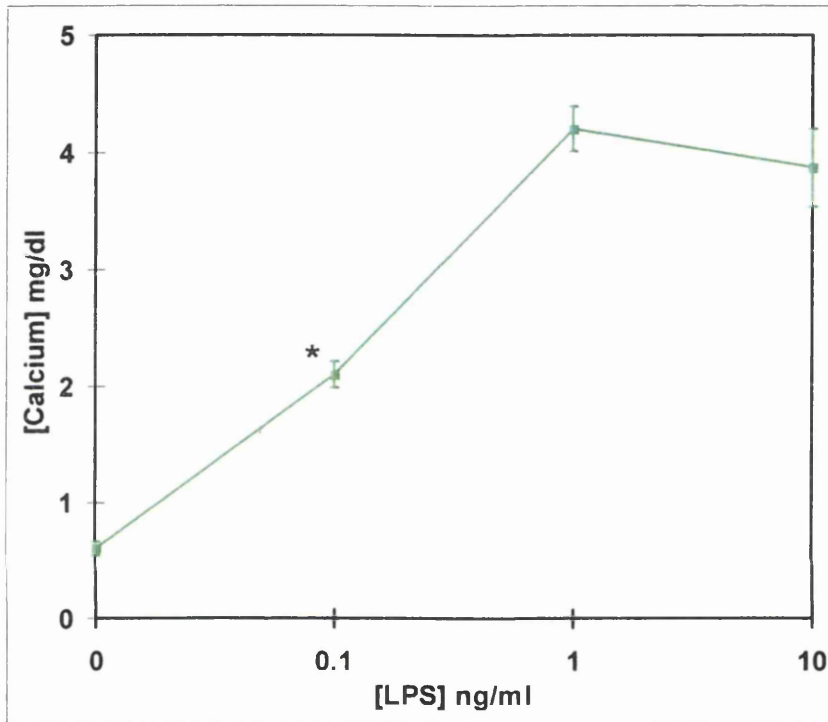
Figure 4. 5 Effects of highly purified *E*. coli LPS on calcium release from neonatal murine calvaria. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

4.3.6. Effect of 5-LOX inhibitors on *E.coli* LPS induced osteolysis.

5-LOX inhibition follows the same pattern established with the above

systemic and local osteolytic agonists. Both BWA70C and BWA4C failed to inhibit

calcium release induced by *E. coli* LPS, except at the very highest concentrations

(Figure 4.6).

This result was unexpected in the light of published reports that indicated a

role for 5-LOX activity in the modulation of LPS activities in cells of the myeloid

lineage. It is well documented that COX 2 is responsive to LPS in murine bone. In

order to check that the metabolism of arachidonic acid was proceeding in calvaria, and that the above result (Figure 4.6) did not reflect an aberration of bones in explant culture, the above experiment was repeated but replacing the 5-LOX inhibitors with the COX inhibitor, indomethacin (Figure 4.7).
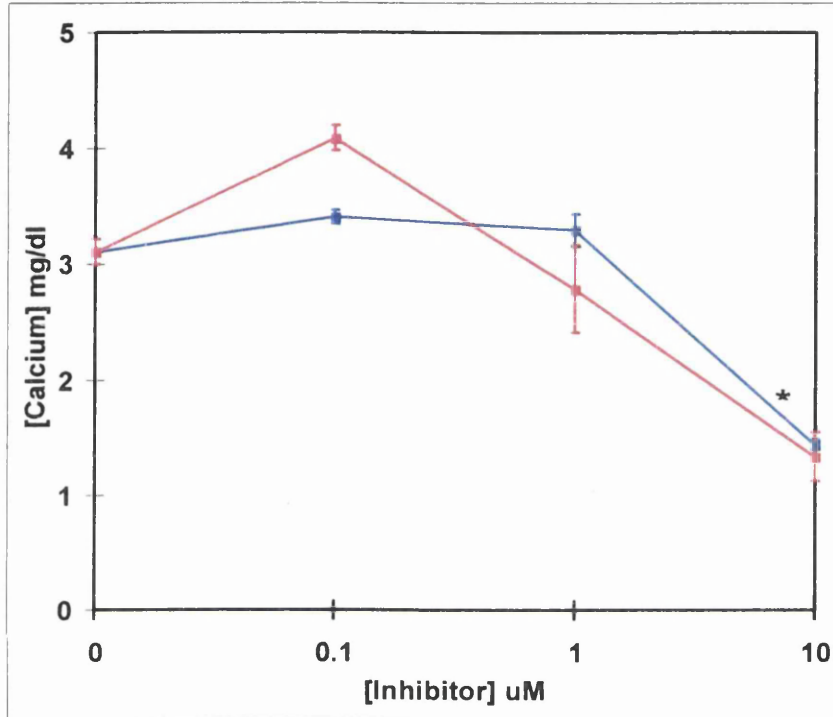
Figure 4. 6 Effects BWA70C and BWA4C on *E. Coli* LPS (0.1ng/ml) induced calcium release from neonatal murine calvaria. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

4.3.7. Effects of COX inhibition on *E.coli* LPS induced osteolysis.

Inhibition of COX with indomethacin revealed almost complete inhibition of calcium release at very low concentrations of inhibitor (Figure 4.7). This indicates that arachidonic acid metabolism in explanted murine calvaria is proceeding in response to LPS.

The lack of response to LOX inhibition is then not due to an artefact in arachidonic acid metabolism in calvaria. However, the response to the COX inhibition by indomethacin indicates the modulation of calcium release induced by

E.coli LPS is via prostanoid production, as has been reported elsewhere for the

hormones and cytokines tested here.
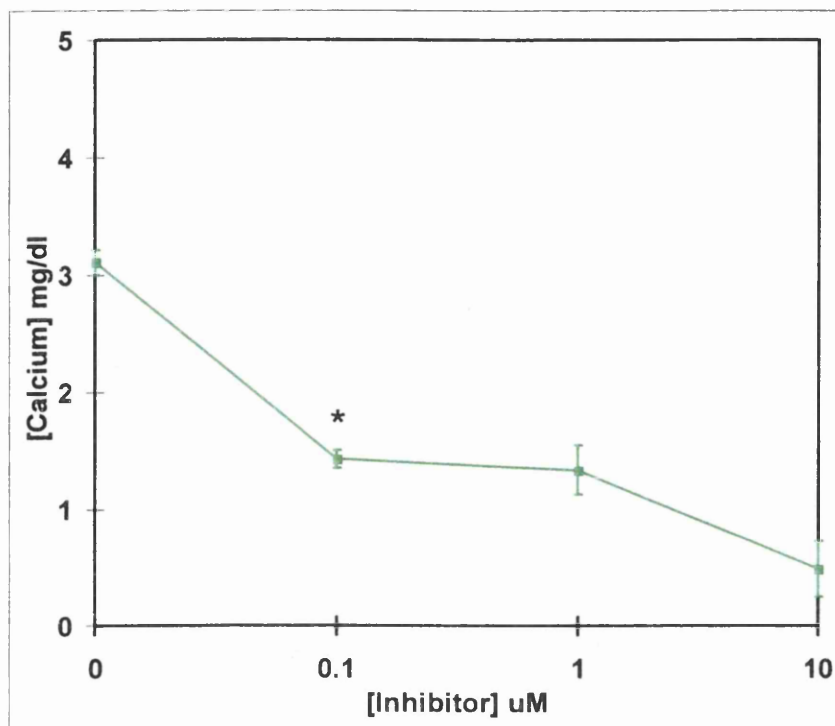
Figure 4. 7 The effect of indomethacin on the calcium release induced by *E. coli* LPS in murine neonatal calvaria. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

4.3.8. Effect of *A. Actinomycetemcomitans* LPS on osteolysis.

Exposure of calvarial explants to graded concentrations of *A. actinomycetemcomitans* LPS over the concentration range 10ng/ml to 1µg/ml resulted in a reproducible dose-dependent increase in calcium release (Figure 4.8). While able to dose dependently stimulate calvarial osteolysis *A. actinomycetemcomitans* LPS was significantly less potent than the LPS from *E. coli*.
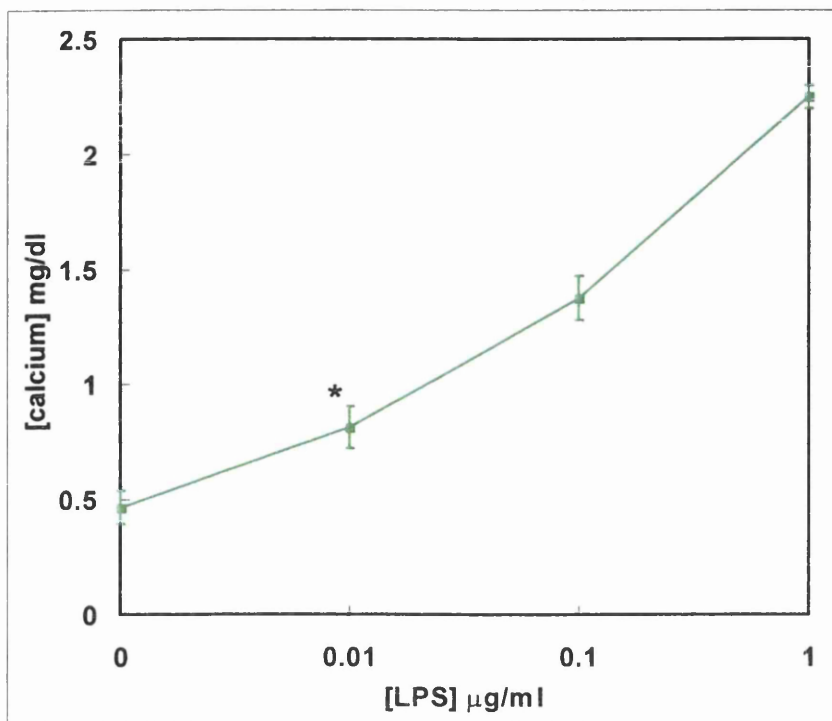
Figure 4. 8 Effects of AA LPS on calcium release from neonatal murine calvaria. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

4.3.9. Effects of 5-LOX and COX inhibition on the osteolysis induced by *A. actinomycetemcomitans* LPS.

BWA4C, and BWA70C caused a reproducible, dose dependent, inhibition of calcium release (Figure 4.9). This was surprising considering the lack of effect these inhibitors had on *E. coli* LPS induced calcium release.

A third inhibitor of 5-LOX activity,MK886 (with a different mechanism of action to BW70C and BW4C), was then used to check this result, and a similar dose dependent inhibition of calcium release was found to occur, confirming the results obtained with BW4C and BW70C.

When the concentration of pLT in the culture media supporting the explants was assessed by EIA, it was found to be abolished in the presence of inhibitors. It was decided to test if other aspects of arachidonic acid metabolism, i.e. COX activity, were also affected differently by *A. actinomycetemcomitans* LPS.
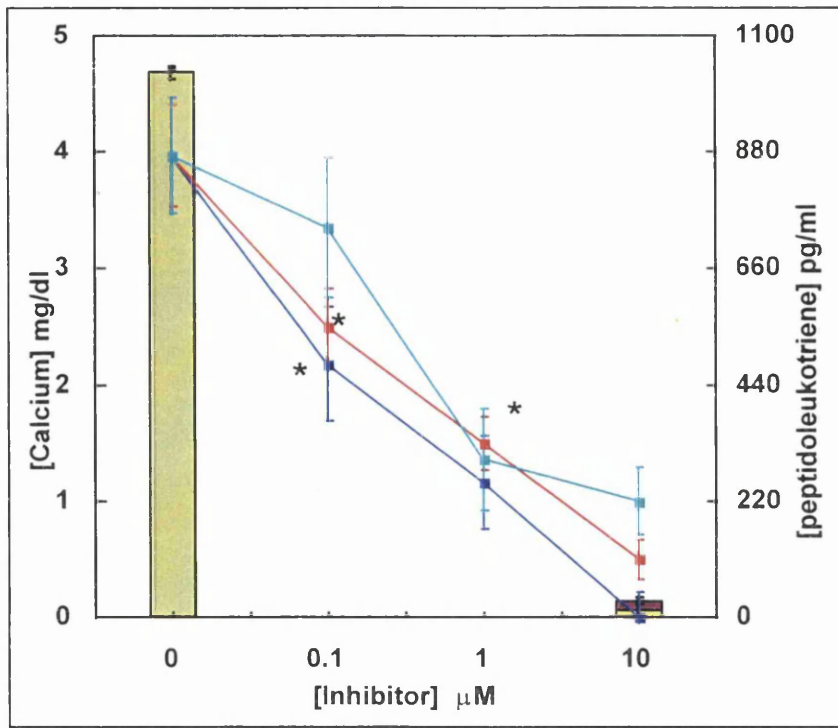
Figure 4. 9 Effects of 5-LO Inhibitors   on AALPS (1ug/ml) osteolysis and pLT generation from calvaria. Calcium is represented by lines (BW4C, BW70C, MK886), pLT generation is represented by bars (LPS, BW70C + LPS and MK886 + LPS). Expressed as mean and SD of quintuplicate cultures (* p< 0.01).
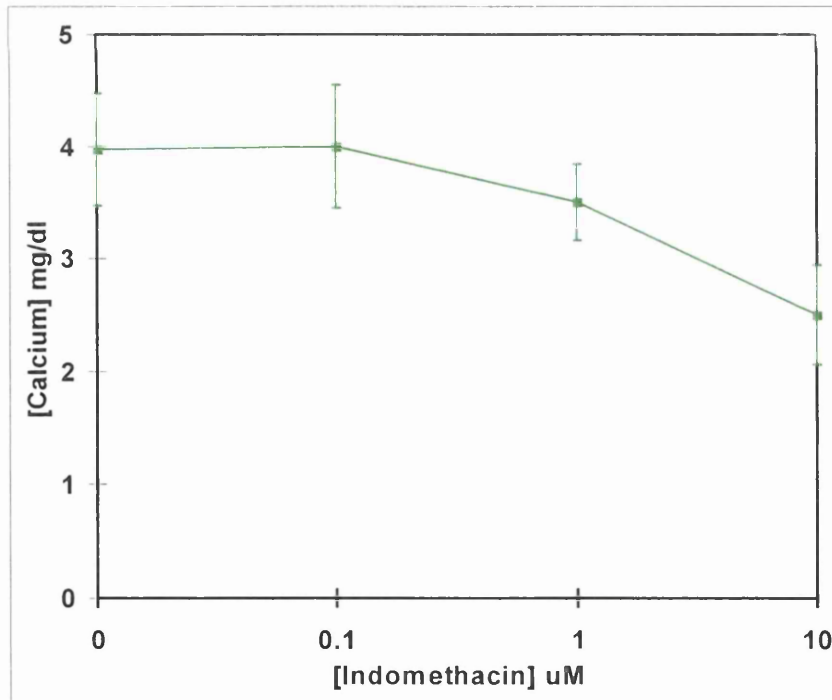
Figure 4. 10 Effect of indomethacin on AA LPS (1ug/ml) induced calcium release. Expressed as mean and SD of quintuplicate cultures .

Indomethacin had little effect on *A. actinomycetemcomitans* LPS induced

osteolysis (Figure 4.10), in contrast to its effects on that induced by *E. coli* LPS.

However *A. actinomycetemcomitans* LPS induced calcium release, unlike *E. coli* induced calcium release, is susceptible to LOX inhibition. Indicating that the bone resorption induced by *A. actinomycetemcomitans* LPS is modulated by 5-LOX activity.

4.3.10. Production of peptidoleukotrienes by calvaria cultured in the presence of *A. actinomycetemcomitans* LPS.

Exposure of bone explants to *A. actinomycetemcomitans* LPS also stimulated the production of 5LOX products and even at concentration of LPS as low as 10ng/ml there was significant production of pLT's (Figure 4.11).
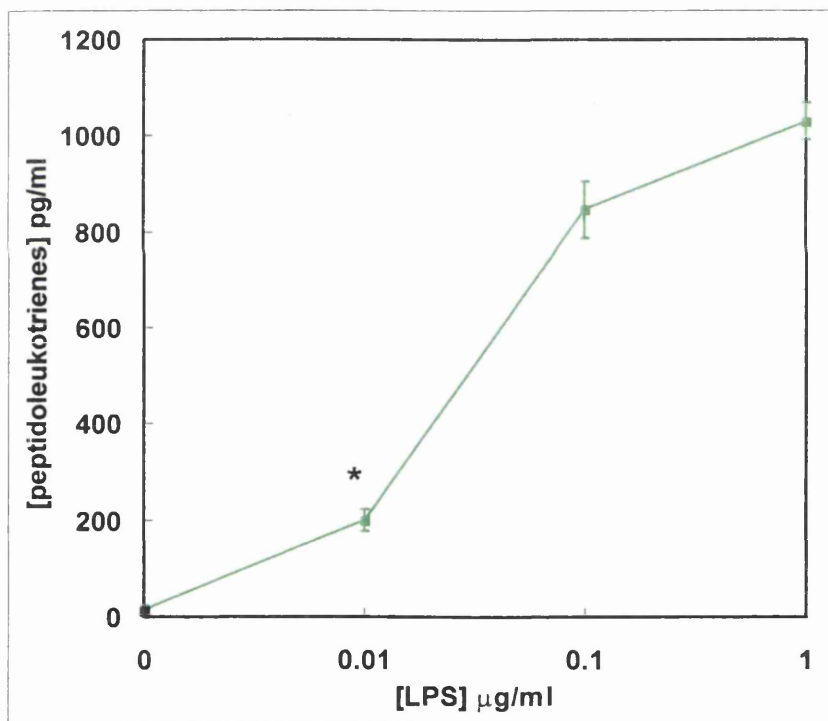
Figure 4. 11 Effects of AA LPS on the generation of pLTs by murine calvarial explants. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

There is a clear rectilinear relationship between the concentration of *A. actinomycetemcomitans* LPS added to cultured calvaria and the amount of pLT produced.

4.3.11. Effect of *A.actinomycetemcomitans* LPS on osteoclastogenesis.

The increase in calcium release from bone explants was reflected in the increase in the numbers of TRAP+ve multinucleated cells in the calvaria, although the numbers of these cells tended to peak at an LPS concentration of 0.1µg/ml (Figure 4.12), which is in contrast to calcium release which was continuing to rise at the highest concentration of *A. actinomycetemcomitans* LPS tested, 1µg/ml (Figure 4.8)
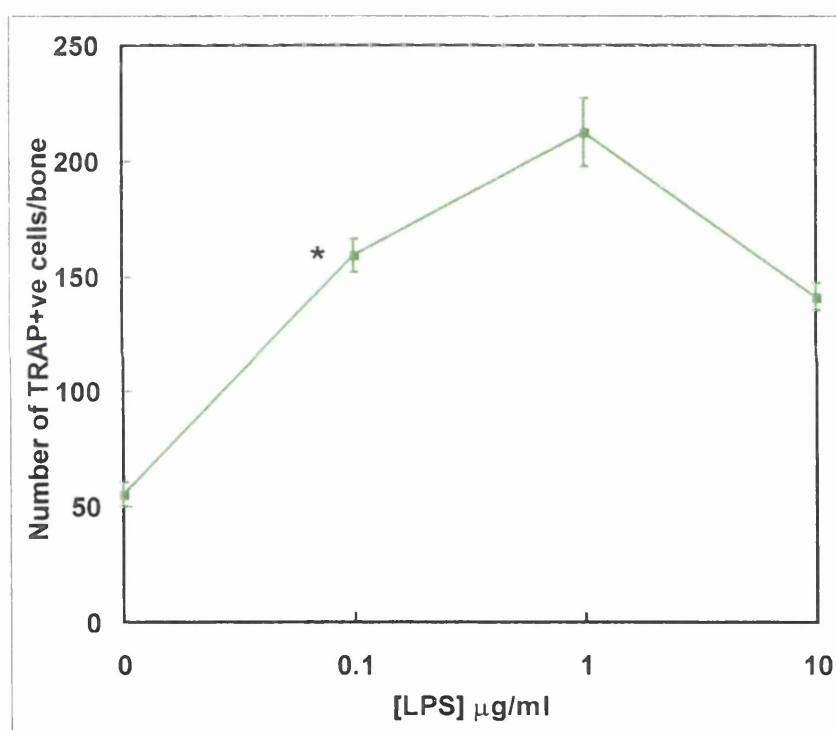
Figure 4. 12 Effects of *A. actinomycetemcomitans* LPS osteoclastogenesis. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

4.3.12. Effect of 5-LOX inhibition on osteoclastogenesis.

The inhibition of *A. actinomycetemcomitans* bone resorption induced by 5-LOX inhibitors (Figure 4.7) was also mirrored by the inhibition in the numbers of TRAP+ve multinucleate cells (MNC) found in the bone explants exposed to *A.*

actinomycetemcomitans LPS and simultaneously treated with BWA70C or MK886 (Figure 4.13).
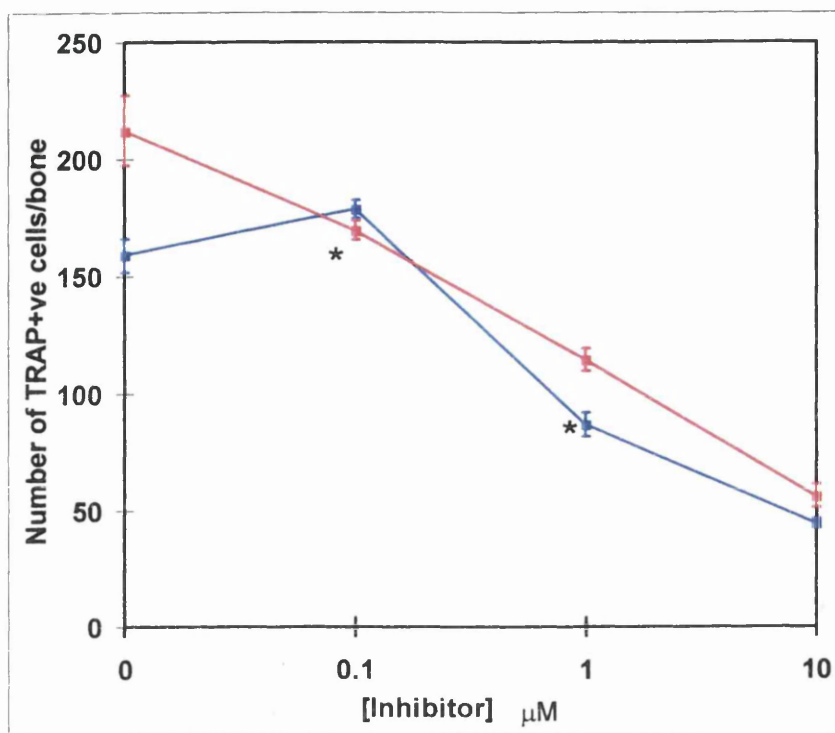
Figure 4. 13 Effects of 5-lipoxygenase Inhibitors (BWA70C and BWA4C) on *A. actinomycetemcomitans* LPS induced osteoclastogenesis in calvaria. Expressed as mean and SD of quintuplicate cultures (* p< 0.01).

CHAPTER 4: 5-LOX AND AGONIST INDUCED OSTEOLYSIS.

4.4. Discussion.

Products of the actions of 5 and 12-LOX on arachidonic acid are as potent as the pro-inflammatory and osteolytic cytokines, interleukin-1 (IL-1) and tumour necrosis factor (TNF), in stimulating the resorption of murine calvaria. Therefore it was expected that the inhibition of 5-LOX would have marked effects on the resorption of calvaria induced by various osteolytic agents. It is known that at micromolar concentrations these compounds can have non-specific effects. However, the hydroxamic acid inhibitors BWA4C and BWA70C failed to inhibit bone resorption induced by PTH, VitD, IL-1 or PGE_2, except at high concentrations. However, these inhibitors were able to block, at much lower concentrations, the bone resorption induced by the lipopolysaccharides from the oral bacterium *A. actinomycetemcomitans* but not that from *E.coli*. This is perhaps due to structural differences, such as acylation or phosphorylation, in the lipid A moieties of these two LPSs. The IC_{50} values of this inhibition is in accord with the reported potency of these compounds against purified 5-LOX and so the results would appear to be due to the selective inhibitory activity of the compounds and not to some non-specific effect. The fact that two mechanistically-distinct classes of 5-LOX inhibitor (BWA70C, BWA4C and MK886) can block bone resorption induced by *A. actinomycetemcomitans* LPS establishes that this enzyme is invoked by this particular osteolytic signal.

It is widely established that LPS is a potent stimulator of the synthesis of osteolytic cytokines such as IL-1 and TNF. Recently, it has been reported that LTB_4 is an extremely potent stimulator of IL-6 transcription and translation in human monocytes (Rola-Plezynski *et al*, 1996). The possibility exists therefore, that LPS may stimulate some bone cell population to produce 5-LOX products

which then stimulate the production of osteolytic cytokines to ultimately promote osteoclastogenesis and osteolysis.

The blockade, by 5-LOX inhibitors, of TRAP+ve cell formation induced by the A. actinomycetemcomitans LPS in calvarial explants confirms a role for 5-LOX products in osteoclastogenesis. However some reports question if 5-LOX metabolites have a direct effect on the osteoclast, making the modulation of osteoclastogenic cytokines an obvious avenue for further investigation. Another possibility is that 5-LOX metabolites stimulate bone lining cells to retract and/or remove the osteoid, so exposing calcified matrix which is thought to be an osteolytic signal in itself (Marshall et al, 1995). Activation of the normally quiescent lining cells might also result in a local lowering of pH (due to increased metabolic activity) which has been shown to increase the activity of isolat d osteoclasts. These possibilities are currently under investigation.

The role of LOXs in bone remodelling is a new area of study and one which has thus far been neglected. The synthesis of selective 5-LOX inhibitors allows the investigation of the role of this enzyme, and its accessory protein FLAP, in agonist-stimulated bone breakdown. The results to date suggest that osteolytically active bacterial products such as certain LPS's, in this case, and Staphylococcus aureus surface-associated material (see chapter 3) act via 5-LOX to induce bone resorption. Their effects certainly seem to be much more sensitive to 5-LOX blockade than physiological mediators of bone resorption or enteric LPS. Considering the differences between the actions of E. coli and A. actinomycetemcomitans LPS, it is tempting to speculate that modulation of bone cells by activation of 5-LOX may be a theme of oral bacteria. The next chapter

shall attempt to verify this by screening of several LPS's from other putative oral

pathogens.

CHAPTER FIVE: 5-LIPOXYGENASE AND OSTEOLYSIS INDUCED BY LIPOPOLYSACCHARIDE ISOLATED FROM ORAL BACTERIA.

5.1. Introduction.

The chronic inflammatory periodontal diseases (CIPDS) are a group of inflammatory diseases mainly caused by Gram-negative oral bacteria including; *Actinobacillus* (*A.*) *actinomycetemcomitans*, *Prevotella* (*P.*) *intermedia*, *Porphyromonas* (*P.*) *gingivalis*, and *Eikenella* (*E.*) *corrodens*.

The mechanisms by which these bacteria cause periodontal pathology are, however, incompletely understood. Nevertheless, the bacteria that are associated with periodontal disease are recognised to produce a number of factors capable of causing, or mediating, destruction of host tissues. The current understanding of the pathogenesis of CIPD's suggests that periodontal tissue is destroyed by modulation of the host's defences by bacterial products.

The host response to bacteria and their products is mediated by the production and release of local factors from inflammatory cells such as lymphocytes and monocytes, and cells of mesenchymal origin such as osteoblasts and fibroblasts. The host factors so produced during periodontal disease have been found in the gingival crevicular fluid and the gingival tissue itself, these have been shown to include cytokines such as interleukin (IL)-1, IL-6 or tumour necrosis factor (TNF) and eicosanoids such as prostaglandin (PG)E_2. Significantly, for the work reported in this thesis, lipoxygenase (LOX) products have also been identified in homogenates of diseased periodontal tissue. Indeed they have been shown to be the major products of arachidonic acid metabolism in these homogenates (El Attar *et al*, 1988). Work presented in the preceding chapter demonstrated a dependency on 5-LOX activity in the bone resorption stimulated by the lipopolysaccharide (LPS) of one known oral pathogen, proposed as an etiological agent for periodontitis, *A. actinomycetemcomitans*.

CHAPTER 5: 5-LOX AND LPS INDUCED OSTEOLYSIS.

As previously described, in chapter 3, work from the Eastman Dental Institute established that products of 5-LOX were potent stimulators of bone resorption in murine calvarial. However other workers reported that leukotrienes (LT's) had little effect on bone, causing interest in this area to flag. Recent work has however supported our initial findings. In the preceding chapter we demonstrated that two mechanistically distinct classes of 5-LOX inhibitor (BWA70C, BWA4C and MK886) can block bone resorption, induced by the LPS of the oral pathogen, *A. actinomycetemcomitans,* establishing that 5-LOX is invoked by this osteolytic signal. Also notable was the lack of effect of 5-LOX inhibition on the osteolysis induced by hormonal and inflammatory mediators, including enteric bacterial LPS. What significance might these observations have for our understanding of periodontal disease pathogenesis and its treatment?

CHAPTER 5: 5-LOX AND LPS INDUCED OSTEOLYSIS.

5.2. Materials and methods.

5.2.1. Preparation of lipopolysaccharides.

The *P. gingivalis, P. intermedia and E. corrodens* LPS preparations used in this work were as described in chapter 2 (section 2.1.3.). They were used at concentrations ranging from 0.01 to 10µg/ml as described in the results section of this chapter.

5.2.2. Assay of osteolytic capacity.

The ability of the LPS's tested here to induce calcium release from neonatal murine calvaria was used as a measure of their osteolytic capacity. This assay was carried out as described in chapter 2 (section 2.6. and its subsections). The LPS's to be tested were added to the culture media of the bones at the concentrations described in the relevant results sections of this chapter. Each LPS was tested at least three times to ensure the reproducibility of results.

5.2.3. Assay of osteoclastogenic capacity.

The ability of the LPS's tested here to induce recruitment of TRAP+ve MNC's in murine calvarial bones was used as a measure of their osteoclastogenic capacity. This assay was carried out as described in chapter 2 (section 2.6.3.). The LPS's to be tested were added to the culture media of the bones at the concentrations described in the relevant results sections of this chapter. The individual examining the explants was unaware of the treatment to which they had been exposed.

5.2.4. 5-LOX inhibitors.

The inhibitor of 5-lipoxygenase activity, BWA70C, and of 5-LOX activating protein, MK886, were used to establish what role 5-LOX may have in the osteolysis and osteoclast recruitment induced by the LPS's of the periodontopathogens under examination.

MK886 and BWA70C were as described in chapter 2 (section 2.4.). These inhibitors were used at concentrations between 0.1 and 10μm, as described in the relevant results sections of this chapter.

5.3. Results.

5.3.1. Effect of *Porphyromonas gingivalis* LPS on bone resorption *in vitro*.

P. gingivalis LPS caused a dose dependent increase in calcium release from neonatal murine calvaria in the concentration range 0.1 to 10μg/ml (Figure 5.1). However at the lowest concentration tested (0.1μg/ml) no significant effect was noted. At 1μg/ml and 10μg/ml there was an approximate 6-7 fold increase in calcium release compared to unstimulated cultures. The potency of *P. gingivalis* LPS is not therefore as great as *A. actinomycetemcomitans* LPS which caused significant resorption at a concentration of 10ng/ml (Figure 4.8). However *P. gingivalis* LPS was quite an efficacious agonist increasing calcium release 7 fold over the unstimulated control at the highest concentration tested (10μg/ml).
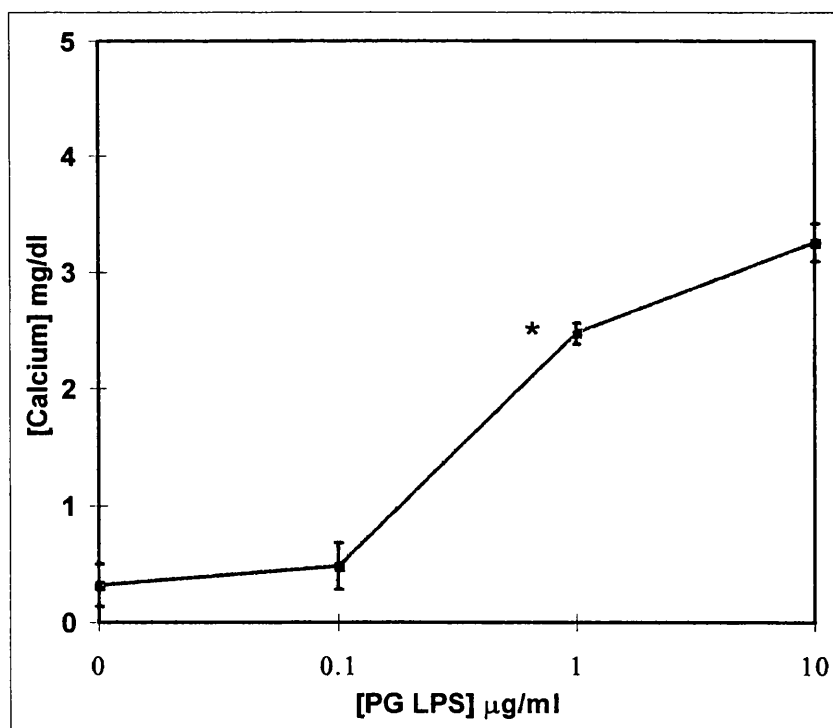
Figure 5. 1 Dose dependent increase in calcium release caused by *P. gingivalis* LPS. Results expressed as mean of 5 replicates +/- SE (* represents a significant difference relative to unstimulated control cultures, p<0.01).

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<u>5.3.1.1. Effect of _Porphyromonas gingivalis_ LPS on</u>

<u>osteoclastogenesis _in vitro_.</u>

P. gingivalis LPS was a potent inducer of TRAP+ve MNC recruitment in murine calvarial explants, when included in the culture media of calvarial explants, at concentrations of 0.1, 1, and 10μg/ml(figure 5.2).

There was a threefold increase, relative to unstimulated control cultures, in TRAP+ve MNC numbers at the lowest concentration tested (0.1μg/ml), and a peak 5 fold increase relative to the unstimulated control at the penultimate concentration tested (1μg/ml). When tested at the highest concentration (10μg/ml) _P. gingivalis_ LPS caused no increase relative to the 1μg/ml cultures.
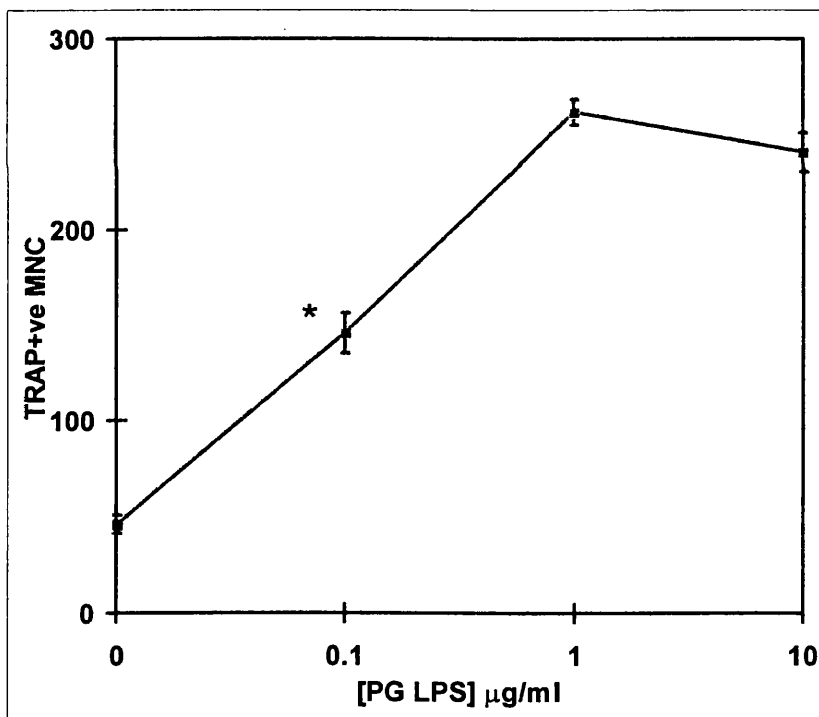
Figure 5. 2 _P. gingivalis_ LPS induced TRAP+ve MNC recruitment. Results are expressed as mean of 5 replicates +/- SE (* Represents a significant difference relative to unstimulated control cultures, p<0.05).

5.3.1.2. Effect of 5-LOX inhibition on *Porphyromonas gingivalis* LPS induced osteolysis *in vitro*.

Addition of the 5-lipoxygenase inhibitors, MK886 and BWA70C, to calvarial cultures stimulated with *P. gingivalis* LPS, at a concentration of 1µg/ml, caused a significant, dose dependent inhibition of calcium release (Figure 5.3). Significant inhibition was noted at the lowest concentrations of inhibitor tested (0.1µM) with calcium release being reduced to unstimulated levels at the highest inhibitor concentrations (10µM). As has been previously observed, the FLAP inhibitor MK886 is more effective than the direct inhibitor of 5-lipoxygenase BWA70C, though in this instance the difference is slight.
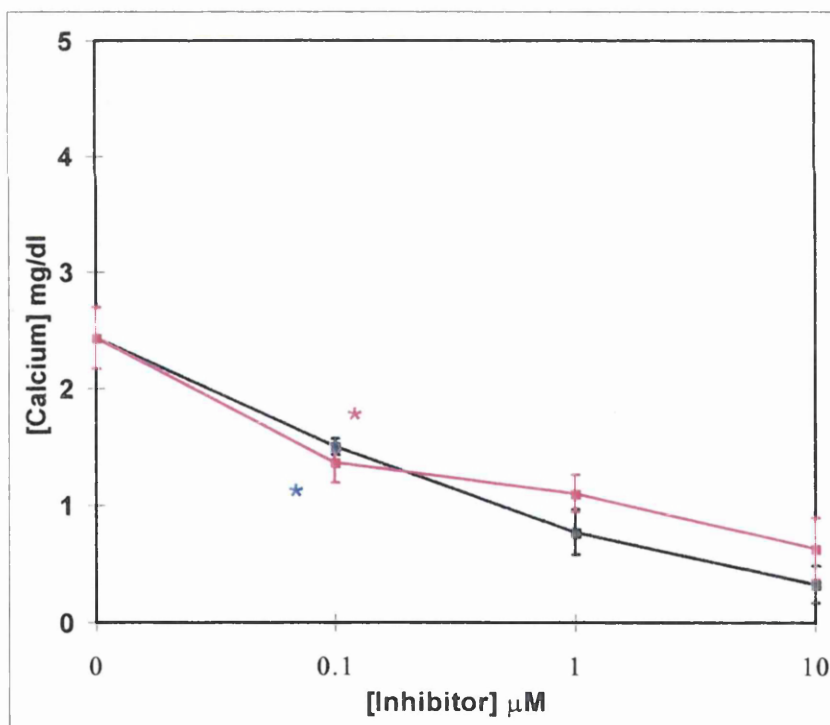
Figure 5. 3 Dose dependent inhibition of *P. gingivalis* LPS (1µg/ml) induced calcium release by BWA70C and MK886. Expressed as mean of 5 replicates +/- SE (*/* represents a significant difference relative to unstimulated control cultures, p<0.01).

5.3.1.3. Effects of 5-LOX inhibition on *P. gingivalis* LPS induced osteoclastogenesis *in vitro*.

The 5-LOX inhibitors are very efficacious at inhibiting TRAP+ve MNC recruitment in murine calvarial bones induced by *P. gingivalis* LPS at a concentration of 1µg/ml (Figure 5.4). Significant inhibition was found at the lowest concentrations of both inhibitors tested (0.1µM). TRAP+ve MNC recruitment was brought back to unstimulated levels at the highest concentrations tested (10µM).
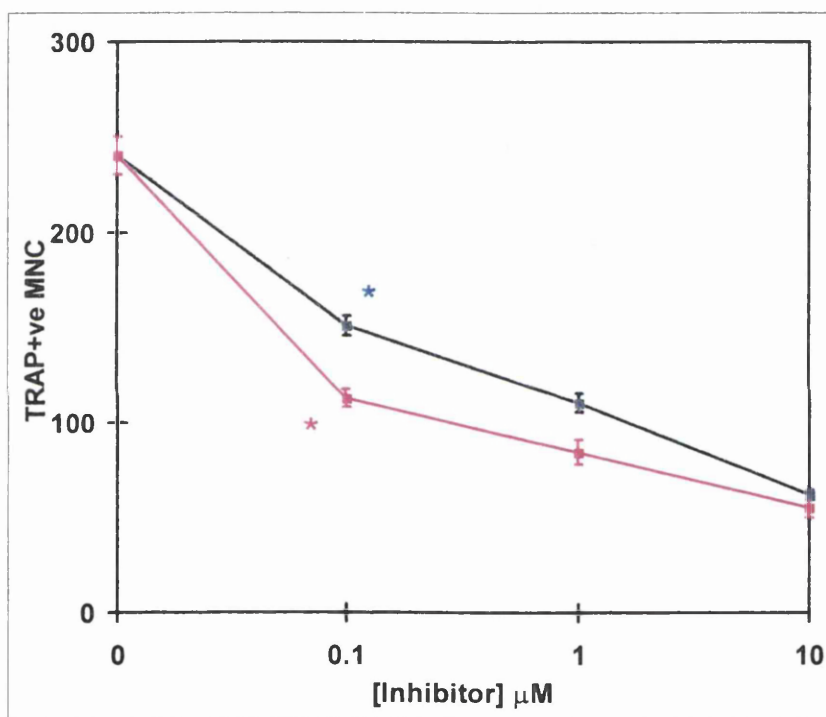
Figure 5. 4 Effects of MK886 and BW70C on *P. gingivalis* LPS (1µg/ml) induced TRAP+ve MNC recruitment. Expressed as mean of 5 replicates +/-SE (*/*significant difference relative to control cultures, p<0.01).

5.3.2. Effect of *Prevotella intermedia* LPS on osteolysis *in vitro*.

P. intermedia LPS, tested over a concentration range of 0.1, 1 and 10 µg/ml, proved to be a poor bone resorbing agonist in this assay both in terms of its efficacy and its potency.

Significant calcium release was only noted at the highest concentration tested (10μg/ml), relative to unstimulated control cultures. The increase in calcium release observed at 10 μg/ml was minimal, compared to the peak calcium release caused by the other LPS's tested, resulting in only a two fold increase over the unstimulated control cultures.
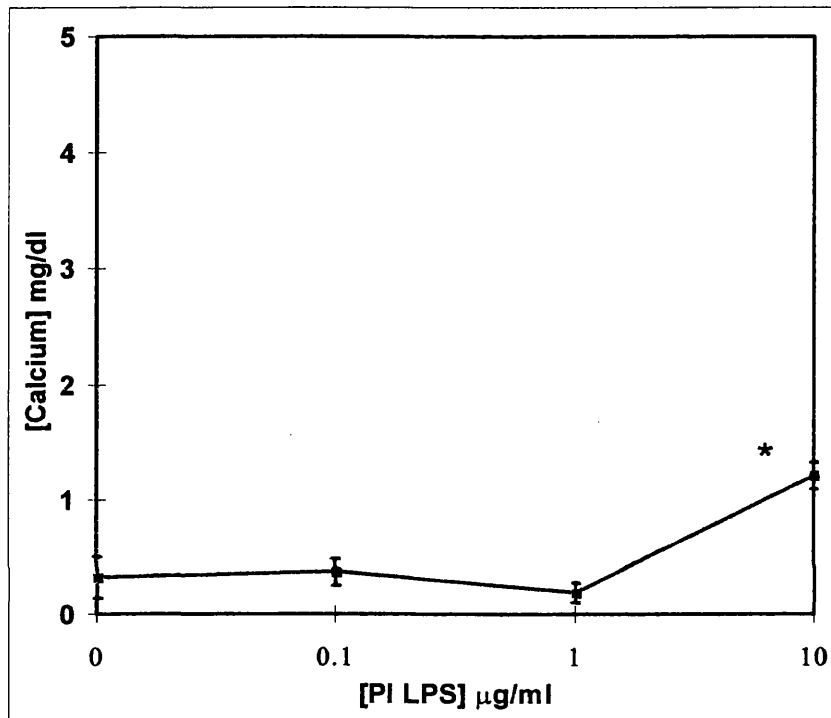
Figure 5. 5 Calcium release caused by *P. intermedia* LPS. The results are expressed as the mean of 5 replicate cultures +/- SE (were * represents a significant difference measured relative to the unstimulated control cultures, p<0.01).

5.3.2.1. Effect of *Prevotella intermedia* LPS on osteoclastogenesis *in vitro*.

In agreement with the weak bone resorbing activity demonstrated by *P. intermedia* LPS, the ability of this material to induce osteoclastogenesis, over the concentration range 0.1, 1 and 10μg/ml, was also minimal.

The recruitment of TRAP+ve MNCs was only found to be stimulated with the highest concentration of *P. intermedia* LPS tested (10μg/ml). Compared to

the peak activities of the other LPS's tested it produced a meagre increase, representing only a two fold increase over the unstimulated control cultures.
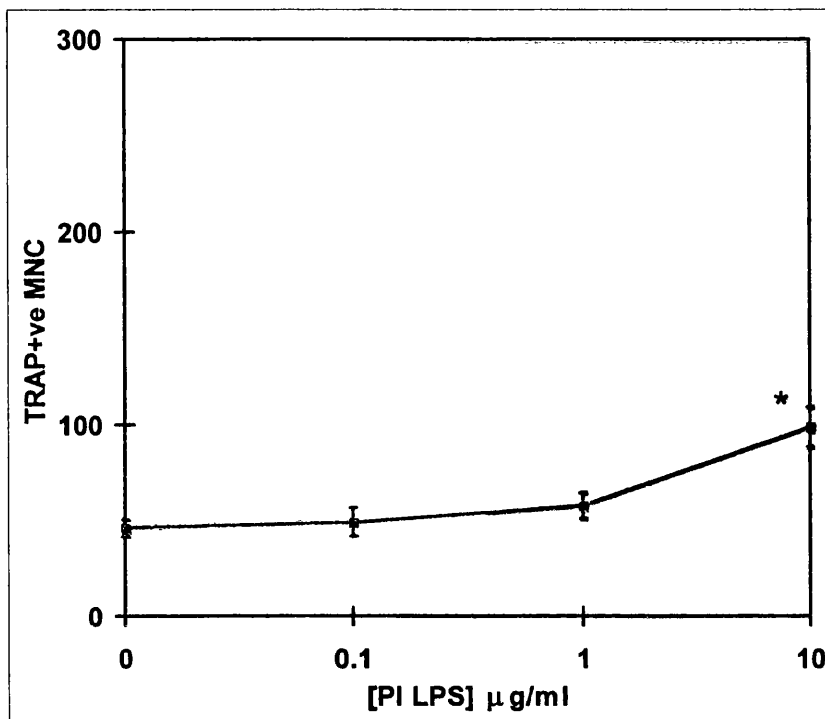
Figure 5. 6 TRAP+ve MNC recruitment caused by *P. intermedia* LPS. The results are expressed as the mean of 5 replicate cultures +/- SE (were* represents a significant difference measured relative to the unstimulated control cultures, $P<0.01$).

5.3.2.2. Effect of 5-LOX inhibition on *Prevotella intermedia* LPS induced bone resorption *in vitro.*

Inhibition of 5-lipoxygenase activity by BWA70C only had significant effect on the release of calcium induced by *P. intermedia* LPS (10μg/ml) at the two highest concentrations tested (1μM and 10μM). MK886 caused a significant effect at all concentrations tested. At the highest concentrations of both inhibitors calcium release was reduced to control levels.

Thus the pattern of MK886 exhibiting a more effective inhibition than BWA70C, as observed in the cases of the other LPS's tested, is repeated here with *P. intermedia* LPS.
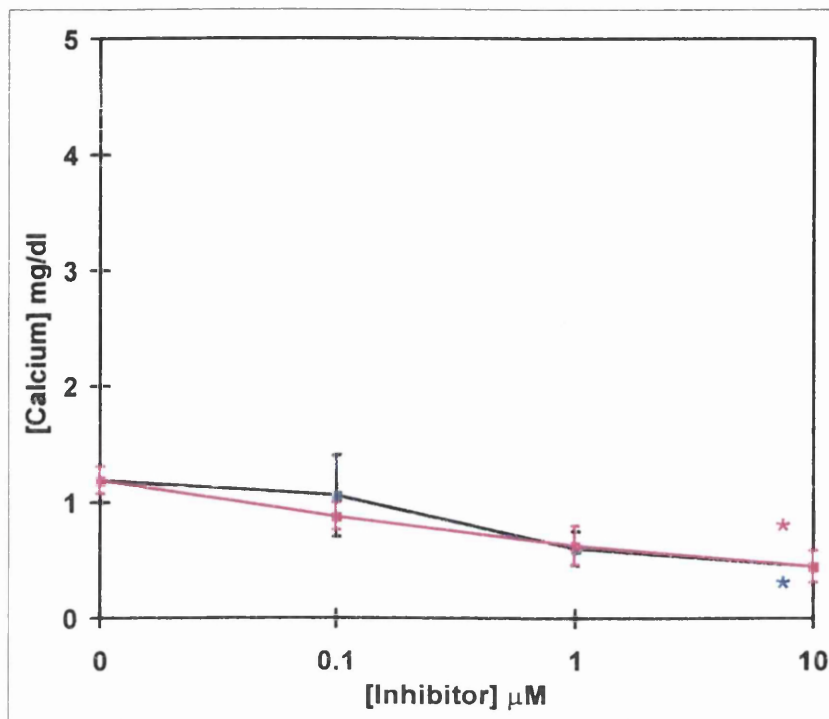
Figure 5. 7 Inhibition of *P. intermedia* LPS (10μg/ml) induced calcium release by MK886 and BW70C. Results are expressed as the mean of 5 replicate cultures +/- SE (*/* represents a significant difference relative to the unstimulated control cultures, p<0.01).

5.3.2.3. Effect of 5-LOX inhibition on *P. intermedia* LPS induced osteoclastogenesis *in vitro*.

Inhibition of 5-LOX activity by BWA70C and MK886 had significant effects on the recruitment of TRAP+ve MNC induced by *P.intermedia* LPS (10μg/ml; Figure 5.8). In contrast to the effects on calcium release both inhibitors had much more marked effects on TRAP+ve MNC numbers. MK886 and BWA70C caused a significant reduction at all concentrations tested. At all concentrations of both inhibitors TRAP+ve MNC numbers were reduced to control levels.
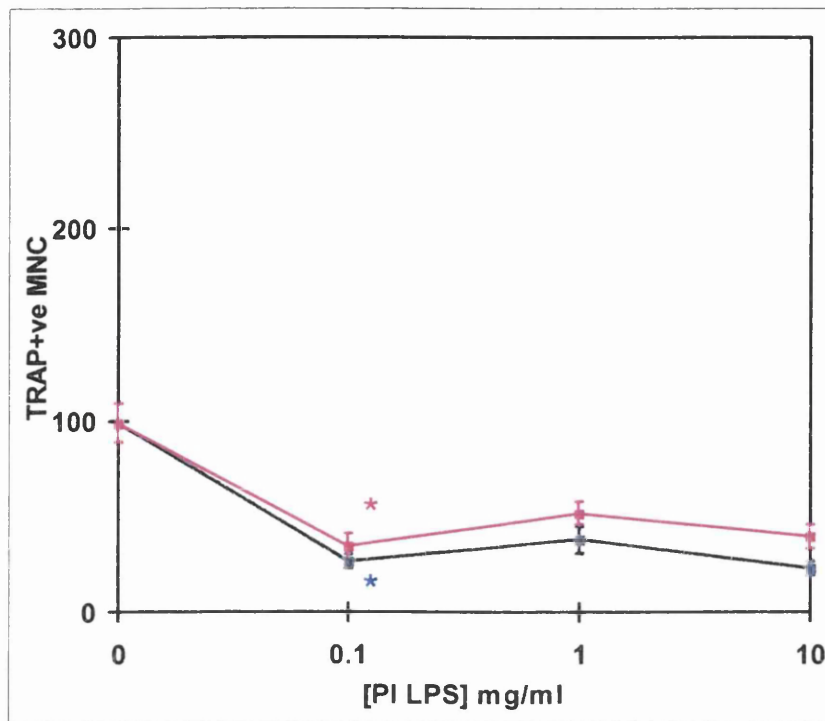
Figure 5. 8 Inhibition of *P. intermedia* LPS (10μg/ml) induced TRAP+ve MNC by MK886 and BW70C. Results expressed as mean of 5 replicates +/- SE (*/* represents a significant difference relative to unstimulated control cultures, p<0.01).

5.3.3. Effect of *Eikenella corrodens* LPS on bone resorption *in vitro.*

E. corrodens LPS, in contrast to *P. intermedia,* and in a similar manner to the LPSs of *P. gingivalis* and *A. actinomycetemcomitans*, was able to induce a dose dependent increase in calcium release from neonatal murine calvaria (Figure 5.9). Significant release of calcium was noted at a concentration of 0.1, 1 and 10μg/ml, relative to unstimulated control cultures. However, the concentration of calcium in the media had plateaued between 1μg/ml and the highest concentration tested (10μg/ml). Peak calcium release at 1μg/ml represented an approximate 8 fold increase in calcium release compared to unstimulated cultures.
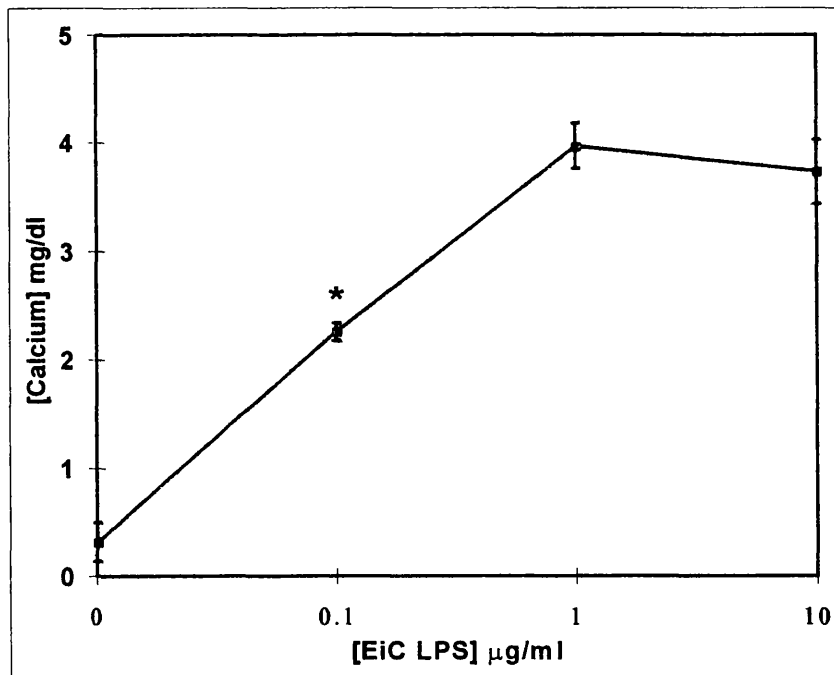
Figure 5. 9 Dose dependent increase in calcium release caused by *E. corrodens* LPS. Expressed as mean of 5 replicates +/- SE (*significant difference, p<0.01).

5.3.3.1. Effect of *Eikenella corrodens* LPS on osteoclastogenesis

Over the range 0.1to 10μg/ml *E. corrodens* LPS caused a rectilinear dose

dependent increase in TRAP+ve MNC formation in murine calvaria (Figure 5.10).
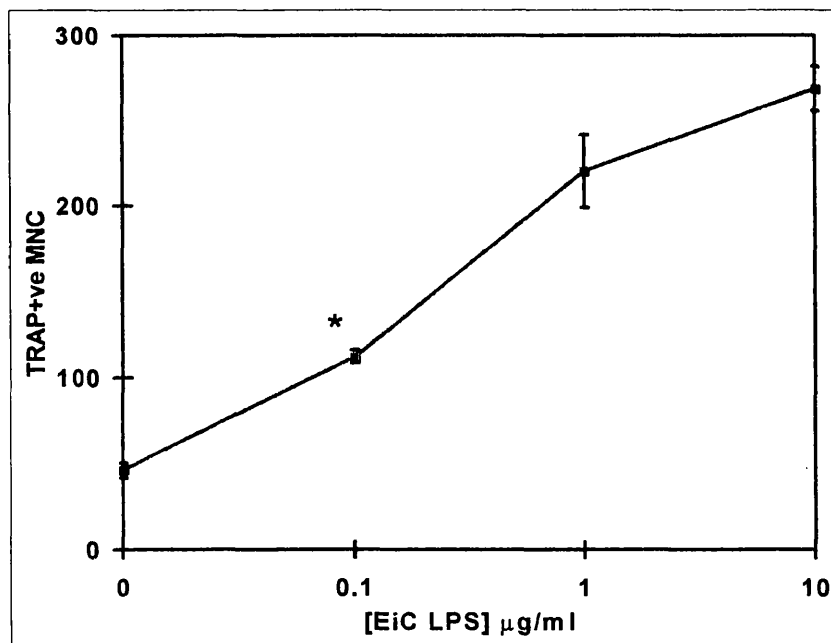
Figure 5. 10 Dose dependent increase in TRAP+ve MNC caused by *E. corrodens* LPS. Expressed as mean of 5 replicates +/- SE (*significant difference, p<0.01).

5.3.3.2. Effects of 5-LOX inhibition on *Eikenella corrodens* LPS induced bone resorption *in vitro.*

Addition of the inhibitors of 5-lipoxygenase activity MK886 and BW70C to calvarial cultures being stimulated with *E. corrodens* LPS caused a significant and dose dependent decrease in calcium release (Figure 5.11). Significant inhibition was noted at the lowest concentrations of inhibitors tested and calcium release was reduced to unstimulated levels with MK886 at the highest concentration.

As has been previously observed, this FLAP inhibitor was more effective than the direct inhibitor of 5-lipoxygenase BW70C. In this instance the difference is much more pronounced than has been observed previously.
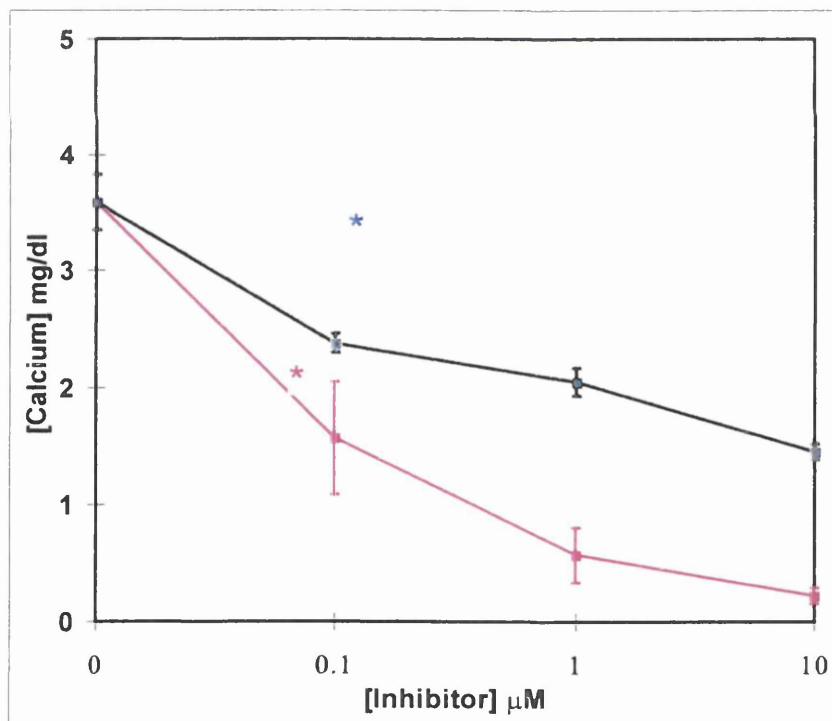
Figure 5. 11 Dose dependent inhibition of *E. corrodens* LPS (1μg/ml) induced calcium release by BWA70C and MK886. Results expressed as mean of 5 replicates +/- SE (*/* represents a significant difference relative to unstimulated control cultures, p<0.01).

5.3.3.3. Effects of 5-LOX inhibition on *Eikenella corrodens* LPS induced osteoclastogenesis *in vitro*.

The 5-lipoxygenase inhibitors were very effective at inhibiting recruitment of TRAP+ve MNC in murine calvarial bones, induced by 1μg/ml *E. corrodens* LPS (Figure 5.12). Significant inhibition is found at the lowest concentration tested for both inhibitors. TRAP+ve MNC recruitment is reduced to unstimulated levels (Figure 5.12) at the highest concentrations tested. The same differences in potency are seen between the two different classes of inhibitor, with BWA70C being slightly less effective than MK886.
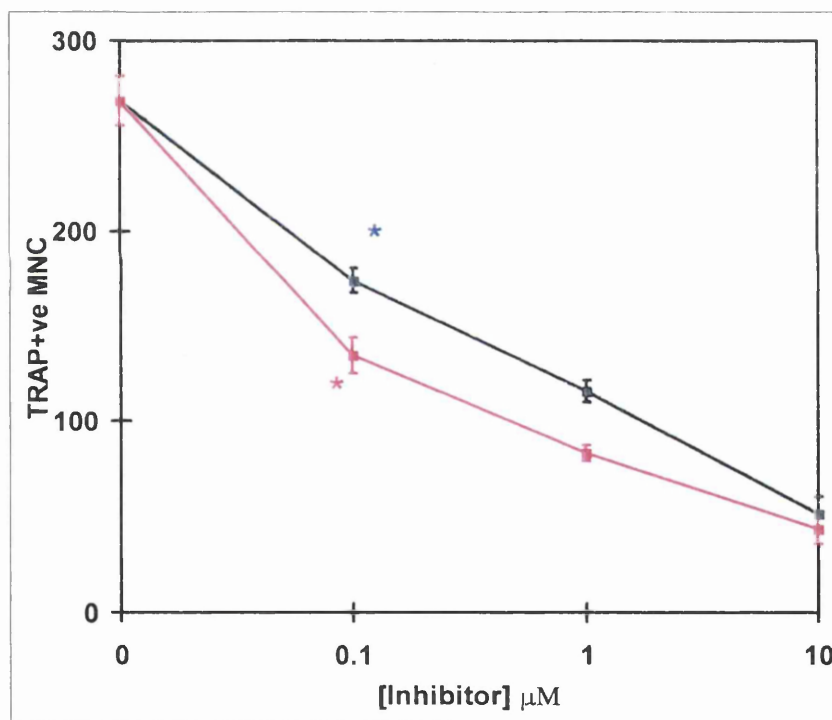
Figure 5. 12 Dose dependent inhibition of *E. corrodens* LPS (1μg/ml) induced TRAP+ve MNC recruitment by MK886 and BWA70C. Results expressed as mean of 5 replicates +/- SE (*/* represents a significant difference relative to unstimulated control cultures, p<0.05).

5.4. Conclusions.

In the preceding chapter it was shown that the LPS from a periodontopathogen, *A. actinomycetemcomitans* was capable of causing bone resorption by modulation of 5-LOX activity. This was in contrast to the LPS from the enteric bacterium *E.coli* and various calciotropic hormones. In this chapter the hypothesis tested was that modulation of 5-lipoxygenase activity might represent a common pathway by which LPS from periodontopathogens induced osteolysis. The results presented in this chapter support this hypothesis. With the exception of *P. intermedia* LPS, which proved to have a very low activity in the assays used, the calcium release and recruitment of TRAP+ve MNC induced by optimal concentrations of the various LPS's tested here could be blocked by the inhibitors of 5-lipoxygenase activity MK886 and BWA70C.

Most of the biological properties linked to LPS are attributable to the lipid A portion, the structure of which is fairly constant among different species, compared to other portions such as polysaccharide side chains (Lynn *et al*, 1992). However, LPS purified from some strains of oral bacteria have unique structural (Ogawa, 1993; Kumada *et al*, 1995) and biological properties (Fujiwara *et al*, 1990; Wilson, 1993) that distinguish them from the better characterised "classical" enterobacterial LPS's. For example, those of the black pigmented anaerobes *P. gingivalis* and *P. intermedia*, can stimulate lymphoid cells from LPS-nonresponsive C3H/HeJ mice as well as LPS-responsive C3H/HeN mice. It is perhaps then not altogether surprising that the LPS's from the oral pathogens tested here, and in the previous chapter, could promote osteoclastic bone resorption via the modulation of arachidonic acid metabolism in a distinct manner to *E.coli* LPS.

The structural requirements of LPS's to activate arachidonic acid metabolism has been investigated by Luderitz at al (1989). In this study it was shown that enterobacterial rough mutants of the Re, Rd1, Rd2 and Rc LPS chemotypes were potent inducers of both LTC_4 and PGE_2 in mouse peritoneal macrophages. This contrasted with wild type strain LPS's, containing the O-specific side chain, which could only cause the release of PGE_2 and not LTC_4. More surprisingly Luderitz *et al* (1989) also found free bacterial and synthetic lipid A were almost incapable of activating arachidonic acid metabolism in macrophages. However, the Re mutant, which differs from free lipid A by the presence of a disaccharide of 2-keto-3-deoxyoctulosonate (KDO) was able to induce the production of LTC_4.

Furthermore, deacylated Re LPS (containing only the KDO disaccharide and lacking the Lipid A fatty acids) was not active in inducing LTC_4 production by macrophages. However coincubation of the deacylated Re LPS with free lipid A rescued the production of arachidonic acid metabolites. Luderitz at al (1989) went on to demonstrate that lipid A represents the essential component in LPS in the induction of lipoxygenase activation and metabolite release. They conclude however that the intrinsic biological potency of lipid A is only expressed if core oligosaccharide components, especially KDO, are simultaneously present. This suggests that KDO residues play a role in the conformation of the molecule leading to an active lipid A. This view is supported by Cavaillon *et al* (1989), who demonstrated that to conserve the full IL1 induction capacity of *B. pertussis* LPS the carboxyl group of the KDO residue must not be substituted.

Luderitz *et al* (1989) also demonstrated an inverse relationship of LPS activity to their phase transition temperatures, indicating that for biological activity

of LPS and free lipid A there is a prerequisite for a relatively high fluidity of the acyl chains and therefore possibly the existence of unique supramolecular structures. It may then be that the periodontopathogen LPS's are able to assume conformations that more closely resemble R type mutants of the enterobacterial LPS's, which can induce lipoxygenase activity than the wild type S forms, which cannot.

Unfortunately there is relatively little data on the structures of the periodontopatogen LPS's studied here. Until a few years ago it was widely accepted that *P. gingivalis* LPS did not contain KDO or heptose (Hanazawa *et al*, 1985; Koga *et al*, 1985; Mansheim *et al*, 1978; Millar *et al*, 1986). However Johne *et al* (1988) detected minute amounts of KDO and heptose in PG LPS. Kumada *et al* (1988) later identified an O-phosphorylated derivative of KDO in *P. gingivalis* and went on to analyse its chemical structure (Kumada *et al*, 1993). Bramanti *et al* (1989) have also confirmed low levels of KDO in *P. gingivalis* LPS. Minute KDO levels have also been detected in *A. actinomycetemcomitans* (Kiley *et al*, 1980) and *Eikenella corrodens* (Progulske *et al*, 1984) LPS's.

Kiley *et al* (1980) suggested that low concentrations of KDO in an LPS may contribute to a weak linkage between the lipid A and core oligosaccharides, resulting in the release of free lipid A and polysaccharide moieties. Such breakage of the LPS molecule could allow the induction of lipoxygenase activity in much the same way that coincubation of deacylated Re LPS with free lipid A rescued the production of arachidonic acid metabolites as described above (Luderitz *et al*, 1989).

LPS is familiar to scientists throughout the whole range of biomedical sciences (for example it is one of the most popular reagents for the stimulation of

cytokine synthesis). This universal usage has led to LPS being regarded as an homogeneous reagent with uniform properties. However In closing this chapter it should be noted that the results presented here, and in the preceding chapter, demonstrate how unpredictable these molecules can be, with only small variations in their chemical constitutions leading to wide variations in their biological effects.

CHAPTER SIX

LPS INDUCED EICOSANOID GENERATION IN

HUMAN OSTEOBLASTS.

CHAPTER 6: EICOSANOID GENERATION IN OSTEOBLASTS.

6.1. Introduction.

The preceding chapters have concentrated on the role of 5-lipoxygenase (LOX) in bone resorption induced by various bacterially derived factors, such as the *S. aureus* surface associated proteins (chapter 3) and oral pathogen lipopolysaccharides (LPS's) (chapters 4 and 5). This work indicates that 5-LOX activity is central in the response of murine bone (*in vitro*) to challenge by bacterial virulence determinants.

The question must therefore be asked - how relevant are the phenomena observed in these murine models to the situations that arise in human bacterially-induced bone disease? It would be unwise to make the assumption, based solely on data obtained using murine models, that human bone cells also respond with modulation of 5-LOX activity. Unwise because differences between species have been documented concerning both the action of hormones in bone (Clemens *et al*, 1997) and the expression of genes in the same cell types from different species (Brum-Fernandes *et al* 1994 versus Kawaguchi *et al*, 1995). Also of interest is how the other enzymes involved in eicosanoid metabolism respond to challenge with bacterial virulence determinants. In addition to 5-LOX there is 12-LOX and 15-LOX. These latter two enzymes have received even less attention from bone biologists than has 5-LOX.

Several lines of evidence indicate the products of 12-LOX may play some role in bone remodelling. Meghji *et al* (1988) have shown that 12-HETE was able to induce bone resorption. Sidhagen *et al* (1982) found that the lipoxygenation of arachidonic acid by explants of inflamed gingival tissue exceeded its metabolism via the cyclooxygenase pathway, this included 12-LOX products. El Attar *et al* (1986 and 1988) found that inflamed periodontal tissue produced large amounts

of 12-HETE's among their major arachidonic acid oxidation products. Thus the behaviour of 12-LOX is relevant to the behaviour of the periodontium.

Both Sidhagen *et al* (1982) and El Attar *et al* (1986 and 1988) in addition to finding 12-HETE's, also found 15-HETE's to be well represented amongst the arachidonate metabolites of inflamed gingival tissue. Whilst the products of 12-lipoxygenation are almost certainly inflammatory, there is much to indicate that 15-lipoxygenation produces anti-inflammatory products and indeed can behave as antagonists to the actions of LTB_4 (Smith *et al*, 1993; Takata *et al*, 1994; Ferrante *et al*, 1997). The presence of these products at bacterially induced inflammatory lesions may therefore represent a mechanism to limit the extent of inflammation, and it would certainly be of interest to know how 15-LOX behaves in human bone cells in the presence of LPS.

Also of significance to this work is the behaviour of the other pathway of arachidonate metabolism. The documented bone resorbing activities of cyclooxygenase (COX) products are legion (Klein *et al*, 1970; Tashjian *et al*, 1972; Goodson *et al*,1973), including their response to LPS in bone cells (Ishihara *et al*, 1990). This makes the lack of effect displayed by indomethacin on *A. actinomycetemcomitans* LPS induced bone resorption (chapter 4) puzzling. Consequently, the role of COX enzymes in human bone is of interest. There are two isoforms of the COX enzyme; a constitutive form (COX-1) and an inducible form (COX-2). How these two isoforms are distributed in human osteoblasts is unclear. There is one report that in human osteoblast-like cells COX-1 is not expressed whereas COX-2 is (Brum-Fernandes *et al*, 1994). This would make human osteoblasts substantially different from murine osteoblasts, which do express COX-1 (Kawaguchi *et al*, 1995). However there are other, conflicting,

reports stating that COX-1 is expressed constitutively in human osteoblast like cells, albeit unresponsive to stimulation with a range of agonists known to operate via a prostanoid dependent mechanism (Min *et al*, 1998). This confusing situation is not unique to human cells, for rat osteoblastic cells the situation is similarly unclear. The rat immortalised osteoblast cell line Py1a fails to yield any detectable COX-1 mRNA whilst expressing COX-2 mRNA, and the opposite situation is found in the rat osteosarcoma cell line ROS 17/2.8 (Pilbeam *et al*, 1997).

In spite of this lack of clarity concerning osteoblasts and COX-1 there is an emerging understanding that the two COX isoforms have quite distinct functions. Normal physiologic functions appear to be maintained by COX-1, while COX-2 appears to mediate the inflammatory response. Activation of COX-1 leads, for instance, to the production of prostacyclin, which when released by the endothelium is antithrombogenic and when released by the gastric mucosa is cytoprotective (Vane *et al*, 1998).

Furthermore COX-1 and 2 products appear to act in different cellular compartments. Thus COX-2 is responsible for modulating responses to signals arriving at the cell membrane and COX-1 products probably function in cellular housekeeping (Crofford, 1997), its activity being only indirectly affected by extracellular signals. This would make COX-2 the more relevant isoform for this work. Nonetheless, given the unclear status of COX-1 expression it would be useful to know how it behaves in the cells used here.

Unfortunately, unlike the mouse, there is no model of human bone resorption that can be applied to the questions this thesis attempts to address. Thus the roles these other enzymes play in bone resorption would not easily be

elucidated by the methods applied to 5-LOX activity. Furthermore *in vitro* models of human osteoclast recruitment are poorly defined and their validation and optimisation remains the subject of an intense research effort (Flanagan *et al*, 1997). No direct method is therefore available to investigate how human bone behaves under the circumstances of interest here.

Nonetheless the results presented in the previous three chapters make it important to at least gain insight into how eicosanoid metabolism progresses in human bone cells under the influence of bacterial virulence determinants. In an attempt to circumvent these problems the work presented in this chapter has used thin layer chromatoraphy to profile the eicosanoids produced by human osteoblasts when they are challenged with the LPS from an oral pathogen. This is followed up with a molecular biological approach to identify the enzymes responsible for the generation of these eicosanoids.

In summary it is uncertain which of the eicosanoid generating enzymes are active in human bone cells. The aim of the work in this chapter was to answer two questions: (i) Is 5-LOX activity in human osteoblasts activated in response to challenge with a bacterial virulence determinant as in the murine models used earlier and (ii) which of the other eicosanoid generating enzymes, if any, is also responsive to such a challenge? To this end use has again been made of LPS from *A. actinomycetemcomitans* and normal human osteoblasts obtained by explant culture of human bone.

CHAPTER 6: EICOSANOID GENERATION IN OSTEOBLASTS.

6.2. Materials and methods.

6.2.1. Culture and isolation of osteoblasts.

Osteoblasts were obtained and propagated as described in chapter 2 (section 2.8; Figure 2.8). Cells to be used in thin layer chromatography or RTPCR were plated and stimulated as described in section 2.8;Figure 2.9.

6.2.4. Polymerase chain reaction.

Total cellular RNA was prepared from appropriately stimulated cells as described in chapter 2 (section 2.10.) and cDNA was prepared as described in section 2.10.2. PCR was then performed as described in section 2.11.3. using the appropriate primer sets as described in table 2.2. Products were then analysed by agarose gel electrophoresis as described in section 2.11.4.

6.2.5. Thin layer chromatography.

Cellular phospholipids were pre-labelled with ^{14}C-arachidonic acid exactly as described in section 2.12.1 (Figure 2.14) prior to stimulation. Extraction of cellular phospholipids was carried out as described in section 2.12.1. (Figure 2.14). TLC plates were loaded, run and developed as described in section 2.12.2. (Figure 2.15).

6.2.6. *A.actinomycetemcomitans* LPS and calcium ionophore.

The *A. actinomycetemcomitans* LPS preparation was as described in chapter 2 (section 2.1.3.) it was used at concentrations ranging from 0.1 to 10μg/ml as described in the results section of this chapter. Calcium ionophore was used at 0.5mg/ml as described in section 2.2.

6.3. Results.

6.3.1. Production of eicosanoids by *A. actinomycetemcomitans* LPS stimulated human osteoblasts.

Exposure of primary cultures of human osteoblasts to graded concentrations of *A. actinomycetemcomitans* LPS for various times revealed time and dose dependent effects on the production and release of PGE_2 , $(LT)C_4$ and 5 and 12/15-hydroxyeicosatetraenoic acids (HETEs). It is not possible to distinguish 12 and 15-LOX products with the system employed here (Figure 6.1).
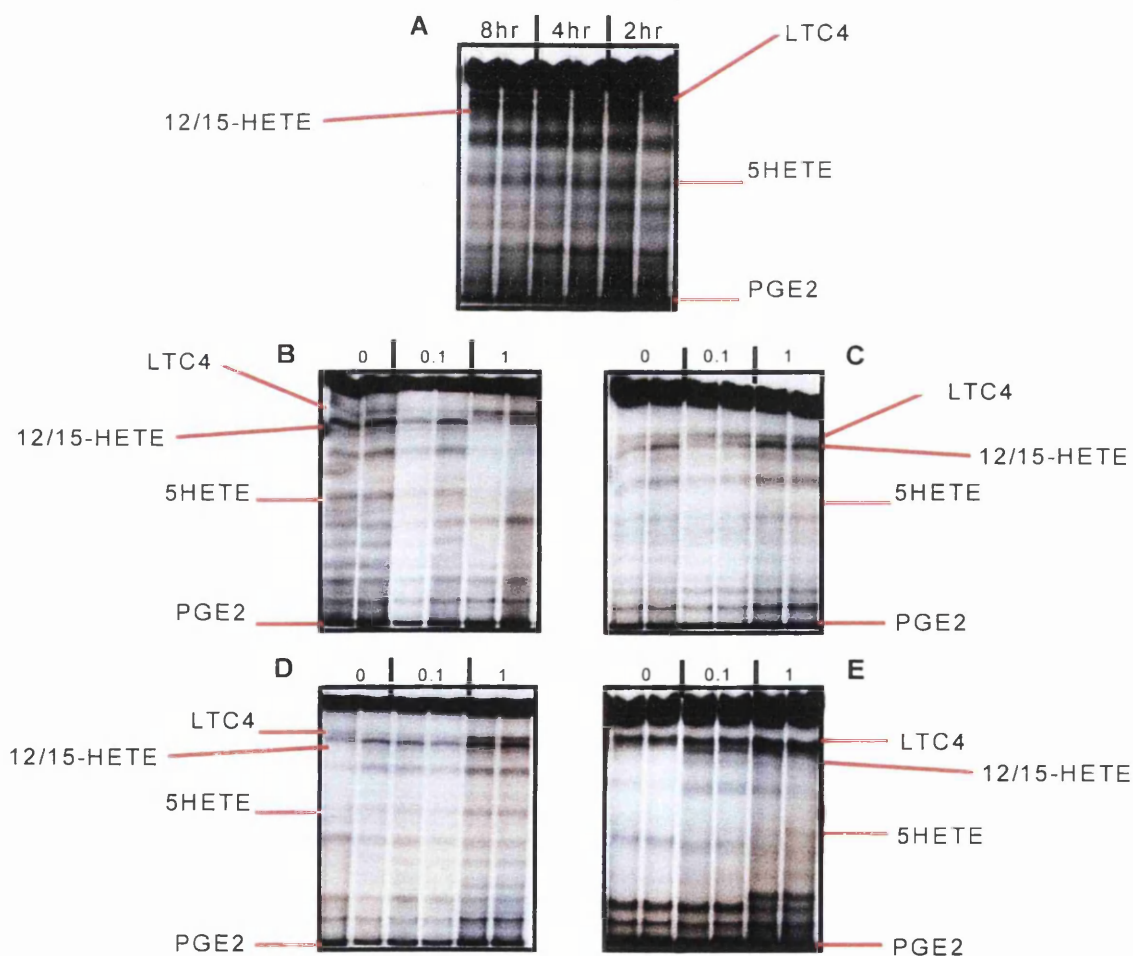
Figure 6. 1 Autoradiograms of TLC plates on which arachidonic acid metabolites extracted from primary human osteoblasts have been run. Products are identified by comparison to standards and are labelled. Plate A; calcium ionophore exposure time in hours at top of each duplicate set of lanes. Plates B to E; concentrations of *A. actinomycetemcomitans* LPS (µg/ml) at top of replicate lanes; B *A. actinomycetemcomitans* LPS 1 hour, C 2 hour, D 4 hour and E 8 hour.

6.3.2. GAPD gene expression in *A. actinomycetemcomitans* LPS stimulated human osteoblasts.

Amplification of the complementary deoxyribonucleic acid (cDNA), to the messenger ribonucleic acid (mRNA), for GAPD in human normal osteoblasts at various times (1 to 8 hours) after exposure to various concentrations of *A. actinomycetemcomitans* LPS revealed that the mRNA for this protein was present in approximately equal amounts in each preparation indicating an equal loading of cDNA per reaction (Figure 6.2).
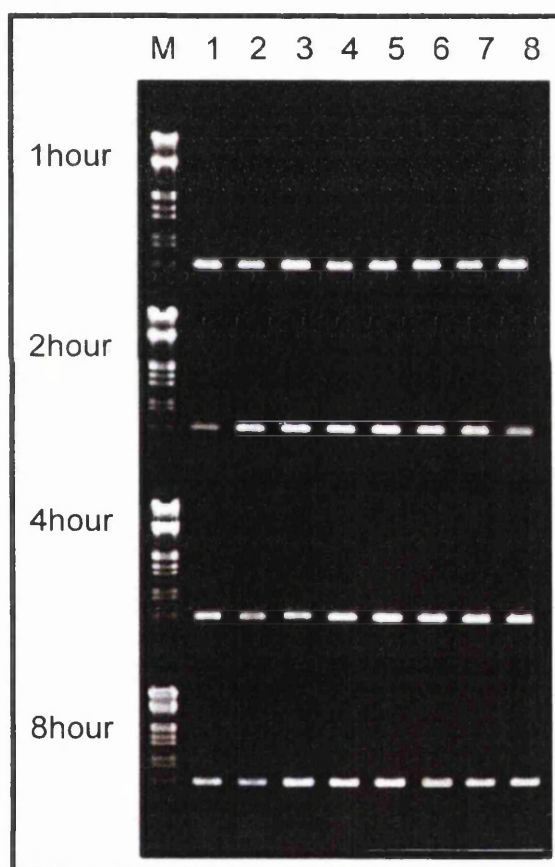
Figure 6. 2 The expression of GAPD mRNA in primary cultures of human osteoblasts. Lane M - EcoR1/Hind3 molecular weight markers. Lanes 1 and 2; mRNA from osteoblasts exposed to 10mg/ml A. actinomycetemcomitans LPS for various periods from 1 hour to 8 hours. Lanes 3 and 4 - osteoblasts exposed to 1mg/ml LPS. Lanes 5 and 6 - osteoblasts exposed to 0.1mg/ml LPS and lanes 7 and 8 - osteoblasts which have not been stimulated with LPS.

6.3.3. 5-LOX gene expression in *A. actinomycetemcomitans* LPS stimulated human osteoblasts.

Amplification of the complementary deoxyribonucleic acid (cDNA), to the messenger ribonucleic acid (mRNA), for 5-LOX in human normal osteoblasts at various times (1 to 8 hours) after exposure to various concentrations of *A. actinomycetemcomitans* LPS revealed that the mRNA for this protein was constitutively expressed in these cells. With increasing time of exposure of cells to the highest concentration of LPS the presence of the mRNA for 5-LOX decreased until at 8 hours it was undetectable (Figure 6.3).
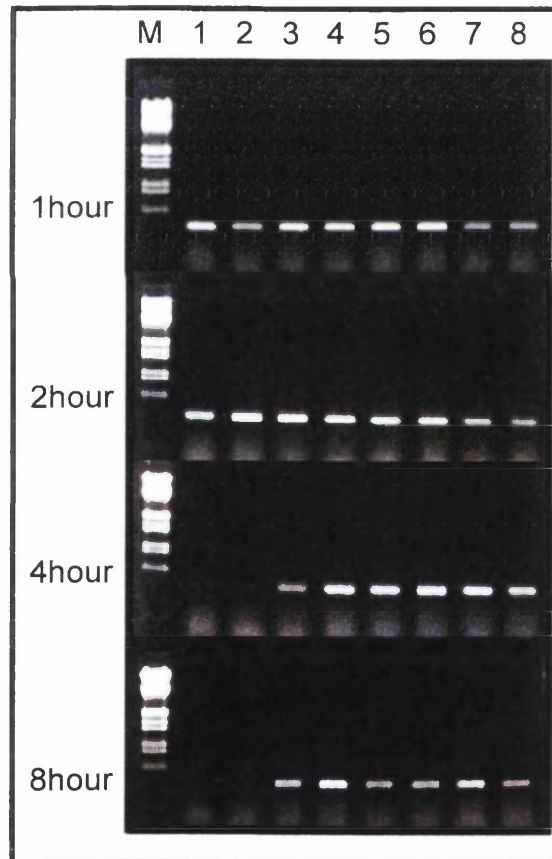
Figure 6. 3 Expression of 5-LOX in normal human osteoblasts. Lane M - EcoR1/Hind3 molecular weight markers. Lanes 1 and 2; mRNA from osteoblasts exposed to 10μg/ml *A. actinomycetemcomitans* LPS for various periods from 1 hour to 8 hours. Lanes 3 and 4- osteoblasts exposed to 1μg/ml LPS. Lanes 5 and 6 - osteoblasts exposed to 0.1μg/ml LPS and lanes 7 and 8 - osteoblasts which have not been stimulated with LPS.

6.3.4. 12-LOX gene expression in *A. actinomycetemcomitans* LPS stimulated human osteoblasts.

Amplification of the cDNA, to the mRNA, for 12-LOX in human normal osteoblasts at various times (1 to 8 hours) after exposure to various concentrations of *A. actinomycetemcomitans* LPS revealed that the mRNA for this protein was expressed in these cells only after long periods of exposure. (Figure 6.4).
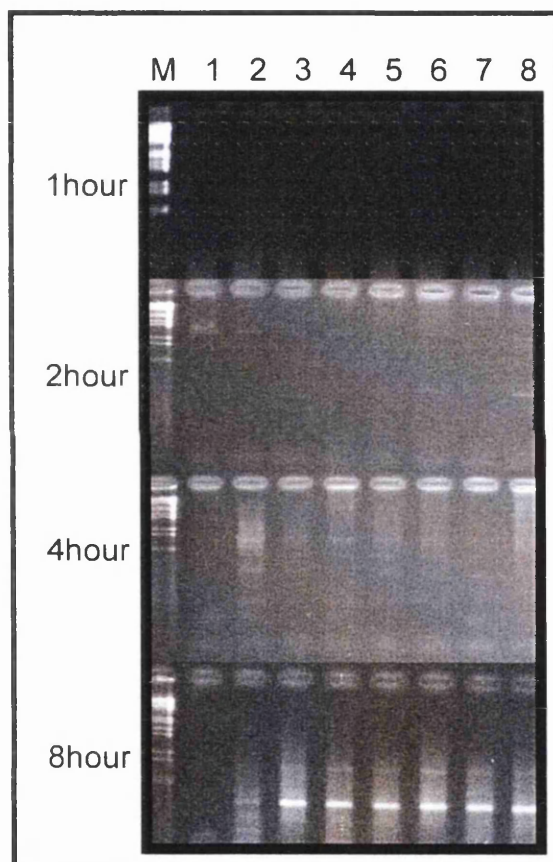
Figure 6. 4 Expression of 12-LOX in normal human osteoblasts. Lane M - EcoR1/Hind3 molecular weight markers. Lanes 1 and 2; osteoblasts which have not been stimulated with LPS. Lanes 3 and 4 - osteoblasts exposed to 0.1μg/ml LPS. Lanes 5 and 6 - osteoblasts exposed to 1μg/ml LPS and lanes 7 and 8 - mRNA from osteoblasts exposed to 10μg/ml *A. actinomycetemcomitans* LPS for various periods from 1 hour to 8 hours.

6.3.2. COX-1 gene expression in *A. actinomycetemcomitans* LPS stimulated human osteoblasts.

RTPCR was used to detect the presence of transcripts for COX-1. All attempts to amplify the complementary deoxyribonucleic acid (cDNA), to the messenger ribonucleic acid (mRNA), for COX1 in human normal osteoblasts at various times (1 to 8 hours) after exposure to various concentrations of *A. actinomycetemcomitans* LPS failed to detect any mRNA for this protein in these cells (Figure 6.2).
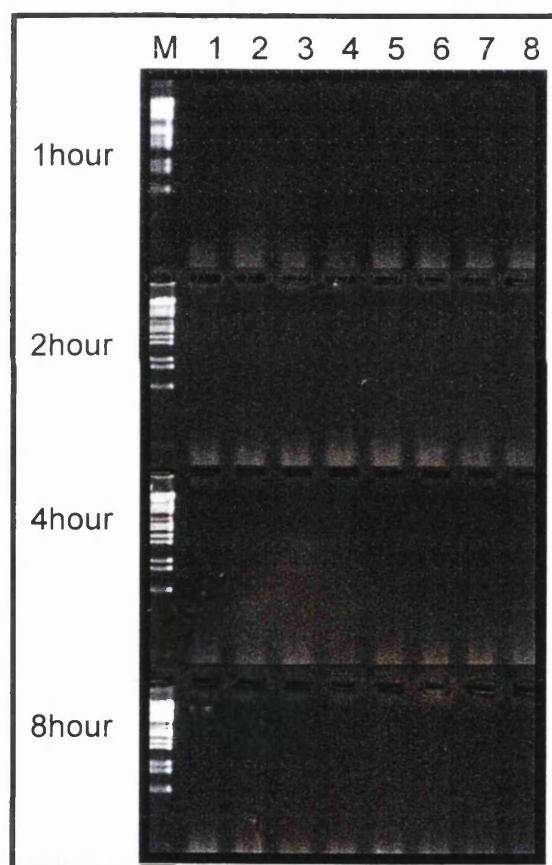
Figure 6. 5 Expression of COX1 in normal human osteoblasts. Lane M - EcoR1/Hind3 molecular weight markers. Lanes 1 and 2; osteoblasts which have not been stimulated with LPS. Lanes 3 and 4 - osteoblasts exposed to 0.1mg/ml LPS. Lanes 5 and 6 - osteoblasts exposed to 1mg/ml LPS and lanes 7 and 8 - mRNA from osteoblasts exposed to 10mg/ml A. actinomycetemcomitans LPS for various periods from 1 hour to 8 hours.

6.3.2. COX-2 gene expression in *A. actinomycetemcomitans* LPS stimulated human osteoblasts.

The reverse transcription polymerase chain reaction (rtPCR) was used to detect the presence of transcripts for COX-2.
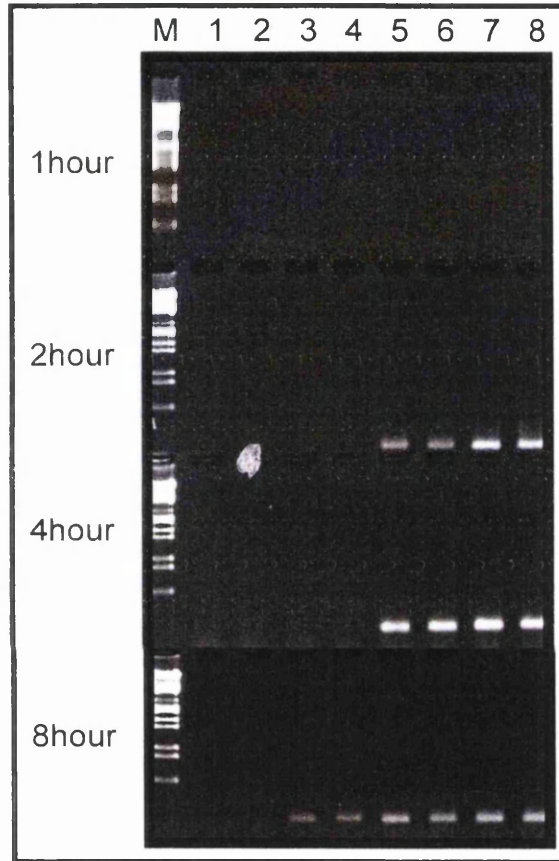
Figure 6. 6 Expression of COX2 in normal human osteoblasts. Lane M-EcoR1/Hind3 molecular weight markers. Lanes 1 and 2; osteoblasts which have not been stimulated with LPS. Lanes 3 and 4; osteoblasts exposed to 0.1mg/ml LPS. Lanes 5 and 6; osteoblasts exposed to 1mg/ml LPS and lanes 7 and 8; mRNA from osteoblasts exposed to 10mg/ml A. actinomycetemcomitans LPS for various periods from 1 hour to 8 hours.

Amplification of the complementary deoxyribonucleic acid (cDNA), to the messenger ribonucleic acid (mRNA), for COX2 in human normal osteoblasts at various times (1 to 8 hours) after exposure to various concentrations of *A. actinomycetemcomitans* LPS revealed that the expression of mRNA for this protein was upregulated in these cells. This upregulation was found to be

dependent on both the dose of *A. actinomycetemconitans* used to stimulate the cells and on amount of time the cells were exposed to the LPS.

6.3.2. 15-LOX gene expression in *A. actinomycetemcomitans* LPS stimulated human osteoblasts.

All attempts to Amplify the complementary deoxyribonucleic acid (cDNA), to the messenger ribonucleic acid (mRNA), for 15-LOX in human normal osteoblasts at various times (1 to 8 hours) after exposure to various concentrations of *A. actinomycetemcomitans* LPS failed to detect any mRNA for this protein in these cells. With increasing time of exposure and dose this situation did not change, up to and including the highest dose and longest time of exposure (results not shown).

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6.4. Discussion.

Are human bone cells able to synthesise 5-lipoxygenase (LOX) in response to an oral bacterial LPS as has been revealed for murine calvaria? Are there other pathways of arachidonate metabolism operating in human bone cells in response to this challenge? To answer these questions the fate of arachidonic acid in early passage cultures of normal human osteoblasts, challenged with A. actinomycetemcomitans LPS, was determined using thin layer chromatography (TLC). Simultaneously, the reverse transcription polymerase chain reaction (RTPCR) was employed to identify the expression pattern of enzymes involved in arachidonic acid metabolism in these cells.

The results presented here show that exposure of human osteoblasts to LPS stimulates the production of a range of arachidonic acid oxidation products. TLC revealed a substantial increase in the production of PGE_2, a number of LOX products, including LTC_4 and HETE's plus several unidentified products. RTPCR revealed the expression of COX-2, 5-LOX and 12-LOX. In agreement with one previous report (Brum-Fernandes *et al*, 1994) no COX-1 gene expression was detected in human osteoblasts, making it probable that COX-2 activity is solely responsible for the production of PGE_2 seen in Figure 6.1. Furthermore, whilst 12 and 15-HETE products observed in Figure 6.1 could not be distinguished with the solvent system used, it seems likely they are exclusively products of 12-LOX activity, since no 15-LOX gene expression could be detected by RTPCR.

Therefore 5-LOX is responsive in human osteoblasts to LPS challenge and it is likely that the activity of this enzyme in the cells examined here would be capable of driving bone resorption. This is supported by the findings of Gallwitz *et al* (1993), who found the production of LT's by stromal cells in giant cell tumours

of bone drove the recruitment and activation of osteoclasts in these tumours and Garcia *et al* (1996), who have demonstrated the presence of LTD_4 receptors on osteoclasts. When the presence of mRNA for 5-LOX was examined using RTPCR it was clear that transcripts of this gene were present constitutively in unstimulated osteoblasts. There was no evidence for increased levels of 5-LOX mRNA in LPS-stimulated cells which could be due to increased gene transcription, increased mRNA stability or a combination of both.

Surprisingly, with increasing time of exposure to LPS, particularly at high concentrations, there was a decrease in the amplifiable 5-LOX mRNA levels in osteoblasts. This is interesting in the light of a recently defined transcriptional silencer region in the 15-LOX gene (Ostareck-Lederer *et al*, 1994). The results presented here may hint at a similar mechanism in operation with the 5-LOX in osteoblasts. This is an attractive possibility suggesting a chronic control mechanism for turning-off the effects of bone-modulating agonists. Presumably the effect of oral pathogen LPS on osteoblasts is not to act as a transcriptional activator of the 5-LOX gene, but is to promote the activation of the 5-LOX protein by stimulating its transfer via FLAP from the cytoplasm to the membrane, where it becomes functional, a mechanism that has been shown to operate in osteosarcoma cells exposed to calcium ionophore (Kargman *et al*, 1992).

Whilst 5-LOX activity in human osteoblasts is responsive to challenge with *A. actinomycetemcomitans* LPS, as predicted by the murine models, COX-2 activity is also induced which was not predicted by these murine models (since indomethacin failed to significantly inhibit the calcium release induced by *A. actinomycetemcomitans* LPS).

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The response of COX-2 activity and gene expression is puzzling at first. However the role of prostaglandins in bone resorption are controversial. Prostaglandins have been shown to have multiple, sometimes opposite, effects in bone cells. Their role in physiological or diseased remodelling is consequently difficult to discern (Chambers *et al*, 1983; Akatsu *et al*, 1989; Chenu *et al*, 1990). Thus PG's are potent agonists that can stimulate and inhibit both bone resorption and formation. *In vivo*, the major effects of exogenous prostaglandins (and therefore COX-2 which is responsible for production of PG's for inter-cellular signalling), particularly PGE_2, is to stimulate resorption and formation, events which seem to involve both the replication and differentiation of both osteoclast and osteoblast lineages. These apparently contradictory effects are however curiously consistent with the tightly coupled nature of the physiological remodelling cycle (see Chapter 1, section 1.4), in which formation is a consequence of resorption. This may explain why indomethacin failed to inhibit the bone resorption induced by *A. actinomycetemcomitans* LPS in the murine bone resorption assay. Therefore even though this oral LPS seems to be inducing prostanoid production and inhibition of this production will result in the loss of the prostanoid resorptive effects, their bone formative effects are also lost. With 5-LOX activity also inducing bone resorption, the net effect would be increased loss of bone.

Furthermore, in a pathological situation (such as the chronic inflammatory periodontal diseases) the equilibrium between formation and resorption established by the actions of COX activity would be shifted to favour resorption by the release of resorptive LOX products; again the net effect would be loss of

bone. Thus the presence of bacteria able to induce 5-LOX activity would lead to the uncoupling of the remodelling cycle.

Considering the potent osteolytic activity the LOX products possess (Meghji *et al*, 1988) the bone loss and eventual loss of dentition found in the CIPD's would not require a large increase in LOX activity, merely a sustained one. This could be achieved by the presence of 12-LOX products, which are also potent resorptive agonists (Meghji *et al*, 1988), and the data presented here indicates this enzyme's activity is also upregulated by the oral LPS tested. This perhaps represents a cascade of osteolytic LOX activity, which could lead to the massive bone destruction caused by the CIPD's.

CHAPTER SEVEN

IDENTIFICATION OF OSTEOLYTIC FACTORS

OF *MYCOBACTERIUM TUBERCULOSIS.*

CHAPTER SEVEN: *M. TUBERCULOSIS* OSTEOLYTIC FACTORS.

7.1. Introduction.

In the course of this thesis we have dealt with the bacteria responsible for osteomyelitis/bacterial arthritis (*S. aureus*) and the CIPD's (oral Gram-negatives). Another serious bone disease is vertebral tuberculosis caused by *Mycobacterium* (*M.*) *tuberculosis*. Tuberculosis is epidemic, accounting for 7% of the annual world wide death toll (Murray *et al*, 1990). Tuberculous infection of bone, particularly of the spinal vertebrae (Pott's disease), is still common in the third world (Boachie-Adjet *et al*, 1996) and with the rise of antibiotic resistant strains of *M. tuberculosis* this organism may again become a scourge of the industrialized nations. This makes understanding the underlying mechanisms of *M. tuberculosis* infectivity of bone, and the consequent tissue destruction, of importance, particularly as it may result in the development of novel therapeutics.

It is not known how infection of bone by *M. tuberculosis* causes bone breakdown. As has been described in earlier chapters, healthy bone is maintained by a dynamic equilibrium between the mesenchymal, bone matrix-forming osteoblast, cell lineage and the myeloid bone-resorbing osteoclast cell lineage (Nair *et al*, 1996). *M. tuberculosis* infection of bone obviously alters this dynamic equilibrium, resulting in the net loss of the extracellular matrix of vertebral bone and collapse of the vertebrae. Whether this loss of bone matrix is the result of the direct action of *M. tuberculosis* components, for example, the LPS like cell surface molecule lipoarabinomannan (LAM) (Fenton *et al*, 1996), on bone cells, or an indirect activation of inflammatory cells leading to bone cell activation, is not established. The subject of this chapter then was work to identify molecules of *M. tuberculosis* that had osteolytic and osteoclastogenic activity.

Several candidate molecules exist on the surface of *M. tuberculosis*. One of these is lipoarabinomannan (LAM). This molecule exhibits immuno-regulatory and anti-inflammatory activities, including suppression of T lymphocyte proliferation (Moreno *et al*, 1988) and inhibition of macrophage activation by gamma-interferon (Sibley *et al*, 1988 and 1990). LAM is also able to stimulate activated macrophages to release TNFα (Moreno *et al*, 1988). Surprisingly polymyxin B, an LPS binding antibiotic, and CD14 can also bind LAM, suggesting some common structure, and by inference, perhaps action.

Another group of molecules that have attracted the attention of the cellular microbiology group at the EDI are the heat shock proteins (HSP's). The main function of these molecules is to ensure the correct folding of proteins. Evidence is, however, beginning to accumulate suggesting molecular chaperones have biological actions in addition to their intracellular protein–folding activity (Coates *et al*, 1996). They are antigenic and have been shown to stimulate cytokine synthesis. The 65kDa HSP of *M. leprae* (the homologue of *E. coli* cpn60) has been found to induce transcription of tumour necrosis factor (TNF) genes in monocytic cells and the release of interleukin (IL)-6 and IL-8 (Friedland *et al*, 1993). The 71 kDa HSP has also been found to have cytokine stimulating activity (Beagley *et al*, 1993). HSPs from a host of bacterial species, including *E. coli*, *Legionella pneumophila*, *M. leprae* and *M. bovis*, have also been found to stimulate cytokine activity (Retzlaff *et al*, 1994). The chaperonins (cpn's), a class of heat shock protein, have received special attention from the cellular microbiology group at the EDI, where it was established that cpn60 of *A. actinomycetemcomitans*, induces both cytokine synthesis and resorption of bone (Kirby *et al*, 1995). The cpn10 molecule, a co-chaperonin for cpn60, has been

found to be an essential growth and immunosuppressive factor in early pregnancy (Cavanagh *et al*, 1994).

Thus chaperonins may induce bone destruction either directly by acting on bone cells or indirectly by the induction of osteolytic cytokines from other associated cells. Of interest is the report that *M. tuberculosis* is able to secrete cpn10 (Orme *et al*, 1992), raising implications for the pathogenic effects of cpn10 in the bone pathology associated with *M. tuberculosis*.

Other components of mycobacteria have been shown to stimulate the synthesis of cytokine implicated in bone remodelling, and may therefore be able to modulate bone remodelling. These include a 58kDa protein able to stimulate monocyte TNFα synthesis (Wallis *et al*, 1993). The purified protein derivative of tuberculin has been reported to stimulate a Th1 pattern of cytokine synthesis in human peripheral blood (Del Prete *et al*, 1991).

CHAPTER SEVEN: *M. TUBERCULOSIS* OSTEOLYTIC FACTORS.

7.2. Materials and methods.

7.2.1. Mycobacterial sonicate.

The mycobacterial sonicate was prepared exactly as described in chapter 2 (section 2.1.). It was used at the concentrations described in the appropriate results section of this chapter.

7.2.2. Monoclonal antibodies.

Monoclonal antibodies SA12, TB78 and CS35 were as described in chapter 2 (section 2.3.1). They were used as described in the appropriate results section of this chapter.

7.2.3. *M. tuberculosis* cpn10 peptides.

Recombinant *M. tuberculosis* peptides were prepared as described in section 2.1.2. They were used as described in the appropriate results section of this chapter.

7.2.4. Assay of osteolytic capacity.

The ability of the osteolytic agents tested here to induce calcium release from neonatal murine calvaria was used as a measure of osteolytic capacity. This assay was carried out as described in chapter 2 (section 2.6. and its subsections). The agents to be tested were added to the culture media supporting calvarial bones at the concentrations described in the relevant results section of this chapter. Each agonist was tested at least three times to ensure the reproducibility of results.

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7.2.5. Assay of osteoclastogenic capacity.

The ability of the agents tested here to induce recruitment of TRAP+ve MNC's in murine calvarial bones was used as a measure of osteoclastogenic capacity. This assay was carried out as described in chapter 2 (section 2.6.3.). The agents to be tested were added to the culture media of the bones at the concentrations described in the relevant results section of this chapter. The individual examining the explants was unaware of the treatment to which they had been exposed.

7.3. Results.

7.3.1. Osteolysis induced by *Mycobacterium tuberculosis* sonicates *in vitro*.
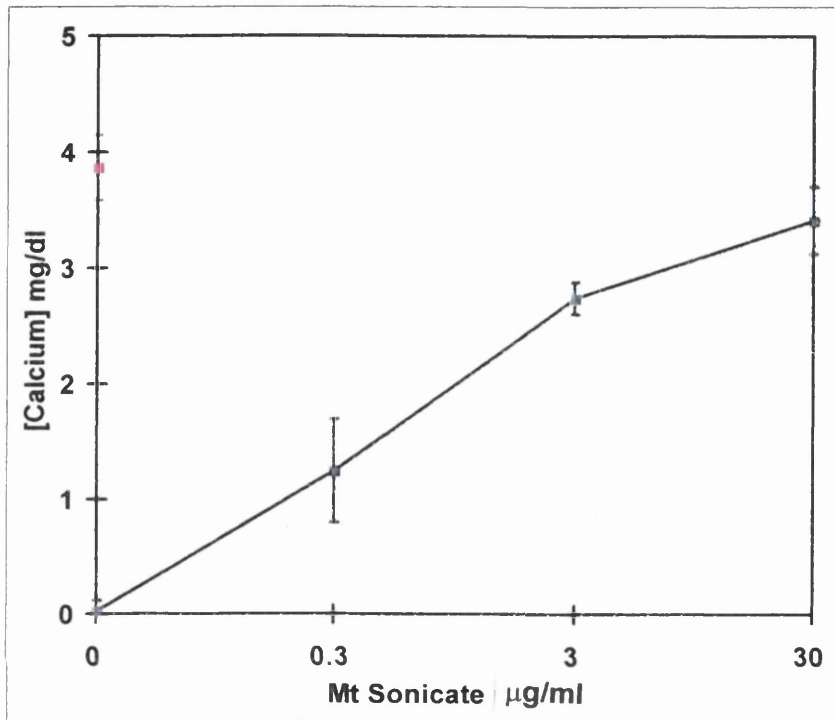
Figure 7. 1 Release of calcium from explants of murine calvaria exposed to a range of concentrations of *M. tuberculosis* sonicate. The PGE2 positive control is also shown. Results are presented as the mean and SE of 5 replicate cultures.

Sonicates of viable *M. tuberculosis* added to explants of murine calvarial bone produced a dose-dependent stimulation of bone resorption, over the concentration range 0.3 to 30 μg/ml, measured as calcium release into the tissue culture medium (Figure 7.1). Calcium release had begun to plateau between 3 and 30 μg/ml. This is in contrast to purified *M. tuberculosis* LAM which had no osteolytic activity (results not shown) when added to this assay system at concentrations up to 1μg/ml.

7.3.1.1. Osteoclastogenesis induced by *Mycobacterium tuberculosis* sonicates *in vitro*.

Sonicated *M. tuberculosis* was also examined for its ability to induce the local recruitment of osteoclasts in the murine calvarial explants. As can be seen in Figure 7.2 there was a dose dependent increase in TRAP+ve MNC numbers over the range 0.3 to 30μg/ml. The effect of PGE_2, used as a positive control, is also shown. This increase in osteoclast number paralleled the concentration-dependent increase in calcium release induced by the bacterial sonicate.
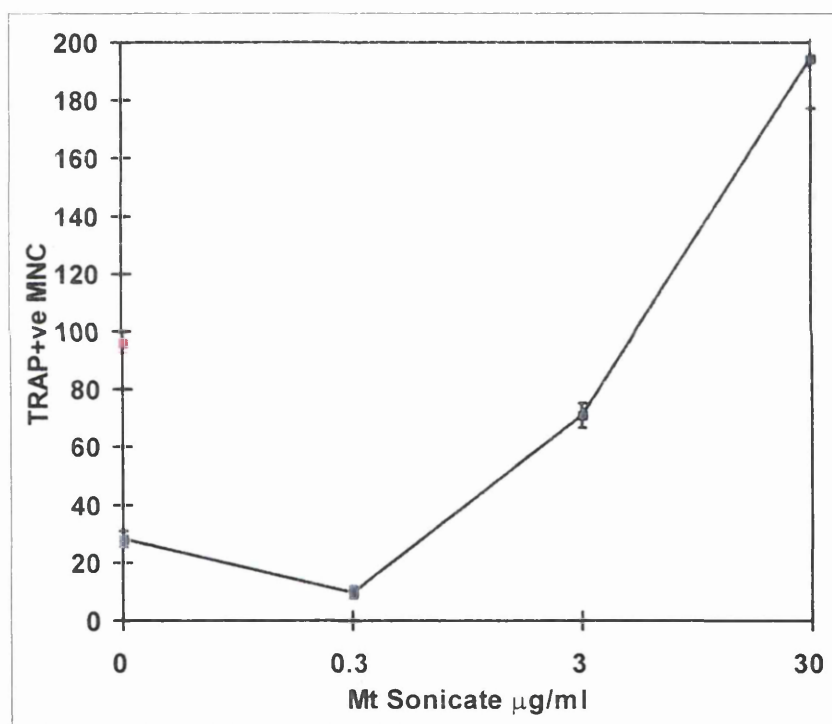
Figure 7. 2 Induction of osteoclast recruitment in murine calvaria exposed to a range of concentrations of *M. tuberculosis* sonicate. The PGE_2 positive control is also shown.

7.3.1.2. Effect of mAb's to mycobacterial components on *M. tuberculosis* induced osteolysis *in vitro*.

The dose-dependent stimulation of calvarial bone resorption induced by sonicated *M. tuberculosis* was completely and concentration-dependently

inhibited by the monoclonal antibody (mAb) to cpn10. In contrast, mAb TB78 which neutralises certain of the actions of *M. tuberculosis* cpn60 had no ability to block the bone resorption induced by the *M. tuberculosis* sonicate. Likewise, the mAb which binds and neutralises the known biological actions of LAM, had no inhibitory effect.
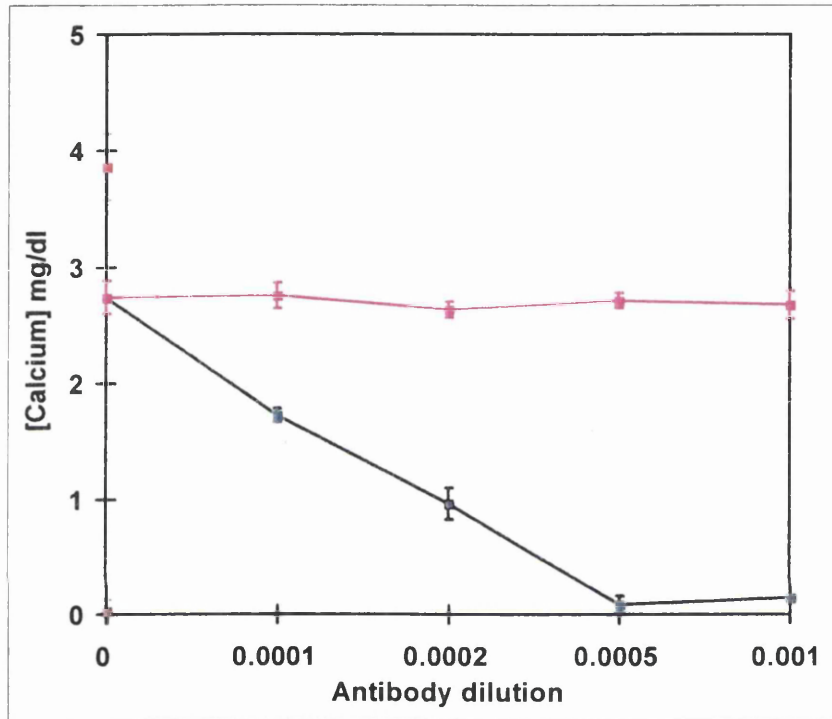
Figure 7. 3 Effects of adding mAb SA12 and mAb TB78 to calvaria cultured with *M. tuberculosis* sonicate (3µg/ml). The PGE₂ positive control is also shown.

7.3.1.3. Effects of monoclonal antibodies to *Mycobacterium tuberculosis* cpn10 on osteoclastogenesis.

Addition of increasing concentrations of the mAb SA12, which binds and neutralises *M. tuberculosis* cpn10, to cultured calvaria produced a dose dependent decrease in the numbers of TRAP+ve MNCs (Figure 7.4), which is interpreted as an inhibition of induced osteoclast formation. These results suggest that the *M. tuberculosis* sonicate tested here achieves its osteolytic

activity by the induction of osteoclastogenesis and that the recruitment of osteoclasts is induced by cpn10.
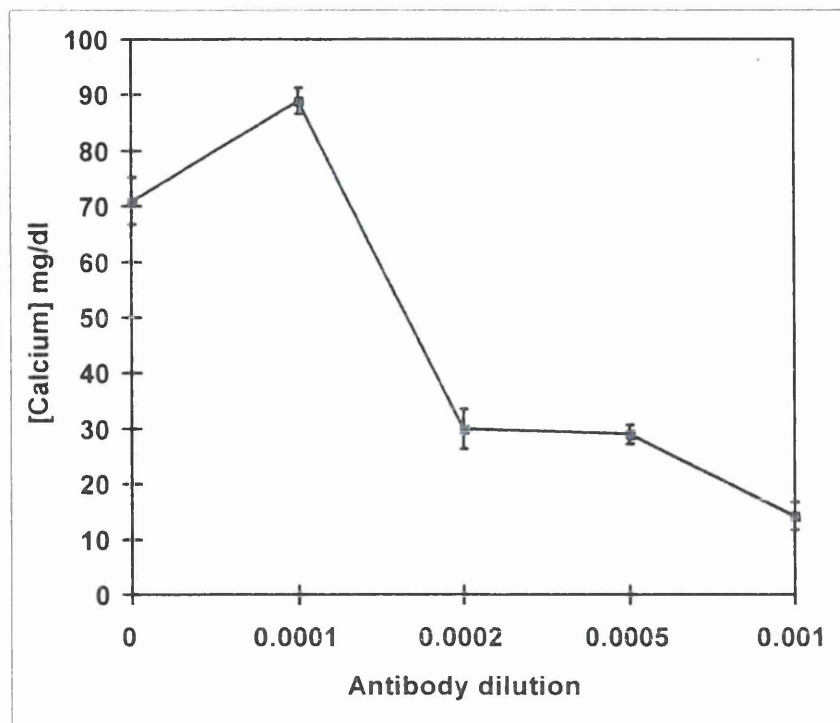
Figure 7. 4 The effect on osteoclast recruitment of adding a range of concentrations of mAb SA12 (against *M. tuberculosis* cpn10) to calvarial explants being cultured in the presence of *M. tuberculosis* sonicate (3μg/ml).

7.3.1.4. Effect of monoclonal antibody SA12 on PGE$_2$ induced calcium release.

In order to determine if the inhibition of *M. tuberculosis* sonicate induced bone resorption by mAb SA12 was specific or non-specific (due to some toxic effect of this particular mAb) the antibody was tested for its ability to inhibit bone resorption caused by PGE$_2$ (1μM). SA12 had no effect on the calcium release induced by this eicosanoid (Figure 7.5) suggesting that its inhibition of *M. tuberculosis* sonicate induced bone resorption is due to binding to cpn10 and inhibition of the activity of this protein.
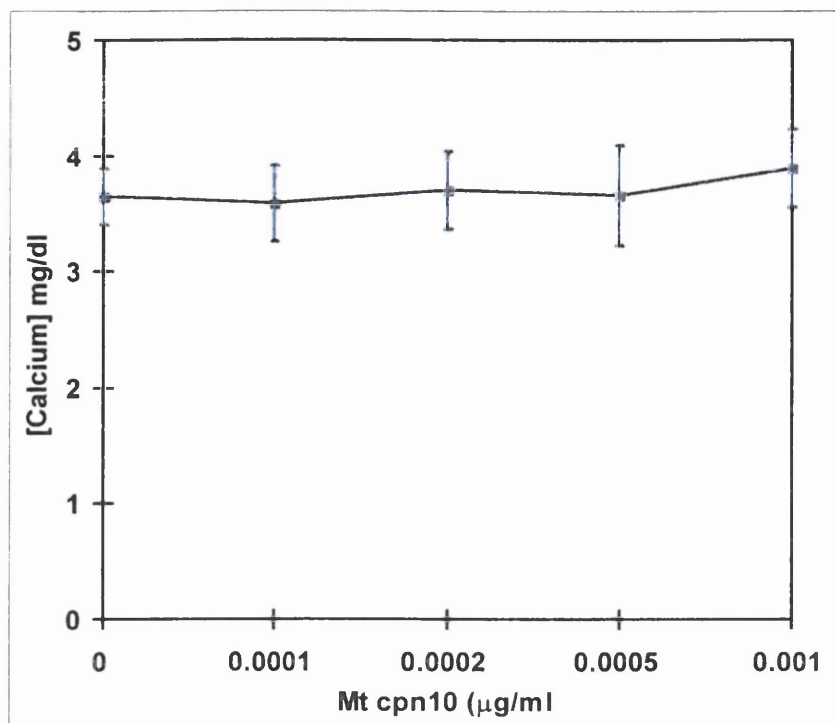
Figure 7. 5 Effect on calcium release of adding mAb SA12 (against *M. tuberculosis* cpn10) to calvarial explants being cultured in the presence of PGE$_2$ (10^{-6}M).

7.3.2. Effect of purified recombinant *Mycobacterium tuberculosis* cpn10 on osteolysis *in vitro*.

Purified recombinant *M. tuberculosis* cpn10 caused a dose-dependent stimulation of calcium release from cultured neonatal murine calvaria over the dose range 1ng/ml to 1μg/ml. Significant and reproducible osteolytic activity (p= 0.01; Figure 7.6) was found with the lowest concentration tested (1ng/ml), this represented an approximate 6-fold increase over the unstimulated control. If the active cpn10 is an oligomer of 7X10kDa subunits then it is active at concentration of 100pmol. The highest concentration of *M. tuberculosis* cpn10 (1μg/ml) tested caused a 24-fold increase in calcium release relative to the unstimulated control.

The purified recombinant *M. tuberculosis* cpn10 proved to be both extremely potent and efficacious in eliciting an osteolytic response in this assay.
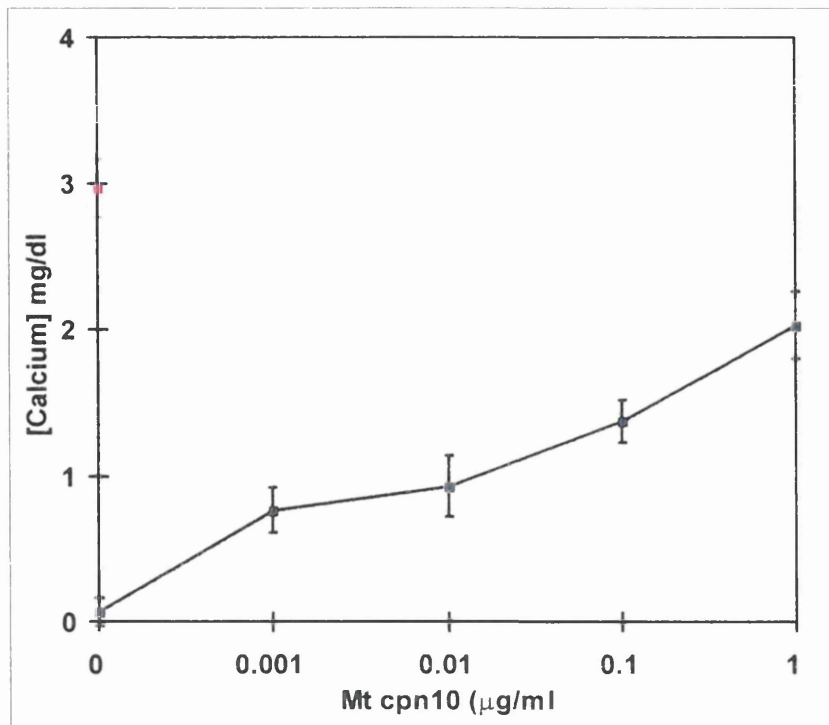
Figure 7. 6 Effect on calcium release of adding a range of concentrations of purified recombinant *M. tuberculosis* cpn10 to calvarial explants. The PGE$_2$ positive control shown.

7.3.2.1. Effect of mAb SA12 on the *in vitro* osteolysis induced by recombinant cpn10 from *Mycobacterium tuberculosis*.

The bone resorbing activity of purified *M. tuberculosis* cpn10 was dose dependently and completely inhibited by mAb SA12 (Figure 7.7). Significant inhibition was noted at the second lowest antibody dilution tested of 1/5000 and was completely abolished at the penultimate dilution of 1/200.

The high potency and efficacy of the inhibition caused by mAb SA12, on recombinant *M. tuberculosis* cpn10 induced osteolysis, supports the hypothesis that the inhibition of *M. tuberculosis* sonicate induced osteolysis (section 6.3.1.2; Figure 7.3) was due to a specific interaction between the mAb SA12 and cpn10 and not some other constituent of the sonicate. Thus it seems cpn10 is the active osteolytic principle in the sonicate of viable *M. tuberculosis*.
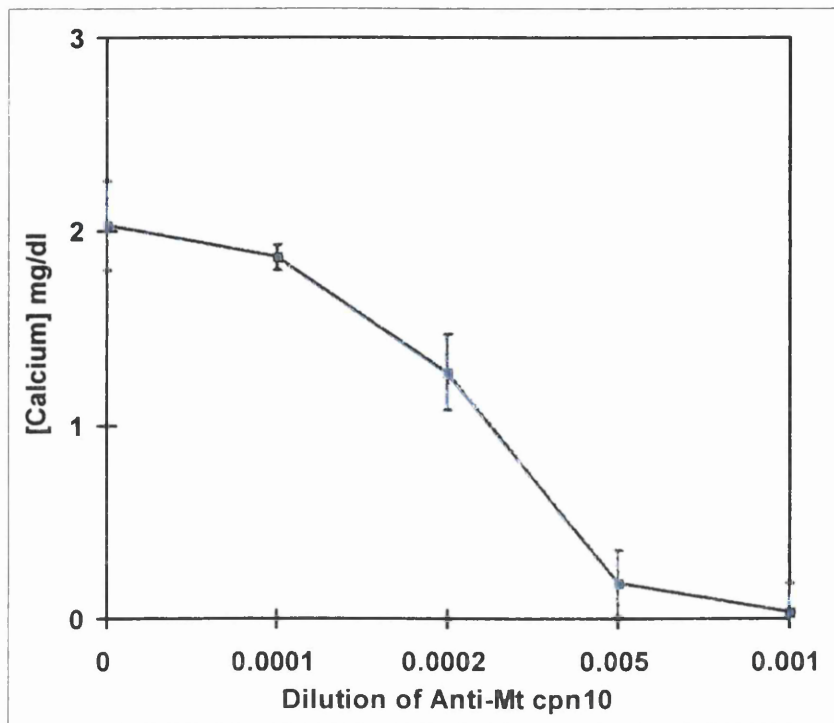
Figure 7. 7 Effect on calcium release of adding mAb SA12 to calvarial explants being cultured in the presence of purified r*M. tuberculosis* cpn10 (100ng/ml).

7.3.3. Antiproliferative activity of *Mycobacterium tuberculosis* cpn10 on a cultured osteosarcoma derived cell line.

Addition of recombinant *M. tuberculosis* cpn10 (over the concentration range 0.001to 10µg/ml) to subconfluent cultures of the human osteosarcoma cell line MG63 caused significant inhibition of cell proliferation at concentrations $\geq$ 1 nM (Figure 7.8). Inhibition was not due to cytotoxic effects of the cpn10 and, surprisingly, could not be blocked by mAB SA12.

These proliferation data together with the osteoclastogenesis data indicates two separate mechanisms involved in cpn10 induced osteolysis; one that decreases the amount of bone formation (by actions on the osteoblast) and another that increases the amount of bone resorption (by actions on the osteoclast). These two mechanisms operating in concert, *in vivo,* would mean a catastrophic uncoupling of the remodeling cycle. Osteoblasts would fail to appear

on cue after the resorptive phase, the reversal phase would therefore not occur. Consequently damage would be unrelentingly rapid and extensive than if only one of these mechanisms were to operate alone.
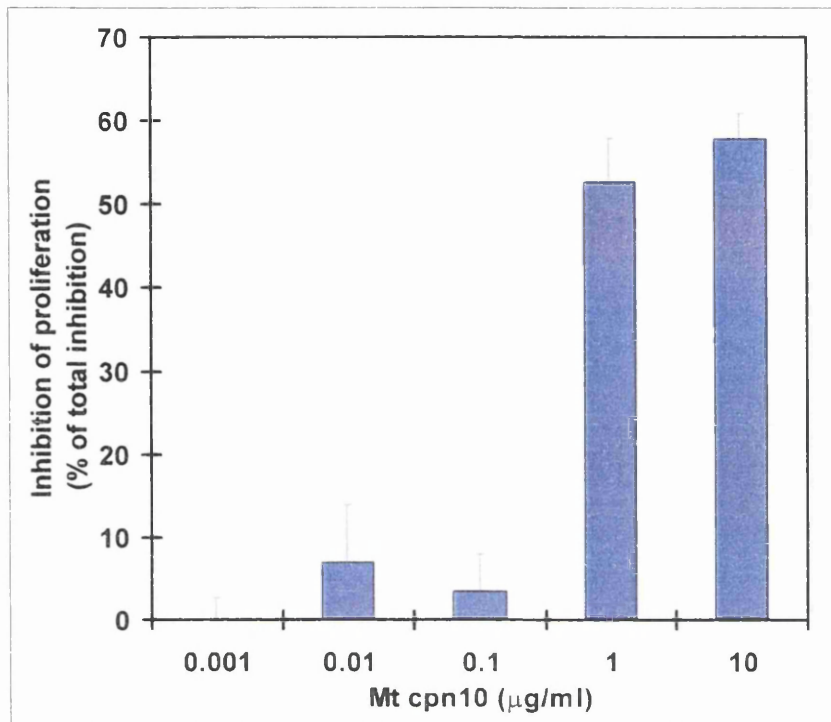
Figure 7. 8 *M. tuberculosis* cpn10 induced inhibition of proliferation. Inhibition was measured as a percentage of absolute inhibition (no thymidine incorporation) calculated from the % decrease in thymidine incorporation for a control cell culture.

7.3.4. Structure-Function relationships of *Mycobacterium tuberculosis* cpn10 to osteolysis.

A panel of 12 endopeptides, truncated NH2-terminal peptides and truncated COOH-terminal peptides, based on the primary sequence of *M. tuberculosis* cpn10 were used to define the specific structural features of recombinant *M. tuberculosis* cpn10 responsible for its osteolytic activities (Figure 7.9). These peptides corresponded to residues 1–25, 1–58, 21-35, 26–99, 46–99, 51–99, 54–99, 59–99, 65–99, 71–86, 75–99, and 91–99.

Three of these eleven peptides, 21-35, 26–99 and 65–99, exhibited reproducible osteolytic activity. Peptides 21-31 and 26–99 contains sequences that are within the flexible loop region of *M. tuberculosis* cpn10 (residues 16–35). An *E.coli* cpn 10 peptide spanning residues 23-33, also within the flexible loop, demonstrated activity.
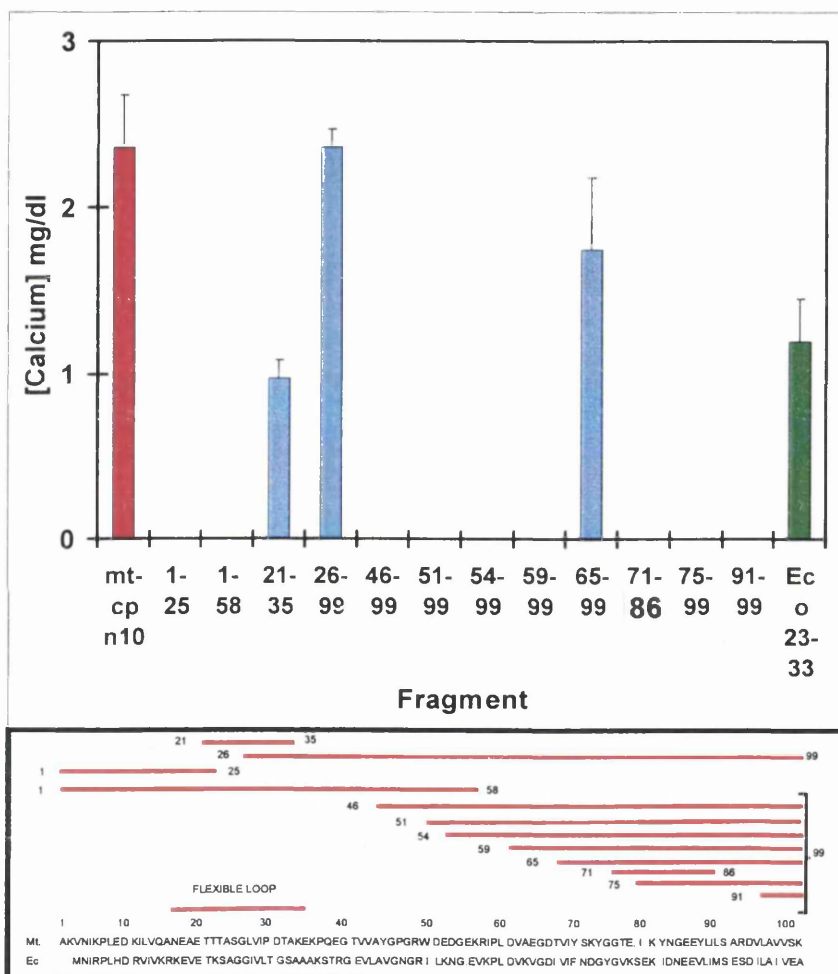
Figure 7. 9 Bone resorbing activity of NH$_2$ and COOH terminal truncated peptides (at 1µg/ml) compared to full length *M. tuberculosis* cpn10. Bone resorption is measured as calcium release from murine calvarial explant cultures stimulated with the peptides, *M. tuberculosis* cpn10 or a peptide fragment from *E.coli* cpn10.

7.3.5. Molecular modeling of cpn10.

This was performed by Dr M. Roberts as described in detail in chapter 2. A molecular model of the heptameric *M. tuberculosis* cpn10 was derived from the

E. coli crystal structure (figure 7.10). The sequences derived from the peptide data that contribute to the osteolytic activity are coloured in red and correspond to the flexible loop (sequence 21-35) at the bottom outer edge of the heptamer which is in close proximity to the sequence 65-70.
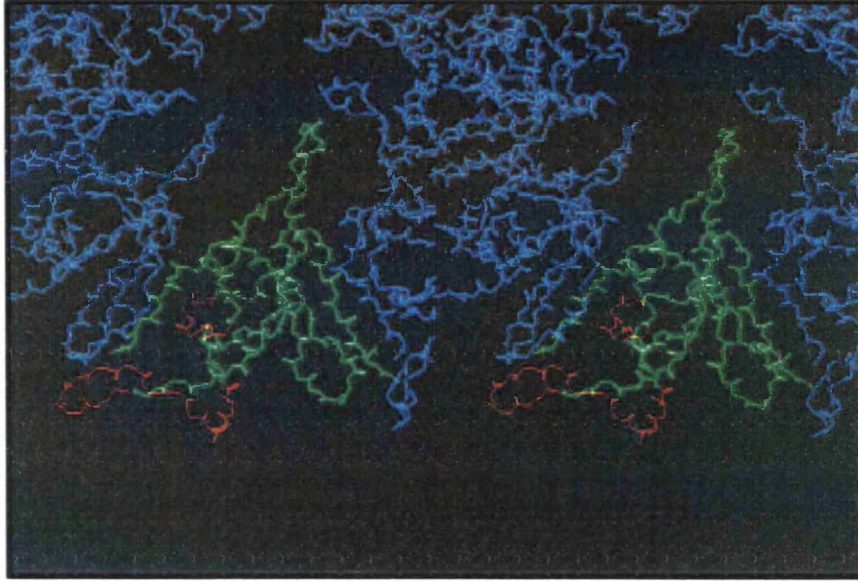
Figure 7. 10 Locations of sequences on the *M. tuberculosis* cpn10 structure that are likely to be important in bone resorption. Stereo view of polypeptide backbone of *M. tuberculosis* cpn10 heptamer model viewed from the top down the sevenfold axis with one subunit highlighted in green. Peptide sequences with osteolytic activity are highlighted in red.

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7.4. Discussion.

Our understanding of the molecular and cellular biology of heat shock proteins molecular chaperones (also termed molecular chaperones or stress proteins) can be traced back to the work of Ritossa (1962). Ritossa found that exposing *Drosophila* to elevated temperatures resulted in new puffing patterns in the giant polytene chromosomes of this insect. During the past decade or so the molecular nature of the proteins which constitute the so-called heat shock or cell stress response has been elucidated and it is clear that cells produce a large number of constitutive and induced proteins which aid in the folding, refolding or transmembrane transport (into organelles such as mitochondria and peroxisomes) of proteins.

Molecular chaperones (the currently favoured name for these proteins) are now known to exist as single chain and oligomeric proteins, with a broad range of molecular masses from 8kDa (ubiquitin) to >100Kda. One of the 'groups' of molecular chaperones which have been most extensively studied are the two heptameric proteins known as the chaperonins. These are two interacting oligomers of 10kDa (chaperonin [cpn] 10) and 60kDa (cpn60). The 60 kDa monomers form a stacked ring-shaped structure defining a central cavity within which proteins are folded in a process requiring the hydrolysis of ATP and the interaction of cpn10. The cpn's most studied are those of *E. coli*, in which species the 60kDa oligomer is called groEL and the 10kDa protein is known as groES. High resolution crystal structures of both molecules have been solved.

In addition to their ability to fold proteins within the cell, it has been known for two decades or so that the cpn's are major immunogens and that infections with bacteria and protozoa results in the infected individual raising large amounts

of antibody and reactive T-cells to the cpn's. This is an interesting finding, given that the primary sequences of the cpn's are very highly conserved between bacteria and man, and has stimulated speculation that the immune response to pathogen derived cpn's may be instrumental in the production of auto-immunity in humans.

In the past few years there have also been reports that the cpn's had the ability to exhibit biological actvities when applied to cultured cells. For example a number of groups have found that cpn60 can stimulate a variety of cells to produce cytokines (Beagley _et al_, 1993; Retzlaff _et al_, 1994) and Cavanagh's group in Australia have suggested that mammalian cpn10 is a hormone known as early pregnancy factor (EPF; Cavanagh _et al_, 1994).

In 1995 the EDI group reported that the potent proteinaceous osteolytic factor found on the surface of the periodontopathogen _A. actionmycetemcomitans_- was the molecular chaperone, cpn60. It was further shown that the cpn60 from _E. coli_ (groEL) was also a potent inducer of bone resorption (Kirby _et al_, 1995). Great care was taken in these studies to exclude the possibility that the osteolytic activity was due to contaminating lipopolysaccharide (LPS). For example use was made of the C3H/HeJ LPS non-responsive strain of mouse. The calvaria of these animals responded to groEL but failed to undergo osteolysis in the presence of _E.coli_ LPS. This pioneering study established that the cpn60 of two bacteria, _E. coli_ and _A. actinomycetemcomitans_, had the capacity to promote resorption of murine bone. In contrast it was found that the cpn60 of mycobacterial species had no osteolytic activity.

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Some of the bacteria involved in bone infections have been described in Chapter 1, and the most horrendous pathology due to bacterial infection of bone must be tuberculosis of the spine, due to the rapid nature of the tissue destruction and the terrible deformity that results. The mechanisms by which *M. tuberculosis* causes bone destruction appear not to have been studied and a very simple approach was taken to this question in the study presented here. Sonicates were made (under carefully controlled conditions) of a pathogenic viable strain of *M. tuberculosis*. The sonicate was then added to murine calvarial bone explants and the release of calcium monitored. As expected, the sonicate had the capacity to promote the release of calcium from the calvaria. It was expected that many of the components of this organism would be able to induce bone resorption. The major osteolytic component was predicted to be the lipoarabinomannan (LAM)- a major cell wall component which can be likened to the LPSs of Gram-negative bacteria. It came as somewhat of a surprise to find that addition of purified LAM to murine calvaria had no osteolytic effect and that a neutralising antibody to LAM had no ability to inhibit the osteolytic activity of the *M. tuberculosis* sonicate.

It came as a greater surprise when it was found that the osteolytic activity of the M. tuberculosis sonicate could be completely blocked by a neutralising antibody (SA12) to *M. tuberculosis* cpn10. This finding was confirmed by the finding the LPS-low recombinant *M. tuberculosis* cpn10 could stimulate murine calvarial calcium release in a dose dependent manner. Furthermore the osteolytic activity of this recombinant molecule could be completely blocked by mAb SA12. This antibody was acting in a selective fashion as it had no influence on the bone resorption induced by PGE_2. In addition a subclass-matched antibody to cpn60 had no effect on bone resorption. Thus it appears that the osteolytic activity of *M.*

tuberculosis, at least in the murine neonatal calvarial bone resorption assay, is due solely to the cpn10 molecule produced by this organism. It is of interest that it has been reported that the cpn10 of M. tuberculosis is an exported protein (Orme *et al*, 1992).

How does *M.tuberculosis* stimulate bone resorption?The full answer to this question cannot yet be given, but in this study the effect of this recombinant molecule on one key cell population, the osteoclast, has been studied. The osteoclast is the cell population responsible for removing the calcified matrix of bone. The method of Holt *et al* (1995), for identifying osteoclasts in murine calvaria was modified and used to determine if the *M. tuberculosis* cpn10 caused the recruitment of osteoclasts directly in calvaria. Blinded measurements were made of the osteoclast numbers in calvaria exposed to *M. tuberculosis* cpn10 and it was found that this bacterial protein was able to induce the recruitment of these cells and that such recruitment was blocked by the mAb SA12. Thus it would appear that *M. tuberculosis* is able to induce the recruitment of osteoclast precursors and then to promote the formation and activation of mature osteoclasts and that it is this effect that is responsible for the loss of bone from the calvaria. This finding has been confirmed by studies of groEL in which it has been shown that this chaperonin has been able to induce the recruitment of osteoclasts in the 'classic' osteoclast recruitment assay using murine bone marrow (Reddi *et al,* 1998).

It was also found that *M. tuberculosis* cpn10 was capable of inhibiting the proliferation of the human osteoblast-like cell-line MG63. As the osteoblast is the cell responsible for the production of the bone matrix this suggests that *M. tuberculosis* not only increases the amount of bone resorption occuring but also

prevents the onset of the reversal phase (when osteoblasts are recruited to replace the matrix removed by the osteoclast) in the remodelling cycle, thus the remodelling cycle is uncoupled. This may account for the rapid and devastating nature of the tissue destruction seen in cases of skeletal tuberculosis.

The work of one of the EDI collaborators, Dr. Paolo Mascagni (working with Professor Anthony Coates), has resulted in the synthesis of a number of peptides based on the primary sequence of *M. tuberculosis* cpn10. It was decided to test these peptides in the hope it would provide some structure/activity information from which one could link particular regions (domains) in the protein with the osteolytic activity and thus infer which parts of the molecule were responsible for osteolysis. The structure of the *M. tuberculosis* cpn10 oligomer is just becoming available as a result of the studies of Dr Michael Roberts and Professor Anthony Coates (St George's Hospital Medical School). However, a high resolution model has been produced of groES (Hunt *et al*, 1996) and a lower resolution structure of the *M. leprae* cpn10 has been reported. These structures reveal the presence of a large flexible loop, made up of residues 16-36, and a second small loop around a conserved tyrosine at position 71. These loop structures are important in the binding of cpn10 to cpn60. The peptides which showed activity in the bone resorption assay were 21-35 (containing the flexible loop), 26-99 (containing the flexible loop), and 65-99 (containing the second loop structure around position 71).

Some of the other peptides also contained these flexible loop structures but showed no activity. Peptide 1–58 was inactive in the bone resorption assay, although it contains the predicted flexible loop. The most likely explanation is that the structure of 1–58 differs from that of whole protein, because peptide 1–58 is a

dimer (Fossati *et al*, 1995), the aggregation of which is unusual as it occurs via the NH2 -terminal region in contrast to the whole protein in which contact between two neighbouring protomers involves the COOH-terminal tail of one protomer and the NH2 -terminal region of the other (Hunt *et al*, 1996). Peptides 46–99, 51–99, 54–99, and 59–99 were also inactive, although they contain the active 65–99 sequence, and again, structural differences are the likely explanation for this discrepancy. Peptide 26–99 is active in the bone resorption assay since most of the mobile loop is part of its NH2-terminal tail and is probably accessible to solvent (and hence a receptor) as often happens to the NH2-terminal and COOH-terminal regions of polypeptides and proteins. For the same reason, amino acids 65–70 would be considered the active sequence in peptide 65–99.

Although the flexible loop is exposed in the heptameric model, the sequence 65–70 is inaccessible at the subunit interface. Furthermore, based on studies with GroES, which dissociates to monomers at concentrations of 1 mM (Zondlo *et al*, 1995), *M. tuberculosis* cpn10 would be expected to dissociate at the concentrations used in all these biological assays. This suggests that an alternative oligomeric form of *M. tuberculosis* cpn10 may be required for osteolytic activity. A tetrameric form of *M. tuberculosis* cpn10 has been reported (Fossati *et al*, 1995), and this may be the osteolytically active form. Alternatively, *M. tuberculosis* cpn10 might assemble as a heptamer only at the putative cell receptor. The flexible loop also binds to cpn60 in the cpn60–cpn10 protein-folding complex (Landry *et al*, 1993), suggesting that the putative cell receptor for *M. tuberculosis* cpn10 has some structural homology with cpn60. If this structural homology is significant, it would require the *M. tuberculosis* cpn10 to assemble as

a heptamer on the receptor. Considering the gross structural rearrangements that occur in the cpn10 subunits of the heptamer when it binds to cpn60 (Saibil, 1996), it may be possible for the two active sequences in *M. tuberculosis* cpn10 to make contact with the receptor. None of the peptide fragments containing the active sequences appear to be involved in the interaction of *M. tuberculosis* cpn10 with the human osteoblast-like cell line MG63, possibly due to their inability to assemble as a heptamer. In this regard, it may be important to note that mitochondrial cpn60 is expressed on the surface of human cells (Soltys *et al*, 1996). These receptors are likely to be of therapeutic importance in the treatment of bone tuberculosis and possibly in other bone diseases.
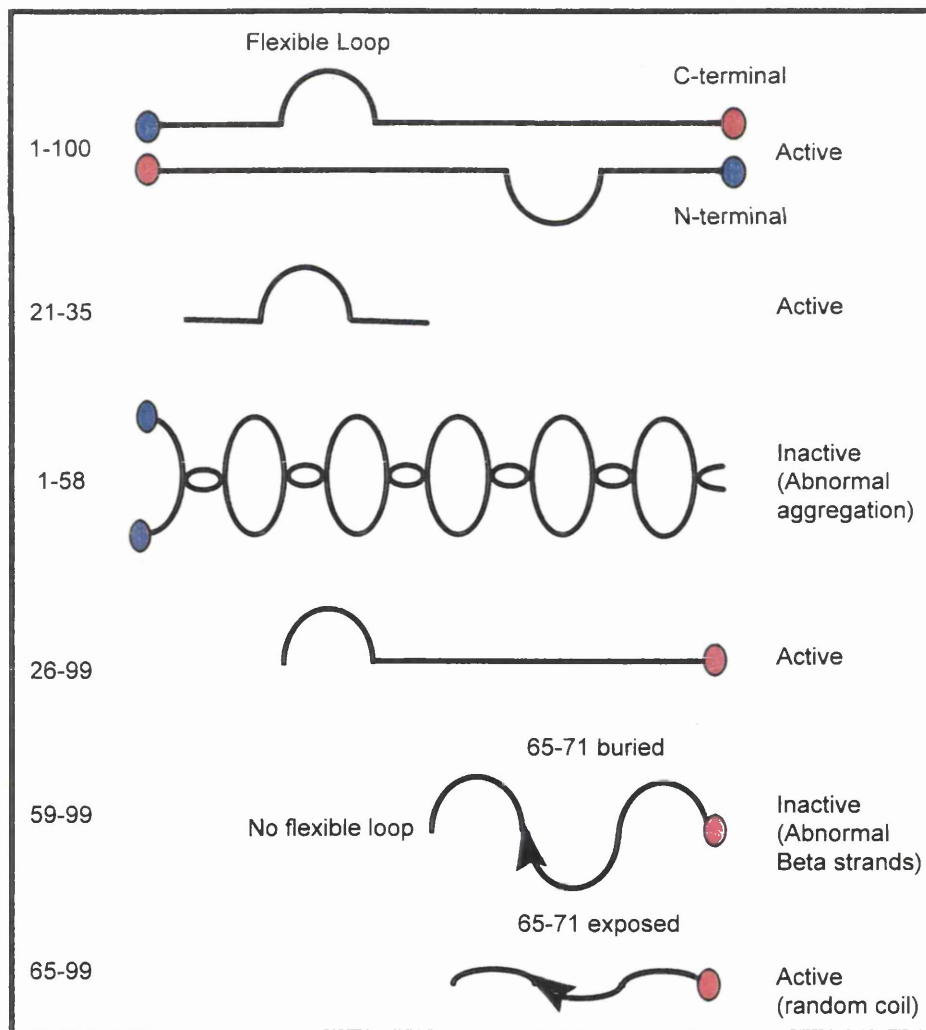
Figure 7. 11 approximate locations of the osteolytically-active sequences on the *M. tuberculosis* cpn10 structure.

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Based upon the peptide activity data, the *M. tuberculosis* cpn10 structure model gives an approximate guide to the location of the osteolytically-active sequences on the *M. tuberculosis* cpn10 structure (Figure 7.11). The precise molecular structure accounting for the bioactivity of *M. tuberculosis* cpn10 will be obtained by ongoing work on solving the *M. tuberculosis* cpn10 structure by x-ray crystallography (Roberts, M.M., A. Coker, G. Fossati, P. Mascagni, A.R.M. Coates, and S.P. Wood, unpublished data) and the use of site-directed mutagenesis.

It is not known which *M. tuberculosis* strains are associated with Pott's disease. In this study we have tested the *M. tuberculosis* strain H37Rv, which is a virulent strain commonly used in research into tuberculosis. We have shown that the obligate protein, *M. tuberculosis* cpn10, is an osteolytically active component produced by this organism. All strains of *M. tuberculosis* must contain this protein and therefore have the potential to induce bone disease. There may be additional factors to consider in the propensity of *M. tuberculosis* to cause Pott's disease, and further studies into this area are clearly necessary.

CHAPTER EIGHT

GENERAL DISCUSSION.

8.1 Introduction.

In 1969 the American Surgeon General, in his address to Congress said, 'you can close the book on infectious diseases'. This reflected the feeling at the time that antibiotics had solved the problems of bacterial infection. Unfortunately, only 30 years later we are now realising that we have not 'defeated' the bacterium and that many organisms that were thought to be of little medical importance are reappearing as causative agents of disease. A topical example of this is *E. coli*, with seven strains now being recognised as pathogens. The most dangerous is *E. coli* 0157, the so-called hamburger bug, which causes haemolytic-uraemic syndrome, an often lethal condition which afflicts the micro-vasculature of the kidneys and brain. Other bacteria, such as *S. aureus* and *M. tuberculosis* are becoming major health problems in the developed world because of the rapid increase in the incidence of antibiotic resistant strains. For example, the only treatment for methicillin (or multiple)-resistant *S. aureus* (MRSA) is Vancomycin. A recent report in Lancet describes the isolation of Vancomycin-insensitive strains of this organism in Japan (Hiramitsu *et al*, 1997). The enormity of the problem of antibiotic resistance has been forcefully described in a recent House of Lords report (House of Lords Select Committee on Science and Technology, 1998). Thus, as we approach the Millennium we are moving into a world of uncertainty as far as bacterial infections are concerned. The problem is accentuated by the fact that our complacence about bacteria and their ability to cause disease over the past few decades has resulted in a dearth of research into the mechanisms by which bacteria produce pathology. One area that has been seriously under-researched is how bacteria infecting bone are able to cause the breakdown or loss of the matrix of bone.

8.1.1. Bacteria Involved in Bone Pathology.

Bone is a useful tissue for the study of the 'history' of bacterial infections. Because bone is a hard tissue, due to its calcified organic matrix, and it is generally the only part of the organism to fossilise, the history of predation by bacteria can be assessed. The earliest record of the effect of bacteria on bone appears to be that of a skeleton of the Permian reptile, *Dimetrodon,* which shows signs of pathology in one of its vertebral spines and has been interpreted as being caused by infection. Many other examples of fossils showing signs of bone infection litter the palaeontological literature (Majno, 1991). Bacterial infections of human bone or the effect of bacterial infections on bone are seen in many conditions and any bacterium is able to infect bone. The most obvious condition associated with bone infection is traumatic injury such as is caused in a motor accident and in which bacteria in the environment can enter into bone. However, it is well established that a number of bacteria seem to have a propensity for infecting bone. The best known example are the staphylococci. Organisms such as *S. aureus* and *S. epidermidis* are well-recognised causative agents of osteomyelitis and osteitis. They also cause bacterial arthritis and are a major problem for the orthopaedic surgeon if they enter metal implants such as those of the hip or knee, either at operation or by the haematogenous route. While antibiotics have been of great significance in the treatment of osteomyelitis, bacterial arthritis and infected implants it is well recognised that bacterial infections of bone are difficult to treat. The increasing longevity of the population is resulting in a larger proportion suffering from joint problems, particularly osteoarthritis and rheumatoid arthritis. These can afflict most of the articulating joints of the body and there are increasing numbers of joints which can be

replaced and, in the process, become infected.

One of the most severe pathology's ensuing from bacterial infection of bone is Pott's disease, described by Percival Pott's of Pott's fracture fame. Pott's disease occurs when the vertebrae are infected with *M. tuberculosis* the causative agent of tuberculosis. Such infection results in massive damage to the vertebrae with resultant severe deformation of the spine. This condition is comparatively rare, with perhaps 5% of patients with tuberculosis having infection of the vertebrae. However, the prevalence of tuberculosis world-wide is enormous and is increasing rapidly giving rise to a large number of individuals afflicted with this horrendous deforming infection.

A range of bacteria can cause infection of the joints and this may be associated with bone damage. Gram-positive cocci are the most frequent causes of bacterial arthritis (*S. aureus*, *Streptococcus* spp) followed by Gram negative bacteria such as *E. coli*, *Neisseria* spp and *Haemophilus influenzae*. Bacteria implicated in sexually-transmitted diseases (gonococci and chlamydia) can also cause arthritis. The most recent example of a bacterium causing arthritis is *Borrelia burgdorferi*, a spirochaete which causes Lyme disease. This condition is caused by infection spread by tics and has become a major health problem in the USA in the past two decades.

Two other extremely well known bacteria: *M. leprae* the causative agent of leprosy and *Treponema pallidum* which causes syphilis are also able to cause destruction of bone. Leprosy is well known for its association with skeletal deformity such as loss of the digits. Such damage is normally secondary to trauma and is caused by the anaesthesia produced by the damage to peripheral nerves by the bacterium. However, in addition to this, patients with leprosy can

exhibit arthritis with bone destruction and loss of bone from the maxillofacial skeleton. Syphilis, in its various forms can affect the skeleton and these affects can be recognised by palaeopathologists from the characteristic changes.

Thus far the bacteria described as causing bone resorption were organisms that had infected the skeleton. However, the most common bacterially-induced group of bone diseases are not caused by infectious organisms but are due to the actions of the normal microflora. The periodontal diseases, which have an estimated world-wide prevalence of 15% (Fox, 1992) are caused by the overgrowth of a range of oral Gram negative bacteria. Organisms implicated in the pathology of these diseases include: *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *F. nucleatum*, *E. corrodens* and *Treponema denticola*. These bacteria grow as biofilms in the pocket formed between the gingiva and the bone of the jaw but the bacteria do not make direct contact with the bone. This suggests that factors released from the bacteria and which diffuse to react with bone cells are responsible for tissue damage. The consequence of this overgrowth of organisms normally resident in the mouth is the destruction of the alveolar bone and periodontal ligament which support the teeth and the eventual loss of the dentition. The bacterial virulence factors contributing to the gingival inflammation and alveolar bone destruction have not been conclusively identified. However, the most likely of such factors is lipopolysaccharide.

8.3. Mechanisms of Bacterially-induced Bone Resorption.

A range of bacteria can infect the skeleton and can produce breakdown of the extracellular matrix of bone. The question which has to be asked is how do bacteria produce such damage? A number of mechanisms are likely. The simplest is that the bacteria release factors which directly damage the skeletal

extracellular matrix. The best, possibly the only, example of this mechanism is dental caries in which cariogenic bacteria release metabolic acids onto the enamel of the teeth causing loss of the calcified matrix. However, this mechanism is unlikely to be responsible for the loss of bone matrix found in the majority of infections of the skeleton. The other potential mechanisms by which bacteria could catalyse bone breakdown include: (i) the release of bacterial molecules which either directly induce bone cells to destroy bone or induce bone cells (or cells in the vicinity of the bone) to produce factors (eicosanoids, cytokines or other mediators) which then feed back onto the bone and induce bone matrix loss or (ii) uptake of the bacteria into cells of bone with consequent effects on skeletal remodelling. These three mechanisms are summarised in Figure 8.1.
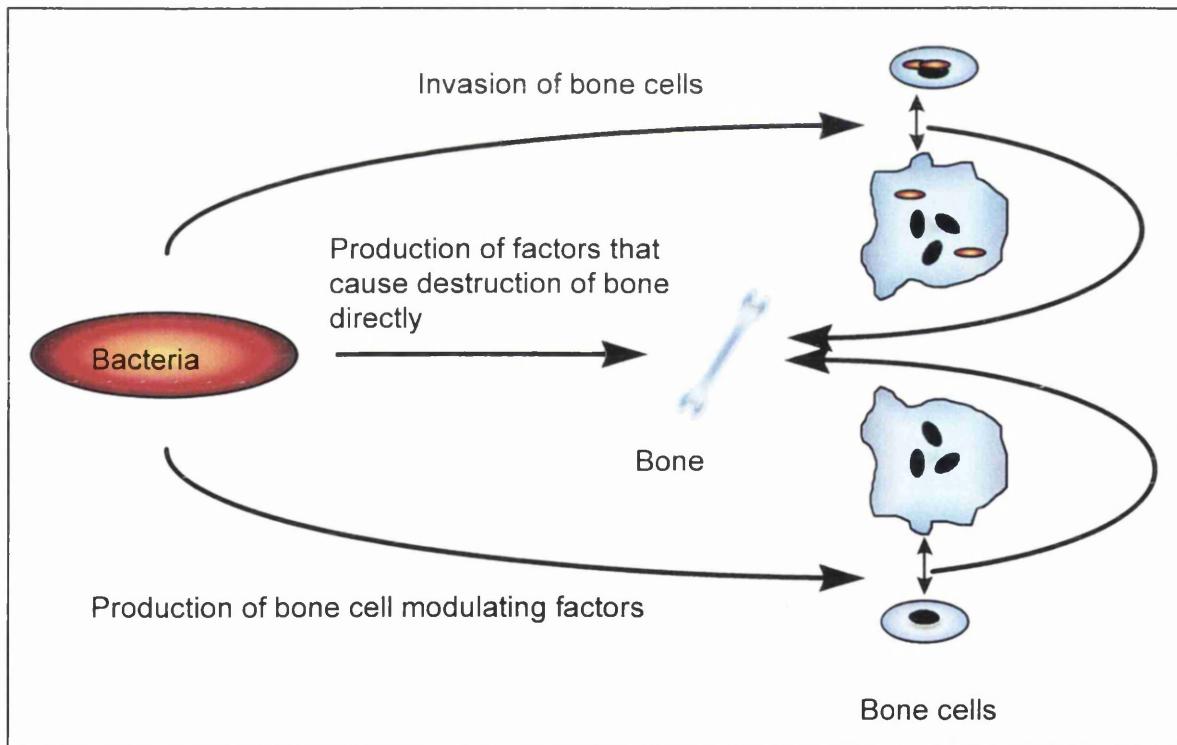
Figure 8. 1 Mechanisms by which bacteria may induce the destruction of bone.

In this thesis mechanism (i) has been examined with specific focus on the ability of bacterial constituents to induce bone cells to produce eicosanoids. The particular bacterial factors which have been examined for osteolytic activity are:

(i) exported proteins which can associate with the bacterial surface, (ii) lipopolysaccharides and (iii) the chaperonins (one family of molecular chaperones).

8.3.1. Eicosanoids and Bone Resorption.

One of the most puzzling aspects of bone remodelling, both normal and pathological is the role played by products of the action of cyclooxygenase (COX) activity. These are the large number of products formed by the oxidation of membrane-derived lipids, the best known of which is arachidonic acid (eicosatetraenoic acid). The most studied products of the action of the COX enzymes are the prostaglandins, the best known being PGE_2. These lipid mediators were initially discovered in terms of their ability to induce bone resorption and were believed to be important in the bone destruction found in rheumatoid arthritis. However, depending on the stimulus and the composition of the cell membranes, a very large number of putative lipid mediators can be produced by the actions of the COXs. It is likely that the complexity of the composition of the mediators that can be produced by COX-1 and -2 is responsible, in part, for the complex nature of the interaction between eicosanoids and bone remodelling. The apparently paradoxical finding that prostaglandins can both stimulate bone resorption and promote bone formation has stretched the imagination of many a scientist (especially the author). However, it is increasingly clear that bone can respond to the same signal in different ways depending on how the signal is presented to the responding bone cell. This is exemplified by the different responses of bone to PTH delivered continuously or in a pulsatile manner.

In addition to the products of the COX enzymes it was shown almost a decade ago that products of another arachidonate-metabolising enzyme - 5-lipoxygenase (5-LOX) could also promote osteolysis. Indeed, using purified 5-LOX products it was found that these lipid mediators were significantly more active than PGE_2 in stimulating bone resorption. This opened up another avenue of exploration which was rapidly closed when other workers claimed (either in print or at meetings) that they could not reproduce the work described by the EDI group. It was only when Greg Mundy's San Antonio group began to explore the mechanism of osteoclast action in giant cell tumours that it was discovered the active principles produced by such tumours were 5-LOX products. They also showed that the likely reason for the failure to repeat the original Eastman findings was the instability of the 5-LOX products which were rapidly oxidised and inactivated. Thus it is only within the past few years that attention has focused on the role of 5-LOX products in bone remodelling and this obviously complicates the potential mechanisms by which arachidonic acid products can modulate bone remodelling.

In this thesis much attention has focused on the role of arachidonic acid metabolism in the mechanisms by which bacteria involved in the major infectious diseases of bone stimulate bone resorption *in vitro*. The focus of the work has been the use of the murine calvarial bone resorption assay for the measurement of osteolysis and the murine bone marrow long-term culture assay for measuring the recruitment of osteoclasts. The calvarial assay has also been used to measure osteoclast recruitment.

8.4. Mechanisms of *S. aureus*-induced Bone Resorption

Prior work from the Eastman had established that material exported from

S. aureus and associated with the bacterium's outer surface is potently osteolytic.

One possible criticism of this work is that it did not account for the possible

contamination with cell wall material with known pro-inflammatory activity such as

peptidoglycan and lipoteichoic acid. In this study the surface-associated material

(SAM) was prepared and the bacteria were checked for possible damage caused

by extraction by use of electron microscopy. This revealed that the resultant

extracted bacteria lacked the fuzzy surface material but showed no signs of

damage which could have resulted in contamination with cell wall components.

However, to check the possible consequences of cell wall contamination the

osteolytic activity of the SAM was compared to that of commercially prepared

peptidoglycan and lipoteichoic acid. In a number of assays it was clear that these

cell wall components lacked significant osteolytic activity compared with the

proteinaceous SAM which exhibited activity, in some assays, at concentrations of

1-10ng/ml. By immunoassay of Vit D_3 in the culture media it was demonstrated

that the effects seen were not due to the up-regulation of macrophage Vit D_3

synthesis. Various physicochemical studies established that the active moiety in

the multi-component SAM was a protein of molecular mass >30kDa. The

question then addressed was what effect did this material have on the induction

of osteoclast formation. Using the murine bone marrow osteoclast generation

assay format it was found that the SAM was a potent inducer of TRAP+ve MNCs

and that these cells could induce pit formation. Furthermore, osteoclastogenesis

was blocked by calcitonin. These various findings strongly support the hypothesis

that the SAM contains a potent osteoclast-inducing activity. The next question to

be addressed was how did the active components in the SAM act to activate

osteoclast recruitment. This complex process was not affected by indomethacin

and was only partially blocked by use of the natural antagonist of IL-1 (the protein IL-1ra)which is a potent inducer of COX2. Inhibition of TNF and IL-6, using neutralising antibodies almost completely blocked osteoclast recruitment. When the two distinct inhibitors of 5-LOX were tested, both showed significant inhibition of osteoclast formation at low concentrations. It is not clear what the interrelationships are between the induction of the transcription of cytokines such as IL-6 and TNF and the synthesis of 5-LOX products. As discussed in Chapter 3 there is literature demonstrating that LOX products can induce IL-6 transcription and LOX inhibitors have been reported to inhibit TNF synthesis (although such compounds are also inhibitors of key kinases). Further work is required to identify the key mechanisms, but this can only be undertaken when the individual proteins responsible for bone resorption have been identified, cloned and expressed. Although this project is going on in the Eastman laboratories it was proving much more difficult than anticipated and so it was decided to examine the role of 5-LOX products in the action of known osteolytic mediators including enteric and oral LPS.

8.6. Role of 5-LOX Products in Agonist-induced Bone Resorption

A number of agents, both hormones and inflammatory mediators have been examined for their ability to induce bone resorption in the presence of selective inhibitors of 5-LOX. Use was initially made of the two hydroxamic acid compounds BWA4C and BWA70C which bind to the iron in the active site of 5-LOX. A further compound, MK886, which inhibits the binding of 5-LOX to FLAP, was also used to show specificity of the effects seen. If both types of compounds inhibited an agonist then it must have been working through 5-LOX. One of these compounds, BWA4C, had significant non-specific effects at high concentration

(Henderson, personal communication). To our surprise neither inhibitor was able to block the osteolytic activity of PTH, Vit D_3, PGE_2, IL-1 or *E. coli* LPS. Some apparent inhibitory effects were seen at high concentrations of BWA4C but these were put down to non-selective effects due to the toxicity of this compound. We also tested the LPS from the periodontopathogen *A. actinomycetemcomitans*. The assumption was that it would show the same response as the LPS from *E. coli*. This oral LPS was less active than the enteric form but, to our surprise, was able to be completely inhibited by all three 5-LOX inhibitors. MK886 which is a potent and very selective inhibitor showed significant inhibitory activity at a concentration of 100nM. To confirm that the effects being seen were due to inhibition of 5-LOX activity, the concentration of 5-LOX products in the culture media supporting explants was measured. This revealed that there was complete inhibition of 5-LOX by both BWA70C and MK886 at concentrations giving maximal inhibition of bone resorption. It was also demonstrated that the LPS from *A. actinomycetemcomitans* was able to dose-dependently stimulate murine calvaria to produce 5-LOX products (specifically peptidoleukotrienes). In addition to stimulating breakdown of murine calvaria, the LPS from *A. actinomycetemcomitans* was able to promote osteoclastogenesis and this process was also blocked by inhibiting the activity of 5-LOX. The LPSs from other oral bacteria also proved able to stimulate calvarial bone resorption and induce osteoclast formation by processes that were inhibited by selective 5-LOX inhibitors.

These data demonstrate that, in contrast to enteric LPS, the LPS from oral bacteria implicated in the pathogenesis of periodontal disease are able to stimulate relevant bone cell populations to produce 5-LOX products and that the

generation of these products is relevant to the osteolytic activity induced by the LPSs. It should be noted that not all the LPS molecules were equiactive. For example, that from *P. intermedia* was relatively inactive. Do these findings have any relevance to the pathology of the periodontal diseases? There have been few comparative studies of the relative contribution of COX and LOX products to inflammatory diseases. In rheumatoid arthritis the major species of eicosanoids are COX products (McMillan *et al*, 1995). However, in the periodontal diseases the major products of arachidonic acid produced by inflamed gingivae are lipoxygenase products (El Attar *et al*, 1983). This suggests that the finding that oral bacterial LPS stimulates bone resorption may be of relevance to the ongoing processes in the lesional gingivae which are generating osteolytic LOX products. The findings reported in this thesis also suggest that blockade of the synthesis of lipoxygenase products by the inflamed gingivae may have therapeutic benefit.

What is the source of these LOX products. No attempt has been made in this thesis to try and identify the cells within, say calvaria, capable of producing these LOX products. The methods that one could employ to do this would be liable to provide ambiguous conclusions. It is not really possible to identify COX or LOX products within cells as these are low molecular mass soluble lipid molecules that would be difficult to identify with immunohistochemistry (although there are now reports suggesting just that). It would be possible to identify the presence of the enzymes within cells but that would not tell you which cells contained the active enzyme. A similar claim can be made for techniques which enable mRNA levels to be assessed by *in situ* hybridisation. Because of these practical difficulties it was decided to address the question specifically to the population of bone forming cells (the osteoblasts) obtained from human bone.

Did these cells, once isolated and exposed to oral bacterial LPS, produce lipoxygenase products. Use was made of thin layer chromatography (TLC) to identify the eicosanoid products produced from radioactive arachidonic acid and RT-PCR was utilised to ascertain presence of mRNA for COX and LOX enzymes in osteoblasts. The finding reported in Chapters 5 and 6 that the bone resorption and osteoclast recruitment induced by the LPSs of aetiological suspects in periodontitis, such as A. actinomycetemcomitans, P. gingivalis and E. corrodens, could be inhibited by 5-LOX inhibitors and that A. actinomycetemcomitans LPS could stimulate human osteoblasts to produce 5-lipoxygenase products is therefore highly significant in terms of the mechanism by which LPSs exert their effects.

Most work concerning the role of eicosanoids in bone and its destruction in periodontitis has concentrated on the prostanoid products of the cyclooxygenase pathway (El Attar, 1976; Goldhaber, 1971; Goldhaber et al, 1973; Gnomes et al, 1976). However homogenates of inflamed gingiva have been shown to metabolise arachidonic acid mainly via the lipoxygenase rather than the cyclooxygenase pathway (El Attar et al, 1983).

The work presented here suggests that the effects of LPS on osteolysis are mediated by the recruitment of osteoclasts, this receives support from the work of Ito et al (1996). Therefore in the case of LPS, this ubiquitous gram-negative polymer probably binds to osteoblasts or other cells in bone through the CD14 receptor and stimulates them to release cytokines and eicosanoids which then induce the recruitment of osteoclasts. This explains the inhibitor effects of nonsteroidal and anti-cytokine agents on LPS-induced bone resorption.

If lipoxygenases, particularly 5-LOX play a key role in bacterially-induced

bone resorption, such as that found in the periodontal diseases, then the possibility of treating such diseases with selective 5-LOX inhibitors is extremely promising. Inhibitors of 5-LOX have been developed over the past 15 years (McMillan et al, 1995) and a range of potent, selective inhibitors now exist and are becoming licensed. For example Abbot Pharmaceuticals have recently launched Zileuton for the treatment of allergic conditions. Could Zileuton be used to treat the periodontal diseases? This is a question which the results in this thesis lead one to ask, however further 'clinical' research would be needed to evaluate this possibility.

8.6. *M. tuberculosis*-induced Bone Resorption.

One of the most severe of infections of bone is that induced by *M. tuberculosis*. Infection of the spine with this organism gives rise to what is termed Pott's disease, a particularly severe osteomyelitis of the spinal vertebrae resulting in horrendous deformity. The work presented here on its molecular chaperonin cpn10 is the first report of an osteolytic principle associated with it.

When sonicates of *M. tuberculosis* were added to murine calvarial bone explants the release of calcium from the calvaria was induced, as expected. Unexpectedly, addition of purified LAM to murine calvaria had no osteolytic effect and a neutralising antibody to LAM did not inhibit the osteolytic activity of the *M. tuberculosis* sonicate. More surprises were to come when it was found that the osteolytic activity of the M. tuberculosis sonicate could be completely blocked by a neutralising antibody (SA12) to *M. tuberculosis* cpn10 and that recombinant *M. tuberculosis* cpn10 could stimulate murine calvarial calcium release. Furthermore the osteolytic activity of this recombinant molecule could be completely blocked by mAb SA12. In addition a subclass-matched antibody to cpn60 had no effect on

bone resorption. Thus it appears that the osteolytic activity of *M. tuberculosis* is due solely to the cpn10 molecule produced by this organism.

Having identified an *M. tuberculosis* osteolytic principle, attention turned to identifying what cells in bone were acted upon to induce osteolysis. The method of Holt *et al* (1995), was used to determine if the *M. tuberculosis* cpn10 caused the recruitment of osteoclasts directly in calvaria. It was found that cpn10 was able to induce the recruitment of these cells and that such recruitment was blocked by the mAb SA12. Thus it would appear that *M.tuberculosis* is able to induce the recruitment of osteoclast precursors and then to promote the formation and activation of mature osteoclasts. It was also found that *M. tuberculosis* cpn10 was capable of inhibiting the proliferation of the human osteoblast-like cell-line MG63. These results indicate that *M. tuberculosis* may not only increase the amount of bone resorption occurring, via the osteoclast but also prevents the onset of the reversal phase of the remodelling cycle, by reducing the proliferation of osteoblasts, thus the remodelling cycle is uncoupled.

In order to characterise which domains in the cpn10 harboured the osteolytic activity It was decided to test peptides derived from it. The peptides which showed activity in the bone resorption assay were 21-35 and 26-99. These two peptides are associated with the large flexible loop structure of cpn10, made up of residues 16-36. A third peptide, 65-99, containing a second small loop structure (around a conserved tyrosine at position 71) was also found to be osteolytically active. These loop structures are important in the binding of cpn10 to cpn60.

<u>8.7. Conclusions.</u>

The results presented in this thesis reveal that bacteria contain and

produce a number of distinct molecules with potent osteolytic activities. In the Gram positive *S. aureus* the key components are exported proteins. In oral Gram-negative bacteria the LPS appears to be a potent factor for certain of the bacteria. With *M. tuberculosis,* surprisingly, there appears to be only one osteolytic component, the molecular chaperone - chaperonin 10. Is there any common mechanism shared by the bacterial bone virulence factors described in this thesis? Many bacteria are capable of stimulating bone matrix loss, and the information available would suggest that each organism possesses different factors which interact with bone in different ways. However what these factors do have in common is their ability to uncouple the bone remodelling cycle. Normal bone remodelling is the sum of the integrated regulation of the differentiation and activity of osteoblast and osteoclast lineages, that is a balance between formation and destruction of bone, each process being a consequence of the other. Understanding this process is central to understanding how bacteria cause bone destruction, since any interference with these integrated cellular systems can result in the loss of bone mass. Such interference is a recurrent theme throughout the work presented here.

8.8. Future work.

In their review paper Nair *et al* (1996) state that the key questions which must be asked of the bacterial bone-modulatory molecules are as follows: (i) what cell population do they bind to, (ii) what is the nature of the receptor and postreceptor events, and (iii) is their action direct or dependent on the induction of secondary extracellular bone-modulating factors such as cytokines and eicosanoids, etc.

LPS was one of the first bacterial molecules to be implicated in

osteoclastic bone resorption (Hausmann *et al*, 1970 and 1972). The work of Sismey-Durrant *et al* (1987) does not lend support for a direct action on osteoclasts. Thus it seems likely that LPS modulates its effects in bone via the osteoblast. LPS is renowned for its ability to stimulate the release of many inflammatory mediators by osteoblasts and to these we can now add 5-lipoxygenase products. However where lipoxygenase products fit into this panoply remains to be defined as does an answer to the second question posed by Nair *et al*.

The results obtained for *S. aureus* SAM also indicated a role for 5-lipoxygenase in the recruitment of osteoclasts, suggesting the possibility of a common receptor for LPS and the active principle in the SAM. Kusunoki *et al* (1995) have demonstrated *S. aureus* does produce molecules that can bind to CD14, it has also been shown that CR3 (CD11b/CD18) expresses one binding site for peptides and another for LPS (Wright *et al*, 1989). However before receptor events can be elucidated the immediate priority for *S. aureus* SAM must be to identify its active constituents. This work is at present underway at the Eastman Institute.

The work presented here on the *Mycobacterium tuberculosis* molecular chaperonin cpn10 is the first report of an osteolytic principle produced by that bacterium. For this molecule all three of Nair's questions must be addressed, and a program of work to this end has now been initiated at the Eastman Institute.

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THESIS.

Mycobacterium tuberculosis Chaperonin 10 Stimulates Bone Resorption: A Potential Contributory Factor in Pott's Disease

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Using a series of NH₂- and COOH-terminal truncated peptides, we have identified sites in Mt cpn10 responsible for the osteolytic activity of this molecular chaperone. We have identified the flexible loop of Mt cpn10 and the sequence 65–70 as regions most probably responsible for the bone-modulating bioactivity of this molecule.

Materials and Methods

Mycobacterial Sonicate. The sonicate was prepared by sonicating a suspension of viable virulent Mt (strain H37Rv) at 4°C for 1 min intervals, followed by a 1 min rest period, for a total period of 1 h. The sonicated material was then centrifuged at 100,000 *g* for 1 h, and the supernatant was filtered through a 0.22-μm membrane filter.

mAbs. Both the mAb to Mt cpn10 (SA12; 12) and the mAb to Mt cpn60 (TB78; reference 13) were obtained from murine ascites in a sufficiently high titer to bind to Mt cpn10 or cpn60 at the dilutions used in this study. SA12 is specific for mycobacterial cpn10 and TB78 is specific for mycobacterial cpn60. Neither of these mAbs are cross-reactive with any other Mt protein (14). The mAb to LAM (CS-35) of isotype IgG3 was obtained from concentrated tissue culture supernatant with a titer of 1:2,000 by Western blot analysis (Belisle, J.T., personal communication). CS-35 was raised against *Mycobacterium leprae* LAM and is cross-reactive with Mt LAM at a dilution of 1:1,000 by Western blot analysis (15). CS-35 was used at a 1:1,000 dilution in the bone resorption assay.

Mt cpn10 Peptides. r-Mt cpn10 was expressed in *Escherichia coli* and purified by reversed-phase HPLC to >97% purity as previously described (16). The synthetic peptide fragments were prepared and purified by isoelectric focusing and by reversed phase HPLC to >95% purity as previously described (17). Before addition to the bone explants, r-Mt cpn10 was passed down a Polymyxin B–agarose column (Detoxigel column; Pierce, Rockford, IL) to remove any contaminating LPS. The composition of the peptides was confirmed by amino acid analysis and mass spectroscopy. All peptides were tested for LPS using the limulus amoebocyte lysate assay (Whittaker M.A. Bioproducts, Inc., Walkersville, MD). All peptides tested negative, indicating the presence of <0.03 endotoxin U LPS.

Calcium Release and Osteoclast Recruitment in Murine Calvaria. The calvarial bone resorption assay was performed as described (18). In brief, calvaria were removed from 5-d-old MF1 mice, adherent connective tissue was dissected away, and the calvarial bone was halved, with each half being cultured separately on stainless steel grids. Calvaria were cultured in groups of 5 replicates in 30-mm dishes with 1.5 ml Biggers, Gwatkin, and Jenkins medium (ICN Biomedicals, Inc., Thame, UK) containing 5% heat-inactivated rabbit serum (GIBCO BRL, Paisley, UK) and 50 μg/ml ascorbic acid (Sigma Chemical Co., Poole, UK). After 24 h in culture, the media was replaced with media containing various concentrations of sonicated Mt, r-Mt cpn10 or Mt cpn10 peptides with or without a range of concentrations of mAb to Mt cpn10 (SA12; reference 12), mAb to Mt cpn60 (TB78; reference 13), mAb to Mt LAM at 1:1,000 dilution. Calvaria were cultured for a further 48 h and then the calcium released into the medium was measured by automated colorimetric analysis (19).

After removal of the media supporting the calvarial explants for calcium assay, the explants were then used for the measurement of osteoclast numbers by a modification of the method of Marshall et al. (20). The calvaria were fixed in 95% ethanol, 5% glacial
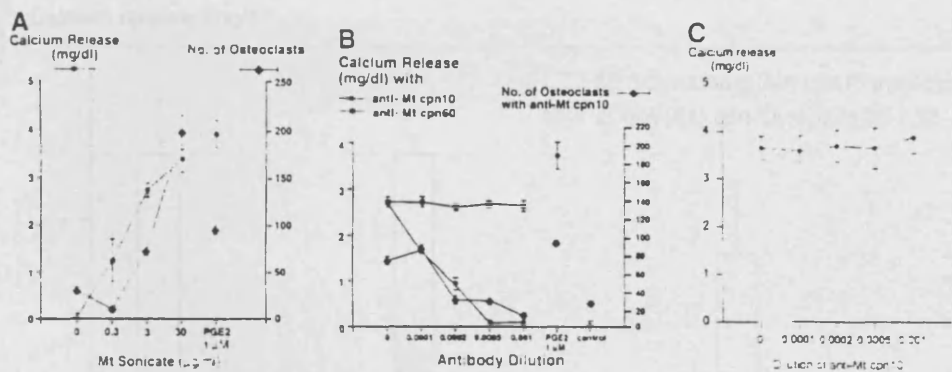
acetic acid for 24 h, and were then washed in PBS containing 1 mg/ml BSA followed by reaction in the histochemical substrate mixture (obtained from Sigma Chemical Co., and used according to the manufacturer's instructions) for the localization of tartrate-resistant acid phosphatase (TRAP) activity, a marker enzyme for osteoclasts. Bones were then washed in PBS, decalcified, and fixed in 12.5% glutaraldehyde (BDH Chemicals, Ltd., Poole, UK) in 1 M hydrochloric acid (BDH Chemicals, Ltd.) for 5 min. Finally, the bone explants were washed and mounted in Aqua-mount (BDH Chemicals, Ltd.). Each calvarial explant was then scanned by transmitted light microscopy and TRAP-positive cells containing three or more nuclei were counted. The individual counting the cells was unaware of the treatment to which each explant had been exposed. Control cultures included unstimulated calvaria (to determine spontaneous release of calcium) and calvarial cultures stimulated with 1 μM prostaglandin (PG; to demonstrate that bone is responsive and to give a measure of the maximal response). r-Mt cpn10 and Mt cpn10 peptides were tested in a minimum of three experiments and gave reproducible results.

Osteoblast Proliferation. The measurement of cell proliferation was as previously described (21). In brief, the human osteoblast-like cell line MG63 (CRL 1427; American Type Culture Collection, Rockville, MD) was cultured at a density of 15,000 cells/well in 96-well plates and incubated overnight at 37°C in DMEM (Gibco) plus 10% FCS (Sigma Chemical Co.) in 5% CO₂/air. The media were then removed and cells were washed twice with sterile Hank's solution (Sigma Chemical Co.). To measure antiproliferative activity, various concentrations of r-Mt cpn10 or truncated peptides were added in DMEM containing 2% FCS to the MG 63 cells. Cells were incubated for 24 h at 37°C. During the last 6 h of culture, 0.05 μCi of [³H]thymidine (Amersham International plc, Amersham, UK) was added to cells. The media were then removed and the cells fixed in 5% trichloroacetic acid. 100 μl of 0.5 M NaOH was used to lyse cells, this being neutralized by an equal volume of 0.5 M HCl. Radioactivity incorporated into nuclear DNA was measured by scintillation spectrometry. The cytotoxicity of the r-Mt cpn10 was determined by lactate dehydrogenase release, measured by the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Heidelberg, Germany). Data has been generated from a minimum of three separate experiments.

Homology Modeling of Mt cpn10. The Mt cpn10 model was generated by homology modeling of the Mt cpn10 sequence onto the atomic coordinates of the GroES structure of the monomer with the flexible loop assigned. The model was energy minimized with QUANTA/CHARMm (Molecular Simulations, Inc., San Diego, CA) using a nonbonded cutoff of 14 Å and a dielectric constant distance dependence until the root mean squared deviations were <0.001 Kcal/Å. The side chains were minimized first, keeping the backbone fixed. This was followed by minimization of the whole monomer. The heptamer was generated from the monomer by the symmetry operations relating the GroES subunits. The same energy minimization procedure was repeated for the final Mt cpn10 heptamer model, which was displayed with the SYBYL molecular modeling package (Tripos UK, Milton Keynes, UK).

Results and Discussion

Sonicates of viable Mt added to explants of murine calvarial bone produced a dose-dependent stimulation of bone resorption, measured as calcium release into the tissue culture medium. Osteoclast numbers in calvarial explants were

A
Calcium Release
(mg/dl)

No. of Osteoclasts

Mt Sonicate (μg/ml)

B
Calcium Release
(mg/dl) with
— anti-Mt cpn10
— anti-Mt cpn60

No. of Osteoclasts
with anti-Mt cpn10

Antibody Dilution

C
Calcium release
(mg/dl)

Dilution of anti-Mt cpn10

Figure 1. Effect of Mt sonicate on bone resorption. (A) Release of calcium from explants of murine calvaria and induction of osteoclast recruitment in the calvaria by dilutions of a sonicate of viable Mt. (B) The effect of adding various concentrations of mAb (12) SA12 (which recognizes Mt cpn10) or a mAb to Mt cpn60 (13) to bone explants stimulated with a fixed concentration of Mt sonicate (3 μg/ml) on the release of calcium from calvarial explants and on the numbers of osteoclasts in the calvaria. Controls with no Mt cpn10 (unstimulated calvaria) and with 1 μM PG are shown for comparison. (C) The effect of increasing concentrations of SA12 on the bone resorbing activity of PG. Results are expressed as the mean and standard deviation of five replicate cultures.

counted and showed a parallel increase (Fig. 1 A). The Mt sonicate–induced stimulation of bone resorption was dose dependently and completely inhibited by a neutralizing mAb to Mt cpn10 (SA12; reference 12), but not by a subclass-matched neutralizing mAb to Mt cpn60 (TB78; reference 13). Likewise, the mAb SA12 caused a dose-dependent decrease in the numbers of osteoclasts present in the calvarial explants (Fig. 1 B). In contrast, SA12 had no effect on the stimulation of bone resorption induced by PG (Fig. 1 C). Purified Mt LAM, added at a concentration of 1 μg/ml, had no osteolytic activity, and neutralizing mAb to LAM did not inhibit the bone resorption induced by the Mt sonicate (results not shown). Addition of polymyxin B

had no effect on the bone resorbing activity of the Mt sonicate.

Purified r-Mt cpn10 caused a dose-dependent stimulation of calcium release from cultured calvaria with osteolytic activity being noted at a concentration of 1 ng/ml (equivalent to 100 pmol) that was reproducible and statistically significant ($P < 0.01$; Fig. 2 A). The bone resorbing activity of Mt cpn10 was dose dependently and completely inhibited by mAb SA12 (Fig. 2 B). The osteoclast-inactivating hormone, calcitonin, at a concentration of 10 ng/ml also blocked r-Mt cpn10-induced bone resorption (results not shown). Addition of polymyxin B had no effect on the bone resorbing activity of r-Mt cpn10.

Addition of r-Mt cpn10 to subconfluent cultures of the human osteoblast-like cell line MG63 caused significant inhibition of cell proliferation at concentrations $\geqslant 1$ nM (Fig. 3). Inhibition of proliferation was not due to cytotoxicity of the r-Mt cpn10.

A panel of 11 NH_2- and COOH-terminal truncated peptides and short peptides (16) were used to define the specific structural features of r-Mt cpn10 responsible for its osteolytic and osteoblast antiproliferative activities. These peptides corresponded to residues 1–25, 1–58, 26–99, 46–99, 51–99, 54–99, 59–99, 65–99, 71–86, 75–99, and 91–99. Graded concentrations of each peptide were tested separately in each assay in three separate experiments. 2 of these

A
Calcium Release
(mg/dl)

Mt cpn 10 (μg/ml)

B
Calcium Release
(mg/dl)

Dilution of Anti-Mt cpn10
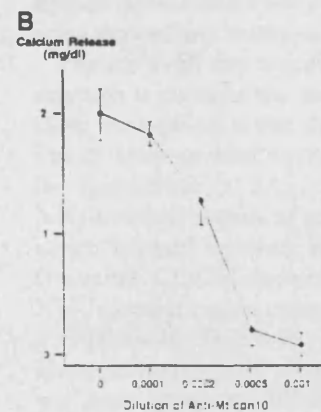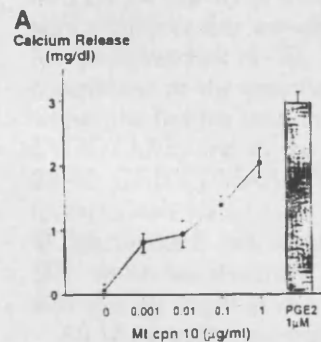
Figure 2. Effect of r-Mt cpn10 on bone resorption. (A) Dose-dependent stimulation of calcium release from murine calvarial explants cultured in the presence of purified r-Mt cpn10. The filled column represents the calcium released from bone explants cultured with 1 μM PG. (B) Effect of adding graded concentrations of mAb SA12 to calvarial bone explants stimulated with 100 ng/ml r-Mt cpn10. Results are expressed as the mean and standard deviation of five replicate cultures.

Inhibition of proliferation
(% of total inhibition)

Mt cpn 10 (μg/ml)
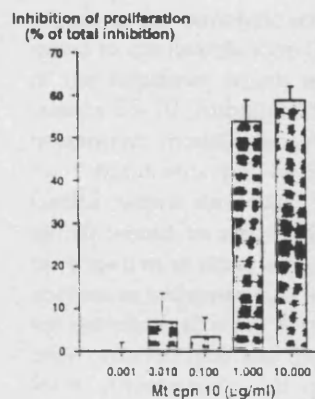
Figure 3. Mt cpn10-induced inhibition of osteoblast proliferation. The effect of purified r-Mt cpn10 on the proliferation of the human osteoblast-like cell line MG 63. Inhibition of proliferation is measured as the percentage of total inhibition (no thymidine incorporation) calculated from the percentage of decrease in thymidine incorporation for a control cell culture.

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Calcium release (mg/dl)

Figure 4. Bone resorbing activity of NH_2- and COOH-terminal truncated Mt cpn10 peptides compared to full-length Mt cpn10. The top graph shows the amount of calcium released in one typical experiment, from calvarial explants cultured with r-Mt cpn10 or truncated synthetic Mt cpn10 peptides at a concentration of 1 μg/ml. The bottom section shows the sequences of the various Mt cpn10 peptides and the sequences of Mt and *E. coli* cpn10. A peptide GGIVLTGSAAA which spans residues 23–3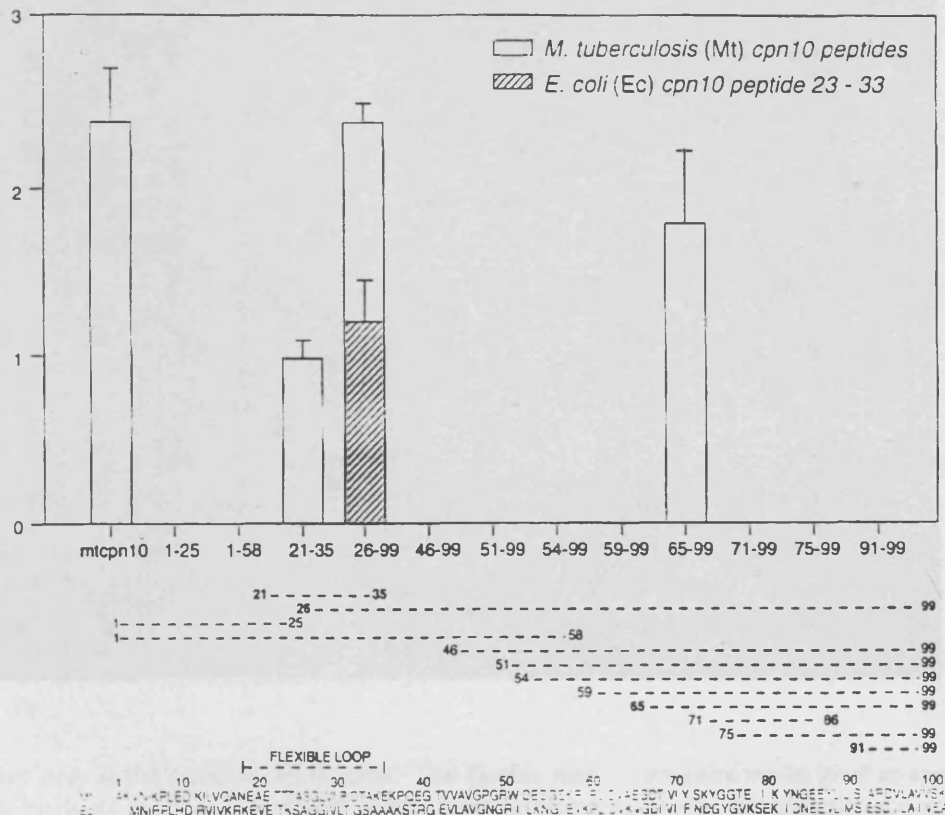3 in the mobile loop of *E. coli* cpn10 also demonstrated osteolytic activity (*shaded column*). The background release of calcium has been subtracted. Results are expressed as the mean and standard deviation of five replicate cultures.

11 peptides, 26–99 and 65–99, exhibited reproducible osteolytic activity (Fig. 4). Polymyxin B had no inhibitory effects on the activity of these peptides. Peptide 26–99 contains sequences that are within the flexible loop region of Mt cpn10 (residues 16–35). To determine if this flexible loop contributed to the osteolytic activity, two short peptides within the flexible loop in Mt cpn10 (21–35: TTTASG-LVIPDTAKE) and in the *E. coli* cpn10 (GroES residues 23–33: GGIVLTGSAAA) were synthesized and were also found to have osteolytic activity in the calvarial assay (Fig. 4). Mt, unlike *E. coli*, is able to secrete extracellular cpn10 (22), which has important implications for the pathogenic effects of Mt cpn10 in vivo.

All 12 Mt cpn10 peptides were repeatedly tested for antiproliferative activity but even at very high concentrations, none showed any ability to inhibit osteoblast proliferation.

Peptide 1–58 was inactive in the bone resorption assay, although it contains the predicted flexible loop. The most likely explanation is that the structure of 1–58 differs from that of whole protein, because peptide 1–58 is a dimer (16) the aggregation of which is unusual as it occurs via the NH_2-terminal region in contrast to the whole protein in which contact between two neighboring protomers involves the COOH-terminal tail of one protomer and the NH_2-terminal region of the other (23).

Peptides 46–99, 51–99, 54–99, and 59–99 were also inactive, although they contain the active 65–99 sequence, and again, structural differences are the likely explanation

for this discrepancy. For example, the structure of the inactive peptide 59–99 has been assigned to that of four antiparallel β strands (24), but circular dichroism spectroscopy data with the active peptide 65–99 (data not shown) indicate that the latter is mainly composed of the random coil conformation. Peptide 26–99 is active in the bone resorption assay since most of the mobile loop is part of its NH_2-terminal tail and is probably accessible to solvent (and hence a receptor) as often happens to the NH_2-terminal and COOH-terminal regions of polypeptides and proteins. For the same reason, amino acids 65–70 would be considered the active sequence in peptide 65–99.

A molecular model of heptameric Mt cpn10 was derived from the *E. coli* cpn10 crystal structure (reference 23; Fig. 5). The sequences derived from the peptide data which contribute to the osteolytic activity are colored red and correspond to the flexible loop (21–35) at the bottom outer edge of the heptamer which is in close proximity to the sequence 65–70. Although the flexible loop is exposed in the heptameric model, the sequence 65–70 is inaccessible at the subunit interface. Furthermore, based on studies with GroES, which dissociates to monomers <1 μM (25), Mt cpn10 would be expected to dissociate at the concentrations used in all these biological assays. This suggests that an alternative oligomeric form of Mt cpn10 may be required for osteolytic activity. A tetrameric form of Mt cpn10 has been reported (16), and this may be the osteolytically active form. Alternatively, Mt cpn10 might assemble as a hep-

Figure 5. Locations of sequences on the Mt cpn10 structure that are likely to be important in bone resorption. Stereo view of polypeptide backbone of Mt cpn10 heptamer model viewed from the top down the sevenfold axis with one subunit highlighted in green. Peptide sequences with osteolytic activity are highlighted in red.

tamer only at the putative cell receptor. The flexible loop also binds to cpn60 in the cpn60–cpn10 protein-folding complex (26), suggesting that the putative cell receptor for Mt cpn10 has some structural homology with cpn60. If this structural homology is significant, it would require the Mt cpn10 to assemble as a heptamer on the receptor. Considering the gross structural rearrangements that occur in the cpn10 subunits of the heptamer when it binds to cpn60 (27), it may be possible for the two active sequences in Mt cpn10 to make contact with the receptor. None of the peptide fragments containing the active sequences appear to be involved in the interaction of Mt cpn10 with the human osteoblast-like cell line MG63, possibly due to their inability to assemble as a heptamer. In this regard, it may be important to note that mitochondrial cpn60 is expressed on the surface of human cells (28). These receptors are likely to be of therapeutic importance in the treatment of bone tuberculosis and possibly in other bone diseases.

Based upon the peptide activity data, the Mt cpn10 structure model gives an approximate guide to the location of the osteolytically-active sequences on the Mt cpn10 structure. The precise molecular structure accounting for the bioactivity of Mt cpn10 will be obtained by ongoing work on solving the Mt cpn10 structure by x-ray crystallography (Roberts, M.M., A. Coker, G. Fossati, P. Mascagni, A.R.M. Coates, and S.P. Wood, unpublished data) and the use of site-directed mutagenesis.

It is not known which Mt strains are associated with Pott's disease. In this study we have tested the Mt strain H37Rv, which is a virulent strain commonly used in research into tuberculosis. We have shown that the obligate protein, Mt cpn10, is the osteolytically-active component produced by this organism. All strains of Mt must contain this protein and therefore have the potential to induce bone disease. There may be additional factors to consider in the propensity of Mt to cause Pott's disease, and further studies into this area are clearly necessary.

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SURFACE-ASSOCIATED PROTEIN FROM *STAPHYLOCOCCUS AUREUS* STIMULATES OSTEOCLASTOGENESIS: POSSIBLE ROLE IN *S. AUREUS*-INDUCED BONE PATHOLOGY

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SUMMARY

Objective. *Staphylococcus aureus* is the cause of bone destruction in osteomyelitis, bacterial arthritis and orthopaedic implant failure. We have previously shown that gentle saline extraction of *S. aureus* has revealed the presence of an extremely potent stimulator of osteoclast activation in both the murine calvarial bone resorption assay and the isolated chick osteoclast resorption assay. In order to investigate the mechanism of action of this surface-associated material (SAM), we have investigated its capacity to recruit osteoclasts.

Methods. The murine bone marrow osteoclast recruitment assay was used. The ability of the recruited cells to resorb dentine slices was also investigated.

Results. The SAM from *S. aureus* dose dependently stimulated tartrate-resistant acid phosphatase (TRAP)-positive osteoclast formation and pit formation on dentine slices. Neutralization of the cytokines tumour necrosis factor alpha and interleukin (IL)-6 totally inhibited, but antagonism of IL-1 only partially blocked, the stimulated maturation of osteoclast-like cells.

Conclusion. These findings suggest that bone destruction associated with local infection by *S. aureus* is due to the stimulation of osteoclast formation induced by the action of the easily solubilized SAM, and could explain the large numbers of osteoclasts found in infarcted bone in osteomyelitis.

KEY WORDS: *Staphylococcus aureus*, Osteomyelitis, Bacterial arthritis, Bone resorption, Osteoclastogenesis.

Staphylococcus aureus is a facultatively anaerobic Gram-positive bacterium found on the skin and in the anterior nares of 10–30% of healthy individuals. This organism is a principal cause of bone destruction in a number of lesions. Haematogenous infection with *S. aureus* is the major cause of pyogenic osteomyelitis [1] and it is the dominant organism associated with infected metal implants [2, 3]. *Staphylococcus aureus* is also the causative agent in >60% of cases of non-gonococcal arthritis [4]. In mice, injection of live *S. aureus* results in rapid destruction of the subchondral bone of diarthrodial joints [5].

In all these conditions, destruction of the calcified extracellular matrix of bone is rapid and severe. Whether the marked involvement of this particular bacterium in bone destruction is due to its greater propensity, relative to other bacteria, for colonizing bone, or to more active bone-modulating activity, is not clear. Indeed, the mechanism by which *S. aureus* stimulates bone destruction, and particularly that associated with osteomyelitis bone necrosis, is still far from clear.

We have been investigating the role of various Gram-negative bacteria in the bone destruction which accompanies chronic inflammatory periodontal disease (CIPD). We have shown that the surface-associated material (SAM) from these bacteria has the capacity to stimulate bone resorption *in vitro* [6, 7]. This very soluble, largely proteinaceous material, removed by a short period of gentle stirring in normal saline [6], is also a potent inhibitor of bone collagen synthesis [8], and has an effect on osteoblasts and other cell populations, including fibroblasts, epithelial cells, macrophages and neutrophils [9]. Indeed, crude extracts of these bacterial SAMs are some 2–3 log orders more active in stimulating bone resorption than the corresponding lipopolysaccharides (LPS) which are normally thought to be responsible for bone destruction in periodontal disease [6].

Staphylococcus aureus is a Gram-positive organism which, by definition, lacks LPS. We have extracted the SAM from this bacterium and have shown that the majority of the material solubilized was protein. The SAM has been shown to be a potent activator osteoclast in the murine calvarial bone resorption assay [10] and the isolated chick osteoclast assay [11]. The possibility that this material stimulates the maturation of osteoclast precursors has been investigated, and has revealed that this surface-associated fraction is a potent inducer of the maturation and activation of osteoclasts. The role of various mediators (cytokines and prostanoids) in the process of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNC) maturation has been investigated. The possibility that this material acts via synthesis of 1,25-$(OH)_2$ vitamin D_3 has also been investigated.

MATERIALS AND METHODS

Growth and harvest of S. aureus

Staphylococcus aureus NCTC 6571 was cultured aerobically at 37°C on Wilkins–Chalgrens agar (Oxoid, Hampshire), containing 10% horse blood, for 24 h. The cultures were routinely Gram stained to detect contaminants and were then harvested by washing plates with sterile saline. The cells were then pelleted by centrifugation, washed once with saline and then freeze-dried.

Extraction of SAM

This was carried out as described by Wilson et al. [6]. Briefly, the freeze-dried bacteria were suspended (1 g/100 ml) in 0.85% (w/v) saline, and the SAM was removed by gentle stirring at 4°C for 1 h. The supernatant was collected after centrifugation at 3000 g for 1 h. The extraction process was repeated twice more, and the SAM was pooled, exhaustively dialysed against distilled water at 4°C, and lyophilized.

In experiments to determine the nature of the active constituents in the SAM, the extract was heat treated by boiling for 1 h or was exposed to trypsin (0.5 mg/ml trypsin incubated with 5 mg/ml SAM) in phosphate-buffered saline (PBS) for 4 h at room temperature. After incubation with trypsin, samples were diluted and soya bean trypsin inhibitor was added, and activity was compared to equivalent concentrations of SAM which had not been trypsin treated, but to which the soya bean inhibitor was added.

To exclude the effect of the medium the bacteria were grown on, a wash of the Wilkins–Chalgrens agar (Oxoid, Hampshire) containing 10% horse blood were extracted in a similar way and tested in the assay.

Electron microscopy

To check on the efficiency of extraction and the integrity of the resultant cells, bacteria were examined by transmission electron microscopy before and after saline extraction as described by Wilson et al. [6]. Briefly, a portion of the bacterial suspension was fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at room temperature (control cells). A second portion was fixed in the same manner, except that the buffer also contained 0.15% ruthenium red. Bacteria were then pelleted by centrifugation at low speed, the fixative decanted and the pellet washed in cacodylate buffer. Control cells were then fixed in 1% osmium tetroxide in cacodylate buffer for 2 h at room temperature in the dark. The test cells were treated identically, except that the buffer also contained 0.15% ruthenium red. The fixative was decanted after centrifugation and the pellets washed in cacodylate buffer. All specimens were then dehydrated in a graded series of concentrations of ethanol, and embedded in araldite. Sections were cut on an LKB ultramicrotome using a diamond knife and examined (unstained) in a JEOL 100CXII electron microscope.

Analytical procedures

The protein content of the SAM was determined by the Lowry method [12] and carbohydrate by the method of Dubois et al. [13] using glucose standards as a control. Lipids were extracted with methanol/chloroform (2:1 v/v), dried and weighed. The DNA content was estimated by UV absorption.

SAM was fractionated into >30 and <30 kDa fractions by passage through an Amicon PM30 membrane. The efficiency of fractionation was assessed by protein measurement and SDS–PAGE analysis; the bioactivity of each fraction was determined and compared with the starting material.

Bone marrow culture

The bone marrow cells were obtained using a modified method of Takahashi et al. [14]. Six-week-old MF-1 mice (Harlan Olac) were killed by cervical dislocation. Femora and tibiae were aseptically removed and dissected free of adherent tissue. The bone ends were removed, the bone was split lengthwise into two, and the bone marrow cells were scooped out of the cavity. The cells obtained were washed twice and suspended (4×10^6 cells/ml) in Dulbecco's Minimum Essential Medium (DMEM; ICN Flow) supplemented with 10% fetal calf serum (FCS; ICN-Flow), L-glutamine (2 mM; ICN-Flow) and penicillin/streptomycin (100 U/ml and 50 µg/ml; ICN-Flow). This suspension was placed (0.5 ml/well) in 24-well plates with or without dentine slices, and incubated at 37°C with various dilutions of SAM or SAM (at 1 or 10 µg/ml) plus added inhibitors. The cultures were incubated for a total of 10 days, the medium being replaced every 2 days.

Tartrate-resistant acid phosphatase staining

The cultures were fixed and stained for TRAP using a commercial histochemical staining kit (Sigma). All the TRAP-positive MNC with three or more nuclei were counted in four replicate wells for each treatment and results expressed as mean and S.D. Each experiment was repeated twice more to ensure that the results were reproducible.

Assessment of resorption activity in marrow cultures

The cells were removed from the dentine slices after 10 days, and the substrate was stained with toluidine blue and examined for the presence of resorption lacunae by light microscopy. The method used for the precise quantification of the resorptive capacity of the osteoclasts involved estimating their surface area by image analysis (Seescan, Cambridge).

Inhibitor studies

The role of prostaglandins in TRAP-positive MNC formation was tested by adding indomethacin (Sigma). The role of interleukin (IL)-1 was assessed by adding the IL-1 receptor antagonist (IL-1ra; Synergen, Boulder, CO, USA). The involvement of tumour necrosis factor alpha (TNF-α) was determined by use of the neutralizing monoclonal antibody TN3-19.12 (produced by Prof. Bob Schreiber, Washington University School of Medicine and manufactured by Celltech Ltd, Slough). The role of IL-6 was determined by adding

(a)   (b)
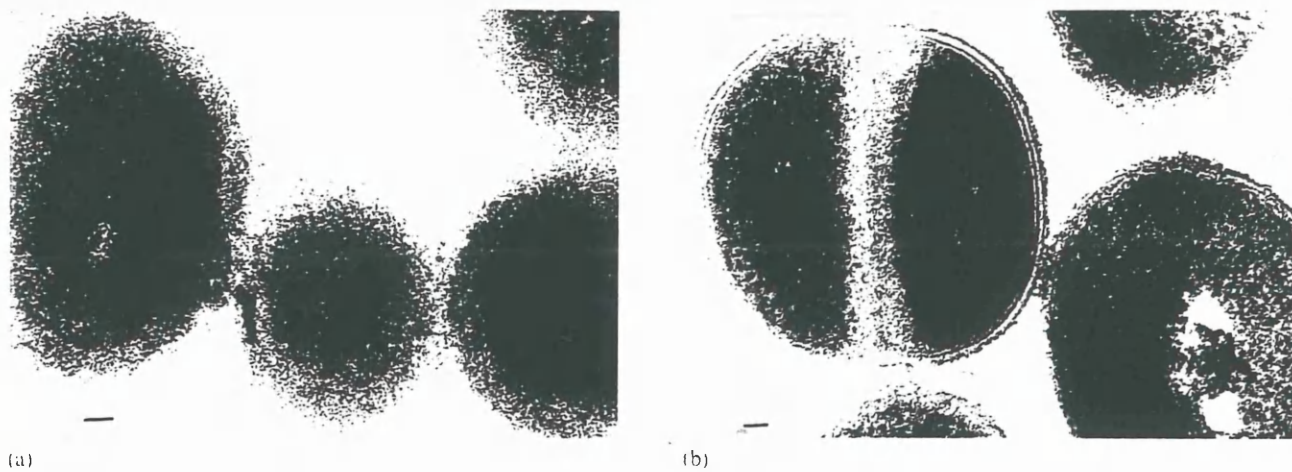
FIG. 1. Transmission electron micrographs showing the appearance of *S. aureus* prior to (a) and after (b) extraction of the surface-associated material. The ruthenium-stained surface-associated material that can be seen in (a) is virtually completely removed by the extraction with saline without any structural damage (b). Scale bar 0.1 μm.

a neutralizing rabbit antibody to murine IL-6 (Genzyme). These inhibitors were added to bone cultures at the same time as the SAM, which was used at a concentration of 10 μg/ml in all of these studies. Calcitonin (10^{-8} M) was also added to cultures to determine the role of this calciotropic hormone in the SAM-induced formation of osteoclast-like cells.

1,25 Dihydroxy vitamin D₃ assay

1.25-(OH)₂ Vitamin D₃ in the bone marrow cultures incubated with a range of concentrations of SAM over 4. 12 and 48 h time periods was assayed by radioimmunoassay [15].

Statistics

The statistical significance of the results was calculated by the use of Student's *t*-test.

RESULTS

Isolation of SAM

Staphylococcus aureus was seen to have a large amount of extracellular material when stained with ruthenium red and viewed by transmission electron microscopy (Fig. 1a). When the cells were extracted with saline. this material was virtually completely removed. as can be seen in Fig. 1b, and this extraction was not associated with the lysis of cells or any easily discernible damage to the cell structure.

Composition of SAM

The protein content of the saline-extractable SAM was 30% (w/w) and the carbohydrate content was 5%. Lipid and nucleic acids constituted only a few per cent of the SAM.

SAM-induced TRAP-positive MNC generation

The SAM dose dependently and reproducibly stimulated TRAP-positive MNC formation in murine bone marrow cultures. There was some variation between experiments in the maximal response seen. but the dose response was reproducible. In the data shown in
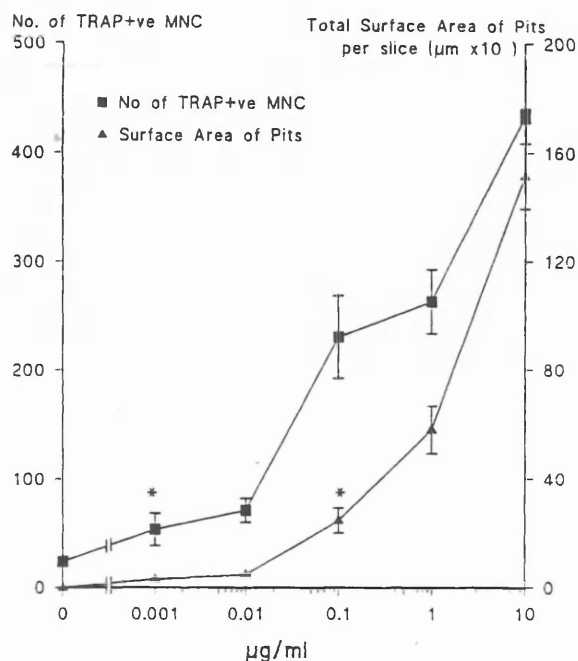

FIG. 2.—The effect of SAM from *S. aureus* showing the number of TRAP-positive multinucleated cells (MNC) in cultures of murine bone marrow following exposure to SAM (0.001-10 μg ml) from *S. aureus* (■). The area of resorption of pits excavated by these cells on each dentine slice was quantified (▲). Results are expressed as the mean and s.ᴅ. of quadruplicate cultures (*$P < 0.01$).

Fig. 2. there was a 3-fold increase in TRAP-positive MNCs at 10 ng/ml and at 10 μg/ml the osteoclast-like cell numbers had increased 18-fold. Heat treatment or exposure to trypsin totally abolished the ability of the SAM to stimulate TRAP-positive MNC formation. Fractionation of the SAM into two molecular weight populations demonstrated that the TRAP-positive MNC-stimulating activity had a molecular weight of >30 kDa.

SAM also stimulated pit formation. i.e. resorption. on dentine slices in a dose-dependent manner. with a
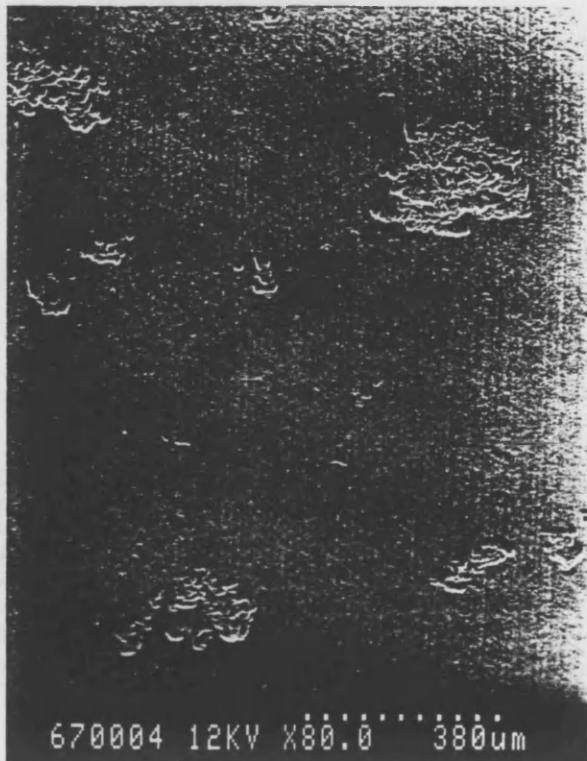
FIG. 3.— Scanning electron micrograph of a dentine slice on which mouse marrow cells were cultured. Mouse marrow cells were cultured with SAM (1 μg ml) on the dentine slice for 10 days. A number of resorption pits are seen.

significant increase in pit area at a concentration of 100 ng ml and above (Figs 2 and 3).

Effects of inhibitors

Calcitonin. This calciotropic hormone, when added at 10^{-9} M, inhibited TRAP-positive MNC formation by 50% ($P < 0.01$) (Fig. 4).

Neutralizing antibodies to cytokines. Inclusion of an anti-IL-6 antibody inhibited TRAP-positive MNC formation, with >50% inhibition being seen at 1 500 dilution of the antisera and complete inhibition at 1 300 dilution (Fig. 5). Similarly, the hamster anti-murine TNF-α antibody completely inhibited the formation of *S. aureus*-stimulated TRAP-positive MNC at 10 μg ml with 50% inhibition being seen at 0.1 μg ml (Fig. 6). Both antibodies also significantly inhibited the spontaneous generation of TRAP-positive MNCs in unstimulated cultures. However, neutralization of IL-1 by IL-1ra only inhibited TRAP-positive MNC formation by a maximum of 40% at the highest concentration used (100 μg ml) (Fig. 7).

Inhibitors of prostanoid synthesis. In all experiments, indomethacin failed to inhibit the *S. aureus*-induced generation of TRAP-positive MNCs (Fig. 8).

1.25-(OH)₂ vitamin D₃

The media supporting bone marrow cultures, taken at 4, 12 and 48 h after initiation of culture, showed
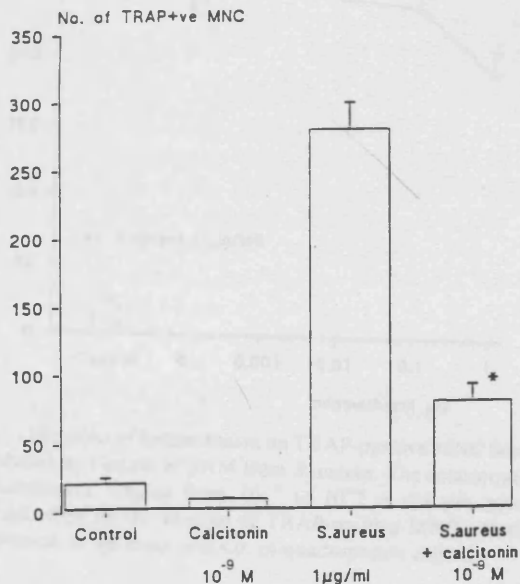

FIG. 4.— The effect of calcitonin (10^{-9} M) on SAM (1 μg ml)-induced osteoclast formation. Data are expressed as the mean and s.D. of quadruplicate cultures (*$P < 0.01$).
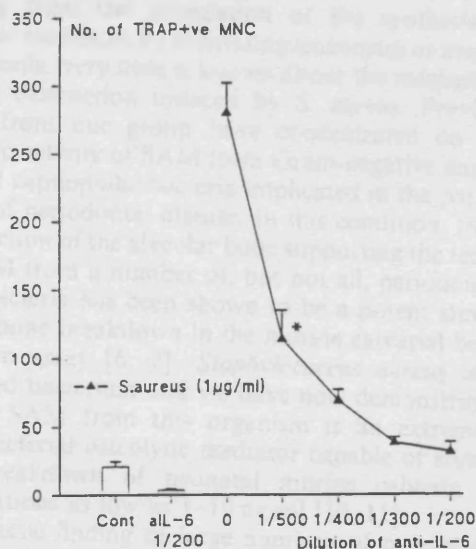

FIG. 5. The dose-dependent inhibition of TRAP-positive MNC formation (induced by 1 μg ml *S. aureus* SAM) by various dilutions of a neutralizing rabbit antibody to murine IL-6. Significant inhibition of TRAP-positive MNC formation was found at a dilution of 1 1000 (not shown) and on this graph >50% inhibition was seen with a 1 500 dilution of the antibody. Results are expressed as the mean and s.D. of quadruplicate cultures. The numbers of TRAP-positive MNCs in unstimulated cultures and in unstimulated cultures exposed to the highest concentration of antibody are also shown (*$P < 0.01$).

insignificant (5-7 pg ml) levels of 1.25-(OH)₂ vitamin D₃.

DISCUSSION

Staphylococcus aureus is the major causative organism of acute and chronic osteomyelitis, and is also the causative agent in around 60% of cases of non-
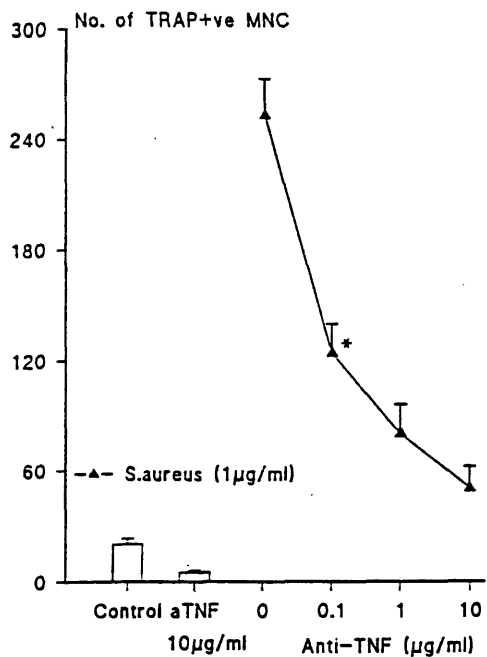
No. of TRAP+ve MNC

FIG. 6.—The dose-dependent inhibition of TRAP-positive MNC formation (induced by 1 μg/ml SAM) by a neutralizing anti-murine TNF antibody: TN3-19.12. Significant inhibition of TRAP-positive MNC formation was found at a concentration of 0.1 μg/ml of antibody. Results are expressed as the mean and s.D. of quadruplicate cultures. The numbers of TRAP-positive MNCs in unstimulated cultures and in unstimulated cultures exposed to the highest concentration of anti-TNF antibody are also shown (*P < 0.01).

No. of TRAP+ve MNC

FIG. 7.—The dose-dependent inhibition of S. aureus SAM (1 μg/ml)-induced TRAP-positive MNC formation by interleukin-1 receptor antagonist (IL-1ra). Results are expressed as the mean and s.D. of quadruplicate cultures. The numbers of osteoclast-like cells in unstimulated cultures with no additives or those exposed to the highest concentration of IL-1ra are also shown (*P < 0.01).

gonococcal bacterial arthritis [4, 16] and of most cases of infected orthopaedic prostheses [3]. In all of these conditions, the major pathological change is the
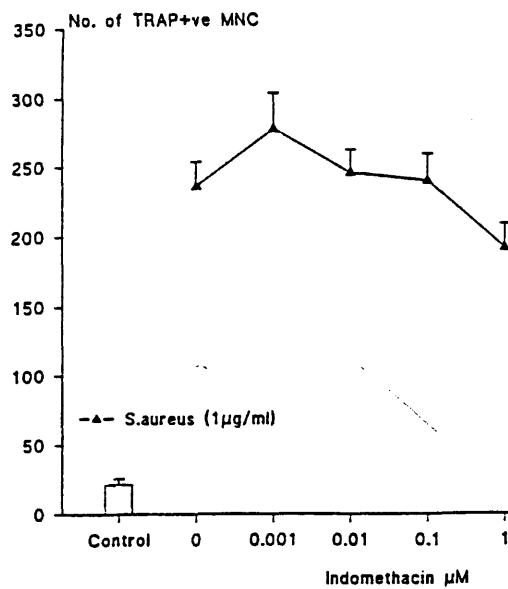

FIG. 8.—The effect of indomethacin on TRAP-positive MNC formation induced by 1 μg/ml of SAM from S. aureus. The concentration of indomethacin ranging from 10^{-9} to 10^{-6} M did not have a significant effect on the number of TRAP-positive MNCs. Results are expressed as the mean and s.D. of quadruplicate cultures.

destruction of bone, which can be a very rapid event. The mechanism of bone resorption induced by bacteria may either be due to the direct activity of bacterial constituents on bone cells or to an indirect effect resulting from the stimulation of the synthesis of osteolytic mediators by infiltrating leucocytes or mesenchymal cells. Very little is known about the mechanism of bone destruction induced by S. aureus. Previous studies from our group have concentrated on the osteolytic activity of SAM from Gram-negative anaerobic and capnophilic bacteria implicated in the pathogenesis of periodontal disease. In this condition, there is destruction of the alveolar bone supporting the teeth. The SAM from a number of, but not all, periodontopathic bacteria has been shown to be a potent stimulator of bone breakdown in the murine calvarial bone resorption assay [6, 7]. Staphylococcus aureus is a capsulated bacterium and we have now demonstrated that the SAM from this organism is an extremely potent bacterial osteolytic mediator capable of stimulating breakdown of neonatal murine calvaria at concentrations as low as 1–10 ng/ml [10, 11].

The classic finding of large numbers of osteoclasts at the periphery of the sequestrum in osteomyelitis led us to investigate the effect of SAM from S. aureus on osteoclast generation in bone marrow cultures. Addition of the SAM to bone marrow cultures produced significant and reproducible increases in TRAP-positive MNC at concentrations as low as 1 ng/ml and reproducible dose responses over the range 10 ng/ml–10 μg/ml. Activity was completely abolished by either heating the SAM or exposing it to trypsin, suggesting that the active component is proteinaneous. Fractionation of the crude mixture of surface components by Amicon filtration revealed that the active
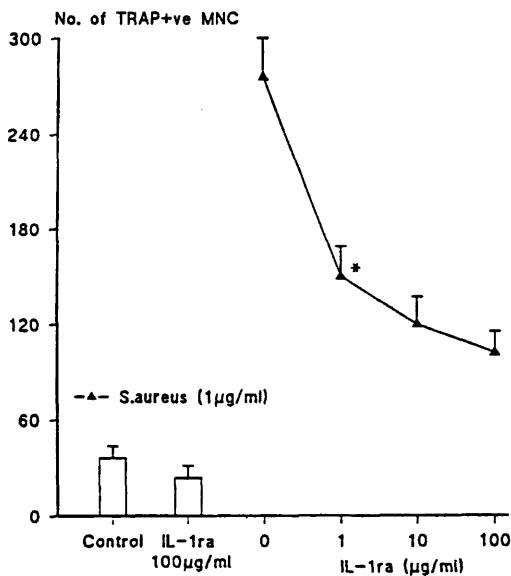
constituent had a molecular weight >30 kDa and may be the 32–34 kDa protein described in our earlier study [10].

SAM from *S. aureus* stimulated TRAP-positive MNC formation in the absence of additional external factors. and appears to be a competence factor both for the proliferation and for the fusion of osteoclast precursors. This is in contrast to many agents, e.g. TGF-α. that increase MNC formation by stimulating proliferation of the osteoclast precursors [17], but depend on the addition of 1,25-(OH)$_2$ vitamin D$_3$ for the fusion of the precursors to form polykaryons [18]. We explored the hypothesis that components in the SAM may have been inducing 1,25-(OH)$_2$ vitamin D$_3$ synthesis. thus accounting for the effects seen. However, assay of the media from the bone marrow cultures revealed negligible quantities of 1,25-(OH)$_2$ vitamin D$_3$.

To determine the mechanism of osteoclast formation. bone marrow cultures were stimulated with a fixed concentration of the SAM and a range of concentrations of 'inhibitors' of major osteolytic mediators such as the prostanoids, IL-1, TNF-α and IL-6 [19]. The activity of the cytokines was inhibited by addition of neutralizing antibodies or IL-1ra. Cyclooxygenase activity was inhibited by addition of indomethacin. The osteoclastogenic activity of the SAM was significantly inhibited by the inclusion of the antibodies to the cytokines. TNF-α and IL-6. Neutralization of IL-6 and TNF generation in bone marrow cultures totally inhibited the generation of TRAP-positive MNCs. In contrast. IL-1ra was a fairly weak inhibitor of osteoclastogenesis. Despite the potent inhibitory activity of indomethacin in the murine calvarial assay [10]. this compound had no effect on TRAP-positive MNC formation in bone marrow cultures. This may. in part. reflect intrinsic differences in both assay systems. However. it should be noted that TNF-α-induced MNC formation is not inhibited by indomethacin [20].

The nature of the active moiety in the SAM has not been defined and. given the complexity of the bone marrow assay. this may prove to be a difficult task. It is clear that the active agent is a protein of >30 kDa. Work is currently in progress to isolate this active constituent.

In conclusion. the SAM of the Gram-positive bacterium *S. aureus* is a potent inducer of osteoclast-like cell formation in the murine bone marrow cultures. Activity is seen at concentrations as low as 1 ng ml (w v). this has a molecular mass of >30 kDa: if one assumes that activity is due to one component. then the minimum effective concentration would be 25 p$_M$. This activity is inhibited by antibodies to IL-6 and TNF. but is only partly inhibited by neutralizing IL-1 but not by the cyclooxygenase inhibitor indomethacin. The ability of the SAM from this bacterium to stimulate osteoclastogenesis. and the activity of these osteoclasts at extremely low concentrations. must contribute to the pathology of the various bone lesions associated with infection by *S. aureus*. The active constituent in this mixture of proteins and carbohydrate represents

an important therapeutic target in view of the increasing numbers of isolates of *S. aureus* resistant to antibiotics [21].

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