THE UNIVERSITY
OF ADELAIDE
AUSTRALIA

CLAST CELL ACTIVITY IN A MODEL OF ASEPTIC ROOT RESORPTION

CRAIG WILLIAM DREYER
B.D.S.(Adel), M.D.S.(Adel), F.R.A.C.D.S.

A research report submitted in partial fulfilment for the Degree of Doctor of Philosophy in Dentistry

DENTAL SCHOOL
FACULTY OF HEALTH SCIENCES
THE UNIVERSITY OF ADELAIDE

2002

TABLE OF CONTENTS

ABSTRACT

Bone resorption by osteoclasts facilitates orthodontic tooth movement. However, an adverse sequela of tooth movement is the possibility of inflammatory root resorption. In order to examine aseptic hard tissue resorption an investigative rat model was developed using *in situ* freezing of the upper right molar by the application of solid carbon dioxide to the tooth crown. Variations in duration and frequency of application permitted the investigation of the cellular reactions responsible for the progression and repair of resorption and periodontal healing using tartrate-resistant acid phosphatase and succinic dehydrogenase enzyme histochemistry and a concert of antibodies directed at specific osteoclast antigens in control and osteoprotegerin-affected animals. In addition, an electron microscopic investigation was undertaken to assess variations in the morphological features of the resorptive cells in the experimental model systems.

The development of the model revealed a pattern of root resorption dissimilar to previously reported patterns caused by orthodontic means. Hard tissue resorption was generated by short-term freezing of molar crowns while ankylosis was a feature of an intense, prolonged insult as well as in all osteoprotegerin-affected rats. TRAP activity was found to be to be a useful clast cell marker but not in areas of ankylosis where ED1 (a macrophage lysosomal marker) proved to be more valuable. ED1 label indicated that macrophages or, at least, clast cell precursors are more involved in hard tissue resorption than previously considered, suggesting that odontoclasts are likely derived from the macrophage lineage of clast cell differentiation.

While osteoprotegerin inhibited osteoclastogenesis and resorptive activity in the skeletal bones and periodontium of unfrozen control molars in rats, it did not appear to inhibit ultrastructural signs of resorption in the frozen rat molars. In addition, TRAP activity was present but diminished while ED1 activity was still evident in the resorptive areas. This study concluded that osteoclasts and odontoclasts, while appearing to be similar cells, are possibly not the only cells responsible for hard tissue resorption. Furthermore, the observation that osteoprotegerin failed to prevent inflammatory resorption in the frozen tooth model suggests that there are possibly other extraclastic mechanisms or factors in this experimental model that influence resorption. Hence, the usefulness of OPG as a potential inhibitor of hard tissue resorption and, in particular, orthodontic root resorption remains to be established.

SUMMARY

The resorption of hard tissues in the periodontium relies on the recruitment, differentiation, maturation and attachment of odontoclasts and osteoclasts to tooth and bone in a precise series of cellular events (Sasaki *et al.*, 1989; Roodman, 1996; Chambers, 2000; Väänänen *et al.*, 2000). Controlling factors governing clast cell differentiation and activity may affect the formative and functional processes at either the systemic or local level principally by osteoblast-osteoclast interaction (Suda *et al.*, 1992a; 1999). A recently discovered osteoclast antagonist, osteoprotegerin (OPG), purports to interfere with osteoclastogenesis by binding with an osteoclast (precursor) membrane-bound receptor, RANK (receptor activator for nuclear factor kappa β), to subsequently interfere with signal transduction of transcription messages (Simonet *et al.* 1997). The administration of OPG therefore competitively inhibits osteoclast activity and so reduces the extent of bone resorption leading to osteopetrosis (Hofbauer *et al.,* 1999a). It might therefore be expected that a similar inhibitory effect would operate on odontoclasts.

The concept that osteoclasts and odontoclasts are the same cells has been accepted based on the morphological and functional similarity between the two (Jones and Boyde, 1988). Both become multinucleate, form ruffled borders when actively ingesting hard tissue and produce a number of secretory products by which these cells might be identified (Athanasou and Quinn, 1990; Athanasou *et al.,* 1991; Athanasou, 1996). However, to date, no direct comparisons have been

made between the two cell types, despite the fact that a number of studies have examined odontoclastic root resorption in various model systems.

Animal models developed to examine the dental resorptive process have mainly relied on the application of a noxious stimulus to a tooth (Cvek and Lindwall, 1985; Lindskog *et al.,* 1987a). A cold thermal stimulus has previously been applied to the periodontium (Wesselink *et al.*, 1986; Tal and Stahl, 1986) but has not been applied directly to the tooth crown in order to mimic the aseptic periodontal inflammation seen in orthodontic root resorption.

Solid carbon dioxide as dry ice (BOC Gases, Adelaide, Australia) was applied to the crowns of 106 Sprague-Dawley rats for varying durations and times. Dissected and processed tissues were examined histologically using haematoxylin and eosin stain and enzyme histochemistry using succinic dehydrogense and tartrate-resistant acid phosphatase, immunohistochemically using a barrage of antibodies directed against osteoclast antigens, and ultrastructurally using the transmission electron microscope. In addition, 32 of the rats were injected with OPG (Amgen Inc., California, USA) at a dose of 2.5 mg/kg of body weight and subsequently examined under the above regime.

Results indicated that freezing of the rat molar crown was an effective way of initiating a sterile inflammatory change in the periodontal ligament (PDL) that led to resorption of periodontal hard tissue surfaces. Root resorption appeared maximal 7 d after the insult and the initiation of repair was evident after 14 d. Multiple freezing episodes and longer duration times increased the extent of the tissue disruption and saw the onset of an ankylotic union between tooth and

bone. The ankylotic union was seen in all OPG-affected rats at minimal exposure times.

Enzyme histochemistry using succinic dehydrogenase was successful only for large and multinucleated clast cells. This further indicated that the citric acid (Kreb's cycle) pathway that utilizes the dehydrogenases, functions in the mature clast cell and is possibly related to the production of digestive enzymes. After 14 d as repair of the damaged teeth occurred, succinic dehydrogenase activity was still evident in bone and on bone surfaces as normal metabolism and physiological tooth drift were re-established.

Tartrate-resistant acid phosphatase staining was evident in the thermally damaged PDL. Staining involved mononuclear and multinucleated cells within resorption lacunae on hard tissue surfaces and within the PDL but not in areas of ankylosis. Both osteoclasts and odontoclasts and their related mononuclear cells appeared to stain similarly.

Success with immunolabelling techniques was largely limited to ED1 antibody which identified macrophage lysosomal activity. Results indicated that there was a high level of macrophage and clast cell activity in the frozen PDL and resorption lacunae. Furthermore, the presence of inflammation within the PDL suggested that odontoclasts might originate from a macrophage lineage rather than from other cell lines.

Ultrastructural evidence suggested that large, multinucleated cells without ruffled borders were intimately involved with hard tissue resorption lacunae. These cells

did not possess the morphological characteristics associated with a fully functioning clast cell. It is suggested that these cells are either clast cell precursors that had yet to differentiate fully or macrophages with hard tissue resorptive capacity. Previous studies have associated macrophages with resorption (Mundy *et al.*, 1977; Alvarez *et al.,* 1991; Brudvik and Rygh, 1993a,b; Lassus *et al.,* 1998; Faust, 1998; 1999) but mainly in an inflammatory role. If macrophages are involved with root resorption, the differentiation of odontoclasts from a macrophage lineage is supported in preference to other haemopoietic stem cell lineages.

OPG was found to inhibit hard tissue resorption in rats at a dosage of 2.5 mg/kg/d administered on alternate days. Interestingly, OPG did not inhibit the inflammatory experimental resorption initiated in this study. It was hypothesized that the PDL inflammation induced by the cold thermal challenge to the molar crown produced mediators and cytokines (such as TNF-α) that had the ability to stimulate clast cell differentiation and antagonize the effects of OPG perhaps in a dose-dependent way. Since hard tissue resorption appeared undiminished, it was further considered that OPG as a possible inhibitor of inflammatory resorption was unlikely to have any beneficial effects or role in aseptic resorption associated with orthodontic tooth movement.

RESEARCH PRESENTATIONS

Articles in refereed journals:

Dreyer, C.W., Pierce, A.M. and Lindskog, S. (2000). Hypothermic insult to the periodontium: a model for the study of aseptic tooth resorption. *Endodontics and Dental Traumatology, 16: 9-15*

Dreyer, C.W. and Pierce, A.M. (2001). The localization of ED1 antibody in the resorbing hard tissues of the periodontium. *Australian Orthodontic Journal, 17: 35-40*

Abstracts:

Dreyer, C.W., Pierce, A.M. and Lindskog, S. (1997). Responses of the rat periodontal ligament to low temperature injury. International Association for Dental Research, Australian and New Zealand Division Annual Meeting, Sydney, Australia. *Journal of Dental Research,* 76: 938.

Pierce, A.M., Haynes, D.R., Loric, M., Findlay, D., Dreyer, C.W. and Vernon-Roberts, B. (1998). The use of human and animal model systems to study tooth and bone resorption *in vitro*. International Association for Dental Research 76th Session, Nice, France. *Journal of Dental Research, 77(B):721.*

Poster presentations:

Pierce, A.M., Haynes, D.R., Loric, M., Findlay, D., Dreyer, C.W. and Vernon-Roberts, B. (1996). Hypothermic insult to the periodontium. International Association for Dental Research, Australian and New Zealand Division Annual Meeting, Sydney, Australia.

Dreyer, C.W. and Pierce, A.M. (2000). The localization of ED1 antibody in the resorbing periodontium. 17th Australian Orthodontic Conference, Brisbane, Australia. Winner of best poster award.

Dreyer, C.W. and Pierce, A.M. (2001). A study of cellular activity in PDL resorption and repair. Australian Society of Orthodontists Foundation for Research and Education Meeting, Sydney, Australia.

Dreyer, C.W. and Pierce, A.M. (2001). An ultrastructural study of experimental root resorption. Australian Society of Orthodontists (South Australian and Victorian Branches) Clinical Meeting, Dunkeld, Australia.

Dreyer, C.W. and Pierce, A.M. (2002). An ultrastructural study of osteoprotegerin effects in a resorptive rat model. 18th Australian Orthodontic Conference, Perth, Australia. Winner of best poster award.

SIGNED STATEMENT

To the best of my knowledge and belief, this report contains no material which has been accepted for any other award or degree or diploma in any university. Furthermore, it contains no information previously published or written by any other person except where due reference is made in the text.

The digital images of histological sections and electronmicrographs have been printed without enhancement. Except where annotations and legends have been added for description and clarity, no alterations or improvements have been made to the scanned images.

Furthermore, if accepted, and when deposited in The University of Adelaide Library, I give my consent for this copy of the thesis to be available for loan and photocopying.

C.W. DREYER, B.D.S.(Adel.), M.D.S.(Adel.), F.R.A.C.D.S.

ACKNOWLEDGEMENTS

I gratefully acknowledge the guidance and supervision of Dr. Angela Pierce, Senior Lecturer in Oral Pathology at The University of Adelaide throughout each phase of this research and in the preparation of this report. Without her drive and inspiration this project might not have been completed.

My thanks are also extended to Professor Wayne Sampson, holder of the Begg Chair in Orthodontics at The University of Adelaide, who relieved me of my teaching duties at crucial times and provided a sounding board for osteoclast theories.

Untiring technical help was provided by Mrs Margaret Leppard, Sandie Hughes, Vicki Tuck and Bijun Cai at various stages of this research. Immunohistochemical assistance was provided by the Bone Laboratory in the Institute of Medical and Veterinary Science, Adelaide and, in particular, Dr. Robert Moore and Jim Melios. Their countless hours of instruction and their perseverence was ultimately rewarded. Cryo-preparation assistance was provided by Chris Leigh and Gail Hermanos in the Department of Anatomy at The University of Adelaide. My gratitude is also directed to Dr Marilyn Henderson and Lyn Waterhouse of the Centre for Electron Microscopic and Microstructure Analysis at Adelaide University for their instruction in transmission electron microscopic techniques

Dr. Colin Dunstan of Amgen Inc., California kindly supplied quantities of osteoprotegerin and technical advice on its dosage and administration. For his assistance I offer my thanks.

I gratefully acknowledge the financial assistance provided by the Australian Society of Orthodontists Foundation for Research and Education (ASOFRE) without whose contribution all university-based orthodontic research would be exceedingly difficult. As the recipient of the Sam Bulkley Travelling Research Award in 1998, the ASOFRE afforded me a great opportunity to pursue my research interests in overseas laboratories. I extend my thanks to G.D. Roodman in San Antonio, Z. Davidovitch in Boston, S. Lindskog in Stockholm and M. Meikle in London for making their facilities and their minds available to me.

My thanks and eternal appreciation goes to my wife, Jan, and my children, Kate and Declan for their support over the past few years. It is indeed difficult not to have a husband or a father for significant periods of time when the demands of experimental protocol take preference. There is a heavy time debt that now needs to be repaid.

ABBREVIATIONS USED IN THIS TEXT

Ab	Antibody
ABC	Avidin-biotin complex
Ag	Antigen
APAAP	Alkaline phosphatase anti-alkaline phosphatase technique
APT	Aminopropyltriethoxysilane
BP	British pharmacopoeia
BSP	Bone sialoprotein
BT	Nitro blue tetrazolium
CA II	Carbonic anhydrase II
Cbfa1	Core binding factor a1
CD	Cluster of differentiation
CFU-GM	Colony forming unit – granulocyte macrophage
CSF	Colony stimulating factor
CTR	Calcitonin receptor
DAB	3´-diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
EACA	Epsilon-aminocaproic acid
EDTA	Ethylenediaminetetra-acetic acid
FBGC	Foreign-body giant cell
FITC	Fluorescein isothiocyanate
GLA	Bone protein, osteonectin
H	Hydrogen
H&E	Haematoxylin and eosin
HRP	Horse radish peroxidase
Ig	Immunoglobulin
IL	Interleukin
IFN-γ	Interferon gamma
IMVS	Institute of Medical and Veterinary Science
IU	International units
JNK	c-Jun N-terminal kinase
K	Potassium
LCA	Leucocyte common antigen
LSAB	Labelled streptavidin-biotin
M	Molar (molarity)
M-CSF	Macrophage colony stimulating factor
MG-CSF	Macrophage-granulocyte colony stimulating factor
MMP	Matrix metalloproteinase
*m*RNA	Messenger ribonucleic acid
NF-$\kappa\beta$	Nuclear factor kappa beta
NO	Nitric oxide
OAF	Osteoclast activating factor
OC	Osteocalcin
ODF	Osteoclast differentiation factor
OFA	Osteoclast functional antigen
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand

OPN	Osteopontin
Osf2	Osteoblast-specific transcription factor 2
PAGE	Polyacrylamide gel electrophoresis
PAP	Peroxidase-antiperoxidase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDL	Periodontal ligament
PGE	Prostaglandin E
PTH	Parathyroid hormone
RANK	Receptor activator for nuclear factor kappa β
RANKL	Receptor activator for nuclear factor kappa β ligand
RGD	Arginyl-Glycyl-Aspartyl molecular attachment sequence
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
Runx2	Runt-related transcription factor-2
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPARC	Secreted protein acidic and rich in cysteine (Osteonectin)
TGF-β	Transforming growth factor - beta
TR1	Tumour necrosis factor receptor-like molecule
TRAF	Tumour necrosis factor receptor associated factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRANCE	Tumor necrosis factor-related activation-induced cytokine
TRAP	Tartrate-resistant acid phosphatase

Abbreviations of length:

m	Metre
mm	Millimetre
µm	Micron
nm	Nanometre

Abbreviations of time:

d	Day
h	Hour
min	Minute
s	Second
wk	Week
y	Year

Abbreviations of volume:

L	Litre
ml	Millilitre

Abbreviations of weight:

g	Gram
kg	Kilogram
mg	Milligram
ng	Nanogram
Da	Dalton
kDa	KiloDalton

CHAPTER 1

INTRODUCTION

The current study develops an animal model system to investigate the cells responsible for aseptic root resorption in the periodontium and their behaviour following administration of OPG. The following review examines the periodontium, osteoclasts and odontoclasts, root resorption, and experimental resorption models.

1.1 The periodontium

Bartold (1995) described the periodontium as a highly vascular collection of discrete and unique connective tissues, each of which conferred their individual structural properties on the overall tissue morphology. The two principal soft tissues consist of the gingival and periodontal connective tissues while cementum and bone comprise the two hard tissue components. After facilitating eruption, collectively these tissues provide homeostatic mechanisms for the support and nourishment of teeth whilst at the same time facilitating physiological drift of the dentition (Berkovitz, 1990; McCulloch, 1995; Bartold, 1995). Furthermore, a healthy, functioning PDL is vital for the orthodontic movement of teeth which is aided by the ability of the component tissues to remodel under the influence of applied forces (Sandy *et al.*, 1993; Davidovitch, 1996). Beertsen *et al.* (1997) contended that, while each of the tissue systems comprising the PDL possesses a unique architecture, composition and function, the extracellular matrix, fibrous proteins and cellular components all have similar characteristics.

1.1.1 Periodontal fibres

1.1.1.1 Collagen

Using histological and transplantation techniques, Grant and Bernick (1972) revealed that the formation and organization of the periodontal ligament arose as a response to the developing dental follicle. Previously, Ten Cate (1969) and more recently Ten Cate (1996) described changes occurring within the dental follicle that were associated with the onset of tooth root formation and the development of the fibre groups of the PDL. These fibres appear as loosely structured collagenous elements which become orientated and more densely packed as the eruptive position of the tooth changes (Grant and Bernick, 1972). According to Melcher and Eastoe (1969), the collagen fibres serve to attach the tooth to its surrounding bone and to act as support during function. Under light microscopy, collagen has been largely identified in discrete bundles. However, due to technical problems associated with histological preparation, Melcher and Eastoe (1969) and Sloan *et al.* (1976) questioned the precise arrangement of these fibrous bundles. The view that collagen exists as gingival, crestal, horizontal, oblique and apical fibre groups was accepted even though it was unclear whether they extended right across the PDL space (Grant *et al.*, 1972). Zwarych and Quigley (1965) and Shackleford (1971a,b) showed that collagen fibrils ramify from fibre to fibre, that fibres branch with frequency and that many of the fibrils are, in part, not orientated to form fibres. Hay (1991) and Sloan *et al.* (1993) explained this concept according to the types of collagen within the ligament and the other functional demands placed on this tissue component.

In order to perform their supportive and cushioning function, morphological descriptions by Melcher and Bowen (1969) revealed that the collagen fibres are

embedded in cementum and alveolar bone. Cohn (1972a,b) provided histological evidence to suggest that some principal collagenous PDL fibres pass as Sharpey fibres through the alveolar process of many animal species leading to the concept that there may be continuity between the fibre systems of adjacent teeth. Furthermore, buccally and lingually, a continuity between fibres of the PDL and those of the gingivae and periosteum is thought to exist. Histological and autoradiographic studies by Burnett (1978) and Dreyer and Sampson (1984) have cast doubt over the concept of transalveolar fibres.

Although collagen is principally considered to be a structural component of the PDL, Hay (1991) suggested a role in tissue adhesion. Collagen types I, II, III, and IV have been shown *in vivo* to provide a substratum for cell adhesion and together with collagen-mediated effects on cell growth and differentiation, there may be a tissue requirement for cell adhesion to collagen. In addition, Postlethwaite and Kang (1976) and Postlethwaite *et al.* (1978) determined that types I, II, and III collagens are chemotactic for fibroblasts and monocytes. It was further reported that the distribution of collagen varies considerably between tissues as well as under differing developmental and pathological conditions. Gay *et al.* (1978) and Narayanan and Page (1983) showed that, during inflammation and wound healing, an increase in type III and IV collagen occurs.

1.1.1.2 Oxytalan, Elaunin and Elastin Fibres

Fullmer (1959) demonstrated oxytalan and elaunin fibres in the PDL by employing special oxidative histological techniques for visualization. Sims (1975; 1976) reported that these fibres form a three-dimensional network linking tooth cementum to the peripheral periodontal blood vessels and, later, Chavrier *et al.* (1988) showed that oxytalan fibres are the likely precursors of elastin fibres. Ross (1973) described mature elastin fibres as comprised of microfilaments, some of which are embedded in an elastin core. Johnson and Pylypas (1992) indicated that grouped elastin microfibrils appear indistinguishable from elaunin and oxytalan. However, Rosenbloom (1993) reported that elastin fibres are only found in the PDL of certain species and tooth types.

The function of oxytalan fibres has not been fully determined but, because of a marked anatomical correlation in the distribution, direction and branching patterns of oxytalan fibres and blood vessels, Chantawiboonchai *et al.* (1998) suggested a specialized physiological role within the PDL. As the oxytalan fibre system provides coupling between neural mechanoreceptors and the microvascular bed, Sims (1983) hypothesized that the oxytalan fibre meshwork forms part of a proprioceptor system for the regulation of PDL vascular flow.

1.1.2 Cells of the periodontal ligament

Unlike many other connective tissues, the PDL has been identified as a highly cellular structure. Berkovitz and Shore (1995) suggested that the cellular components might be classified as having synthetic, destructive, or epithelial characteristics.

1.1.2.1 Synthetic cells

Berkovitz (1990) and Beertsen *et al.* (1997) indicated that the synthetic cells of the PDL comprise fibroblasts, osteoblasts and cementoblasts.

- **The fibroblast**

Ross (1968) reported that fibroblasts are found between the collagenous fibres of the PDL and are the most common and functionally important cells. As the producers of the fibrous and matrix components, their appearance is essentially governed by the surrounding tissues. Beertsen *et al.* (1974) described a variety of shapes depending on the cell's position within the PDL and the plane of histologic section. It has been generally agreed that these cells are either fusiform or tripolar in nature with long fine processes, or stellate with numerous short cytoplasmic processes (Berkovitz, 1988). Beertsen *et al.* (1997) further described these cells as orientated parallel to the PDL collagen fibres and interconnected with one another by numerous gap- and adherence- type of junctions. Shore and Berkovitz (1979) reported a single nucleus as appearing like a flattened disc with a smooth outline. Multinucleated fibroblasts which, according to Sasaki and Garant (1993), arose by fusion of mononuclear cells or by faulty division, have been reported in the aged PDL. At the ultrastructural level Garant and Cho (1979) revealed that fibroblasts contain the necessary polarized cytoplasmic organelles

involved in the manufacture and degradation of proteins associated with the enzymes, fibres and matrix of the PDL.

- **The osteoblast**

Osteoblasts are derived from mesenchymal precursors in a complex series of differentiation steps involving a transcription factor termed core binding factor a1 (Cbfa1), otherwise known as osteoblast-specific transcription factor-2 (Osf2) or runt-related transcription factor-2 (Runx2) (Wise *et al.*, 2002). Ducy et al. (1997) and Ducy (2000) showed that Cbfa1 binds to and governs the expression of multiple extracellular matrix genes in osteoblasts and Cbfa1 overexpression can induce osteoblast-specific gene expression in fibroblasts and myoblasts.

Osteoblasts within the PDL, like osteoblasts elsewhere in the body, line the surfaces of (alveolar) bone. Holtrop (1990) described them as a sheath or layer of plump cuboidal cells aligned closely together between Sharpey fibres over a layer of osteoid on the bone surface. In addition, he observed that active cells reacted intensely to basic stains due to an abundance of ribonucleic acid (RNA) located in an eccentrically placed nucleus. However, inactive osteoblasts, resting osteoblasts, or bone lining cells contain very little cytoplasm and change to an elongated shape parallel to the bone surface. At an ultrastructural level, Schenk *et al.* (1993) described these cells as similar to fibroblasts as they contain prominent rough endoplasmic reticulum, mitochondria and vesicles. In addition, they possess a more extensive Golgi apparatus and numerous and prominent microtubules and microfilaments. Roberts *et al.* (1987) identified cells that might be osteoblast precursors beneath the osteoblastic bone layer. The precursors possess reduced cytoplasm and fewer organelles, and appear to be related to

blood vessels. As differentiation progressed, Saffar *et al.* (1997) described an unusual migratory pattern in which these cells move away from the surface of bone into the body of the PDL before assuming a functional position. Holtrop (1990) stated that cytoplasmic processes allow bone-forming cells, whether they were early or late in their differentiation or functional life, to maintain communication with each other and so provide a functional network. Mariotti (1993) detailed the important role of osteoblasts in the production of bone matrix components, such as collagenous and non-collagenous proteins in the form of osteocalcin, osteonectin, osteopontin, matrix gla protein, plus numerous other phospho-, sialo-, and glyco-proteins. Of significance, however, Chambers and Fuller (1985) and Greenfield *et al.* (1999) ascribed an important role for osteoblasts in the regulation of the osteoclast and its bone resorptive activity.

- **The cementoblast**

Diekwisch (2001) indicated that early cementogenesis arose from follicular mesenchymal cells which penetrate Hertwig's epithelial root sheath. Thesleff (2000) reported that these processes were controlled by genes affecting signalling pathways, and Runx2 has been identified as a principal controlling factor of cementoblastogenesis (Gaikwad *et al.,* 2001).

Bosshardt and Selvig (1997) reported that cementoblasts line cementum in much the same fashion as osteoblasts line bone. In addition, their arrangement is more irregular compared with osteoblasts and their appearance is highly dependent on their activity. According to Yamasaki *et al.* (1987), cementoblasts are often indistinguishable from fibroblasts and these authors suggested that cemental cells contribute to tissue matrix production within the PDL. Yamamoto *et al.*

(1996) described these cells ultrastructurally as having wing-like processes that partly encircle the principal collagenous fibres of the PDL. Moreover, it was revealed that microtubules and collagen secretory granules within the cell processes are orientated specifically for the secretion of intrinsic and principal collagen fibres. However, Bosshardt and Schroeder (1996) stated that virtually nothing was known about cementoblast origin, differentiation and cell dynamics during normal development, repair and regeneration. Tenorio *et al.* (1993) further suggested that cementoblasts and osteoblasts share many phenotypic characteristics and therefore may be derived from the same progenitor cells. Although cementoblasts and osteoblasts both express major osteoblast markers, the responses of these cells to collagen 1, osteocalcin (OC) and bone sialoprotein (BSP) genes clearly differentiates the two cell phenotypes (Pavlin and Gluhak-Heinrich, 2001). In addition, Lindskog *et al.* (1987b) showed that cementoblasts do not appear to respond to parathyroid hormone in the same manner as osteoblasts.

- **Epithelial Cells**

Ten Cate (1969; 1996) provided evidence suggesting that remnants of Hertwig's epithelial root sheath remain as aggregations of epithelial cells within the PDL. Brunette *et al.* (1979) described these cells as unique because, unlike other epithelia in the body, the epithelial cell rests in the PDL are surrounded by connective tissue cells. Hamamoto *et al.* (1991) stated that the cell rest structure is supported by a basement membrane containing laminin which was considered to play a role in the formation, differentiation and maintenance of the epithelial rest. In routine histological sections, Valderhaug (1974) described epithelial rests as clusters of cuboidal cells located closer to the cementum than alveolar bone. Wesselink and Beertsen (1993) showed a change in distribution with age as the number of cells decreased with time. Furthermore, higher concentrations of cells were reported in the apical region in the young, but with age the distribution became more cervical and gingival (Reeve and Wentz, 1962). Spouge (1980) contended that the epithelial cells serve a function that remains undetermined. However, Brice *et al.* (1991) suggested that they are possibly involved in reparative cementogenesis, while Lindskog *et al.* (1988b) indicated that they might play a role in the maintenance of the periodontal ligament space

1.1.2.2 Destructive cells

Osteoclasts and odontoclasts have been identified as the prime cells involved with bone and tooth resorption (Domon *et al.,* 1998; Greenfield *et al.,* 1999). Comprehensive reviews of these cells are presented in later sections.

1.1.3 Cementum

Although a hard tissue structure, Bosshardt and Selvig (1997) considered cementum to be a part of the periodontium. Cementum lines tooth root dentine, provides attachment for periodontal ligament fibres and is a highly responsive tissue. Nevertheless, Bosshardt and Schroeder (1996) recorded that it was one of the least-known mineralized tissues. Because cementogenesis and periodontal fibre attachment occurs at the same time, Bosshardt and Schroeder (1996) found that a cellular distinction between the two processes was difficult. Lindskog and Blomlöf (1994) provided evidence suggesting that osteoblasts, as well as cementoblasts, might contribute to cementum formation, particularly in reference to reparative cementum.

Birkedal-Hansen *et al.* (1977) considered cementum to be similar to dentine and bone and characterized equal proportions of inorganic and organic content. Selvig and Selvig (1962) indicated that bone is comprised of 70% inorganic salts whereas cellular cementum contains only 46%. In addition, Butler *et al.* (1975) reported that collagen type I is found in bone while collagen type I and a small percentage of collagen type III is present in cementum. Although difficulties existed in obtaining suitable quantities of cementum for analysis, research directed at the non-collagenous protein content by Somerman *et al.* (1990; 1991) identified at least three RGD-associated adhesion proteins, bone sialoprotein, tenascin, osteonectin and proteoglycans. Bartold *et al.* (1988; 1990) confirmed the presence of hyaluronan, dermatan sulphate and chondroitin sulphate and, further, showed the distribution of the proteoglycans to be closely associated with cementoblasts. However, Hammarström *et al.* (1996) reported that the precise biochemical composition of cementum remains obscure in relation to other

periodontal tissues as does the precise mechanism of formation of both cellular and acellular cementum. A review of the origin of cementum by Hammarström *et al.* (1996) reported increasing evidence that the epithelial sheath of Hertwig plays a significant role. However, Ten Cate (1997) indicated that there is also evidence implicating dental follicular cells in the development of acellular cementum. Additional evidence suggests that cellular cementum might be derived from cells that are extrinsic to the periodontal ligament (McCullough *et al.,* 1987; Lang *et al.,* 1995)

1.1.4 Blood vessels

Berkovitz (1990) indicated that blood vessels comprise a substantial proportion of the periodontal ligament in contradistinction to other connective tissue systems. Estimates vary, but Sims (1980) determined that 11% of human PDL volume is occupied by vascular elements but a range of 1-20% accounted for species, site and tissue preparation variables. Johnson and Pylypas (1992) showed that, at the light microscope level, the PDL appears to be more vascular in the apical region and less so towards the cervical and gingival regions. Ultrastructurally, Weekes and Sims (1986), Lee *et al.* (1990) and Lee *et al.* (1991) revealed a complex network of vessels with a distribution that varies across, as well as along, the length of the PDL. Although the main entry for vessels is from the apical area, Kindlova (1965) described a considerable collateral circulation through alveolar bone and gingivae which adds to the nutritive and hydrostatic tooth supporting mechanisms of the vascular bed.

1.2 The osteoclast

Roodman (1996) described bone resorption as a lifelong physiological process, initially involved with the processes of growth and modelling and continuing as remodelling of the mature skeleton. The main cellular agent involved in the removal of bone has been identified as the osteoclast, and extensive reviews, as well as entire books, have been written on this unique cell (Arey, 1920; Chambers, 1980; 1985; 1988; 2000; Hall, 1975; 1991; Pierce *et al.*, 1991; Rifkin and Gay, 1992; Zaidi *et al.,* 1993; Baron, 1995; Athanasou, 1996; Roodman, 1996; Greenfield *et al.,* 1999; Lerner, 2000; Väänänen *et al.,* 2000; Suda *et al.,* 2001). Lucht (1972a;1980) described the osteoclast as a large multinucleated cell usually containing between 10 and 20 nuclei. Meunier *et al.* (1980) indicated that osteoclasts are uncommonly seen in bone, but might be located either on endosteal surfaces within Haversian systems or on periosteal surfaces. However, osteoclast numbers have been shown to increase at sites of active bone tumours (Roodman, 1996).

1.2.1 The origin of osteoclasts

Kölliker (1873) is credited as the first to identify an association between the osteoclast and bone. It is currently believed that this multinucleated cell is derived from the pluripotential haemopoietic stem cell and remains the principal cell involved in bone resorption (Burger and Nijweide, 1992).

Early reports by Arey (1920) suggested that osteoblasts possessed the dual capabilities of osteogenesis and bone resorption. This impression was based on histological evidence of the close relationship between osteoblasts and osteoclasts. Arey (1920) further suggested that osteoblasts were mononuclear

precursors that coalesced to form the multinucleated osteoclasts. Subsequent parabiotic research by Walker (1972; 1973) indicated that osteoclasts are of haemopoietic origin. Osteoporosis was found to be cured when affected mice were joined in parabiotic union with normal littermates. Additional experiments by Walker (1975) using parabiotic rats, one of which was exposed and the other shielded from irradiation, demonstrated that osteoclasts form in and are derived from bone marrow tissue from the protected animal. This important finding was utilized by Coccia *et al.* (1980) in the management of infantile and juvenile osteopetrosis by bone marrow transplantation. Further transplantation studies by Kahn and Simmons (1975) using quail-chick chimeras revealed that osteoclasts are host derived and clearly indicated that osteoclast precursors are present in marrow, spleen and also detectable in circulating peripheral blood.

While there is general agreement that osteoclasts form from mononuclear cells of extraskeletal origin (Marks, 1983), debate exists as to whether this originating stem cell is part of the mononuclear phagocyte system. Zambonin-Zallone *et al.* (1984) showed that circulating blood monocytes fused to form osteoclasts in tissue culture and osteoclasts have differentiated from proliferating bone marrow mononuclear phagocytes *in vitro* (Burger *et al.,* 1982). Whereas monocytes have been shown to be capable of causing bone resorption (Mundy *et al.,* 1977), transplantation studies have revealed a bone marrow (Ash *et al.,* 1980) and/or a splenic (Marks and Walker, 1981) origin for the osteoclast precursor. By examining cell surface antigens, Oursler *et al.* (1985) determined that osteoclasts share certain determinants with multinucleated giant cells and monocyte derived macrophages.

In contrast, Chambers and Horton (1984) demonstrated the uniqueness of osteoclast resorption by the incubation of monocytes, macrophages, inflammatory polykaryons and myeloid cell lines on slices of human cortical bone. A scanning electron microscopic examination determined that none of the above cells produced a detectable change in the surface of bone and it was therefore concluded that mononuclear phagocytes are unable to function as bone resorptive cells. However, Quinn *et al.* (1996) altered the experimental conditions and incubated mouse and rat monocytes, macrophages, spleen and marrow haemopoietic cells as well as foreign body macrophages and macrophage polykaryons, with UMR 106 osteoblast-like cells on bone slices in the presence of 1,25-dihydroxyvitamin D_3. It was noted that numerous tartrate-resistant acid phosphatase-positive cells formed in these co-cultures and extensive lacunar resorption was observed on bone surfaces. Quinn *et al.* (1996) reported that a bone-derived stromal cell element is necessary for the differentiation of monocytes and macrophages into osteoclast-like cells capable of bone resorption. Quinn *et al.* (1996) also suggested that osteoclasts are members of the mononuclear phagocyte system.

1.2.2 Osteoclast morphology

A schematic representation of the morphologic features of the osteoclast appears in Diagram 1.

Bone biologists have described osteoclasts as large, multinucleated cells found in resorption bays (Howship's lacunae) on the surface of bone (Kölliker 1873; Arey, 1920; Hancox, 1972; Barnes, 1987; Holtrop, 1992). Under high power light microscopy, infoldings of the plasma membrane termed "ruffled borders" are observed in close contact with the hard tissue surface. The dramatic and revealing ultrastructural appearance of the ruffled border was first described by Scott and Pease (1956) as a complex series of finger-like cytoplasmic folds and projections. Väänänen (1996) described the ruffled border as the true resorptive organ of the active osteoclast and was further reported as an ultrastructural feature not seen in other giant cells (Lucht, 1972a; Holtrop and King, 1977; Gay, 1992). Väänänen *et al.* (1990) contended that the ruffled area was formed by the fusion of intracellular vesicles whose membranous proteins matched those present in the osteoclast's plasma membrane. Baron *et al.* (1988) suggested that the ruffled border was a specialized form of lysosomal membrane based on the localization of a mannose-6-phosphate receptor and a lysosomal proton pump. However, Akamine *et al.* (1993), using an immunocytochemical label for a lysosomal sialoglycoprotein, considered that the ruffled border proteins are more consistent with endosomal rather than lysosomal membranes. Holtrop and King (1977) reported that bone resorption and degradation of bone matrix occurs in a resorptive compartment beneath the ruffled border, as a result of the cellular release of proteolytic enzymes and hydrogen ions.

On either side of the ruffled border, Lucht (1972b; 1980) reported an organelle-free area of cytoplasm assocated with a portion of cell membrane that is in intimate contact with the hard tissue surface. Gay (1992) suggested that this intimate contact created a sealing zone which anchors the osteoclast to bone via specific adhesion molecules collectively termed integrins. This adhesion zone effectively isolates the resorptive compartment from the extracellular environment and so creates a resorptive micro-environment. The adjacent intracellular cytoplasmic clear zone was shown to contain actin-like filaments (β-actinin, talin, vinculin) but was devoid of organelles (Marchisio *et al.*, 1984).

The remaining cytoplasm of the osteoclast contains the necessary intracellular organelles to fulfil its digestive functions. Lucht (1980) reported extensive endoplasmic reticulum involved in protein (enzyme) synthesis in company with ribosomes, occurring singly or as polyribosomes. Perinuclear Golgi bodies were identified for vesicle production and numerous mitochondria provided the cell's energy requirements. Gay *et al.* (1983) and Baron *et al.* (1985) revealed that osteoclast mitochondria are associated with adenosine triphosphatase (ATPase) release as a by-product of the conversion of water and intracellular carbon dioxide to H^+ and HCO_3^- ions under the catalytic action of carbonic anhydrase II. Lucht (1972b) identified numerous intracellular vesicles carrying secretory products towards the ruffled border and in the transcytotic process of moving bone dissolution products through the cell towards the basolateral membrane for excretion into the extracellular space (Baron *et al.*, 1988; Salo *et al.*, 1997). Apart from a role in cell adhesion, the clear zone filaments were hypothesized by Teitelbaum *et al.* (1997) to act as intracellular guides for the vacuolar transcytotic process. Multiple nuclei were seen but it was other unique features of the

osteoclast such as the ruffled border and intracellular organelles that distinguished this cell from other polykaryons.

Scanning electron microscopic studies by Jones and Boyde (1977) and de St Georges *et al.* (1989) revealed the complexity of the osteoclast's plasma membrane. Prominent microvilli or pseudopodia were located over the central portion of the cell and were associated with excavation cavities. de St Georges *et al.* (1989) believed that, in the resorptive areas, osteoclasts appeared to be connected to adjacent cells and suggested the formation of a functional syncytium. Because of their pseudopodia and observations using time-lapse video photography (Kurihara, 1988), osteoclasts were reported to be highly motile cells that contracted when exposed to calcitonin or prostaglandin E_2 (Chambers *et al.*, 1985a).

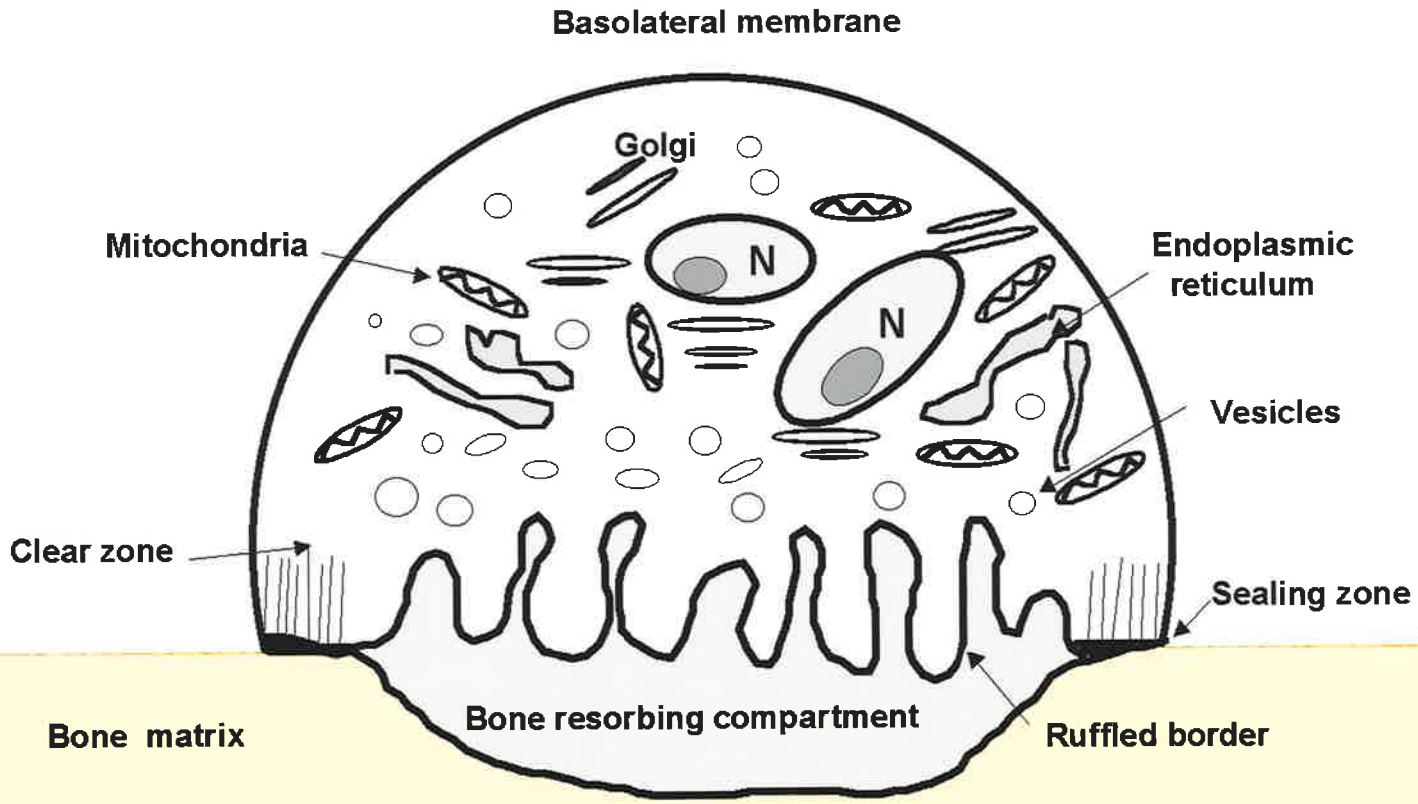


Diagram 1. Schematic ultrastructural representation of the osteoclast (N = nucleus)

1.2.3 Mechanisms of resorption

The process of bone resorption requires a series of events leading from the differentiation and recruitment of mononuclear osteoclast precursors, to their multinucleation and attachment to the hard tissue surface, before the removal of the inorganic and organic hard tissue components (Loutit and Nisbet, 1979; Chambers, 1985; Marks and Popoff, 1988; Suda *et al.,* 1992a). Many studies have identified the osteoblast as the principal regulator of resorption (Rodan and Martin, 1981; Chambers, 1980; McSheehy and Chambers, 1986; Greenfield *et al.*, 1999).

Vaes (1988) and Delaissé and Vaes (1992) reported that osteoclasts have the capacity to digest mineralized matrices such as bone, mineralized cartilage and dentine. Burger *et al.* (1984) revealed that an initial step in the sequence of events leading to bone digestion is the formation of the osteoclast from mononuclear progenitor cells from the monocyte lineage. Fusion of the mononuclear precursors to form osteoclasts at the site of resorption is followed by attachment to the bony surface which is facilitated by membrane-bound integrins and bone-associated proteins (Lakkakorpi *et al.*, 1991; Horton *et al.*, 1991; Helfrich *et al.*, 1992). Chambers *et al.* (1985b) suggested that osteoblast-derived collagenase (MMP-1) plays a prime role in facilitating osteoclast attachment by the degradation of surface osteoid. Pretreatment of bone with mammalian collagenase predisposed surface tissue to osteoclastic resorption which lead to the concept that the layer of osteoid acts as a protective barrier against osteoclastic contact with the underlying, resorption-stimulating bone mineral (Chambers *et al.,* 1985b). In between episodes of resorption, Chow and Chambers (1992) showed that the surface of bone is largely covered by a layer of

non-mineralized collagen fibrils which have the effect of making bone resistant to osteoclastic attack. In addition, it was shown that cells isolated from the tissues in close apposition to bone and subsequently seeded on to bone slices possess the capacity to render the mineralized surface accessible to osteoclasts. Chambers *et al.* (1984; 1985b) and Chambers and Fuller (1985) concluded that bone needs to be cleared of non-mineralized collagen prior to osteoclastic attack, and further, that bone-tissue derived osteoblast-like cells have the capacity to strip the surface of bone of its collagenous fringe. Meikle *et al.* (1991; 1992) suggested that osteoblasts are responsible for the removal of non-mineralized collagen and, further, indicated that collagen elimination depends on the activity of matrix metalloproteinases (MMP) which are manufactured and secreted by the osteoblast.

Further evidence implicating the involvement of osteoblasts in bone resorption was provided by Jilka (1989) and Delaissé and Vaes (1992) who demonstrated that the incubation of osteoblast-like cells with parathyroid hormone (PTH) results in a decreased amount of non-mineralized collagen. It was established that PTH stimulates the breakdown of the investing collagenous layer by increasing the activity of PTH-sensitive cells such as the osteoblast, and possibly the fibroblast.

Everts *et al.* (1992) indicated that enzymes other than MMPs, of which collagenase is a member, are possibly involved in bone resorption. The cysteine proteinases (cathepsins B and L) were shown to play a crucial role in the digestion of phagocytosed fibrillar collagen. This involvement was supported by Reynolds and Meikle (1997) who examined the role of MMPs and cysteine proteinases in mediating pathophysiological bone resorption and found that both

groups of enzymes play a part depending upon the environmental pH. Delaissé *et al.* (2000) reviewed the various proteinases involved in bone resorption and indicated that they may be of importance in determining the site of bone resorption and possibly whether bone formation will eventuate.

1.2.3.1 Osteoclast recruitment

Väänänen (1996) stated that the determining factors governing sites of resorption are largely unknown, as are the factors guiding osteoclast precursors to resorptive sites. Mangham *et al.* (1993) suggested that complement mediates the recruitment of mononuclear osteoclasts whereas Kukita *et al.* (1997) believed that macrophage inflammatory protein-1α plays an attracting role. Osteoclasts are seen in the vicintiy of osteoblasts producing macrophage inflammatory protein-1α which lead Kukita *et al.* (1997) to believe that this chemokine is involved in haemopoiesis as well as bone remodelling. Alternatively, Hentunen *et al.* (1995) suggested that human osteogenic protein-1 in combination with 1,25,dihydroxyvitamin D_3 has a profound effect on osteoblastic growth as well as the recruitment of osteoclasts as judged by vitronectin receptor and carbonic anhydrase activity. Blavier and Delaissé (1995) used proteinase inhibitors to show that MMPs play a major role in determining where and when osteoclasts attack bone. Importantly, tumour necrosis factor receptors types 1 and 2 are found to differentially regulate osteoclastogenesis (Abu-Amer *et al.*, 2000) while interleukin-1 and tumour necrosis factor, by stimulating the inflammatory process, lead to osteoclast recruitment and bone resorption (Assuma *et al.*, 1998). Rody *et al.* (2001) established that osteoclast recruitment also occurs at sites of PDL compression as a result of orthodontic tooth movement due to the likely release of local mediators. Suda *et al.* (1995) suggested that osteoclast recruitment is

more likely to be related to local factors which Zaidi *et al.* (1993) established are governed by hormonal, cytokine, growth and colony-stimulating factor interaction. Once at a site of resorption, a complex multistep series of events occurs which Lakkakorpi and Väänänen (1996) described in terms of osteoclast attachment and polarization, the formation of a sealing zone followed by active bone resorption, and finally cell detachment and death.

1.2.3.2 Osteoclast attachment

Teitelbaum *et al.* (1997) described the physical intimacy between the osteoclast and bone as essential in order to create and isolate an acidic extracellular resorptive microenvironment. According to Baron (1995), the most striking and unique feature of the osteoclast cytoskeleton is found at the site of cell contact with the substratum. Baron (1995) identified these specialized sealing areas as a prominent peripheral ring of filaments containing F-actin, orientated parallel to the plane of the substrate. In addition, numerous punctate structures of F-actin filaments orientated perpendicular to the substratum have been described and termed podosomes (Marchisio *et al.*, 1984). Marchisio *et al.* (1987) reported that podosomes occur in cells of monocytic origin and also in cells that have been transformed by *src*, *fps* and *abl* oncogenes. Furthermore, Teti *et al.* (1991) indicated that podosomes contain other proteins which have been associated with sites of cell-substratum and cell-cell interaction. These were spatially described as rosette structures surrounding podosome cores. Teti *et al.* (1991) also identified several tyrosine kinases and substrates localized to focal adhesions and to the sealing zone in osteoclasts.

A dynamic view of the attachment of osteoclasts to bone matrix was provided by Kanehisa *et al.* (1990) who described highly motile cells with few podosomes located mainly at the cell's leading edge. Upon osteoclast attraction and attachment, Kanehisa *et al.* (1990) and Lakkakorpi and Väänänen (1991) observed an increase in the number of podosomes arranged in a peripheral ring. The establishment of a seal results in the replacement of the punctate podosomes by two concentric protein rings of vinculin and talin that surround a central zone of F-actin. Baron (1995) suggested that these observations provided a distinction in time and in specific cell-matrix interactions between a motile cell and one that stops at a prospective bone-resorbing site.

According to Baron (1995), the mediation of cell-substratum interaction at the interface between the osteoclast and bone is facilitated by proteins of the integrin family. Nesbitt *et al.* (1993) described integrins as heterodimeric molecules containing α and β subunits which, when combined, have specific, receptor-like, extracellular binding sites that recognize the Arginyl-Glycyl-Aspartyl (RGD) sequence. Ruoslahti and Pierschbacher (1987) ascertained that the RGD sequence represents a core ligand for all members of the integrin family and, furthermore, found that the amino acid sequence surrounding this motif determined which integrin would recognize and bind to a specific matrix protein.

Nesbitt *et al.* (1993) showed that osteoclasts express multiple integrin proteins, some of which are involved in cell adhesion to bone matrix. Teti *et al.* (1989a; 1991) identified several RGD-bone matrix proteins, of which collagen type I, osteopontin, and bone sialoprotein are the most likely candidates to fill the integrin-binding role. The important use of integrins in osteoclast attachment and

function was shown by Fisher *et al.* (1993) who demonstrated that several RGD-containing proteins inhibit bone resorption *in vitro* and *in vivo*.

1.2.3.3 Osteoclast polarization

Lakkakorpi *et al.* (1989) and Lakkakorpi and Väänänen (1991) determined that osteoclasts experience strict organization of their cytoplasmic elements during resorptive activation. They become more highly polarized via changes in their plasma membrane morphology (Väänänen and Horton, 1995; Väänänen, 1996) with the sealing area of cell attachment to bone separating the ruffled border and the basolateral membrane into distinct basal membrane specializations. Salo *et al.* (1996) suggested that there are at least two functionally different basal membrane domains and the intervening central area is postulated to be involved with the transcytotic movement of degraded bone products. Salo *et al.* (1997) found that both organic and inorganic bone degradation products are transported in vesicles through the osteoclast and liberated into the extracellular environment via the specialized and polarized basal membrane area. Nesbitt and Horton (1997) and Mostov and Werb (1997) also reported the transcytosis of proteins liberated from mineralized matrix to the basolateral membrane for extracellular release. Baron (1989b) described an intracellular polarization of organelles which accumulated adjacent to the basolateral region of the cell away from the ruffled border and sealing zone. These polarized organelles synthesized the lysosomal enzymes and vesicles for vectorial transport towards the resorptive compartment.

1.2.3.4 Dissolution of bone mineral

The crystalline salts comprising the inorganic component of bone are calcium and phosphate in the form of hydroxyapatite (Buckwalter *et al.,* 1995). Blair *et al.* (1986) reported that osteoclasts degrade bone mineral and collagen with temporal asynchrony suggesting that the inorganic phase is removed prior to collagenolysis. Bone mineral dissolves in a low pH environment and the acidic nature of the resorptive lacunae was demonstrated by Fallon (1984a,b). Blair *et al.* (1989) showed that the ruffled border of actively resorbing osteoclasts contains a vacuolar-type of proton pump involved in the acidification of the resorptive compartment. According to Baron (1995), protons are pumped across the ruffled border into the sealed extracellular microenvironment via a complex ionic-balance process requiring co-ordinated electrogenic ion pumps, ion channels and electroneutral ion exchangers to maintain cytoplasmic pH. In addition to protons, Teti *et al.* (1989b) and Salo *et al.* (1996) indicated that the ruffled border plasma membrane expresses chloride channels that are an essential requirement of the acidification process.

Teitelbaum *et al.* (1997) summarized a series of intracellular processes that produce the protons for exchange. Cytosolic hydration of carbon dioxide to carbonic acid is catalysed by mitochondrial carbonic anhydrase II (Hall and Kenney, 1985a,b). This is followed by dissociation of carbonic acid to produce protons and bicarbonate ions. The protons are secreted into the resorption lacunae in an energy-dependent manner while at the basolateral surface opposite the resorbing zone, the bicarbonate ions are exchanged for chloride ions to maintain homeostasis (Teti *et al.,* 1989a,b). In addition, Ravensloot *et al.*

(1995) found that intracellular pH recovery was assisted by a Na^+-H^+ exchange process.

Baron *et al.* (1988) considered that the osteoclast is similar to acid-secreting epithelial cells found in other areas of the body such as the gastrointestinal tract and the kidney. However, Wang and Gluck (1990) demonstrated subtle differences in vacuolar-type ATPases in chicken osteoclasts compared with kidney intercalated epithelial cells suggesting that the osteoclast proton pump is pharmacologically unique.

1.2.3.5 Removal of organic matrix

The organic matrix of bone consists predominantly of type I collagen and non-collagenous matrix components comprising structural glycoproteins, proteoglycans and specific bone-related proteins (Buckwalter *et al.,* 1995; Robey 1996). Meikle (1997) indicated that organic matrix contains numerous growth factors which provided bone with a remarkable ability to repair and regenerate itself. In contrast, dentine contains type I as well as a small percentage of type III collagen. Matrix components include glycoproteins and proteoglycans of a similar nature but not identical with those of bone (Mjor, 1984). An odontoblast-produced phosphoprotein and dentine sialoprotein appear to be specific for dentine (Butler, 1998). Cementum also has a similar organic composition compared with bone but an adhesion molecule and a growth factor possibly remain unique to this material (Saygin *et al.,* 2000)

Väänänen (1996) stated that an understanding of the removal of matrix proteins is as far from clear but that several major classes of lysosomal proteolytic

enzymes are likely to be involved. Meikle (1997) divided these enzymes into four groups; namely, matrix metalloproteinases (MMPs) and serine, cysteine and aspartic proteinases. MMPs are qualitatively and quantitatively the most important, because they function at neutral pH and are apparently capable of digesting all of the bone matrix proteins (Meikle, 1997). Murphy and Reynolds (1993) reported that biochemical and clonal studies subgrouped MMPs into collagenases, gelatinases and stromelysins, all of which are active on matrix components synergistically and with broad specificity.

In 1993, Delaissé *et al.* demonstrated the presence of collagenase in rat osteoclasts and in their resorption lacunae using immunohistochemistry. Similarly, Okamura *et al.* (1993) showed that odontoclasts are capable of expressing *m*RNA for collagenase. In contrast, Fuller and Chambers (1995) were unable to find *m*RNA for collagenase in rat osteoclasts, despite its presence in other bone cells. Reponen *et al.* (1994) and Okada *et al.* (1995) also reported that MMP-2 and MMP-3 are not found in osteoclasts, and that possibly MMP-9 is expressed at *m*RNA and protein levels, but not in the resorption lacunae. Okada *et al.* (1995) revealed MMP-9 as an enzyme localized exclusively in osteoclasts and perhaps involved in the degradation bone collagen below the ruffled border in concert with cysteine proteinases. Multinucleated giant cells from osteoclastomas and osteoclasts from patients with Paget's disease were reported to have high levels of MMP-9 activity (Wucherpfennig *et al.*, 1994) while Roodman (1996) indicated that MMP-9 is expressed early in osteoclastic differentiation.

Etherington (1972) noted the ability of cysteine proteinases (cathepsins / caspases) to degrade type I collagen in an acidic environment and suggested the involvement of these enzymes in bone resorption. Subsequently, Kirschke *et al.,* (1982) and Goto *et al.* (1993; 1994) identified a number of cysteine proteinases in intracellular lysosomes located in osteoclasts as well as in their resorption lacunae. Burleigh *et al.* (1974) demonstrated that cathepsin B1 degrades collagen in solution at an optimal pH of 4.5-5.0 and also degrades insoluble collagen at a pH lower than 4. In addition, collagen degradation by cathepsin L was five times faster at a pH of 3.5 compared with higher pH levels and its specific activity was five to ten times greater than that of cathepsin B (Kirschke *et al.,* 1982). That these enzymes directly and effectively participated in the degradation of bone matrix was shown by Debari *et al.* (1995) and Inui *et al.* (1997) who employed a general cysteine proteinase inhibitor (E-64) and a selective cathepsin L inhibitor (Z-Phe-Phe-CHN_2). These cysteine protease inhibitors reduce resorption pit formation by osteoclasts in a concentration-dependent manner. The inhibition of resorption by the other cysteine proteinase inhibitors utilized by Delaissé *et al.* (1980; 1984) provided direct evidence for the importance of these enzymes in bone resorption where they are able to function in the acidic microenvironment beneath the osteoclast where neutral collagenases (MMPs) cannot (Meikle, 1997).

Murata *et al.* (1991) suggested that cathepsin L was the main cysteine proteinase responsible for bone collagen degradation since the epoxysuccinyl peptide inhibitor, CA074, specific for the inactivation of cathepsin B, failed to inhibit bone resorption. Kakegawa *et al.* (1993) supported the contention that cathepsin L was the principal proteinase involved in resorption by inhibiting this enzyme with

chymostatin or pig leucoctye cysteine proteinase inhibitor. Resorption lacuna formation on ivory slices was completely blocked and cathepsin L activity in osteoclasts was reported to be much higher than other cathepsin varieties.

However, more recently, Inaoka *et al.* (1995) cloned human cysteine proteinase (cathepsin K) which was shown to be predominantly expressed in osteoclasts, although not exclusively (Goto *et al.*, 1994). Goto *et al.* (1994) identified cathepsins B, D and L as important in the breakdown of extracellular matrix during osteoclastic bone resorption and therefore possible markers for resorptive activity.

1.2.3.6 Removal of degradation products

Väänänen (1996) observed that breakdown products from the resorptive process need to be continuously removed from the extracellular resorptive compartment. As there is no experimental evidence to support leakage of material through the sealing zone, he suggested that vesicular transcytotic passage through the osteoclast is the most likely pathway. Nesbitt and Horton (1997) used confocal microscopic analysis to show that released matrix proteins, including degraded type I collagen, are endocytosed along the ruffled border and transcytosed through the osteoclast to the basolateral membrane. Earlier ultrastructural studies suggested that bone mineral may be phagocytosed by osteoclasts (Pierce, 1989a) and subsequently removed in the low pH of lysosomes as degradation products are transcytosed. The transcytotic mechanism was supported by Salo *et al.* (1997) who reported that bone degradation products, both organic and inorganic, are transported in vesicles through the cell to the middle of the basolateral membrane domain where they are released into the extracellular

space. Mostov and Werb (1997) suggested that the osteoclast is similar to epithelial cells in this metabolite transport mechanism but that the process was significantly more complicated. These authors considered that the endocytic and exocytic processes are dissimilar to those previously encountered in other cell types and require a specialized area of the osteoclast basolateral membrane.

1.2.3.7 The fate of osteoclasts

Lakkakorpi and Väänänen (1991) showed that osteoclasts are able to pass through more than one resorption cycle but their fate is uncertain once resorption had ceased. Väänänen (1996) considered that a mechanism exists to remove or destroy multinucleated cells *in situ*. Hughes *et al.* (1995) studied the effects of bisphosphonates *in vitro* and *in vivo* and discovered a 4- to 24- fold increase in the number of apoptotic osteoclasts. This lead to the suggestion that osteoclasts, formed by the aggregation of mononuclear cells rather than by mitosis, were removed by apoptosis. Confirmation was provided by Kameda *et al.* (1995) who examined osteoclast death ultrastructurally and in a culture system, and showed that the majority of cells exhibited morphological nuclear changes, DNA fragmentation, and biochemical changes similar to those found in apoptosis in other cell systems.

Savill (1997) indicated that similar mechanisms have been implicated in the clearance of eosinophils, lymphocytes and monocytes and in the resolution of the acute inflammatory response. A study by Yuan *et al.* (1993) revealed that the activation of proteolytic enzymes, including the cysteine proteinases, is a characteristic feature of apoptosis.

An alternative concept to apoptosis is multinucleated cell fission back to mononuclear constituents which, according to Väänänen (1996), has heretofore been considered improbable for osteoclasts. However, using an *in vitro* osteoclast differentiation model and fluorescent markers injected into mature multinucleated cells, Solari *et al.* (1995) demonstrated that mononuclear cells bud from their giant cell parent. Furthermore, it was shown that the newly budded mononuclear cells are able to later coalesce to re-form multinucleated cells. Solari *et al.* (1995) concluded that our understanding of the ultimate fate of osteoclasts is, at best, confused.

1.2.4 Osteoprotegerin and the regulation of osteoclasts

Greenfield *et al.* (1999) indicated that the control of osteoclast activity and hence hard tissue resorption is of primary importance in the overall regulation of bone metabolism. Many controlling factors have been identified including hormones, cytokines, growth factors, ions and arachidonic acid metabolites. With the notable exception of calcitonin (Hattersley and Chambers, 1998), the majority of these factors operate on osteoclasts via surface receptors on osteoblasts acting as intermediaries (Orlandini *et al.,* 1995). Extensive reviews have been written by Osdoby *et al.* (1992b), Mundy (1989; 1993a,b; 1996), Heymann *et al.* (1998), Greenfield *et al.* (1999), Suda *et al.* (1999), Manolagas (2000), Hofbauer *et al.* (2000), Chambers (2000), Khosla (2001) and Duong and Rodan (2001).

A summary of regulatory factors involved in clast cell activity is presented in Table 1.

Factor	Stimulatory	Inhibitory	Reference
Hormones			
Amylin		+	Alam *et al.* (1993)
Androgens		+	Bellido *et al.* (1995)
Calcitonin		+	Zaidi *et al.* (1991)
Calcitonin gene-related peptide		+	Alam *et al.* (1991)
Glucocorticoids	+		Delany *et al.* (1994)
Oestrogen		+	Oursler *et al.* (1993)
Parathyroid hormone	+		Talmage (1967)
PTHrP	+		Moseley and Gillespie (1995)
Thyroid hormone	+		Mundy *et al.* (1979)
1,25$(OH)_2$vitamin D_3	+		Reichel *et al.* (1989)
Cytokines and Growth Factors			
Bone morphogenic proteins, BMP	+		Udagawa (2002)
Colony stimulating factors, CSF-1	+		Hattersley *et al.* (1991a)
Endothelin-1	+		Tarquini *et al.* (1998)
Epidermal growth factor, EGF	+		Tashjian *et al.* (1986)
Fibroblast growth factor, FGF	+	+	Shen *et al.* (1989) Chikazu *et al.* (2001)
Granulocyte macrophage colony stimulating factor, GM-CSF	+		Kurihara *et al.* (1989)
Insulin-like growth factor, IGF-1	+		Mochizuki *et al.* (1992)
Interferon-γ, IF-γ		+	Gowen *et al.* (1986)
Interleukin-1, IL-1	+		Lorenzo *et al.* (1987)
Interleukin-4		+	Shioni *et al.* (1991)
Interleukin-6	+		Peters *et al.* (1996)
Interleulin-8		+	Fuller *et al.* (1995)
Interleukin-10		+	Burger and Dayer (1995)
Interleukin-11	+		Manolagas and Jilka (1995)
Interleukin-18		+	Udagawa *et al.* (1997)
Kinins	+		Lerner *et al.* (1987)
Macrophage inflammatory protein 1-α, MIP-1α	+		Choi *et al.* (2000)
Nitric oxide		+	MacIntyre *et al.* (1991)
Platelet-derived growth factor, PDGF	+		Canalis *et al.* (1989)
Prostaglandins	+		Akatsu *et al.* (1991)

Factor	Stimulatory	Inhibitory	Reference
Transforming growth factor alpha, TGF-α	+		Tashjian *et al.* (1986)
Tissue inhibitors of metalloproteinases, TIMP	+		Sobue *et al.* (2001)
Transforming growth factor β, TGF-β	+		Udagawa (2002)
Tumour necrosis factor, TNF-α	+		Kobayashi *et al.* (2000)
Tumour necrosis factor β, TNF-β	+		Bertolini *et al.* (1986)
Substance P	+		Lotz *et al.* (1988)
Vasoactive intestinal peptide, VIP	+		Hohmann *et al.* (1983)
Drugs			
Bisphosphonates		+	Fleisch *et al.* (1969)
Genes			
ADAM8	+		Choi *et al.* (2001)

Table 1. A list of stimulatory and inhibitory factors associated with osteoclast activity.

Recent literature has described a newly discovered regulator of clast cell activity termed osteoclast differentiation factor / osteoprotegerin ligand (ODF/OPGL /RANKL) and its antagonist osteoprotegerin / osteoclast inhibitory factor (OPG /OCIF). Hofbauer *et al.* (1999a,b; 2000) indicated that many of the above-listed regulatory factors operate via stimulation of the RANKL/OPG system that links the osteoblast with the osteoclast.

1.2.4.1 Osteoprotegerin

A schematic diagram of the RANKL/RANK/OPG interaction between osteoblasts and osteoclasts appears in Diagram 2.

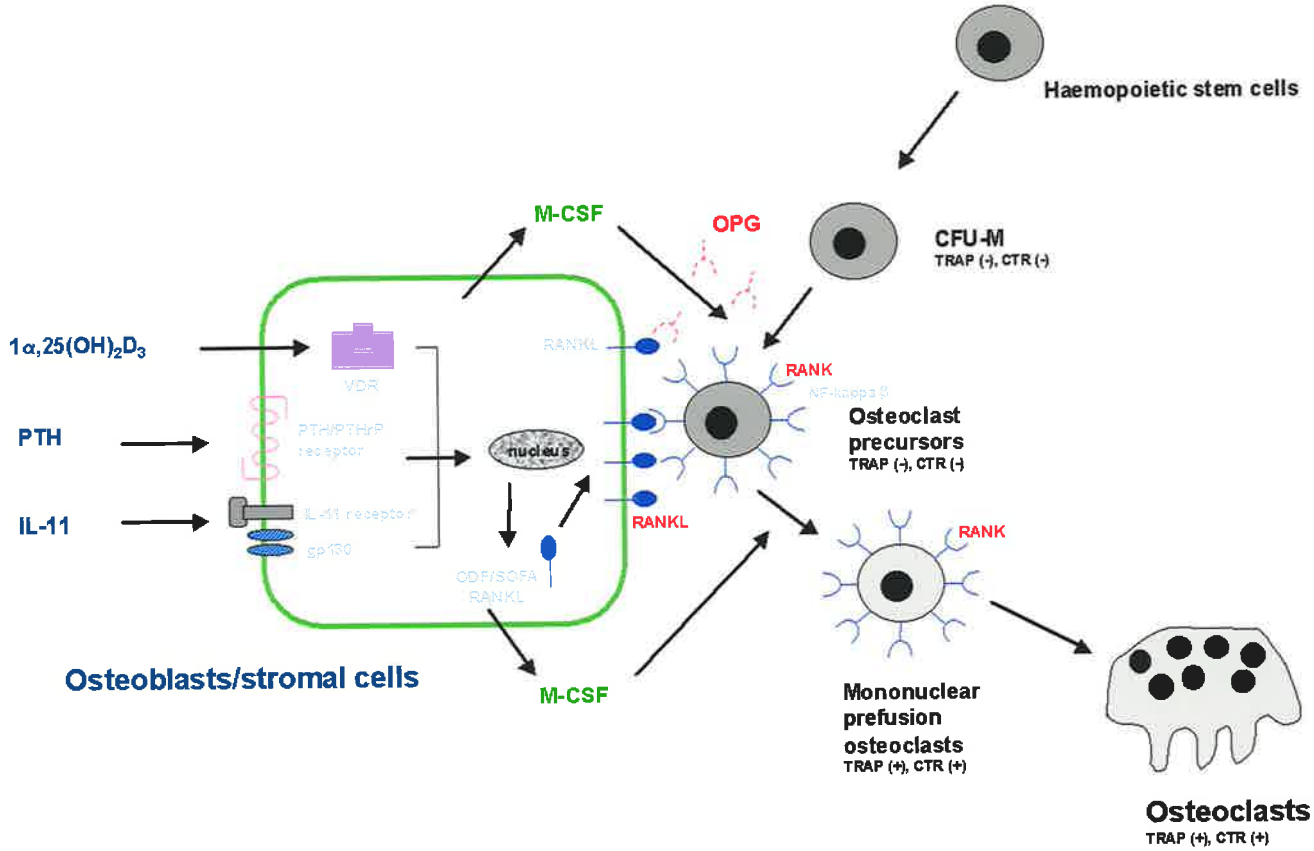


Diagram 2. **Schematic representation of the relationship between the osteoblast and the osteoclast with the TNF family of receptors and ligands (Modified from Suda *et al.*, 2001). (Not to scale)**

Simonet *et al.* (1997) and Tsuda *et al.* (1997) almost simultaneously identified a novel secreted glycoprotein that regulates bone resorption, and they termed it osteoprotegerin. It was reported to be a member of the tumour necrosis factor receptor superfamily that was able to cause a profound but non-lethal osteopetrosis in transgenic mice. Also reported was its coincident decrease in the later stages of osteoclast differentiation. Simonet *et al.* (1997) considered that OPG might act as an important and significant factor in the regulation of bone mass and therefore could be a useful adjunct in the treatment of osteoporosis and increased osteoclastic activity. Tsuda *et al.* (1997) independently isolated the same protein which they termed osteoclastogenesis inhibitory factor (OCIF). By the cloning of a complimentary DNA of human OCIF, Yasuda *et al.* (1998a) ascertained that OCIF and OPG were identical. Recombinant OCIF and OPG specifically acted on bone tissues to increase bone mineral density and bone volume associated with a decrease in active osteoclast number in normal rats. Yasuda *et al.* (1998b) further reported that OCIF/OPG blocked the usual interactions between osteoblasts (or bone marrow-derived stromal cells) and osteoclast precursors by interrupting the signalling through OCIF/OPG cell binding sites.

Tan *et al.* (1997) identified a new member of the tumour necrosis factor receptor family termed TNF receptor-like molecule (TR1) and also found it to be identical to OPG. In a co-culture system, osteoclast formation was inhibited, as was pit formation and bone resorption in organ culture of foetal mouse long bones. For simplicity, Suda *et al.* (1999) proposed that the term OPG be adopted for molecules that include OCIF and TR1.

While the structure of OPG was described by Simonet *et al.* (1997) and Yasuda *et al.* (1998a), its physiological roles were outlined by Mizuno *et al.* (1998) and Bucay *et al.* (1998). These authors showed that OPG-deficient mice suffered from severe osteoporosis and accelerated osteoclast development and function. This, in turn, reduced the strength and mineral density of bones. In a histomorphometric examination of tibial and vertebral bone in OPG-deficient mice, Bucay *et al.* (1998) reported that both the osteoblast and osteoclast surface areas were increased and concluded that OPG is a physiological regulator of bone formation in the postnatal animal. Furthermore, because OPG inhibited the terminal stages of osteoclast development but did not affect the development of osteoclast precursors, the osteoporosis in OPG-deficient mice was considered to be most likely due to dysfunction in osteoclast recruitment and activation.

An additional development by Emery *et al.* (1998) was the report of a tumour necrosis factor related ligand (TRAIL) that induced apoptosis upon binding to its death domain-containing receptors. It was further reported that OPG bound to TRAIL which then blocked the osteoclastogenesis inhibitory activity of OPG in coculture. The results of Emery *et al.* (1998) suggested a potential cross-regulatory mechanism involving OPG and TRAIL which may also participate in the regulation of osteoclastic bone resorption.

Yasuda *et al.* (1998a) purified and molecularly cloned OPG and also reported the expression cloning of a ligand for OPG from a complimentary DNA library of mouse stromal cells. These authors verified that this protein ligand found on the surface of osteoblasts and stromal cells is a member of the membrane-associated tumour necrosis factor ligand family which induces osteoclast

formation from its progenitors. From cell culture experiments, Yasuda *et al.* (1998b) determined that the membrane-bound protein is an osteoclast differentiation factor (ODF) which had previously eluded identification but was known to exist. ODF was thought to provide an essential signal to osteoclast progenitors to induce differentiation into osteoclasts, and it was found to be identical to TRANCE / RANKL which enhances T-cell growth and plays an important role in the regulation of the immune system.

Lacey *et al.* (1998) were also successful in the molecular cloning of a ligand for OPG from an expression library of myelomonocytic cell line and ascertained that the OPG ligand (OPGL/RANKL) was identical to ODF. When a soluble form of OPGL was administered to mice, hypercalcaemia was induced despite little change in the numbers of osteoclasts. The results indicated to Lacey *et al.* (1998) that OPGL stimulates osteoclast function as well as differentiation. Research by Kong *et al.* (1999) succeeded in producing OPGL knockout mice which exhibit osteopetrosis with total occlusion of bone marrow spaces within endosteal bone. Osteoclasts were lacking but normal osteoclast progenitors were present and able to differentiate into functionally active osteoclasts in a co-culture with osteoblasts or stromal cells.

Molecular cloning of ODF/OPGL by Wong *et al.* (1997) provided supporting evidence that this molecule was identical to TRANCE and RANKL of the TNF ligand family. TRANCE was cloned during a search for apoptosis-regulatory genes in T-cell hybridomas and was shown to activate the c-Jun N-terminal kinase enzyme in these cells.

Anderson *et al.* (1997) cloned a new member of the TNF receptor family termed RANK from a cDNA library of human dendritic cells. In searching for a binding molecule of RANK, these authors cloned RANK ligand (RANKL) from thymoma cells and found it to be identical to TRANCE. The various members of the TNF receptor superfamily interacted with signalling molecules to activate nuclear factor kappa beta (NF-κβ) via c-Jun N-terminal kinase (JNK) pathways. Darnay *et al.* (1998) characterized the intracellular domain of receptor activator of NF-κβ and determined that RANK interaction with a TNF-receptor-associated factor (TRAF) was necessary for NF-κβ activation but not for activation of the JNK pathway. Wong *et al.* (1998) indicated that the TRAF family of signal transducers mediated NF-κβ activation via the TRANCE receptor. This was shown to be a critical step in the differentiation and activation of dendritic cells and osteoclasts. Wong *et al.* (1998) suggested that RANKL/TRANCE directed differentiation and activation of osteoclasts through RANK by stimulating NF-κβ through TRAFs.

The nomenclature at the present time appears confused and incomplete. An attempt to clarify terminology was provided by Suda *et al.* (1999) who indicated that ODF, OPGL, TRANCE and RANKL are the same molecules important for the development and function of osteoclasts. RANK appears to be the transmembrane signalling receptor for ODF/OPGL/TRANCE/RANKL. OCIF/OPG/TR1 is a soluble receptor for ODF/OPGL/TRANCE/RANKL and appears to function as a decoy receptor. It was further indicated that these TNF-related ligands and receptors have a diverse range of functions and effects on cells other than osteoclasts and osteoblasts.

In order to standardize the nomenclature for the new tumor necrosis factor members, the American Society for Bone and Mineral Research President's Committee on Nomenclature recommended the names of receptor activator of NF-κβ (RANK) for the membrane receptor, RANK ligand (RANKL) for the ligand and OPG for the decoy receptor (The American Society for Bone and Mineral Research, 2000). This nomenclature will be followed hereinafter and is outlined below in Table 2.

Structure	Proposed nomenclature	Other nomenclature
Ligand	**RANKL** Receptor activator of NF-κβ ligand	**ODF** Osteoclast differentiation factor **OPGL** Osteoprotegerin ligand **TRANCE** TNF-related activation-induced cytokine
Receptor	**RANK** Receptor activator of NF-κβ	
Decoy receptor	**OPG** Osteoprotegerin	**OCIF** Osteoclastogenesis inhibitory factor **TR1** TNF receptor-like molecule 1

Table 2. Accepted current nomenclature for the tumour necrosis factor family of receptors (The American Society for Bone and Mineral Research, 2000).

Horowitz *et al.* (2001) and Khosla (2001) published comprehensive reviews of the tumour necrosis factor family of receptors and ligands, including a summary of their possible influence in a range of medical conditions. However, to date, little research has appeared in the literature regarding the relationship between OPG and the dentoalveolar complex. Shiba *et al.* (2000) examined the changes in expression of secreted proteins (SPARC/osteonectin and OPG/OCIF) by aged periodontal cells and found no change was found in the levels of OPG/OCIF *m*RNA with cellular ageing but increased levels of SPARC. Through determinations of cell population doubling, these authors suggested that an impairment in periodontal ligament repair with ageing was possibly due to a decrease in the proliferative ability of the cells involved while the increase in SPARC expression might be explained by changes in cellular metabolism.

Rani and MacDougall (2000), in an examination of odontoblasts and osteoblasts, detected OPG, RANKL and M-CSF *m*RNA in both cell types. In a co-culture system, it was determined that odontoblast and dental pulp cell lines inhibited osteoclast formation from spleen and bone marrow precursors. It was concluded that dental cells possess the ability to regulate osteoclastogenesis and bone resorption by the balanced secretion of stimulatory RANKL and M-CSF as well as inhibitory OPG.

Despite the many recent publications examining OPG and bone resorption, little research has been performed on the relationship between dental resorption and the presence of OPG. Sakata *et al.* (1999) used reverse transcriptase polymerase chain reaction techniques to determine that OPG/OCIF synthesized by dental mesenchymal cells regulates the resorption of dental hard tissues. One

of the aims of the current study was to examine the effects of OPG on the cells involved in an experimental dental resorptive and repair model.

1.2.5 Identification of osteoclasts

Apart from the uniqueness of the calcitonin receptor (Hattersley and Chambers, 1989a; 1998), traditional methods of osteoclast identification have relied on enzyme histochemistry and immunolabelling to distinguish this cell from other multinucleated giant cells (Athanasou, 1996). Apart from unique morphological differences, Athanasou and Quinn (1990) reported that osteoclasts contain a number of phenotypic features that enable their detection.

1.2.5.1 Osteoclast enzyme histochemistry

1.2.5.1.1 Tartrate-resistant acid phosphatase

Yam (1974) described the acid phosphatases as a class of enzymes that catalyse the hydrolysis of phosphate esters in an acid environment. Martland *et al.* (1924) first demonstrated phosphatase isoenzymes in erythrocytes and bone based on their respective pH optima. Erythrocyte phosphatase activity was optimal below a pH of 7 whereas the phosphatase of bone had an optimal activity at a pH of 9. After surveying a variety of enzyme sources, Kutscher and Wolbergs (1935) identified an elevated concentration of acid phosphatase in a number of tissues but particularly the prostate gland. Since that time, elevated serum acid phosphatase activity has been considered a diagnostic marker of prostatic cancer. Abu'l-Fadl and King (1949) distinguished prostatic acid phosphatase from erythrocyte acid phosphatase by an inhibitory effect on L-tartrate. For diagnostic reasons, the tartrate-sensitive acid phosphatase was referred to as the "prostatic

fraction" whereas the tartrate-resistant acid phosphatase has often been overlooked.

Lucht (1971) showed immunohistochemically that osteoclasts contain high levels of acid hydrolases which Hammarström *et al.* (1971) and Chen *et al.* (1979) recognized as acid phosphatases. Acid phosphatases were also identified in Gaucher cells by Li *et al.* (1973) and in hairy cells by Lam and Yam (1977), and further, appeared so closely related that they were considered to be antigenically identical. However, Vaes and Jacques (1965), Wergedal (1970) and Anderson and Toverud (1977) distinguished two different types of acid phosphatase in bone, one of which is sensitive, and one that is resistant, to the activity of tartrate.

The term purple acid phosphatase was also applied to this class of enzymes which, according to Vincent and Averill (1990), makes them readily distinguishable from other mammalian acid phosphatases. This terminology arose because acid phosphatase isolated from bovine spleen was shown to migrate as a purple band on electrophorectic gels by Glomset (1959). The metal stoichiometry was established by Campbell *et al.* (1978); however, at that time, the exact structure of the metal chromophore remained undetermined. Antanaitis and Aisen (1983) and Doi *et al.* (1988) reported that these enzymes existed in two forms, one containing an oxidized, catalytically inactive purple diferric centre and the other containing a reduced, enzymatically active, mixed-valent pink iron centre. Resonance Raman investigations by Averill *et al.* (1987) indicated that the pink or purple colour arose from a tyrosine phenoxide to ferric iron charge transfer band.

Ek-Rylander *et al.* (1991) considered that purple acid phosphatase was a member of the TRAP group, all of which possess the ability to catalyze the hydrolysis of phosphate esters. It was further considered that TRAP plays a vital role in the regulation of physiological levels of inorganic phosphate and possibly in regulating the activity of certain phosphorylated proteins. Robinson and Glew (1981) considered that TRAP likely serves a catabolic function rather than a regulatory role as a phosphoprotein phosphatase.

Further electrophoretic differentiation of tartrate-sensitive from the tartrate-resistant acid phosphatase was performed by Lam *et al.* (1973). Moss (1986) showed by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions at acidic pH that there are five major bands of acid phosphatases in tissues and body fluids. Band 5 was revealed as consisting of an acid phosphatase resistant to inhibition by tartrate but Ramponi *et al.* (1989) determined that there are at least two tartrate-resistant acid phosphatases which do not migrate in the PAGE system but are expressed in the cytosol of mammalian cells. Furthermore, Clark *et al.* (1989) showed that band 5 tartrate-resistant acid phosphatase is highly enriched in osteoclasts which caused Minkin (1982) to regard it as a marker for these cells. Using organ cultures of newborn mouse calvaria, Minkin (1982) stimulated bone resorption *in vitro* by the addition of parathyroid hormone or $1,25(OH)_2$vitamin D_3. Tartrate-resistant acid phosphatase activity was largely found over osteoclasts while tartrate-sensitive activity was associated with other bone cells.

Whereas Wergedal and Baylink (1969) reported the distribution of acid and alkaline phosphatase activity in undemineralized sections of rat tibial diaphysis

was on endosteal-resorbing surfaces, Hammarström *et al.* (1971) revealed that the activity was present in osteoclasts and extracellularly in the lining of resorption lacunae. In addition, Reinholt *et al.,* (1990) located tartrate-resistant acid phosphatase (TRAP) in osteoclast lysosomes, Golgi bodies, extracellular channels of the ruffled border and in the resorptive compartment. The ultrastructural immunolabelling by Reinholt *et al.* (1990) further localized TRAP to bone surfaces facing the ruffled border of osteoclasts and more diffusely in adjacent bone areas. TRAP was not seen related to osteoblasts nor their adjacent bone.

Lundy *et al.* (1988) disputed the lack of acid phosphatase activity in osteoblasts and reported that osteoblasts and osteoclasts, but not fibroblasts, stain for fluoride-sensitive acid phosphatase activity in chicken bone. However, the activity of acid phosphatase in osteoblasts appeared to be sensitive to the concentration of fluoride. Furthermore, van de Wijngaert and Burger (1986) reported TRAP presence in a subset of bone marrow mononuclear cells which Baron *et al.* (1986) considered as osteoclast precursors. An increase in the number of marrow TRAP-positive cells was detected prior to the formation of osteoclasts to the point where Udagawa *et al.* (1990) considered that even mature monocytes and macrophages were capable of differentiating into osteoclasts under a suitable microenvironment established by bone marrow-derived stromal cells.

Evidence that TRAP was associated with bone metabolism was provided by Zaidi *et al.* (1989) who showed that the immunological inhibition of TRAP was able to prevent bone resorption confirming that the enzyme was involved in the resorptive process. Vaes (1968) had previously suggested a relationship by

demonstrating that TRAP activity in osteoclasts and bone resorption was increased after treatment with parathyroid hormone. A spectrophotometric assay of serum TRAP levels established by Lau *et al.* (1987) was considered a clinically useful marker of bone resorptive activity. Increased TRAP activity and increased blood serum levels were shown to occur during bone growth (Chen *et al.*, 1979) and in a number of disease states (Whitaker *et al.*, 1989; Lau *et al.*, 1987; de la Piedra *et al.*, 1989).

Because of its association with bone resorption, Andersson *et al.* (1989) believed that TRAP was a useful marker for osteoclast ontogeny and function. In addition, Andersson and Marks (1989) considered that tartrate-resistant acid ATPase was a valid and useful cytochemical label for osteoclasts which had the additional benefit of leaving bone surfaces unstained. Further, Andersson *et al.* (1986) and Andersson and Marks (1989) suggested that tartrate-resistant nucleotide tri- and di-phosphatase (TrATPase), as members of the TRAP family, were more specific osteoclast markers that provided the energy for acidification of the bone resorptive microenvironment. However, it was further revealed that TRAP is present in other tissues and is not unique to osteoclasts (Bianco *et al.*, 1988; Reddy *et al.*, 1993).

1.2.5.1.2 Succinic dehydrogenase

Barka and Anderson (1963) described succinic dehydrogenase as a readily soluble iron flavoprotein that catalyses the reversible oxidation of succinic acid to fumarate. Walker (1961) reported succinic dehydrogenase as a member of a group of oxidative enzymes involved in the Krebs citric acid cycle which is known to be active in osteoblasts and osteoclasts. Their studies revealed intense

dehydrogenase presence in osteoclasts which indicates high levels of citrate and lactate production by these cells. Burstone (1960) similarly found high succinic dehydrogenase levels in osteoclasts and concluded that the Krebs citric acid cycle is involved in the resorptive process.

Hess *et al.* (1958) reported that most dehydrogenase activity is present in mitochondria. Rustin *et al.* (1997) verified the mitochondrial involvement by revealing that inborn errors of the Krebs cycle result in a number of mitochondrial diseases mostly affecting the neuromuscular system. In addition, Blanco *et al.* (1988) reported succinic dehydrogenase activity in skeletal muscle fibres, while Levrat *et al.* (1991) indicated that tumour necrosis factor is important in the activation of mitochondrial succinic dehydrogenase.

Fullmer (1964) examined the dehydrogenases in rat developing bone via histochemical techniques requiring the use of tetrazolium salts as hydrogen ion acceptors. His results indicated that all bone cells possess a fully functioning citric acid cycle, pentose cycle and the capacity to metabolize fatty acids and carbohydrates. It was further discovered that osteoclasts exhibit greater enzymatic activity compared with osteoblasts or osteocytes.

Gibson and Fullmer (1966) found particularly strong dehydrogenase reactions in osteoblasts, osteocytes, osteoclasts, cementoblasts and fibroblasts in a study which assessed the reliability of various methods of localizing dehydrogenases in the PDL. Osteoclasts were found to label intensely for many dehydrogenases after demineralization in 4% EDTA and cryostat cutting of frozen specimens. Takimoto *et al.* (1966) detected succinic dehydrogenase in osteoclasts following

experimental tooth movement in rats. Osteoclasts were revealed on the periodontal surface of the pressure side and also on the marrow surfaces of the tension side. In addition, Lilja *et al.* (1983) studied the histochemistry of enzymes associated with tissue degradation incident to orthodontic tooth movement. The findings of this study indicated that lactate dehydrogenase activity is not affected by low orthodontic forces; however, under high forces, a zone of lactate dehydrogenase and acid phosphatase inactivity is seen in the most compressed areas of the PDL. Lilja *et al.* (1983) considered, along with Takimoto *et al.* (1966), that dehydrogenase labelling of osteoclasts is a useful marker of bone resorptive activity.

1.2.5.1.3 Carbonic anhydrase II

Minkin and Jennings (1972) and Hall and Kenny (1985a,b) associated high levels of carbonic anhydrase II (CA II) with bone resorption and considered that this enzyme is useful as an osteoclast identifying marker. Simasaki and Yagi (1960) first identified carbonic anhydrase in osteoclasts which was later located throughout the cytoplasm and associated with the inner surface of the ruffled border (Anderson *et al.,* 1982). However, Zaidi *et al.* (1993) indicated that other acid secreting cells of the body exhibit CA II activity and therefore questioned its uniqueness as an osteoclast marker.

Tong *et al.* (1994) employed more sophisticated reverse-transcriptase polymerase chain reaction (RT-PCR) techniques to distinguish osteoclasts from macrophage polykaryons and showed that osteopontin *m*RNA, carbonic anhydrase II, and a calcitonin receptor are present in osteoclasts. Additionally, it

was demonstrated that *m*RNA's associated with the osteoblast phenotype, such as alkaline phosphatase and osteocalcin, are absent.

1.2.5.1.4 Miscellaneous enzymes

Dorey and Bick (1977a) used the ultrastructural histochemical distribution of aryl sulphatase in the rat periodontal ligament as a marker of osteoclast activity. It was noted that this enzyme is localized specifically in osteoclasts and in perivascular macrophages but not in macrophages associated with bone formation. This finding was interpreted as providing a potential marker for osteoclast differentiation from macrophages or as an indication of the ability of macrophages to modulate their enzymatic complement in response to the environment. Macrophages were therefore identified as a significant participant in bone remodelling by Dorey and Bick (1977b) who further isolated cellular sites of glycosaminoglycan hydrolysis by the localization of a key enzyme, N-acetyl-beta-glucosaminidase in the rat periodontal ligament. Reaction product is seen in osteoclasts, osteoblasts, fibroblasts and macrophages in areas of bone formation as well as resorption.

1.2.5.2 Immunolabelling of osteoclasts

The immunobiochemistry of osteoclasts was investigated by Osdoby *et al.* (1992a) in an attempt to clarify the developmental relationship between circulating mononuclear cells, macrophages and osteoclast precursors in haemopoietic tissues. An incomplete biochemical and molecular characterization of the mature osteoclast had previously impeded understanding of the unique capabilities of this bone-resorbing cell.

Immunolabelling research by Horton *et al.* (1984) determined that osteoclasts fail to express granulocyte-monocyte, common leucocyte or other haemopoietic determinants thereby prompting the suggestion that osteoclasts were antigenically effete. The monocyte antigens, My-7, MCS.2 and DüHL60.4, detected on osteoclasts by Horton *et al.* (1984) are also expressed by tissues outside the haemopoietic system. At the time, these findings created doubt that osteoclasts were specialized bone-resorbing macrophage-derived giant cells and supported an hypothesis that they were the end-product of fusion of an unidentified circulating mononuclear cell type whose lineage was separate from those originating from the conventional multipotential haemopoietic stem cell.

Horton *et al.* (1985a) implemented monoclonal antibody techniques to examine the relationship between osteoclasts and other bone marrow-derived cells in an attempt to determine whether osteoclasts arose from a separate cell line. By innoculating mice with osteoclasts from osteoclastomas, these authors were able to produce hybridomas secreting osteoclast-reactive monoclonal antibodies, several of which were shown to recognize membrane antigens, whereas others reacted with cytoplasmic determinants. Horton *et al.* (1985a,b) further reported a number of antibodies that showed no cross-reactivity with macrophages and therefore provide a means of differentiating between macrophages and osteoclasts. They concluded that these antibodies would prove useful in the identification of osteoclasts in tissues and in the separation of their circulating precursors.

Horton and Chambers (1986) developed a series of monoclonal antibodies that reacted specifically with human osteoclasts in foetal bone and also with a range

of neoplastic and non-neoplastic bone lesions. Several of the antibodies reacted with non-human osteoclasts suggesting possible experimental routes for the isolation and subsequent study of osteoclasts and their precursors. Furthermore, these authors suggested that the retention of some osteoclast antigens during phylogeny might indicate their functional importantance in the regulation of osteoclast activity.

Immunolabelling performed by Athanasou *et al.* (1988a) examined the monoclonal antibody, anti-L-35, and its reactivity in a wide variety of tissues. Anti-L-35 is known to react with human osteoclasts in foetal and adult bone as well as display high specificity for cells of the mononuclear phagocyte system. A high number of other tissues failed to label which lead Athanasou *et al.* (1988a) to conclude that monocytes, macrophages and osteoclasts contain common cellular antigens.

Further studies by Athanasou *et al.* (1986; 1991) attempted to identify surface antigens that might be specific for osteoclasts. These authors showed that monoclonal antibodies against human β_1- and β_3- integrins, as well as human macrophage-associated CD68 antigen, react with osteoclasts from several animal and avian species. Avian osteoclasts react with CD11a/18 and CD14 which are absent from human osteoclasts. Furthermore, Athanasou and Quinn (1990) revealed that Fc receptors were absent from osteoclasts in contrast to macrophage polykaryons.

Continuing work by Athanasou *et al.* (1988b,c) compared the antigenic phenotype of human foetal osteoclasts with that of human tissue macrophages

and polykaryons in foreign body lesions. A large number of monoclonal antibodies directed against myeloid antigens were used and the results showed that osteoclasts express a restricted range of macrophage-associated antigens including CD13, CD15a, CD44, CD45, CD54, ICAM-1, CD68 and CD71. Moreover, it was reported that the above antigens are found on macrophages and macrophage polykaryons both of which also strongly express CD11a,b,c, CD14, CD18, CD31, CD36, CD37, CD39 and CD43. Athanasou *et al.* (1988b,c) suggested that the presence of some macrophage-associated antigens on osteoclasts was consistent with their origin from the mononuclear phagocyte system. However, it was considered that the numerous differences in antigenic phenotype between osteoclasts and macrophage polykaryons indicated that their development and differentiation were not identical. It was further suggested that these differences should provide a method of distinguishing the two cell types in normal and diseased tissues, thereby supporting the earlier conclusions of Horton *et al.* (1985a,c).

Athanasou *et al.* (1991) identified osteoclasts in routinely processed bone specimens by using monoclonal antibodies directed against leucocyte common antigen (LCA) (PD7/26, 2B11), CD68 (KP1), and gpIIIa (Y2/51) but not against HLA-DR (CR3/43 and Ta11B5). It was shown that mononuclear cells on resorbing surfaces and occasionally against or near resting bone surfaces exhibit a similar pattern of reactivity, thereby providing further support for a phagocytic osteoclast precursor.

Monoclonal antibodies developed against osteoclasts associated with osteoclastomas (giant cell bone tumours) identified two antibodies termed 23C6

and 13C6 which were found to react with the vitronectin receptor (Horton *et al.*, 1985b). Athanasou (1996) also indicated that osteoclasts reacted with the $ß_3$-chain of the vitronectin receptor (CD51) as a member of the integrin family of glycoprotein membrane antigens. Originally, Horton *et al.* (1985b) and Horton and Davies (1989) believed that 23C6 was specific for osteoclasts but subsequently Athanasou *et al.* (1990), Athanasou and Quinn (1990) and Athanasou (1996) reported that there is no convincing evidence of a cell-surface antigen that is osteoclast specific. Kukita *et al.* (1989) and Athanasou *et al.* (1990) also found sites of vitronectin immunoreactivity, detected with 13C6 and 23C6, on macrophages and macrophage polykaryons.

Kukita and Roodman (1989) developed a monoclonal antibody termed Kn22 which reacted with osteoclasts and osteoclast precursors but is also found to react with cells early in the macrophage lineage. Kukita *et al.* (2001) further described a cell surface antigen which is considered to be a reliable immunological marker for identifying osteoclast precursors in rats. The Kat-1 antigen was immunohistochemically and ultrastructurally detected on mononuclear cells which share the same morphological features as preosteoclasts. Additionally, Kukita *et al.* (2001) assessed the relationship between the Kat1-antigen and the calcitonin receptor and determined that almost 100% of mononuclear cells in culture possess both characteristics. However, a significant number of calcitonin-positive mononuclear cells do not express the Kat1-antigen which indicated to Kukita *et al.* (2001) that subpopulations of preosteoclasts exist.

Nelson *et al.* (1991) characterized two monoclonal antibodies (211D and 312G) raised against human osteoclastomas by hybridizing spleen cells of osteoclast-immunized mice with plasmacytoma cells. These monoclonal antibodies showed specificity for membrane determinants on osteoclasts present in frozen sections of foetal bone. Furthermore, they showed no reactivity with either rabbit or rat osteoclasts, suggesting that they recognized unique epitopes on human osteoclasts.

Osdoby *et al.* (1992a) and Collin-Osdoby *et al.* (1995) reported an osteoclast-specific monoclonal antibody termed, 121F, which was described as a 200+ kDa glycoprotein located in the osteoclast plasma membrane with an associated peptide region highly homologous to superoxide dismutase. Because high levels of superoxides were released by osteoclasts resorbing bone, it was believed that the glycoprotein served in a novel way to protect the osteoclast from the lethal effects of superoxides generated during extracellular matrix resorption of bone.

The current use of monoclonal antibodies that attempt to identify osteoclasts was summarized by Athanasou (1996). Because there is no evidence of an osteoclast-specific cell-surface antigen (Athanasou *et al.*, 1990), this author recommended the use of the leucocyte common antigen (CD45) as well as other macrophage and granulocyte antigens including CD13, CD15, CD68 and CD54. These were recommended for the detection of osteoclasts and their precursors in routinely prepared histological specimens of human material (Athanasou, 1996), and were therefore considered for use in the present study.

- **KP1 (CD68)**

At the third Workshop on Leucocyte Differentiation Antigens several antibodies were grouped together based on their pan-macrophage activity. Antibodies coded Y2/131, Y1/82A, EBM11, Ki-M6 and Ki-M7 all precipitated an antigen of 110 kDa. KP1 antibody reacted with the same antigen but was also shown to label macrophages in paraffin-embedded tissue. This entire group of antibodies was classed as anti-CD68 by the Fourth Workshop (Micklem *et al.*, 1989). Pulford *et al.* (1989) described KP1 as a new monoclonal antibody raised against a lysosomal fraction of human lung macrophages. It was able to recognise a fixation-resistant epitope in a wide variety of tissue macrophages such as Kupffer cells, splenic and lamina propria macrophages as well as granulocyte precursors and osteoclasts. Tests on routinely processed samples of normal and reactive lymphoid tissues reveal a broad reactivity with cells of the monocyte-phagocyte lineage (Pulford *et al.,* 1989).

Evidence for the low specificity of KP1 as a histiocyte/myeloid marker was supplied by Tos *et al.* (1993) who examined KP1 expression in benign neural tumours. All tumours examined showed strong granular cytoplasmic positivity for KP1. In particular, Schwann cells reacted more positively which Tos *et al.* (1993) suggested was attributable to a high lysosome content. This finding supported previous evidence by Tsang and Chan (1992) who also questioned the specificity of macrophage reaction with KP1. These authors and Facchetti *et al.* (1991) performed immunohistochemical studies of non-histiocytic tumours (granular cell neoplasms and melanomas) that were rich in cells containing lysosomes. All reported positive immunostaining in tumours unrelated to the mononuclear

phagocyte lineage thereby suggesting that KP1 might be more appropriately viewed as a marker for lysosomes rather than for cells of histiocytic derivation. Sismanidou *et al.* (1996) used KP1 as one of a panel of selected immunohistochemical markers to study resorption and regeneration of periodontal tissues incident to orthodontic human tooth movement. Two populations of KP1-positive cells were identified in association with resorption areas. The first consisted of mononuclear cells located at a distance from the tooth surface, and the second were a multinucleated cell population in resorption lacunae in close contact with the tooth. The mononuclear cells were found in clusters with uniform staining for KP1 throughout their cytoplasm while the multinucleated cells stained preferentially in that part of the cell closest to the dentine surface. Sismanidou *et al.* (1996) suggested that the KP1-positive cells in the periodontal ligament might represent either precursors of odontoclasts or phagocytic scavenger cells of the macrophage lineage.

As KP1 has been shown to be one of a number of antibodies that recognise the CD68 antigen (Micklem *et al.*, 1989), it has been used to identify the CD68 antigen on mast cells (Horny *et al.*, 1990) and in neoplasms that have a high histiocyte, granulocyte or giant cell component (Warnke *et al.,* 1989; Watanabe *et al.*, 1997; Chang *et al.*, 2000; Femiano *et al.*, 2001). The ability of KP1 to identify a wide variety of tissue macrophages therefore allowed Athanasou *et al.* (1991) to successfully use the antibody to label osteoclasts in routinely-processed human tissues.

- **Cysteine proteinases (cathepsins)**

As described in an earlier section, cysteine proteases have been implicated in bone matrix degradation by osteoclasts (Delaissé *et al.,* 1980; 1984). Delaissé *et al.* (1991) identified cathepsins B, L and a cathepsin L-like 70 kDa proteinase via a cell fractionation procedure of mouse calvarial tissue. Multiple forms of cathepsin B were isolated from human osteoclastomas by Page *et al.* (1992) whereas Sasaki and Ueno-Matsuda (1992;1993) used a polyclonal antibody and immunoelectron microscopy to localize cathepsins B, G and L in both osteoclasts and the sub-osteoclastic resorption zone. Goto *et al.* (1993) similarly employed light microscopy of semi-thin cryosections immunolabelled for cathepsins B, D, and L, and reported that cathepsins B and L are localized within osteoclastic granules or vacuoles and also within the resorption lacunae. However, although cathepsin D is found within the osteoclast, it is not seen in the resorptive compartment suggesting that cathepsins B and L are directly involved in bone matrix degradation. Subsequent studies indicated that cathepsin L is the main cysteine proteinase responsible for the degradation of bone collagen during resorption (Murata *et al.,* 1991)

Various authors have described the immunohistochemical localization of cathepsin L in liver (Ii *et al.*, 1985) and kidney (Bando *et al.*, 1986; Yokota *et al.*, 1988). No reports were found of its immunolocalization in dental tissues.

- **The vitronectin receptor**

Athanasou *et al.* (1988b) indicated that osteoclasts express several antigens that are also involved in platelet aggregation and adhesion. Identified and named the vitronectin receptor (CD51) and a glycoprotein IIIa (gpIIIa), they are described as

members of the integrin family of membrane antigens which are involved in cell-to-cell and cell-to-matrix adhesion (Hynes, 1987; Ginsberg *et al.*, 1988). Hynes (1987) described integrins as non-covalently associated alpha (α)- and beta (β)-subunits in the form of α/β- heterodimers of which the vitronectin receptor is designated integrin β_3. Horton and Davies (1989) suggested that the vitronectin receptor and other matrix receptors might be involved in osteoclastic function as mediators of osteoclastic adhesion to bone, cell motility and bone resorption. Furthermore, Davies *et al.* (1989) identified an "osteoclast functional antigen" (OFA) which was determined to be biochemically and functionally related to the vitronectin receptor. The OFA is an abundant surface antigen in human and animal osteoclasts that is characterized by monoclonal antibodies 13C6 and 23C6 (Horton *et al.*, 1991). Davies *et al.* (1989) further characterized the OFA as a member of the cell adhesion receptor family that recognizes Arg-Gly-Asp (RGD) containing peptides.

In order to determine whether osteoclasts express other membrane receptors for matrix proteins, Quinn *et al.* (1991) analysed the antigenic phenotype of osteoclasts for the presence of integrins and other platelet-associated antigens involved in adhesion. It was revealed that osteoclasts and foreign body giant cells expressed the α-chains of the vitronectin receptor as well as reacting strongly with other antibodies against fibrinogen, fibronectin and vitronectin.

- **ED1**

A panel of three monoclonal antibodies considered to recognize cells belonging to the monocyte/phagocyte system in rats were termed ED1, ED2 and ED3 by Dijkstra *et al.* (1985). According to these authors, ED1 recognizes a cytoplasmic antigen in monocytes and most macrophages, but ED2 and ED3 are specific for membrane antigens. This enabled discrimination between different macrophage subpopulations which was confirmed by Beelen *et al.* (1987) who examined the different stages of macrophage differentiation.

By using cytochemical and immunolabelling techniques that monitored peroxidase activity, Beelen *et al.* (1987) determined that ED1 recognizes almost all members of the mononuclear phagocyte system, including blood monocytes, peritoneal macrophages, alveolar and tissue macrophages, and osteoclasts, and was subsequently considered a general monocyte/macrophage marker in rats. ED2 recognizes a membrane antigen found at a later stage of macrophage differentiation while ED3 only recognizes a particular macrophage found in lymphoid organs. Since that time, Damoiseaux *et al.* (1989) isolated several additional monoclonal antibodies (ED4, ED7, ED8 and ED9) raised principally against granulocyte subpopulations of macrophages.

While early work localized ED1 to the macrophage cytoplasm, Damoiseaux *et al.* (1994) revealed that the antigen is located on both the cell surface and on cytoplasmic granules in the form of lysosomes. The antigen appeared to be a heavily glycosylated protein of 90-110 kDa molecular weight. Harms *et al.* (1990) was able to use ED monoclonal antibodies to identify glycosyl receptors in macrophage subpopulations in the spleen and lymph nodes. Furthermore,

Damoiseaux *et al.* (1994) showed that the amount of ED1 expression is related to the phagocytic activity of the particular cell type.

The heterogeneity of the macrophage population in the gastro-intestinal tract in the rat, was demonstrated by Sminia and Jeurissen (1986) by using ED1, ED2 and ED3. In addition, Sminia and Dijkstra (1986) used tissue labelling with these antibodies to investigate the origin of osteoclasts in embryonic rat bone primordia. ED1-positive cells were found in the perichondrium/periosteum of developing bone and started to infiltrate the primordia when the cartilage became hypertrophic. As bone formed, multinucleated ED1-positive cells were detected in marrow cavities. Because monocytes and macrophages shared a common antigen with the large multinucleated cells, it was concluded that osteoclasts were derived from blood monocytes.

Jäger *et al.* (1993) used ED1 and ED2 to analyse the role of the mononuclear phagocyte system in orthodontic rat tooth movement. Using routinely processed, paraffin-embedded sections and the avidin-biotin-peroxidase-complex (ABC) technique, they showed that only ED1 proved suitable as it identified both mononuclear and multinucleated cells within the periodontal ligament. Akamine *et al.* (1994a) studied the nature of macrophages in experimentally-induced periapical lesions (apical periodontitis) in rats using a non-specific esterase and ED1. Macrophages were seen in control sections near regularly arranged osteoblasts and near detached osteoblasts at the initiation phase of bone resorption. In addition, numerous macrophages were widely distributed throughout the periodontium at the activation phase of bone resorption. However, macrophages were rarely seen near bone-forming surfaces and were localized in

the micro-abscesses at the start of bone formation. Akamine *et al.* (1994a) suggested that macrophages play an important role in activating osteoclastic bone resorption and in the inhibition of complete repair in bone remodelling.

An additional study by Akamine *et al.* (1994b) indicated that macrophage levels increase rapidly after initiation of the periapical lesion and remain high for 60 d. After 90 d, plasma cell populations increase and tissue repair begins to occur. The results suggested that macrophages have a close relationship to bone resorption and that plasma cells might participate in tissue repair rather than in the development of periodontal lesions.

- **Leucocyte common antigen (LCA)**

Thomas (1989) described the leucocyte common antigen (LCA) as a group of high molecular weight glycoproteins uniquely expressed on the surface of all leucocytes and their haemopoietic progenitors. Members of this group differ in both protein sequence and carbohydrate structures and are expressed in leucocyte populations in specific patterns. The literature refers to LCA under different names including T200 (Trowbridge and Mazauskas, 1976), B220 for the B cell form (Coffman and Weissman, 1981), the mouse allotypic marker Ly-5 (Komuro *et al.*, 1975), and more recently CD45 (Cobbold *et al.*, 1987) which remains the most accurate and widely used descriptive title.

The antigen has been localized to the cell membrane of leucocytes including thymocytes, lymphocytes granulocytes and macrophages and carries much of the carbohydrate of these cells. The abundance of carbohydrate allowed Trowbridge *et al.* (1975) to easily detect LCA using polyacrylamide gel

electrophoresis (SDS-PAGE) of lymphocyte membranes, while Ralph *et al.* (1987) described a number of structural variants. The function of LCA remains obscure but studies using antibodies to LCA have implicated this family of antigens in lymphocyte activation (Seaman *et al.*, 1981).

Because early reports by Horton *et al.* (1985b) failed to detect LCA on osteoclasts, it was considered that osteoclast precursors were not derived from multipotential haemopoietic stem cells. Furthermore, it was believed that osteoclasts were unlikely to be members of the mononuclear phagocyte system. Athanasou *et al.* (1987a) examined foetal and adult osteoclasts for the presence of LCA using a large panel of anti-LCA monoclonal antibodies, and showed that LCA is located on the surface of osteoclasts in specimens of fixed and decalcified osteoarthritic bone, as well as in bone imprints and cryostat sections. The intensity and pattern of osteoclast reactivity was similar to foreign-body type of macrophage polykaryons in inflammatory lesions. Consequently, Athanasou *et al.* (1987a) considered that osteoclasts and their precursors are derived from the multipotential stem cell that produces peripheral blood leucocytes and argued against their origin from a separate stem cell.

The leucocyte common antigen has also been used as a diagnostic discriminant between haemopoietic and non-haemopoietic neoplasms in paraffin sections. Kurtin and Pinkus (1985) employed antibodies PD7/26 and 2B11 to show that haemopoietic neoplasms are consistently highly reactive for LCA, whereas non-haemopoietic neoplasms are unreactive. It was concluded that LCA is an effective cell marker to enable the differentiation of tumour types.

- **CD13**

The CD13 antigen, or aminopeptidase N, is recognized as a 150 kDa membrane glycoprotein located on most cells of myeloid origin and is principally used in the identification of the myeloid leukaemias (Griffin *et al.*, 1981). In addition, CD13 has been shown to be present on fibroblasts, endothelial cells, bone marrow stromal cells, osteoclasts and epithelial cells of renal tubules and intestinal brush borders (Koch *et al.*, 1990; 1991). Athanasou *et al.* (1991) reported that CD13 is expressed by macrophages and provides an identifying mechanism for clast cells.

- **CD15**

The CD15 antigen is a 220 kDa carbohydrate structure also known as the X-hapten located in cell membrane glycoproteins and glycolipids and also in paranuclear granules. CD15 is described as a granulocyte-associated antigen but is also found on eosinophils and to a lesser degree on monocytes. Hsu and Hsu (1994) reported that the antigen is thought to play a role in inflammation and in the mediation of phagocytosis and further, is useful in the identification of Hodgkin's disease. As a granulocyte marker Athanasou *et al.* (1988c) employed this antigen in the identification of clast cells and their precursors. These authors considered that clast cells and macrophages shared many immunolabels.

1.3 Root resorption

Whereas deciduous root resorption is a normal physiological phenomenon associated with natural exfoliation (Sahara *et al.,* 1992), permanent tooth root resorption is invariably associated with pathological situations (Hammarström and Lindskog, 1985; Tronstad, 1988; Andreasen, 1988; Pierce, 1989a,b). Internal resorption of dentine from within the dental pulp has been shown to be associated with trauma and infection (Wedenberg and Lindskog, 1987). Additionally, an undesirable problem facing the clinician is the treatment of external inflammatory root resorption subsequent to trauma (Andreasen and Andreasen, 1992).

Andreasen (1988) and Andreasen *et al.,* (1990) described several types of resorption based on the PDL reaction. Inflammatory resorption was observed if large, active resorption lacunae are present along with an intense, usually pulpally-derived inflammatory response, and replacement resorption is noted if ankylosis occurs. The site of the resorption may vary from apical, lateral to cervical dependent of the type and aetiology of the resorptive process (Vlaskalic *et al.,* 1998). Invasive cervical resorption may be observed subsequent to dental trauma, tooth bleaching, orthodontic treatment or other factors detailed by Heithersay (1999a,b).

Root resorption is an unfavourable sequela of orthodontic tooth movement first reported by Ketcham (1927). The application of tooth moving forces and their mechanotransduction by the tissues and cells of the PDL results in a sterile inflammatory response that facilitates bone resorption and deposition in the direction of movement (Davidovitch, 1991). The tissue damage and cellular

breakdown, with the subsequent release of inflammatory mediators and cytokines produce in a complex series of destructive and reparatory events that often leads to the loss of root structure. Comprehensive reviews of orthodontic resorption have been written by Linge and Linge (1991), Brezniak and Wasserstein (1993a,b; 2002a,b), Vlaskalic *et al.* (1998) and Bosshardt *et al.* (1998).

The cells implicated in root resorption have been identified as clastic in nature since they have been identified as large, multinucleated and possessing properties similar to the osteoclast (Addison, 1976).

1.3.1 The odontoclast

Based on the identification of the osteoclast and odontoclast cell types as both resorptive, located in Howship's lacunae and possessing similar cytological features, Jones and Boyd (1988) stated that there was no reason to believe that the cell types differed except in their relative substrata. These authors reported that multinucleated odontoclasts are polarized with respect to dental tissues and possess a ruffled border within an annular clear zone that is closely adherent to mineralized tissues. Wesselink *et al.* (1986) described odontoclasts as capable of having two ruffled border areas that resorbed bone and tooth surfaces simultaneously. Accordingly, Jones and Boyd (1988) suggested that if an odontoclast was defined as an eukaryotic cell that is capable of resorbing mineralized dental tissues, "osteoclasts became odontoclasts with alacrity".

Addison (1976; 1978a; 1978b; 1979; 1980) examined odontoclasts and osteoclasts in a number of species-comparative studies. In a quantitative investigation of odontoclasts from resorbing roots of human primary teeth

subjected to dehydrogenase and acid phosphatase, Addison (1978a) showed that the mean number of nuclei per odontoclast was 7.8 (SD ± 4.8) with a median of 6.4. This author suggested that the median is a more important measure of central tendency as the distribution of cells with varying numbers of nuclei is asymmetric with a positive skew. In addition, it was revealed that 5% of odontoclasts have 15 nuclei or more which led to the conclusion that there is an optimum size for maximum resorptive efficiency. Addison (1978a) further considered that the size of the odontoclasts might significantly affect the cell's ability to form an effective ruffled border system.

Another comparative study by Addison (1979) examined the enzyme histochemical characteristics of human and kitten odontoclasts and osteoclasts. Enzyme profiles suggested that odontoclasts have similar properties and metabolic functions to those of osteoclasts and that species differences appear to be minor. Addison (1979) further contended that both odontoclasts and osteoclasts are rich in enzymes concerned with energy production, particularly phosphatase activity, as well as those related to catabolic functions. Consistent with this finding was the further observation that large numbers of mitochondria and lysosyme-like organelles are present in both cell types.

In a later study, Addison (1980) described the effects of low dose PTH on feline odontoclasts and, in particular, on the number of odontoclast nuclei. PTH administered intravenously was found to have a dramatic and almost instantaneous effect on increasing numbers of nuclei. Addison (1980) did not offer an explanation for the increase in number of nuclei but noted that

parathyroid extract was able to affect cells involved in active resorption by stimulating dehydrogenase activity.

Earlier ultrastructural studies by Freilich (1971) examined the effects of PTH on both the fine structure of odontoclasts and on their acid phosphatase activity. It was determined that active odontoclasts possess cytoplasmic processes that enter dentinal tubules, and that acid phosphatase is present both intra- and extra-cellularly. However Freilich (1971), by using a modified Gomori acid phosphatase reaction, determined that the subcutaneous administration of PTH increased the number of extracellular dense bodies showing acid phosphatase activity but did not appear to influence intracellular phosphatase activity. Freilich (1971) considered that the identification of odontoclasts might therefore be possible by the localization of certain plasma proteins. Subsequently, immunofluorescent staining by Okamura *et al.* (1980) demonstrated albumin, α-antitrypsin, α_2-HS glycoprotein, transferrin and several immunoglobulins in the cytoplasm of odontoclasts and also within human dentine.

Odontoclast research has mainly utilized the physiological resorption of deciduous teeth as an experimental model. Matsuda (1992) conducted an ultrastructural and cytochemical study of odontoclasts gathered from trypsin-treated dentine and cemental surfaces. Extensive ruffled borders were described along with multiple phagosomes containing tannic-acid stainable amorphous inclusions. It was further noted that odontoclasts did not phagocytose collagen fibrils but they exhibited acid phosphatase activity. Matsuda's (1992) observations led to the conclusion that odontoclasts resorb the non-collagenous

component of the dental organic matrix via the release of hydrolytic enzymes and have the capacity to demineralize hard tissue by H^+- K^+- ATPase activity.

Sahara *et al.* (1994), employing a similar model, expanded Matsuda's (1992) study and reported that odontoclasts are capable of resorbing the superficial non-mineralized layer of predentine. Light and electron microscopy indicated that, as root resorption was nearing completion, multinucleated cells were observed between degenerative osteoblasts on the predentine surface of the coronal dentine. These cells had the same ultrastructural characteristics as odontoclasts and excavated resorption lacunae in the non-mineralized dentine. In addition, histochemical demonstration of tartrate-resistant acid phosphatase activity revealed intense staining in intracellular lysosomes. Sahara *et al.* (1994) concluded that multinucleated odontoclasts are capable of resorbing non-mineralized predentine matrix *in vivo,* probably in a similar fashion to the manner in which they resorb demineralized dentine matrix.

Domon *et al.* (1994) reported the existence of a mononuclear odontoclast *in vitro*, but these authors were uncertain as to its existence *in vivo*. Using an exfoliating deciduous tooth model and an azo dye for detecting TRAP activity, results indicated that TRAP activity was present in both multinucleated and mononuclear odontoclasts. Ultrastructurally, a smaller ruffled border area was observed on the mononuclear cell leading Domon *et al.* (1994) to conclude that cells with ruffled borders and clear zones may be identified as odontoclasts or osteoclasts irrespective of the number of nuclei.

Okamura *et al.* (1993) employed *in situ* hybridization techniques to identify collagenase-producing cells and to determine the role of collagenase in root resorption of deciduous teeth. A digoxigenin-labelled, non-radioactive RNA probe detected collagenase *m*RNA expression in odontoclasts, macrophages, fibroblasts, odontoblasts and cementoblasts around bovine resorbing tooth roots. In addition, TRAP activity and interleukin-1 *m*RNA expression was also observed in odontoclasts, fibroblasts and macrophages leading Okamura *et al.* (1993) to conclude that collagenase produced by odontoclasts might play a role in dentine collagen degradation and that interleukins could be an important factor in promoting root resorption.

Sasaki and Uneo-Matsuda (1992) demonstrated cathepsins B and G in lysosomes, vacuoles and within the extracellular channels of the ruffled border in odontoclasts using ultrastructural immunogold labels. The presence of these proteolytic enzymes suggested to the authors that they are of prime importance in the intra- or extra-cellular degradation of collagen and other non-collagenous matrix proteins in the resorption of deciduous teeth.

Histological and histochemical observations of deciduous teeth by Sahara *et al.* (1992) determined that odontoclastic resorption usually occurred at the pulpal surface of coronal dentine, and in a specific time-related pattern. During physiological root resorption, coronal pulpal tissue retained its normal structure until root loss was almost complete. Multinucleated odontoclastic resorption was seen to proceed from predentine to dentine on the pulpal surface at the cervical areas of the crown before spreading towards the pulp horns.

Further research by these authors examined the cytodifferentiation of odontoclasts during root resorption (Sahara *et al.,* 1996). In this light and electron microscopic study, odontoclasts differentiated from TRAP-positive mononuclear cells which were presumed to originate from circulating progenitors. Ruffled borders, clear cytoplasmic zones and multinucleation occurred only after contact with the substrate surface. Sahara *et al.* (1996) confirmed that odontoclasts resorb predentine before dentine and suggested that the processes are similar to those responsible for the resorption of bone. It was also noted that the end of the resorptive process was characterized by loss of the odontoclast ruffled border and detachment from the resorbed surface.

Shigeyama *et al.* (1996) examined resorption at the cemento-enamel junction in feline teeth by using specific antibodies and immunohistochemical analyses to localize adhesion molecules associated with mineralized tissues. Osteoclast/odontoclast numbers were increased in resorptive lesions, bone sialoprotein (BSP) and osteopontin (OPN) were identified in tissues, and a complementary clast cell surface receptor (integrin α_v β_3) was linked to these molecules. Shigeyama *et al.* (1996) considered that OPN was localized to resorption fronts and reversal lines whereas BSP was localized to reversal lines only. Odontoclasts were found in juxtaposition to mineralized surfaces not associated with OPN and the cell surface integrin receptor, $\alpha_v\beta_3$, was localized to odontoclastic surfaces. Shigeyama *et al.* (1996) concluded that this integrin receptor is involved in the resorptive process and facilitates the attachment of clastic cells to their substrate.

Dentinoclast as a term has been used to describe multinucleated clastic cells that resorb dentine and they have been isolated from rat molars and cultured successfully by Pierce and Lindskog (1989) and Gilles *et al.* (1994). Pierce (1989a) suggested that osteoclasts and dentinoclasts were possibly indistinguishable cell types and that both resorb mineralized substrata. Pierce and Lindskog (1987; 1989) showed that dentinoclastic tooth resorption *in vitro* may be controlled by the early administration of calcitonin and prednisolone or exacerbated by the introduction of hydrocortisone. Scanning electron microscope evidence indicated that all substances inhibited dentoclast spreading and attachment and hence resorption. Pierce and Lindskog (1989) considered that the direct effect of steroids on resorption was one of inhibition but that local secondary effects might moderate a systemic *in vivo* effect.

Lindskog and Pierce (1988) showed that dentinoclasts spread and colonize surface dentine in a time-related fashion. Scanning electronmicroscopy at predetermined time intervals determined that dentinoclasts follow a general pattern of attachment and spreading on solid substrata. Lindskog and Pierce (1988) produced a spreading model characterized by filopodial exploration of the substrate surface, cell enlargement and gradual disappearance of the filopodial fringe before final adaptation and active resorption are seen.

Pierce and Lindskog (1987) provided evidence for direct inhibition of inflammatory root resorption by the use of a corticosteroid/antibiotic paste. The culture of isolated dentinoclasts with a steroid paste inhibited cell spreading which suggested that the intrapulpal *in vivo* application of a steriod paste could

arrest inflammatory root resorption by the detachment of dentinoclasts from the root surface.

1.4 Models of root resorption

Experimental models for the study of bone resorption are numerous and principally based on osteoporosis in humans (Frost and Jee, 1992) and in normal and ovariectomized animals (Kalu, 1991, 1995; Jee *et al.*, 1993). The use of a dental model for the study of root resorption has also involved human investigation (Barber and Sims, 1981) and numerous animal studies. The examination of root resorption accompanying the exfoliation of primary teeth has provided a physiological model for both tooth and bone resorption (Sasaki *et al.*, 1988a; Sahara *et al.*, 1996).

Sasaki *et al.* (1988a), Sahara *et al.* (1993) and Sahara *et al.* (1996) examined the cells and resorptive processes in extracted deciduous teeth using light and electron microscopic morphological, enzyme histochemical and immunocytochemical techniques. Their studies revealed the dominant role of the odontoclast in the removal of calcified dental tissues.

Pathological inflammatory root resorption has been simulated by Andreasen (1980a,b; 1981b) by the extraction and replantation of teeth. Ocherse (1959) indicated that the incisors of the vervet monkey are easy to extract and similar to human teeth, and these animals were used by Andreasen (1980b; 1981b) as a human-like model system. Reimplantation of an incisor after extraction and thirty minutes of drying, simulated the clinical situation of avulsion, and offered a reliable inflammatory resorptive model that histologically revealed a number of

dental aspects of clastic cell activity (Andreasen, 1975; 1987; Andersson *et al.,* 1987). Andreasen (1975) and Andreasen and Kristerson (1981a) outlined a number of periodontal ligament reactions which could result 8 wk after the procedure. These were described as (1) no root resorption, (2) resorption and repair with cementum, (3) active resorption with inflammation, and (4) ankylosis. It was further determined that the period of time that the tooth was out of the mouth and allowed to dry has a significant effect on the histological response (Andreasen, 1980a; 1980b). In addition, the trauma of tooth removal affected the results with a slow traction causing minimal resorption by tearing the periodontal ligament fibres rather than by creating a crushing injury (Andreasen and Andreasen, 1992).

While the trauma of the extraction process and the drying of the tissues determined the type of resorptive response, Andreasen and Kristerson (1981b) attempted to control the resorption process by performing endodontic therapy on the extracted teeth prior to reimplantation. According to these workers and Andreasen (1981a), pulp removal prevented the development of inflammatory resorption but left ankylosis and surface resorption as the possible procedural sequelae. These responses were further related to the extent of cell death along the PDL where it was revealed that larger areas lead to ankylosis and smaller areas tended to repair. Andreasen (1981a) and Andreasen and Kristerson (1981a) explained this finding by a more complex and competitive healing process involving bone marrow-derived cells entering the large areas of injury and producing the ankylotic response. The smaller areas of cellular death appeared to be managed by more limited local response. As a result of

Andreasen's (1980a,b; 1981a,b) research, a reliable resorption model was developed in monkeys that could control the extent and type of response created.

The extraction-reimplantation model was further utilized by Lindskog *et al.* (1983) and Blomlöf *et al.* (1987) who, in addition to extracting incisors in monkeys *(Macaca fasicularis)* deliberately caused further damage to the PDL and root surfaces. Scraping of root-adhering tissue or the mechanical drilling of experimental cavities in root surfaces prior to the quick reimplantation of the tooth exacerbated the resorptive and reparative responses. After 8 wk, reparative cementum was observed within the traumatic defects but was easily separated from the previously denuded dentine. However, firm reparative cementum attachment was established to dental cementum at the periphery of the resorption cavities. Lindskog *et al.* (1983) further observed that over the experimental period the reparative cementum did not fill the root surface cavity in the reimplanted teeth and there appeared to be a corresponding adjustment in alveolar bone morphology in order to maintain the width of the PDL. The surrounding tissue contained many epithelial and endothelial cells and it was therefore hypothesized that these cells contributed to the integrity of the PDL and prevented ankylosis and resorption of the tooth root.

The application of mechanical forces to teeth has provided models of aseptic root resorption in both animals and humans (Barber and Sims, 1981; Langford and Sims, 1982). Langford and Sims (1981) reported that orthodontic forces can have dire consequences with respect to tooth root integrity and severe resorption may lead to tooth loss. Davidovitch (1991) established that many factors were involved with the generation, extent and prognosis of root resorption as a result of

orthodontic tooth movement. Tooth movement was achieved by Rygh (1977; 1984; 1992) via the cementation of an orthodontic finger spring to the incisors of rodents in order to apply a lateral force to the molars. Williams (1984) and Brudvik and Rygh (1993a;b) altered the mechanical design to allow a coil spring attachment between the two groups of teeth. This resulted in approximation of the incisors and molars and therefore permitted a study of the PDL cellular changes and the resorption-repair mechanisms of tooth and bone (Brudvik and Rygh, 1994b; 1995a).

A simpler tooth movement system was designed by Yen *et al.* (1992) who stretched an "alastic" orthodontic module between the anterior and posterior teeth of mice in order to generate tooth movement. More complicated systems were developed by Clark *et al.* (1991) who constructed a complex extra-oral extrusion device that extended intra-orally to contact the first molars. This device was capable of delivering an axial load of constant magnitude for a short period of time which allowed Clark *et al.* (1991) to examine the periodontal vasculature under stressed conditions.

Magnets in intra-oral holders have also been an effective way of generating extrusive or intrusive orthodontic forces. Crowe (1989) and Weir (1990) investigated the combined effects of endodontic therapy and orthodontic extrusion on marmoset incisors but for differing periods of time. Their efforts attempted to duplicate a clinical situation in which traumatic tooth fracture (Andreasen, 1981a) was managed by these combined techniques. Magnets embedded in the fractured marmoset incisor and in the metallic holder cemented to the other teeth supplied the extrusive load. Importantly, these studies revealed

that long term stabilization and review of the periodontal ligament structures indicated a return to normal of the previously challenged tissues.

Apart from mechanical trauma delivered by orthodontic mechanisms, other root resorption models have aided research. Cvek and Lindvall (1985) examined the use of hydrogen peroxide as a bleaching agent for human teeth that had previously been subluxated or luxated through injury. It was suggested that damage to the periodontium caused by the chemical irritant might result in healing or ankylosis of resorptive areas. When complicated by bacterial contamination, progressive root resorption associated with persistent inflammatory change was evident in the periodontal tissues. Chemical irritation was also employed by Wedenberg and Lindskog (1985) who studied internal resorption of monkey incisors. The teeth were opened, injected with Freund's complete adjuvant and either sealed or left open to the oral cavity. Following varying observational periods and employing a variety of detection systems, macrophage-like cells were observed colonizing the walls of the pulp chambers and tartrate-resistant acid phosphatase activity characteristic of hard tissue resorbing cells was evident in pulp tissue. From this study, Wedenberg and Lindskog (1985) proposed that internal tooth resorption is transient or progressive in nature depending on the continuity of the insult.

Birkedal-Hansen (1973) used instrument luxation techniques to study external root resorption pattern in rats. Surface root resorption of the mandibular molars occurred primarily on the sides where pressure was exerted over a 3-wk period. During that time, resorption increased considerably and no definitive evidence of repair was observed. Hellden (1972) created a resorptive area by drilling into the

vestibular root surfaces of human teeth *in situ*. Cementum repair was seen after 23 d, indicating the ability of the PDL to heal following a severe traumatic event. A modification of Hellden's (1972) technique was used by Nakane and Kameyama (1987) who mechanically injured the rat PDL by inserting a flat, needle-like instrument into the gingiva and cervical periodontal ligament of maxillary molars. Root resorption developed from day 1 and increased over the observational period. These workers concluded that mechanical injuries to human gingivae accidental or incidental to dental treatment may cause resorption of the adjacent root surfaces.

Thermal insult has also provided a model for the study of root resorption. Wesselink *et al.* (1986) studied the processes leading to the resorption and ankylosis of teeth by the local application of liquid nitrogen to the outer surface of the mouse lower jaw causing freezing of the incisor teeth and surrounding tissues. Subsequent light and electron microscopic examinations revealed periodontal ligament cell death followed by repopulation by fibroblasts and macrophages. Seven to 12 d following cold exposure, root resorption and some ankylosis was seen.

Tal and Stahl (1986) modified the freezing technique by using a gas expansion micro/cryoprobe that generated temperatures of –81°C. The tip of the cryoprobe was placed directly on exposed buccal bone over the roots of rat first molars after reflection of the gingivae. Early tissue changes reported were similar to those described by Wesselink *et al.* (1986). However, root resorption was marked at 5 to 7 wk in conjunction with reparative cementum and areas of ankylosis.

Dogs were used as the experimental animal by Tal *et al.* (1991) who froze the buccal plate over canine teeth. A cryoprobe freeze-thaw cycle was repeated three times and the tissues histologically examined at 1 h, 48 h and 30 d. Although the 1-h specimens were indistinguishable from controls, by 48 h, the cellular elements of the PDL could not be identified but inflammatory response was minimal and the collagenous framework of the PDL formed a continuum between bone and cementum. By 30 d the frozen periodontal ligament did not differ significantly from surrounding tissues and neither bone resorption nor ankylosis was seen in the experimental sites. This differed from earlier reports by Wesselink and Beertsen (1988) in which extensive root resorption was detected subsequent to a cold thermal insult.

CHAPTER 2

AIMS

2.1 Justification

Resorption in the dentoalveolar apparatus is a complex and only partially understood process. Alveolar bone is normally resorbed as teeth erupt into the oral cavity (Wise, 1998) or move under the influence of either physiologic migration (Picton, 1976) or an orthodontic force (Davidovitch, 1991). Skeletal bone in health undergoes appositional and resorptive processes of growth and calcium homeostasis that are governed by metabolic, physiologic and functional needs (Frost, 1989). Bone may also be lost due to disease, as occurs with tooth loss, periodontal disease, or with local and systemic bony pathologies, including the common debilitating condition of osteoporosis in the elderly (Christiansen, 1991; Marcus, 1996).

Even though much research has been undertaken (Reitan, 1974; Rygh, 1977; Bosshardt *et al.*, 1998) and comprehensive reviews have been published in the orthodontic literature (Brezniak and Wasserstein, 1993a,b; 2002a,b; Vlaskalic *et al.,* 1998; Mah *et al.*, 2000), the current lack of understanding of root resorption as an adverse consequence of tooth movement, persists. Neither predisposing nor initiating factors, or factors that might control this undesirable sequela of orthodontic treatment have been fully determined. Recently hypothesized as being of importance is the possibility of a systemic propensity due to pre-existing inflammatory medical disorders (Davidovitch *et al.,* 2000), or a genetic or individual biological susceptibility. Mechanical factors related to the type,

magnitude and duration of the applied force have also been shown to be of significance (Linge and Linge, 1991; Sandy *et al.*, 1993).

Once initiated, the prevention and control of root resorption remains an important and major goal of dental science. While considerable attention has been given to the minimization of force delivery to physiologically acceptable levels, pharmacological agents also have been employed to influence the implicated inflammatory process. Inflammatory inhibitors such as steroid hormones (Ross *et al.*, 1994) and non-steroidal anti-inflammatory drugs (Park *et al.*, 2000) and osteoclast inhibitors including the bisphosphonates (Cecchini and Fleish, 1990; Sato *et al.*, 2000) and bafilomycin A1 (Sundquist *et al.*, 1990) have been administered to both experimental animals and patients, but only with equivocal success.

Although pathological resorption of permanent teeth is rare, evidence to date indicates many similarities in the mechanisms of pathological resorption of both bone and dentine. The inflammatory bone resorption that facilitates tooth movement is considered to be a similar process to that which results in resorptive damage to the teeth (Jones and Boyde, 1988). That there is not more root resorption due to mechanical stress is fortuitous and beneficial to orthodontic practice. Considered to be of a protective nature within the periodontal ligament (PDL), is the cellular blastic layer lining hard tissue surfaces (Andreasen, 1988), and lining layers of unmineralized osteoid or cementoid matrix (Jones and Boyd, 1988; Tronstad, 1988). Elevated levels of matrix metalloproteinases in cementum (Morris *et al.*, 1993) or the presence of a cementum anti-invasion factor (Lindskog and Hammarström, 1980; Wedenberg and Lindskog, 1987) have also

been considered to be local protective factors. The epithelial cell rests of Malassez have also been suggested as a protective barrier preventing the vascular ingress of clast cells (Spouge, 1980). Despite the lack of detailed knowledge on the nature of this protection, it is apparent that root resorption is initiated when these factor(s) break down or are overcome.

The cells responsible for root resorption, odontoclasts, have been reported to be morphologically similar, if not identical, to osteoclasts (Furseth, 1968; Freilich, 1971; Nilsen, 1977; Lucht, 1980) but do not appear to reach the size or nuclear numbers of their osseous counterparts. Research to date indicates that both cell types share similar enzymatic properties and resorb hard tissue in a similar fashion by producing large surface pits or lacunae as they move between sites of resorption. However, little work has been done to examine the relationships between odontoclasts and osteoclasts, and establish whether they are phenotypically the same cells.

Examination of the PDL at sites of inflammatory root resorption often reveals simultaneous alveolar bone and root resorption occurring on either side of the inflamed ligament. The dentoalveolar complex therefore becomes a useful system for the study of mineralized tissue resorption. Most of the different types of resorption seen therein are the result of one or more of a concert of aetiological factors, causing similar resorptive processes mediated by similar clastic cells. Hence, by studying one of these situations, valuable information applicable to the other types may be gained and data relevant to the pathogenesis of dentoalveolar resorption revealed. In order to further research these mechanisms and increase our understanding of normal and pathological

hard tissue resorption and remodelling in the dentoalveolar complex, a reliable yet simple animal model of hard tissue resorption in the periodontium would be advantageous. Not only would this allow a comparison between the contemporaneous and allied processes of root and bone resorption, but the study of basic clastic cell functions may offer the possibility of exploring therapeutic interventions for all pathological dentoalveolar resorptions, for which there are currently no ideal preventive nor management regimes.

As most of the orthodontic models used to examine aseptic root resorption and the accompanying cellular activity have been cumbersome, a simple model based on the application of a cold thermal insult directly to the tooth may be a way of conveniently creating resorptive activity in the PDL that would allow for the study of clast cell behaviour. Unlike bone resorption, currently there is no clear understanding of the cellular processes and mechanisms involved in root resorption. Most researchers believe that osteoclasts attack cementum and dentine with the same zeal as they resorb bone (Jones and Boyd, 1988) although there are clear differences between the structure and composition of the two hard tissues (Saygin *et al.,* 2000).

Furthermore, control of the root resorption process would assist in minimizing the hard tissue destruction and so preserve tooth integrity. In this regard, a recently discovered bone resorption inhibitor, osteoprotegerin, may be useful in preventing dental resorption. This study aims to assess the effects of osteoprotegerin on odontoclasts in a new model of aseptic root resorption.

2.2 Aims of the study

The aims of this study are:

1. To develop a reliable, reproducible and predictable model of aseptic dentoalveolar resorption in the rat.
2. To investigate the cellular reactions responsible for the progression and repair of resorption and periodontal healing in this model, using light and electron microscopy, light microscopic immunocytochemistry and enzyme histochemistry.
3. To identify the cells responsible for tooth root and bone resorption (odontoclasts and osteoclasts), with particular emphasis on characterising the odontoclast and establishing its relationship to the osteoclast, using transmission electron microscopy and light microscopic immunocytochemistry.
4. To use the dentoalveolar resorption model to study the effects of an osteoclast inhibitor (osteoprotegerin) on osteoclast and odontoclast activation using light microscopic immunocytochemistry and transmission electron microscopy.
5. To examine the use of osteoprotegerin as a possible mechanism for the therapeutic management of dental resorption seen as an adverse sequela of orthodontic treatment.

2.3 Null Hypothesis

The prevention and management of osteoporosis by the administration of osteoprotegerin has proven to be beneficial in animal model systems (Simonet *et al.*, 1997; Bucay *et al.*, 1998). A similar effect in a dental model would have profound implications for tooth longevity in situations where orthodontic or periodontal osteolytic inflammatory stimuli are acting.

The null hypothesis being investigated in this study states that **"Osteoprotegerin, as a clast cell inhibitor, prevents osteoclastogenesis and hence root resorption in an experimental rat model that utilizes a cold thermal stimulus".**

CHAPTER 4

MATERIALS AND METHODS

3.1 Materials

A total of 106 male Sprague-Dawley rats were used throughout this project. Seventy-four animals were used in the first phase of the study involving the establishment of the resorption model and accompanying immunolabelling and electron microscopy examinations. The remaining 32 rats were used in a resorption inhibition experiment involving OPG. Each animal weighed between 250 and 300 g and was aged between 8 and 10 wk, at which time their dentitions had fully developed with minimal root hypercementosis (Schour and Massler, 1971). The rats were divided into experimental groups of 6 or 8 animals for the various procedures. All were fed a diet of commercially manufactured standard rodent pellets (Parastoc Feed, Ridley AgriProducts, Murray Bridge, Australia) and water *ad libitum,* and were housed in the Animal House facility of the Medical School of The University of Adelaide. Approval for the experimental procedures was provided by the Ethics Committee of The University of Adelaide under ethics numbers S/28/95A,B,C,D,E and SO/61/96,B,C,D.

3.2 Methods

3.2.1 Anaesthesia

A pilot experiment was conducted in order to determine the most appropriate anaesthetic to stop all reflex activity and keep the animals unconscious for at least 1 h during and after the various procedures. It has been reported that the thaw following tissue freezing is a painful procedure (Gage, 1982) and an attempt was made to keep the rats as comfortable as possible.

The anaesthetic pilot study involved freezing the upper right first molar of 6 rats with dry ice (CO_2, -81°C) for 10 min. Two animals were sacrificed by carbon dioxide inhalation at periods of 2, 7 and 14 d after the thermal insult and their maxillae dissected out and processed for routine histological examination.

The anaesthetics trialed with their recommended dosages are presented below. The anaesthetics used were:

1. Saffan (alphaxalone/alphadolone acetate, Pitman-Moore Australia Ltd. North Ryde, Australia), injected intramuscularly at a dosage of 12 mg/kg of body weight.
2. A combination of xylazine (3 mg/ml) and ketamine (10 mg/ml, Parke-Davis, Caringbah, Australia) injected intraperitoneally.
3. Nembutal® (pentobarbitone sodium, 60 mg/ml, Boehringer Ingelheim Pty Ltd, Artarmon, Australia) injected intraperitoneally at a dosage of 20 mg/ml per 100 g of body weight.
4. A combination of Hypnorm® (fentanyl citrate, 0.315 mg/ml and fluanisone 10 mg/ml, Janssen-Cilag Ltd., High Wycombe, Buckinghamshire, UK) and Hypnovel® (midazolam hydrochloride, 5 mg/ml, Roche, Berne,

Switzerland). The two drugs were diluted 1:1 with sterile water for injection, combined, and then administered intraperitoneally at a dosage of 2.7 ml/kg of body weight.

3.2.2 Thermal insult

Following weighing and Hypnorm®/Hypnovel® anaesthesia, individual rats were placed on their back on a specially constructed rack. The mouth was propped open by metal rings looped around the upper and lower incisors. The rings were individually attached by elastic bands to the supportive rack so that there was a diametrically opposite pull on the incisors which gently stretched the mouth open. The tongue was placed into the lower ring and therefore protracted from the operative field. A small spatula was used to retract the right cheek and so expose the upper right first molar. The restraining rack and retracting mechanism allowed one operator to perform the experimental procedures.

The upper right first molar was frozen for varying periods and frequencies by the continuous application of customized pellets of dry ice (CO_2 at –81°C, BOC Gases, Adelaide, Australia) held in tweezers. Large tubular pellets provided by the manufacturer were quartered using a sharp chisel producing smaller pellets with diameters approximating the crown size of the rat upper first molar. Care was taken to apply the dry ice to the occlusal aspect of the teeth and to avoid contact with surrounding soft tissues. Following the application of cold, the tissues thawed slowly under anaesthesia and the animals allowed to recover. The upper left first molar was left unfrozen and served as a control. A schematic representation of the freezing process appears in Diagram 3 below.

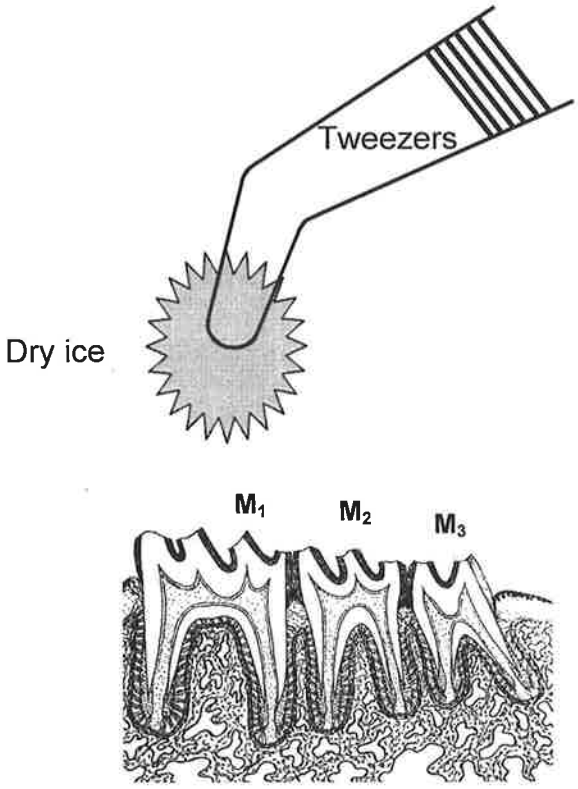


Diagram 3. Schematic representation of dry ice application to the rat upper first molar crown drawn approximately to scale. M_1 – first molar, M_2 – second molar, M_3 – third molar

3.2.3 Sacrifice

The animals involved in pilot studies were sacrificed in an enclosed chamber by carbon dioxide inhalation. The jaws were immediately dissected out and placed in appropriate fixative.

Subsequent experimental groups were sacrificed during the following perfusion fixation procedures.

3.2.3.1 Carotid perfusion

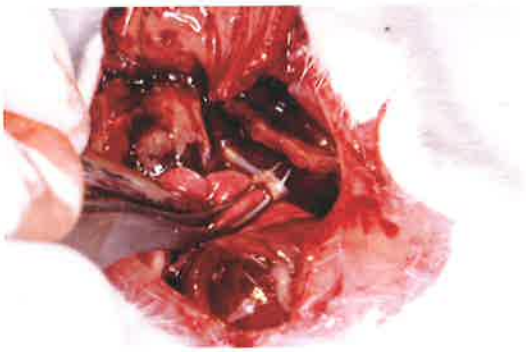
In order to adequately fix the jaws a perfusion technique via the carotid arteries was performed. Animals were anaesthetized with Hypnorm®/Hypnovel® as previously described. Surgical anaesthesia was determined by the loss of both plantar and corneal reflexes. Each animal was placed on its back in a dissecting tray and a subdermal incision was made obliquely along the medial surface of the right hind limb. The skin was reflected and the overlying fascia was blunt dissected to expose and clear the femoral vein. An injection of heparin B.P. (David Bull Laboratories, Mulgrave, Australia) was administered intravenously at a dose of 0.02 ml/100 g of body weight (Appendix 1).

A carotid cannula was assembled prior to experimentation (Appendix 2). It was flushed with heparin to avoid undesirable coagulation of blood entering the tubing. The dissection of the neck and the isolation and cannulation of the right and left carotid arteries required 2 operators and was performed according to the following procedure.

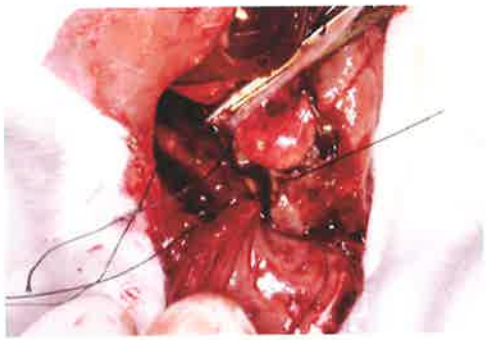
The rostral end of the sternum was palpated and a horizontal subdermal incision was made to 15 mm either side of the midline with curved dissecting scissors. A midline vertical incision was made to the level of the hyoid bone. The overlying fascia was dissected away in the midline with care taken to avoid the large external jugular veins (Greene, 1971). Using a fine pair of curved scissors, the fibres of the sternohyoid muscle were blunt dissected in the midline in a vertical direction to reveal the trachea beneath. The sternohyoid muscle was retracted with tweezers while the carotid bundle was located lateral and deep to the trachea. The fine beaks of the curved tweezers were hooked beneath the bundle and the vagus nerve was carefully separated. Two black silk sutures were passed around the carotid artery and loosely tied.

Isolation of the right carotid artery preceded the left. The 2 sides of the cannula were taped to the sides of the animal with micropore adhesive so that the tips of the cannula were at the level of the neck dissection. The passive positioning of the cannula prior to insertion into the vessel helped to minimize difficulty and ensured an uneventful procedure.

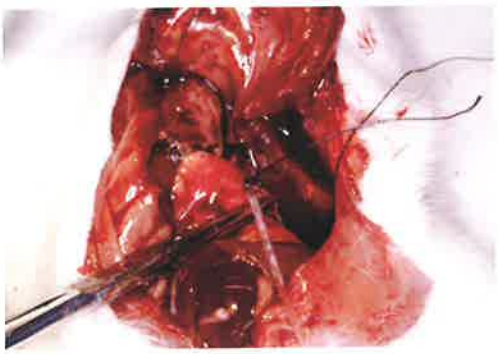
The right carotid artery was elevated and held taut by rostral and caudal tension placed on the silk sutures by an assistant. The length of artery exposed measured approximately 10 mm. Using iris scissors, a fine, incomplete oblique cut was made in the vessel approximately 3-4 mm from the caudal end of the exposure. Before shock and vasoconstriction of the vessel occurred, one beak of a fine pair of tweezers was inserted into the lumen of the partially severed vessel.



a. Neck dissection and identification of left carotid artery



b. Isolation with silk ligatures



c. Insertion and tying off of cannula

Figure 1. Diagrammatic procedures for carotid cannulation.

The tweezers were closed so that the artery was firmly held and the tweezer tips acted as guide for the insertion of the cannula. Once inserted, the cannula was tied in place by the tightening of the rostral silk suture around both the vessel and its inserted cannula. The caudal silk suture was tied around the vessel to occlude the blood flow (Fig. 1).

The same procedure was followed for the left carotid artery while syringe pressure was maintained on the heparin within the cannula in order to prevent the back flow of blood. With both carotid arteries tied and occluded, rapid draining of the perfused fixative was achieved using the following procedure:

Working quickly, a horizontal incision at the caudal end of the sternum was made to expose the upper abdominal cavity just below the diaphragm. Lateral relieving incisions in an axillary direction allowed reflection of the rib cage, and dissection of the diaphragm exposed the heart and great vessels. The vena cavae were identified and cut using a fine pair of curved scissors. Once venous return had been severed, 20 ml of fixative was injected through the cannula into the carotid arteries. A mouth prop was placed into order to assist later dissection of the jaws in the fixed head. Once perfusion had been completed, the maxilla and mandible were dissected out and immersed in fixative for a further 24 h.

The procedures took approximately 45 min in total per rat.

3.2.3.2 Cardiac perfusion

When fixation of both the head and the body organs was required, cardiac perfusion of fixative was employed using the following method.

After anaesthesia and the administration of heparin via the femoral vein as previously described, a long midline vertical incision was made from the neck to the abdomen. Additional relieving incisions were made into the axillary area and the skin and superficial fascia reflected to expose the underlying thoracic and abdominal muscles. The thoracic cavity was entered by sharp scissor dissection at the caudal end of the sternum and at the level of the diaphragm. With sharp forceps, the rib cage was vertically bisected in the midline and the two halves reflected outwards to expose the beating heart. The superior and inferior vena cavae were clamped and cut so that there was no venous return and immediately 50 ml of fixative was slowly injected into the left ventricle of the heart. On completion of perfusion, the maxilla plus the required abdominal organs (spleen, liver, kidney, gut) used as immunolabelling control tissues, were dissected out and placed in the appropriate fixative for 24 h.

3.2.4 Fixation

The fixative solutions employed were dependent upon the ultimate processing and viewing techniques for the tissues. The aim of fixation was to preserve the tissues as closely as possible to their life-like state for optimum microscopy and/or immunolabelling.

For electron microscopy, rats were perfused and the jaws immersed in Karnovsky's fluid (Karnovsky, 1965, Appendix 3). For light microscopic immunocytochemistry, Zamboni's fixative (Zamboni and De Martino, 1967; Appendix 3) or neutral buffered formalin (Appendix 3) was employed.

3.2.5 Decalcification

All specimens and tissues were decalcified prior to further processing. The aim was to remove the inorganic content from the tissues and leave the organic component for histological review. The type of decalcifying solution employed depended on the subsequent tissue preparation and experimental protocol. For the rapid removal of inorganic salts "Decal" (Institute of Medical and Veterinary Science, Adelaide, Australia), a combination of ethylenediaminetetra-acetic acid (EDTA) and hydrochloric acid (Appendix 4) was used. Decalcification of the tissues for immunocytochemistry protocols used 4% EDTA in phosphate buffer (Appendix 4). For electron microscopy, 4% EDTA in 0.06M cacodylate buffer and 2.5% glutaraldehyde (Appendix 4) was used. Determination of the completion of decalcification was established via radiographs of the specimens taken before and after the procedure. In the case of "Decal", decalcification generally occurred in 4-5 d but EDTA in the various buffers took up to 5 wk. All decalcification procedures were undertaken at 4°C with constant stirring, and the decalcifying solutions were changed daily.

3.2.6 Tissue processing

3.2.6.1 Histology and immunocytochemistry

After decalcification, light microscopy specimens were washed in phosphate buffered saline (Appendix 3) and excess tissue was trimmed with a scalpel from the maxillae and teeth. The tissues for examination were placed in 70% alcohol before automatic processing in a Shandon Citadel 2000 automatic tissue processor (Shandon Industries, Pittsburgh, Pennsylvania). Dehydration through graded alcohols was undertaken before paraffin wax impregnation (Appendix 5). Specimens were embedded in paraffin wax using a Reichert-Jung Histostat with

care to orientate each maxilla so that sectioning would occur in a coronal plane and permit viewing of both experimentally frozen and control molars.

Paraffin blocks were mounted in a Leitz 1512 Microtome and ribbons of 5 μm sections cut. Sections were floated on a water bath thermostatically set at 37°C and two or three sections at a time were lifted on to aminopropyltriethoxysilane (APT) coated, labelled glass slides (Appendix 6). The slides were allowed to air dry. Every tenth slide was stained with Mayer's haematoxylin and eosin (Appendix 7) to check for orientation and the suitability of adjacent slides for immunolabelling.

3.2.6.2 Transmission electron microscopy

Decalcified tissues were washed in three 30 min rinses of 0.06M sodium cacodylate buffer (Appendix 3). Under an Olympus stereo dissecting microscope and using a No. 15 scalpel blade, excess tissue was removed from the maxilla. The right and left dental arcades were removed by an excision in the sagittal plane adjacent to the palatal aspect of the teeth and parallel to their long axes. The right and left first molars were sectioned longitudinally into thirds in the bucco-palatal plane. Each resulting specimen contained part of the tooth crown and root plus the investing periodontal tissues. Specimens were maintained in 0.06 M sodium cacodylate buffer in labelled glass vials.

LR White resin was used as the embedding medium (London Resin Co., London, England). Specimens for routine transmission electron microscopy were post fixed in osmium tetroxide, dehydrated through graded alcohols and progressively infiltrated with resin (Appendix 8). The processed tissue was placed in gelatin

capsules half-filled with resin before complete filling and sealing to prevent air contact. Capsules were placed in a 60°C oven for 24 h to allow polymerization to proceed to completion. Tissue blocks were removed from their capsules, placed in individual, labelled plastic vials and stored at room temperature until required.

3.2.7 Tissue sectioning and staining

3.2.7.1 Resin embedded material

Glass knives were prepared using an LKB knife maker Type 780 1B. Specimen blocks were secured in a Reichert specimen holder chuck and mounted in a Reichert-Jung Om U4 ultra-microtome. A mesa was prepared with a No. 11 scalpel blade and 1 µm sections were cut using the glass knife and collected in a water bath. The sections were flattened with chloroform vapour and placed onto clean glass slides using a wire loop and dried on a 60°C thermostatically controlled hot plate. For orientation, sections were stained with 0.05% toluidine blue solution and 1% borax (Appendix 7) at 60°C for 30 s. Sections were jet washed with millipored, double distilled water and allowed to dry. They were subsequently viewed in an Olympus EHT light microscope in order to check specimen orientation and ensure that both the tooth and surrounding alveolar bone were present. Serial sections were collected from the tooth cervical and interradicular areas, and, where areas of interest were encountered, small mesas were prepared for silver sectioning using glass knives so that as much tissue as possible was preserved.

Sections in the silver interference range (approximately 750 nm) were cut using a diamond knife (RMC-HD Scientific Supplies, Bayswater, Australia) at a knife angle of 6°. Silver sections were collected on clean 3 mm, 150 square mesh

copper grids which were subsequently stored on Whatman grade 1 filter paper in labelled, plastic petri dishes until needed. Selected sections were stained with uranyl acetate and Reynold's lead (Appendix 9) and viewed in a Philips CM100 electron microscope operating at 80 KvP. Digital electronmicrographs of specimens were generated by a MegaView II high resolution digital camera (Soft Imaging System GmbH, Munster, Germany). Images were stored in a Tagged Image File Format (.tif file).

3.2.7.2 TRAP enzyme histochemistry

A pilot study was conducted to determine the optimum histochemical protocol for demonstrating TRAP in paraffin sections. Sections of maxillae showing areas of physiological bone resorption as determined by orientational haematoxylin and eosin (H&E) staining were used. Various methods requiring different types of couplers were employed to stain for TRAP activity. The following methods were evaluated:

Method 1. The first method used a kit developed by Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany. The reagents included: naphthol AS-BI phosphoric acid at 12.5 mg/ml in N,N'-dimethyl formamide as a substrate, Fast Garnet GBC salt at 15 mg/capsule as a coupler, L (+) tartrate buffer at 0.67 mol/L, and acetate buffer at 2.5 mol/L. The solution pH was 5.2 when diluted as indicated below:

Materials	TRAP solution	Control solution
37°C Distilled water	44.0 ml	42.0 ml
Acetate solution	2.0 ml	2.0 ml
Naphthol AS-BI solution	2.0 ml	-
Tartrate solution	2.0 ml	2.0 ml

One capsule of Fast Garnet GBC salt was added to each beaker and stirred on a magnetic mixer for 60 s. Solutions were rapidly filtered through Whatman No. 54 paper into separate staining jars, each of which contained 5 slides. Slides were incubated for 2 h at 37°C in the dark. After incubation, solutions were decanted and slides rinsed in water for 10 min before being stained with 1% Fast Green FCF (Sigma-Aldrich) for 5 min and mounted using DePeX (BDH Laboratories, Poole, United Kingdom).

Method 2. The second method used Burstone's complete medium, as described by Cole and Walters (1987) and modified by Farrell *et al.* (1990). Naphthol AS-BI phosphate substrate (Sigma-Aldrich) was used as the substrate (Janckila *et al.*, 1978) along with acetate buffer at 0.1-0.2M. Fast Red Violet LB salt (Sigma-Aldrich) served as the coupler. Tartrate L (+) and other solutions were prepared as indicated below:

Material	TRAP solution	Control solution
Acetate (0.2 M)	50 ml	50 ml
Fast Red Violet LB	70 mg	70 mg
Tartrate L (+) (0.67mol/L)	1ml	1ml
Naphthol AS-BI (12.5mg/ml)	1ml	-
10% $MnCl_2$	0.25 ml	0.25 ml

Media were filtered into acid-clean Coplin jars and incubated with slides for 2 h at 37°C. Following incubation, counterstaining was performed as described in Method 1.

Method 3. The third method for demonstrating TRAP was performed with hexazotized basic fuchsin using the modified technique of Goldberg and Barka (1962). TRAP solutions comprised the following:

50 ml of 2% sodium acetate.$3H_2O$, pre-warmed to 37°C

3ml of hexazotized basic fuchsin (see below)

2ml of 0.67mol/L L (+) tartrate solution (Sigma-Aldrich)

2ml of 12.5 mg/ml naphthol AS-BI phosphoric acid (Sigma-Aldrich)

The final solution was poured into Coplin jars, each containing 10 slides. Control solutions were prepared by omitting the substrate naphthol AS-BI phosphoric acid solution and increasing sodium acetate solution to 52 ml. Slides were incubated for 2 h at 37°C, after which solutions were decanted and slides washed for 10 min in running water and left to air dry. Slides were counterstained with 1% Fast Green FCF (Sigma-Aldrich) for 5 min, washed in running water for 1 min, and dried before being mounted using DePeX. Sections were studied under the light microscope for evidence of TRAP stained cells.

Hexazotized basic fuchsin solution was prepared by mixing equal volumes of basic fuchsin solution (Sigma-Aldrich) and 4% sodium nitrite solution ($NaNO_2$). Basic fuchsin solution was prepared by dissolving 800 mg of basic fuchsin in 16 ml of distilled and 4 ml of concentrated (36%) hydrochloric acid. This was mixed well on a heated plate until dissolved and subsequently filtered. An equal volume was mixed thoroughly with sodium nitrite solution immediately before use.

3.2.7.3 Immunolabelling of sections for light microscopy

Antibodies employed in the study were either raised against osteoclasts, macrophages or monocytes/granulocytes, and comprised:

- KP1 antibody (Zymed, San Francisco, USA, Appendix 10)
- Anti-cathepsin L antibody (Santa Cruz Technologies, Santa Cruz, USA, Appendix 11)
- ED1 antibody (Serotec, Oxford, United Kingdom, Appendix 12)
- Anti-CD13 antibody (Dako, Carpinteria, USA, Appendix 13)
- Anti-CD15 antibody (Dako, Carpinteria, USA, Appendix 14)
- Anti-CD45 antibody – Anti-Leucocyte common antigen (Serotec, Oxford, United Kingdom, Appendix 15)
- Anti-CD61 - β_3 integrin receptor antibody (Serotec, Oxford, United Kingdom, Appendix 16)
- Anti-carbonic anhydrase II antibody (Serotec, Oxford, United Kingdom, Appendix 17)
- AE1/AE3 antibody (Cell Marque, Austin, USA, Appendix 18) – a cytokeratin marker for epithelial tissues

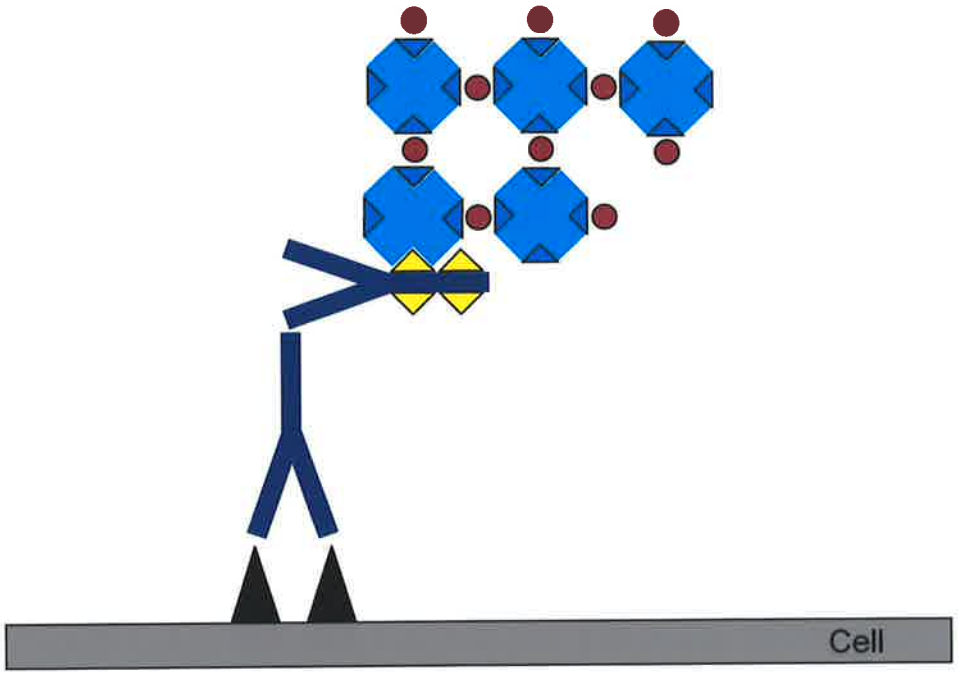
A detailed account of the immunolabelling protocol is found in Appendix 19. Immunological procedures followed the avidin-biotin peroxidase complex (ABC) technique (Signet, Dedham, Massacheusetts, USA) which is schematically presented in Diagram 4 below. Five micron sections on silane-coated glass slides were dewaxed and placed in a solution of H_2O_2 in methanol in order to block endogenous peroxidase activity. A pilot study was undertaken to establish the most suitable method of antigen retrieval either by trypsin digestion of sections for 3 min at 37°C, or by microwaving specimens in citrate buffer at pH 6.0 for 15 min (Wu *et al.,* 1992, Appendix 20). Treated sections were washed in phosphate buffered saline prior to incubation in normal horse serum for 30 min. After further washing, slides were incubated with the primary antibody overnight. Successive incubations in biotinylated linking or secondary anti-mouse antibody (30 min) and streptavidin peroxidase (1 h) occurred prior to the application of diaminobenzidine (DAB) as a peroxidase substrate (Appendix 21). Slides were washed between each stage before a light counterstaining with haematoxylin and mounting in aquatex (Merck, Darmstadt, Germany). Negative controls were provided by sections to which primary antibody had been omitted and positive controls were provided by sections of rat spleen, liver, gut or kidney according to, and depending on, the nature of the primary antibody used.

Except where manufacturers provided antibodies diluted for immediate use (for example, KP1, Zymed), all supplied concentrated antibody solutions were titrated to determine optimal concentrations for label visualization. Solutions were diluted with phosphate buffered saline (Appendix 3) in a chequerboard approach beginning with a 1:1 dilution and progressing through 1:2, 1:5, 1:10, 1:50, 1:100, 1:250, 1:500, 1:1000, 1:5000 and 1:10000. Antibody titrations were monitored

over varying time periods beginning at 1 h, and then progressing through 2 h, 4 h, 8 h, 12 h (overnight) and finally, 24 h (Appendix 22).

Immunologic reaction was determined by the microscopic appearance of brown-coloured diaminobenzidine substrate attachment to the antigen-antibody complex. The site of the label was dependent on the specific location of the antigen in the cell that is a component of the cell membrane or of the cytoplasm. In the latter case, the entire cell labelled diffusely.

All immunolabelling procedures were performed at least twice in order to minimize the possibility of experimental error affecting the results. In addition, positive and negative control tissues were verified by the Bone Laboratory of the Institute of Medical and Veterinary Science, Adelaide, Australia,



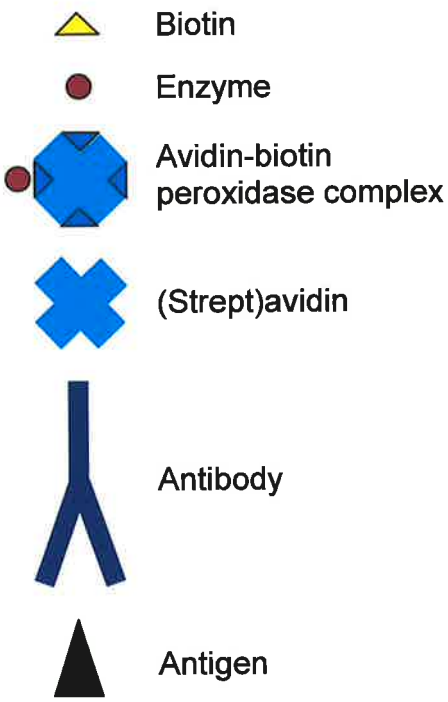


Diagram 4. Schematic representation of the antigen-antibody reaction utilized in this study.

3.2.8 Photography and image capture

Histological slides were viewed under an Olympus BH2 microscope to which a 35mm camera was attached. An automatic control unit (Olympus PM-10) provided consistent exposure of 35mm Kodak Ektachrome 64T EPY 135-36 slide transparency film at an ASA of 64 and a reciprocity of 2. A light balancing tungsten filter (LBT) provided colour balance. Areas of interest were photographed at lens magnifications of 10, 20 and 40 times with a photo eyepiece magnification of 2.5 times. This resulted in 25, 50 and 100 times magnifications of the original view in the final slides.

Slide transparencies were scanned using Adobe Photoshop 5.0LE software on a Canon FB 1200S scanner with an FAU S10 transparency adaptor at a resolution of 600 x 600 dots per inch. Scanned images and electronmicrograph image files were imported into Adobe Pagemaker 6.5 where they were arranged and annotated without digital enhancement before being printed using an Epson 760 colour injet printer on Epson S041140 photo quality paper.

3.3 Experimental studies

The experimental procedures employed are summarized in Table 2 below.

Experiment	No. of animals	Duration of CO_2 application	No. of applications	Days sacrificed	LM	TEM	TRAP	Succinic dehydro-genase	Immuno-labelling	OPG
Anaesthesia	6	10 min	1	7	✓					
1	8	10 min	1	2,7,14,28	✓					
2	8	20 min	1	2,7,14,28	✓					
3	8	20 min	3	2,7,14,28	✓					
4	16	10 min	3	7,14	✓	✓	✓		✓	
5	8	20 min	1	7	✓	✓	✓		✓	
6	8	10 min	1 plus mechanical insult	7	✓					
7	12	10 min	1	2,7,14	✓			✓		
8	32	10 min	1	7	✓	✓	✓		✓	✓

Table 2. Summary of experimental procedures

3.3.1 Experiment 1 – To determine the effects of freezing of the tooth crown on periodontal tissues.

The upper right first molar of 8 rats was frozen by a 10 min application of dry ice. Two rats were sacrificed at each period of 2, 7, 14 and 28 d after the insult and tissues were processed for routine histology.

3.3.2 Experiment 2 – To determine optimal freezing time.

The upper right first molar of 8 rats was frozen by a 20 min application of dry ice. Two rats were sacrificed at each period of 2, 7, 14 and 28 d after the insult and tissues were processed for routine histology.

3.3.3 Experiment 3 – To determine optimal number of applications of freezing medium.

The upper right first molar of 8 rats was frozen 3 times over the period of 1 wk by a 20 min application of dry ice. Two rats were sacrificed at each period of 2, 7, 14 and 28 d after the insult and tissues were processed for routine histology.

3.3.4 Experiment 4 – To determine and assess resorption and repair mechanisms using immunohistochemistry, enzyme histochemistry and ultrastructural evaluations.

The upper right first molar of 16 rats was frozen 3 times over the period of 1 wk by a 10 min application of dry ice. Rats were sacrificed at either 7 or 14 d after the insult and the tissues were processed according to protocols required for later tissue examination. Two rats at each time frame were used for routine histology while the other 12 were sacrificed by and during the fixation procedure via carotid or cardiac perfusion of fixative. Four animals at each time frame were fixed using Zamboni's fluid (Appendix 3) and specimens retrieved were used for light microscopic immunocytochemistry and TRAP enzyme histochemistry, while 2 animals were fixed for ultrastructural examination using Karnovsky's fluid (Appendix 3) at each of the 7 and 14 d periods.

3.3.5 Experiment 5 – To assess the effect of a single prolonged thermal insult using enzyme histochemistry, immunohistochemistry and ultrastructural evaluations.

The upper right first molar of 8 rats was frozen once by a 20 min application of dry ice. All rats were sacrificed during perfusion fixation at a period 7 d after the

insult. Six animals were fixed with Zamboni's fluid for enzyme histochemistry and immunohistochemistry while the remaining 2 animals were fixed with Karnovsky's fluid for transmission electron microscopy.

3.3.6 Experiment 6 – To determine the combined effects of mechanical and thermal insults to the periodontium.

The upper right first molar of 8 rats was frozen once by a 10 min application of dry ice. In addition, a mechanical insult was delivered by a sterile, sharp probe, forcibly inserted into the periodontal ligament space and scraped along the cementum surface. All rats were sacrificed at a period of 7 d after the insult and the tissues processed for routine histology.

3.3.7 Experiment 7 – To determine succinic dehydrogenase activity in the periodontium after cold thermal insult.

Resorption was generated in the teeth of 12 rats by a 10 min application of dry ice to the upper right first molar. Four rats were sacrificed by carbon dioxide inhalation at time intervals of 2 d, 7 d and 14 d. The maxillae were immediately dissected out and placed, without fixation, in 10% EDTA as a decalcifying solution (Appendix 4). The solution was continually agitated and changed regularly until decalcification was completed after approximately 14 d.

Decalcified maxillae were trimmed of excess soft tissue and attached to a cryostat chuck using cryo-embedding medium (O.C.T. Compound, Miles Laboratories, Illinois, USA). Specimens were left to freeze for before sections 20 μm thick were cut on a Kryostat 2000 (Leitz, Germany) at -25°C and placed on silane-coated glass slides. Slides were stored in a freezer until required.

Selected slides were stained for succinic dehydrogenase activity using the method of Takimoto *et al.* (1966) (Appendix 23). Slides containing sections were incubated for 45 min at 37°C in a substrate medium for succinic dehydrogenase (Appendix 23). Sections were fixed in 10% neutral buffered formalin (Appendix 3) for 30 min prior to washing in distilled water and mounting in aquatex. No counterstaining was used.

3.3.8 Experiment 8 – To determine the effects of osteoprotegerin on the resorption model.

This procedure used osteoprotegerin (FcOPG, Amgen, Thousand Oaks, California, Appendix 24) in an effort to determine its effect on the dental apparatus and investing tissues in the model resorption system. The upper right first molar of 32 rats was frozen with pellets of dry ice for 10 continuous min. This was designated as day 1. On days 1, 3 and 5 after freezing of the tooth, a 2.5 mg/kg dose of osteoprotegerin was administered subcutaneously.

Sacrifice and dissection of maxillae and selected long bones (femur and tibia) occurred on day 7. Tissues were prepared for light and electron microscopic histology, light microscopic immunolabelling, and TRAP staining as previously described.

CHAPTER 4

RESULTS

4.1 Animals

The 106 animals used in this study survived the application of the cold thermal stimulus, gained weight, were active and remained free of stress during the observation periods. They appeared to suffer no lasting ill effects from the experimental procedures except for the occasional rat that had right side facial swelling. This was likely related to inadvertent dry ice contact with the buccal mucosa during the experimental procedures. The swelling subsided within 24 to 36 h and the animals quickly returned to normal activity and function. It was noted that facial swelling was usually observed in animals that sustained multiple thermal insults. In addition, sloughing of gingival marginal tissues was also occasionally associated with multiple applications of dry ice.

4.2 Results of method trials

4.2.1 Anaesthesia

The results of the pilot anaesthetic study in Table 3 below indicated that the Hypnorm®/Hypnovel® combination was excellent at providing swift surgical anaesthesia with muscle relaxation that permitted experimental procedures to be performed without duress to the animal or the operator. A single dose of Hypnorm®/Hypnovel® was adequate to maintain anaesthesia during the various operations and for a sufficiently lengthy period of time thereafter. This allowed tissues to recover and thaw thus minimizing any immediate and short-term

discomfort to the animal. This drug combination had the additional benefit of being reversible by naloxone hydrochloride (0.4 mg/ml, David Bull Laboratories, Mulgrave, Australia) administered subcutaneously at a dose of 0.1 mg/kg in the event of anaesthetic complications. No anaesthetic complications were encountered with Hypnorm®/Hypnovel®; however, the other anaesthetics trialed caused difficulties and inconsistencies in anaesthesia onset, depth and duration when used at recommended dosages and often additional dosages were required. Because of its efficiency, safety and reliability Hypnorm®/Hypnovel® anaesthesia was used throughout all of the main experimental procedures in the current project.

Animal	Weight	Anaesthetic	Dose	Duration of anaesthesia	Comments	Sacrifice
1	260g	Hypnorm + hypnovel	0.7 ml	120 min	Swollen face Recovered at 24 hr	At 2 d
2	258g	Nembutal	0.4 ml	130 min	Minor swelling	At 7 d
3	260g	Saffan	0.26 + 0.3 ml	150 min	Respiratory difficulty	At 14 d
4	262g	Rompun + Ketamil	0.05 + 0.075 + 0.05 + 0.05 ml	85 min	Onset delayed	At 14 d
5	260g	Hypnorm + hypnovel	0.8 ml	90 min	Onset delayed	At 2 d
6	265g	Saffan	0.3 + 0.3 + 0.2 + 0.2 + 0.2 + 0.2 ml	Unable to anaesthetise	Animal deferred to following day – given hypnorm 0.7ml	At 7 d

Table 3. Results of pilot anaesthetic study indicating the efficacy of Hypnorm®/Hypnovel® for producing swift and prolonged surgical anaesthesia.

4.2.2 Pilot study of TRAP enzyme histochemistry

Method 1 using Fast Garnet GBC salt as a coupler produced a light, reddish, granular, poorly localized precipitate that was difficult to observe under light microscopy. **Method 2** using Fast Red Violet LB salt as the coupler produced a fine purplish reaction product, which was also hard to distinguish under light microscopy. Localization of the precipitate was vague and imprecise.

The third option employed in **Method 3** using hexazotized basic fuchsin proved to be the coupler of choice. It yielded a fine granular red reaction product which was readily visible under light microscopy. This method was therefore chosen to detect TRAP activity in selected sections from specimens prepared in the main experimental procedures.

4.2.3 Antigen retrieval techniques

The two antigen retrieval techniques used were dependent on the recommendations suggested by the manufacturers of the antibodies. Enzyme digestion by trypsin was less destructive to the tissues and appeared to adequately unravel the cross-linking of the aldehyde fixatives and so expose some antigens under examination. Microwave retrieval methods using citrate buffer did not lead to enhanced results for the antibodies tested. The prolonged heating of tissue sections in buffer caused tissue loss from slides unless prior subbing of slides was adequate. The protocol for APT subbing (Appendix 6) was altered to reduce the time of alcohol washing and therefore leave a heavier film of APT on each slide. While this improved tissue adherence, immunologic reaction was not noticably enhanced.

4.3 Results of experiment studies

4.3.1 Experiment 1 – Single 10 min episode of freezing.

To determine the effects of freezing of the tooth crown on periodontal tissues.

4.3.1.1 Light microscopy

A. Control teeth

The histology of the rat molar and its investing periodontium noted in control unfrozen molar teeth is presented in Figure 2. The orientation of the principal fibres of the periodontal ligament was clearly visible as they coursed from the alveolar bone surface to the tooth root. The orientation of these fibres appeared dependent on their level of hard tissue attachment (Fig. 3). Near the alveolar crest, fibres ran from bone in a slightly upward direction towards the tooth before running more horizontally and then more obliquely as sections were viewed further down the periodontal ligament towards the root apex. The principal fibres associated with the appositional bone surfaces were large in size and appeared to form from the aggregation of smaller fibres. Principal fibres adjacent to resorptive bone surfaces appeared smaller, finer and less aggregated (Figs. 3 and 4). In haematoxylin and eosin stained sections the majority of cells within the ligament appeared to be ovoid fibroblast-like cells interspersed with blood vessels, and the occasional small cluster of epithelial-like cells adjacent to the root surface (Figs. 4 and 5).

Alveolar bone exhibited both appositional and resorptive changes. A single layer of lining cells was evident on buccal-facing appositional bone surfaces which were relatively smooth in nature (Fig. 4). An irregular outline and bone resorption

lacunae were found on palatal-facing bone surfaces, often in association with multinucleated cells (Fig. 6).

Interradicular alveolar bone contained osteocytes, a few trabecular spaces and exhibited a compact appearance characteristic of woven bone (Fig. 7). The interradicular bony surfaces appeared similar to the cribriform plate lining the outer alveolar walls. Buccal-facing bone surfaces were relatively smooth yet punctured by the insertion of large principal (Sharpey) fibres. The depth of fibre penetration into bone was variable and appeared to terminate at darkly staining reversal lines that ran at regularly spaced intervals parallel to the bone surface (Fig. 7).

Palatal-facing interradicular bony surfaces possessed a rough irregular outline. Principal fibres did not show obvious bony penetration but appeared to make surface contact. Interrupting fibre attachment were resorption lacunae exhibiting the occasional presence of multinucleated cells (Fig. 6). The cementum lining root surfaces was covered by a single layer of cells interspersed with surface fibre attachments (Fig. 5). The roots were covered by a fine layer of intact acellular cementum except in the apical region where an increased thickness of cellular cementum was seen (Fig. 8). Shallow surface resorption lacunae and related multinucleated clast cells were occasionally seen on tooth root surfaces (Fig. 9). These were, however, infrequent, and were situated more apically. They were not observed in every control section.

The dental pulp presented a fine connective tissue reticulum with a lining layer of darkly staining elongated odontoblasts adjacent to dentinal surfaces. Many blood

vessels were apparent within the tissue stroma which also contained numerous connective tissue cells along with some macrophages and lymphocytes (Fig. 10). Pulp stones were commonly seen.

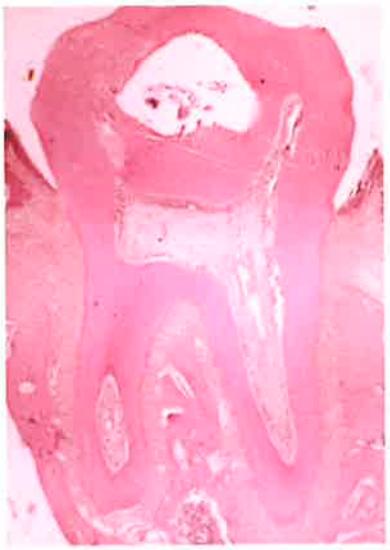


Figure 2. Experiment 1, light microscopy (LM), haematoxylin and eosin (H&E), magnification x10. Coronal control section of a control unfrozen rat first molar showing two roots and investing periodontium.

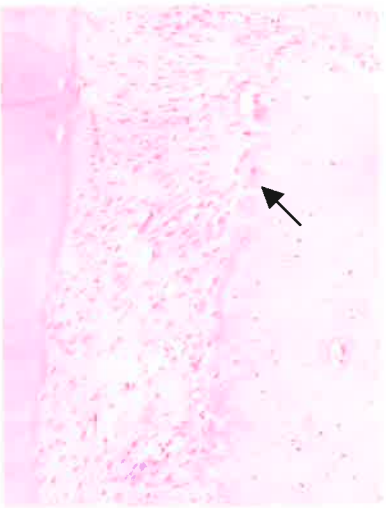


Figure 3. Experiment 1, LM, H&E, magnification x100. Principal periodontal fibre orientation and Sharpey fibres (arrow) entering appositional alveolar crestal bone in control unfrozen periodontium.

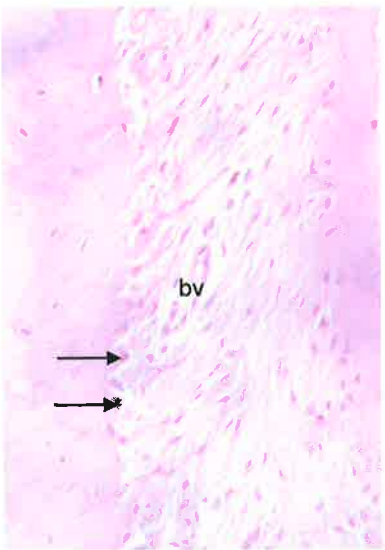


Figure 4. Experiment 1, LM, H&E, magnification x100. Control unfrozen appositional buccal-facing bone surface with a single layer of lining cells (arrows). Periodontal ligament cells appear ovoid and orientated with fibres. bv - blood vessel

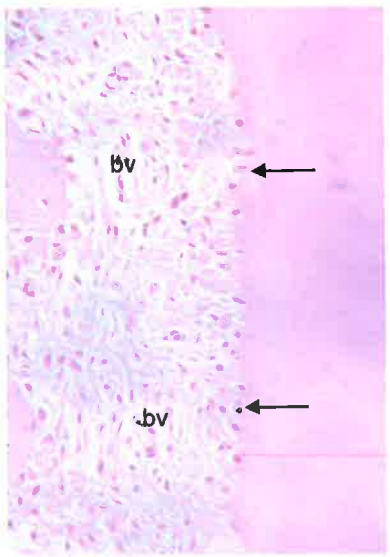


Figure 5. Experiment 1, LM, H&E, magnification x100. Control unfrozen periodontal ligament and cementum surface with a layer of lining cells (arrows). bv - blood vessel

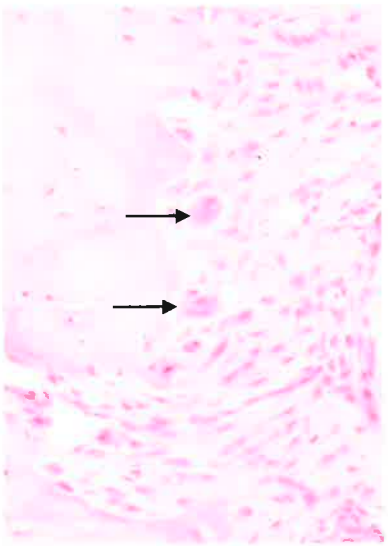


Figure 6. Experiment 1, LM, H&E, magnification x100. Control unfrozen palatal-facing bone surface showing resorption lacunae and multinucleated cells (arrows).

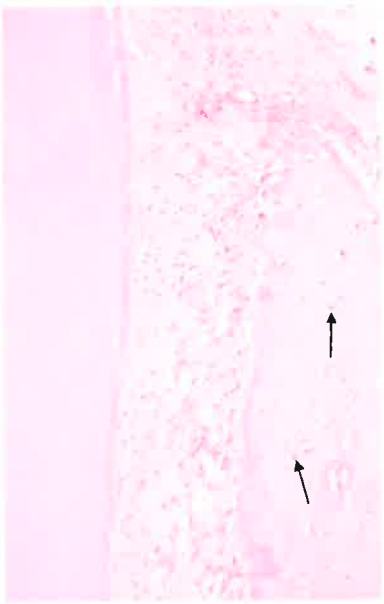


Figure 7. Experiment 1, LM, H&E, magnification x50. Control unfrozen appositional alveolar bone surface with penetrating PDL (Sharpey) fibres ending in reversal lines. Osteocytes (arrows) are apparent in bone along with the occasional marrow space.

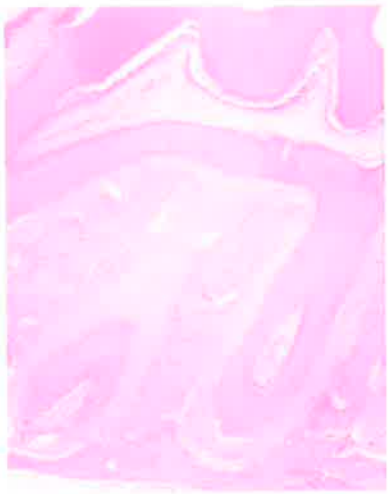


Figure 8. Experiment 1, LM, H&E, magnification x25. Photomicrograph showing the control unfrozen molar interradicular area and an increase in cementum thickness towards the apex.

Figure 9. Experiment 1, LM, H&E, magnification x100. Control unfrozen tooth root showing resorption lacunae into dentine (arrows).

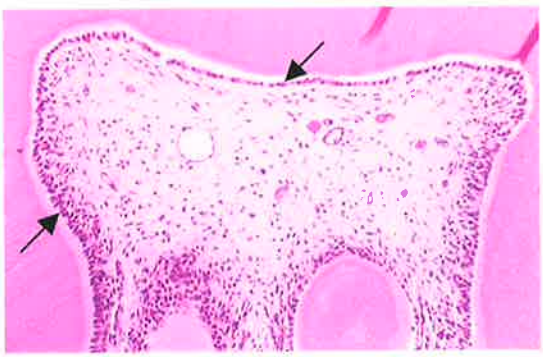


Figure 10. Experiment 1, LM, H&E, magnification x100. Photomicrograph of control unfrozen pulp tissue indicating a fine vascular connective tissue reticulum and a prominent odontoblastic layer (arrows).

B. Experimental teeth

Two days after the application of dry ice to the upper right first molar no overt signs of root resorption and clastic cell activity were seen on the root surfaces except for small areas near the root apices. At the level of the alveolar crest the periodontal ligament exhibited signs of disorganization, fibre disaggregation and a mild degree of hyalinization. Swollen cells showed signs of freezing injury but no significant inflammatory infiltration was evident (Fig. 11).

At day 7, shallow resorption lacunae were visible along the external surfaces of the root but were mainly concentrated in the interradicular and cervical areas. Large multinucleated cells appeared in association with the resorption bays on both cementum (Figs. 12 and 13) and bone surfaces (Fig. 14). The periodontal ligament fibres and cells showed marked disorientation and more extensive areas of hyalinization were present (Fig. 15). The cells of the periodontal ligament appeared to be fewer in number and the odontoblasts lining the pulp chamber underwent necrosis and were obliterated (Fig. 16).

Fourteen days after freezing, dental resorption lacunae appeared larger and more widespread. Multinucleated giant cells were still in evidence (Fig. 17), while active resorption and osteoclasts were present in the adjacent bone (Fig. 17). Partial or incomplete cellular cementum repair was observed in resorption bays (Fig. 18).

At day 28, active root resorption was absent and few clast cells were seen. Complete repair of the previous resorption lacunae was observed with cellular cementum filling the defects (Fig. 19).

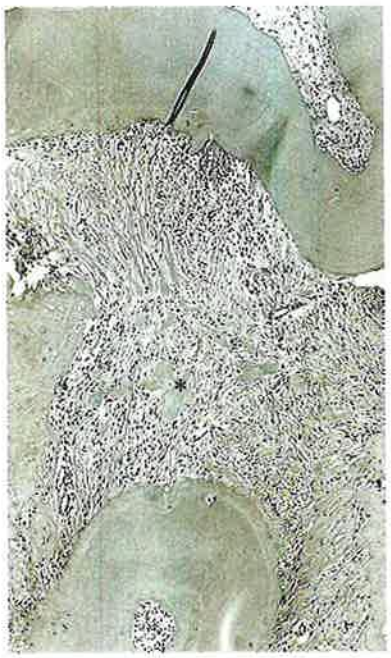


Figure 11. Experiment 1, LM, H&E, magnification x50. Photomicrograph taken 2 d after 10 min of dry ice application showing mild disruption and areas of hyalinization (*) in the frozen interradicular PDL. No significant inflammatory infiltration was detected.

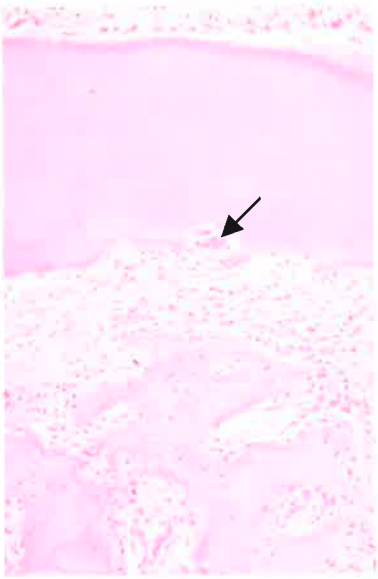


Figure 12. Experiment 1, LM, H&E, magnification x50. Photomicrograph of frozen tooth showing resorption lacunae and odontoclast activity (arrow) in the interradicular area 7 d after 10 min of thermal insult.

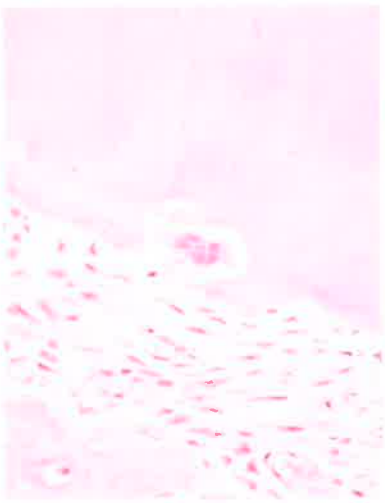


Figure 13. Experiment 1, LM, H&E, magnification x100. Higher power photomicrograph frozen tooth showing an odontoclast in the dental resorption lacunae shown in Fig. 12. Multinucleation is clearly apparent.

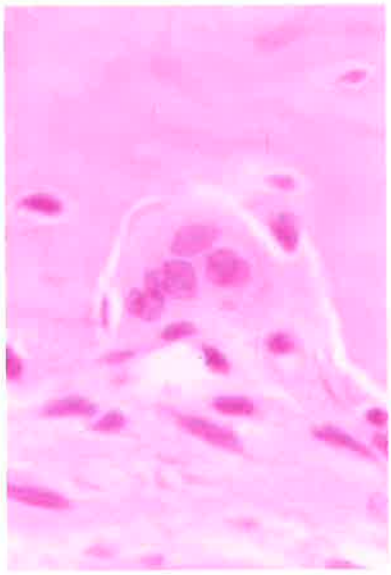


Figure 14. Experiment 1, LM, H&E, magnification x120. Photomicrograph of a molar frozen for 10 min showing an osteoclast with at least 7 nuclei in a bone resorption lacuna.

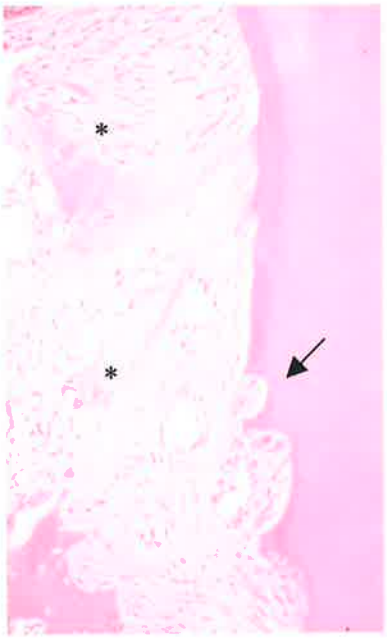


Figure 15. Experiment 1, LM, H&E, magnification x50. Photomicrograph of PDL 7 d after 10 min of freezing showing more widespread hyalinization (*) and resorption lacunae (arrow) in the cervical area of the tooth adjacent to the alveolar crest.

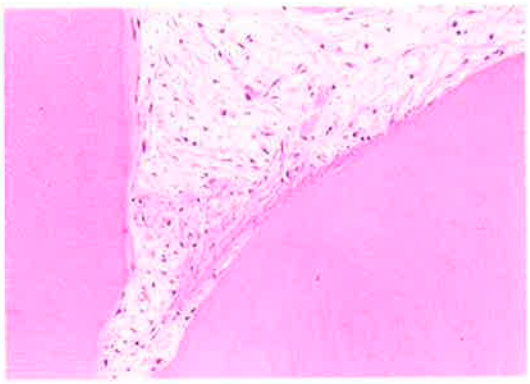


Figure 16. Experiment 1, LM, H&E, magnification x100. Photomicrograph of the pulpal effects of 10 min of freezing resulting in obliteration of the odontoblast layer and disruption of the cellular and connective tissue stroma.

Figure 17. Experiment 1, LM, H&E, magnification x50. Resorptive activity in tooth and cervical alveolar bone 14 d after 10 min of freezing. Odontoclast and osteoclast presence is evident as well as hyaline change.

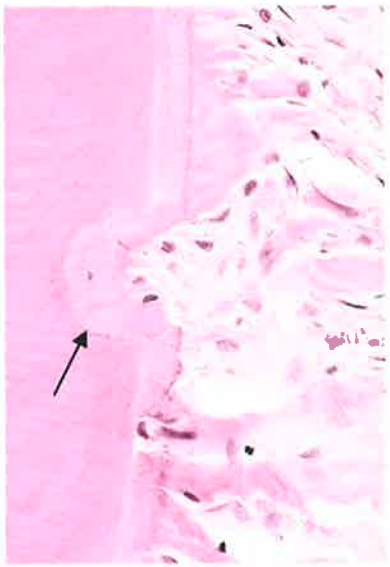


Figure 18. Experiment 1, LM, H&E, magnification x100. Photomicrograph of molar root 14 d after 10 min of freezing showing partial and incomplete repair (arrow) of resorption bay in interradicular site.

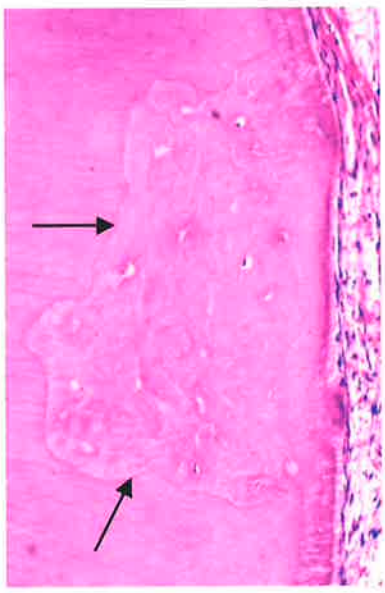


Figure 19. Experiment 1, LM, H&E, magnification x100. Photomicrograph of rat molar 28 d after 10 min of freezing showing cellular cementum repair of previous resorption bay (arrows).

4.3.2 Experiment 2 - Single freezing episode – 20 min.

To determine optimal freezing time.

4.3.2.1 Light microscopy

A. Control teeth

Results were similar to those described in experiment 1.

B. Experimental teeth

Experimental teeth subjected to longer freezing times generally showed more extensive injuries. Greater disruption in the form of hyalinization within the periodontal ligament was observed and the areas of resorption were more pronounced but still largely confined to the cervical third of the root (Fig. 20). Fourteen days after freezing for 20 min, some areas showed a cessation of active resorption and a reduction in clast cell presence. Cementum deposition was noted in many resorption lacunae and an ankylotic union was often seen in the interradicular area (Fig. 21). Twenty-eight days after molar freezing active resorption appeared to have ceased, repair of resorption defects had occurred, but ankylosis persisted.

4.3.3 Experiment 3 - Multiple freezing episodes – three times of 20 min.

To determine optimal number of applications of freezing medium.

4.3.3.1 Light microscopy

A. Control teeth

Results were similar to those described in experiment 1.

B. Experimental teeth

Multiple freezing episodes over a period of 1 wk produced a similar sequence of resorptive events compared with the single freezing insult. The discernable difference was the extent of the resorptive changes which extended further down the PDL in the 7 and 14 d animals. The resorptive effects involved interradicular and cervical areas as well as the upper one third of the tooth root (Fig. 22) and the lacunae appeared to be more numerous. Clast cell presence was also noted in the neighbouring alveolar bone. By 28 d, repair of the majority of the previous extensive resorptive lacunae appeared to be completed

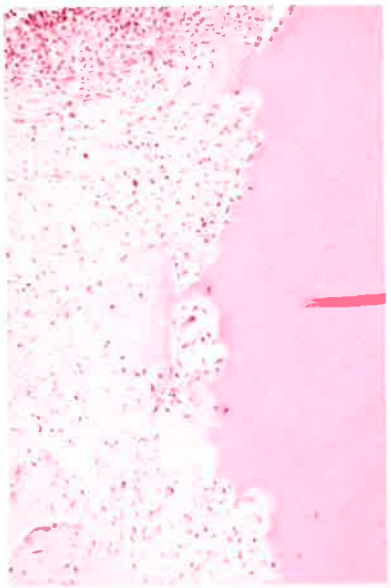


Figure 20. Experiment 2, LM, H&E, magnification x50. Extensive cervical resorption and PDL hyalinization in a rat 7 d after being subjected to a longer molar freezing time of 20 min.

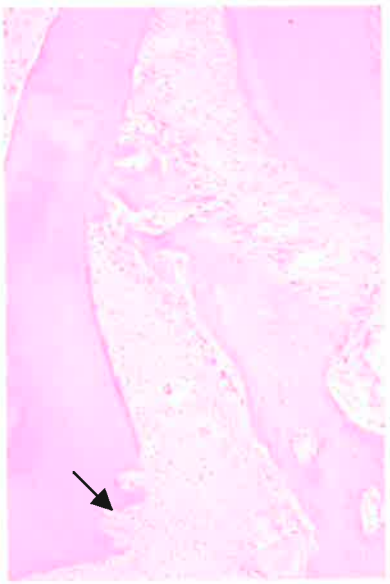


Figure 21. Experiment 2, LM, H&E, magnification x25. Photomicrograph of interradicular area 14 d after prolonged freezing insult. While areas of resorption are still present (arrow) ankylotic union has occurred.

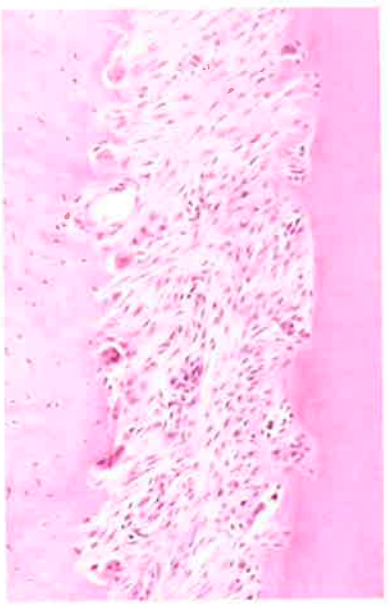


Figure 22. Experiment 3, LM, H&E, magnification x50. Multiple freezing episodes and sacrifice after 7 d revealed more extensive resorptive lacunae extending further down the tooth root. Osteoclast activity was also evident in neighbouring alveolar bone.

4.3.4 Experiment 4 - Multiple freezing episodes – sacrifice at 7 and 14 d.

To determine and assess resorption and repair mechanisms using immunohistochemistry, enzyme histochemistry and ultrastructural evaluations.

4.3.4.1 Light microscopy

A. Control teeth

Results for control teeth were similar to those described in experiment 1.

B. Experimental teeth

Multiple freezing episodes over the period of 1 wk with animal sacrifice 7 d later produced dental resorption and clast cell activity in the interradicular and cervical areas which involved the cervical third of the root (Fig. 23). Adjacent to active resorption, ankylosis was observed in the interradicular region in several animals. Intense inflammation and bony sloughing was a rare finding (Fig. 24).

The examination of tissue 14 d after 3 thermal insults over the period of 1 wk revealed more extensive injuries but the resorptive/repair response provided mixed results. In many animals clast cell activity occurred on dental (Fig. 25) and bony surfaces while, at the same time, repair of previous resorption lacunae was apparent. Ankylosis was also a frequent observation in the interradicular area (Figs. 25, 26 and 27) and, in some instances, the ankylotic union appeared to be quite widespread (Fig. 27). An additional finding was pulp necrosis and abscess formation in two animals (Fig. 28). The latter was accompanied by an increased number of clast cells resulting in substantial resorption of root structure and communication between the pulp and periodontal ligament (Fig. 29).

4.3.4.2 TRAP enzyme histochemistry

A. Control teeth

TRAP-positive cells were absent in the stained control sections in which the substrate was omitted, but were present in sections incubated in TRAP medium. TRAP activity was demonstrated by fine red granules in the cytoplasm while nuclei, TRAP-negative cells and background matrix stained green. TRAP-positive cells were routinely found in association with lacunae on hard tissue surfaces. In unfrozen sections, infrequent and isolated TRAP activity was apparent on root surfaces but a line of TRAP positive cells was seen on bone surfaces relating to the direction of physiological tooth drift (Fig. 30). More resorption and TRAP activity was noted in the apical area of teeth and surrounding alveolar bone compared with the interradicular crestal region (Fig. 31).

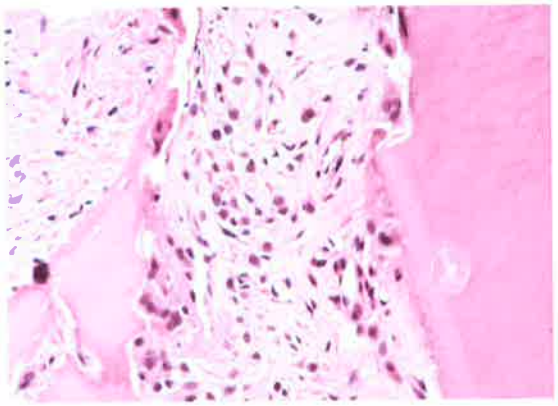


Figure 23. Experiment 4, LM, H&E, magnification x100. Photomicrograph of extensive alveolar crest and cervical resorption extending down root surface in a rat molar 7 d after multiple freezing insults.

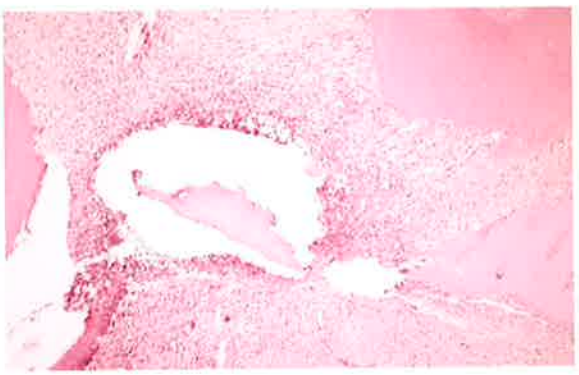


Figure 24. Experiment 4, LM, H&E, magnification x50. Photomicrograph of a bony sequestrum in the PDL after multiple freezing insults and 7-d sacrifice. Surrounding inflammation is evident.

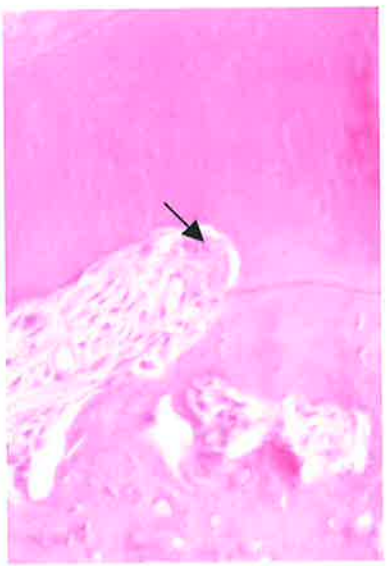


Figure 25. Experiment 4, LM, H&E, magnification x100. Photomicrograph of rat interradicular region 14 d after 3 freezing episodes showing odontoclast activity (arrow) adjacent to an area of ankylotic union.

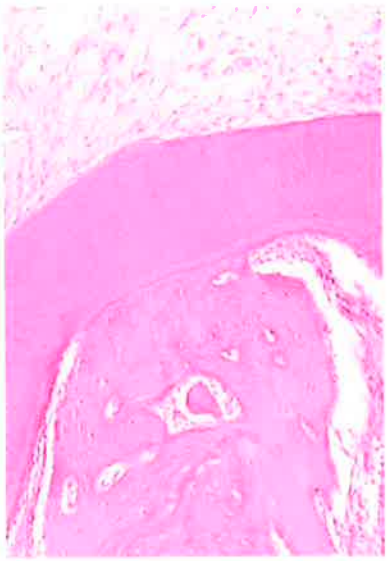


Figure 26. Experiment 4, LM, H&E, magnification x100. Ankylosis in the interradicular area of a rat molar after freezing 3 times for 20 min and sacrifice 14 d later.

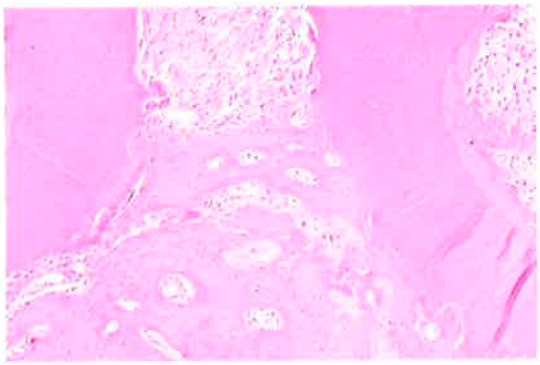


Figure 27. Experiment 4, LM, H&E, magnification x100. Obliquely cut interradicular section showing extensive ankylosis forming a bony bridge between adjacent roots of a rat molar after being subjected to multiple cold thermal insults and sacrifice 14 d later.

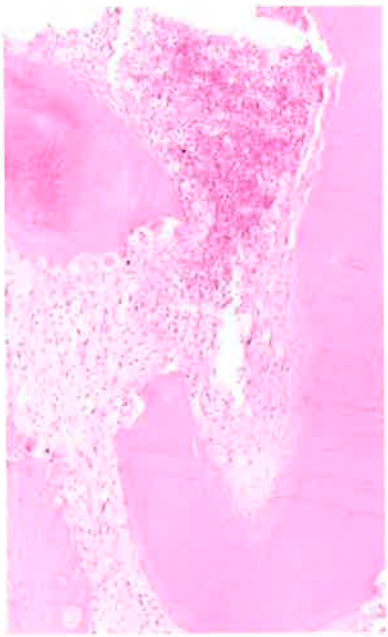


Figure 28. Experiment 4, LM, H&E, magnification x50. Photomicrograph of rat molar after multiple freezing insults and 14-d sacrifice showing pulp abscess formation with inflammatory change resulting in massive root resorption and clast cell activity.

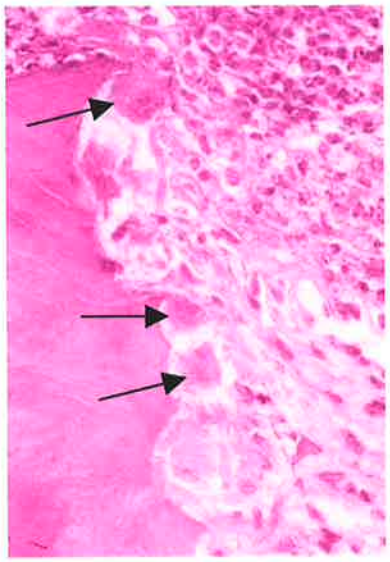


Figure 29. Experiment 4, LM, H&E, magnification x100. Many large clast cells (arrows) lining a resorbing root secondary to a pulpal abscess in a molar subjected to multiple freezing insults.

Figure 30. Experiment 5, LM, Fast Green, magnification x25. Photomicrograph of unfrozen control interradicular area revealing TRAP stain distribution (arrows) in direction of tooth drift.

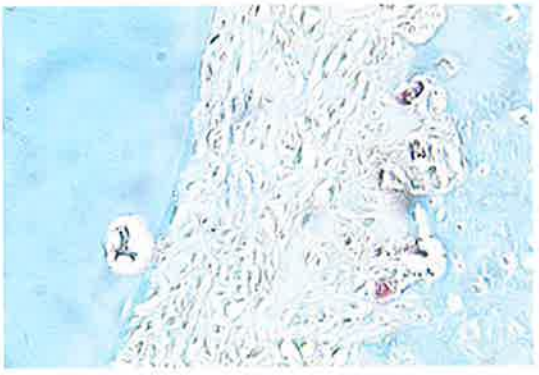


Figure 31. Experiment 5, LM, Fast Green, magnification x100. Photomicrograph of periapical PDL of unfrozen control molars showing positive TRAP reactivity in resorption lacunae.

B. Experimental teeth

Experimental sections showed positive TRAP staining along bone surfaces undergoing resorption particularly at the crestal level adjacent to the root furcation (Fig. 32). A line of positively-labelled cells covered the alveolar crest (Fig. 32), extending down the wall of the interradicular bone (Fig. 33), in addition to lining marrow cavities (Fig. 32). TRAP activity was also noted in resorption lacunae of affected teeth (Fig. 34) and occasionally in the periodontal ligament at a distance from tooth and bone (Fig. 35).

Many of the resorption lacunae in experimental teeth contained small TRAP-negative cells in addition to collagen fibres extending from the PDL to the base of the lacunae. Several lacunae were in the process of repair, with reparative cementum being laid down in the base of the resorption bay. Associated with a small number of lacunae were either mono- or multinucleated TRAP-positive cells. Most TRAP-positive cellular activity was located at the cervical areas of the molar as well as in the interradicular crestal area (Fig. 36).

Teeth that had been subjected to severe thermal insult and had subsequently undergone ankylosis revealed no TRAP activity in and around the ankylotic areas (Figs. 37 and 38). Weak TRAP-positive staining seen in isolated cells within the PDL but the overall lack of stain in ankylotic areas was a consistent finding in sections from animals exhibiting hard tissue union (Figs. 39 and 40).

Figure 32. Experiment 4, LM, Fast Green, magnification x50. Photomicrograph of interradicular area of molar frozen for 7 d showing TRAP activity on bone surface and in marrow spaces.

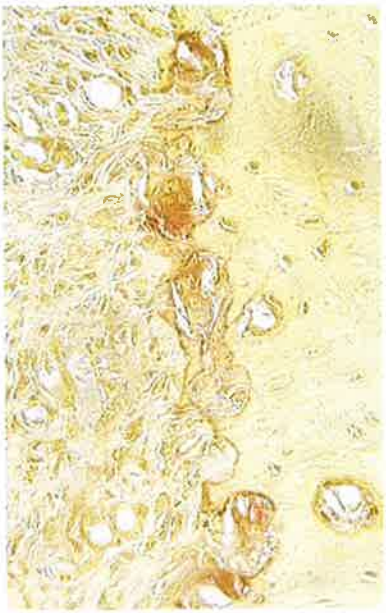


Figure 33. Experiment 4, LM, Fast Green, magnification x100. Photomicrograph of frozen alveolar bone showing TRAP activity in multinucleated osteoclasts situated in resorption lacunae.

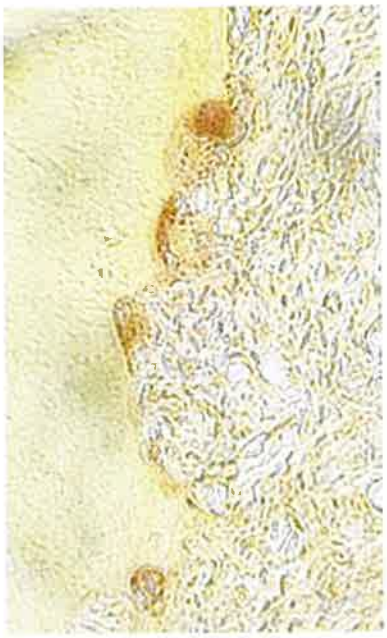


Figure 34. Experiment 4, LM, Fast Green, magnification x100. Photomicrograph of frozen molar resorption lacunae showing positive TRAP reaction of odontoclasts.

Figure 35. Experiment 4, LM, Fast Green, magnification x100. Photomicrograph of PDL and alveolar bone of a frozen molar showing TRAP staining of clast cells and a cell in the PDL (arrow).

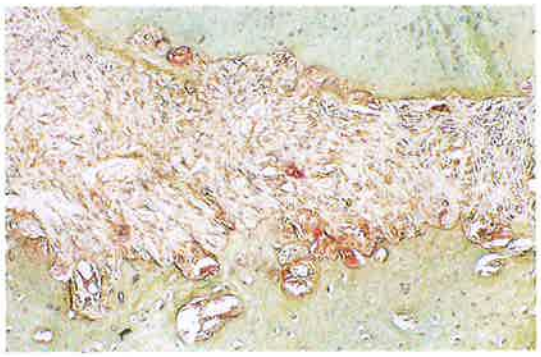


Figure 36. Experiment 4, LM, Fast Green, magnification x50. Photomicrograph of TRAP stain distribution in the interradicular PDL and resorption lacunae of a frozen molar.

Figure 37. Experiment 4, LM, Fast Green, magnification x50. Photomicrograph of interradicular area of frozen rat molar showing ankylotic union but no TRAP staining.

Figure 38. Experiment 4, LM, Fast Green, magnification x50. Photomicrograph of cervical area of frozen rat molar showing little TRAP activity. Isolated mononuclear cells show positive staining (arrows).

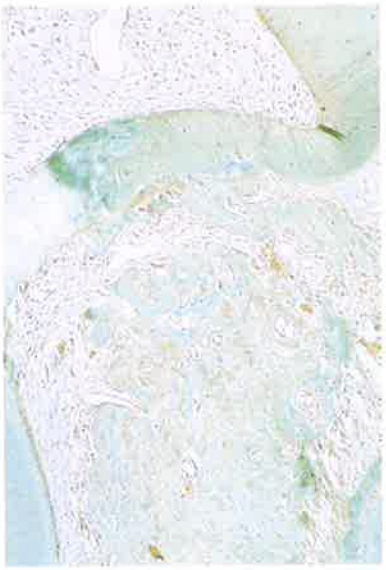


Figure 39. Experiment 4, LM, Fast Green, magnification x50. Photomicrograph of interradicular area of a frozen rat molar revealing ankylosis and no TRAP staining.

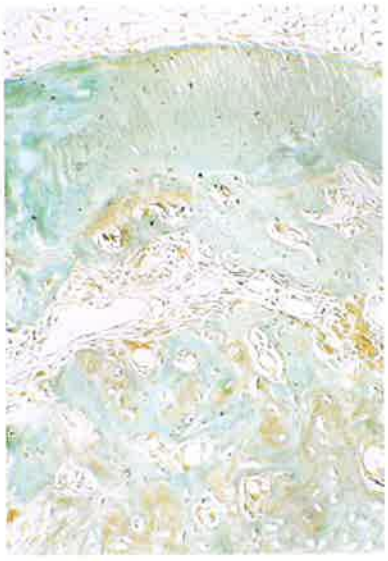


Figure 40. Experiment 4, LM, Fast Green, magnification x100. Higher power photomicrograph of Fig. 39 showing ankylotic union and no TRAP activity.

4.3.4.3 Immunolabelling

- **KP1 antibody**

A. Immunollabelling control tissue sections

Anti-CD68 as the KP1 antibody was used to reveal macrophages in tissues and specifically Kuppfer cells in the liver (Pulford *et al.,* 1989). A positive staining reaction was seen in sections of rat liver. However, the label appeared to be extracellularly located and formed radiating lines from hepatic vessels (Fig. 41). The brown label was patchy in its distribution throughout the sections but appeared in association with central blood vessels of hepatic lobules (Fig. 42). The vascular nature and the normal histological features of the lobulated liver stained with haematoxylin and eosin are presented in Figure 43.

Negative control sections of rat liver to which KP1 antibody had not been applied also labelled with a similar patchy distribution but to a less intense level (Fig. 44). Staining again appeared to be related to vessels in a radiating fashion.

Positive control sections of human skin (IMVS positive control tissue) revealed weakly staining cells in the dermal layers (Figs. 45 and 46).

No label could be detected in rat spleen (Fig. 47), gut or kidney.

B. Control teeth - unfrozen

No label was detected in the PDL of unfrozen rat molars.

C. Experimental teeth – frozen

No label was detected in the PDL of frozen rat molars (Figs. 48, 49). Inconsistent and weak labelling was observed in the necrotic dental pulp

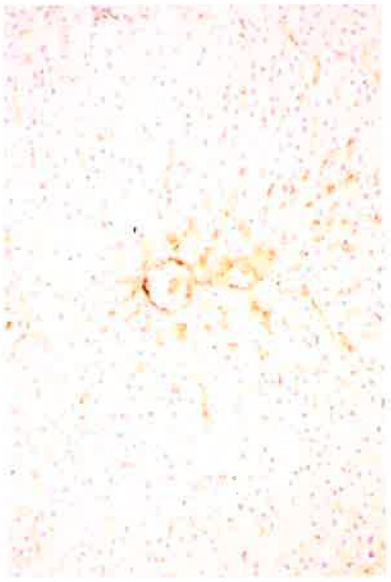


Figure 41. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x25. Photomicrograph of staining reaction of KP1 in liver section. Label appears to radiate from hepatic lobule vessels.

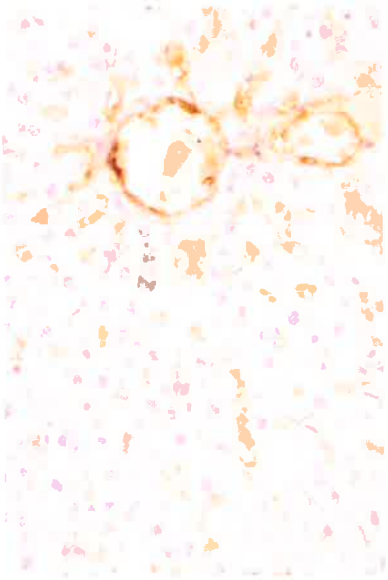


Figure 42. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Higher power photomicrograph of Fig. 41 revealing the extracellular deposition of stain.

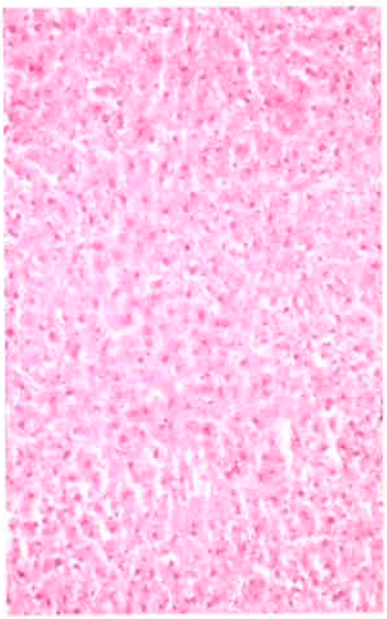


Figure 43. Experiment 4, LM, H&E, magnification x50. Photomicrograph of control liver histological section revealing normal hepatic structure.

Figure 44. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of negative control liver section. No KP1 antibody was applied but label is evident in a radiating pattern.

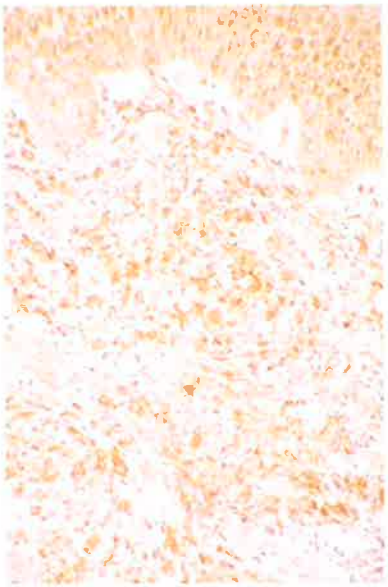


Figure 45. Experiment 4, LM, microwave antigen retrieval, haematoxylin, magnification x25. Photomicrograph of KP1 antibody label in human skin as a positive control tissue revealing macrophage distribution.

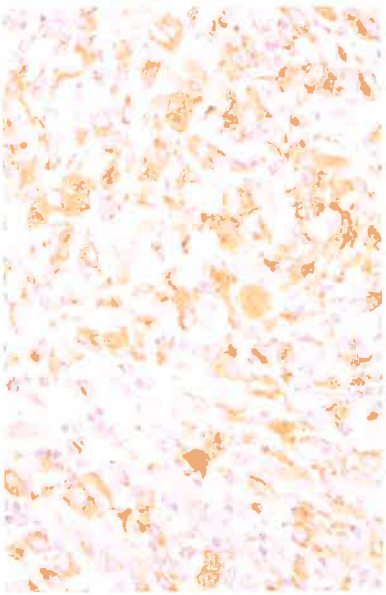


Figure 46. Experiment 4, LM, microwave antigen retrieval, haematoxylin, magnification x50. Higher power photomicrograph of Fig. 45 showing KP1 and macrophage distribution in skin.

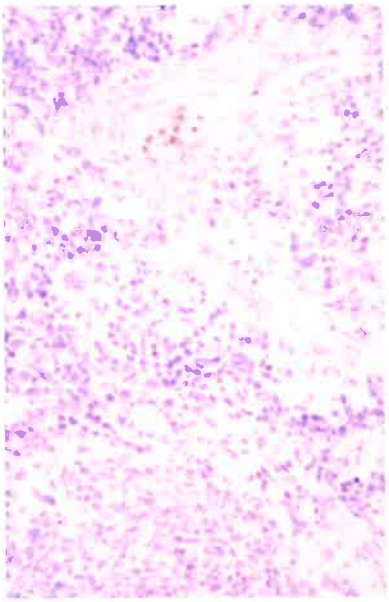


Figure 47. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of spleen sections. No KP1 label was apparent.

Figure 48. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of dental distribution of KP1. No label was noted in the periodontal ligament of a frozen molar but some stain in the necrotic dental pulp.

Figure 49. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Higher power photomicrograph of Fig. 48 showing frozen molar PDL and only a minor reaction in dental pulp.

- **ED1 antibody**

A. Immunolabelling control tissue

As a macrophage marker, the ED1 antibody produced a positive DAB brown-stain reaction localized in the cytoplasm of the cells in the tissues examined.

Sections of spleen as haemopoietic tissue were used as positive immunologic controls and brown DAB-staining cells were seen in the haemopoietic areas that surround lymphoid cell germinal centres (Fig. 50). At higher magnification, the positive marker was identified in the cytoplasm of the labelled cells (Fig. 51).

Negative control tissue was provided by sections of spleen to which ED1 antibody had been omitted but all other labelling steps completed. Brown staining was not seen in any areas of the spleen (Fig. 52) irrespective of the antigen retrieval method.

A positive ED1 reaction was identified in isolated cells of the rat kidney. This reaction was limited to cells between nephron glomeruli and appeared less intense compared with that seen in spleen tissue. The brown immunolabel appeared associated with the cytoplasm of the affected cells (Fig. 53).

Negative labelling controls using kidney sections, to which ED1 antibody had been deleted from the immunolabelling protocol, showed no reaction (Fig. 54). No ED1 stain was seen in unfrozen molar sections that were used as negative immunologic controls (Figs. 55 and 56). The omission of ED1 antibody from the immunolabelling procedures resulted in the detection of no immunologic reaction on tooth or bone surfaces. In addition, sections of experimentally frozen rat molar

which were used as immunological negative controls failed to stain when ED1 primary antibody was omitted from the staining protocol (Fig. 57).

However, positive ED1 staining was evident in all liver sections irrespective of whether ED1 antibody had been added (Fig. 58). Both positive and negative control sections labelled.

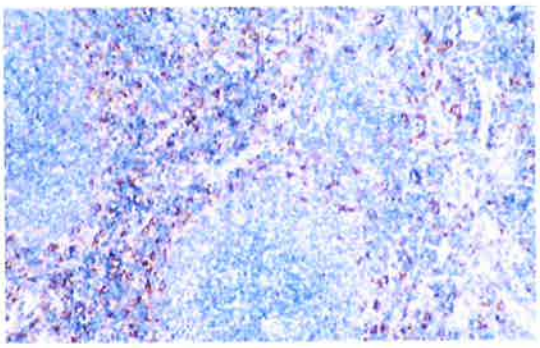


Figure 50. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of ED1 staining in a positive control section of rat spleen.

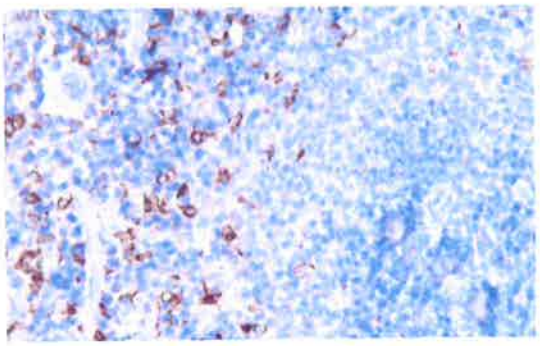
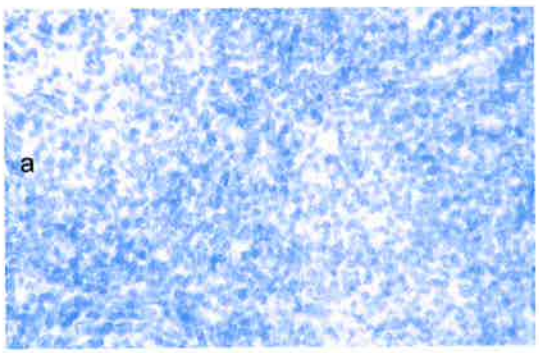


Figure 51. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. A higher power photomicrograph of positive rat spleen tissue revealing the cytoplasmic distribution of the ED1 label.



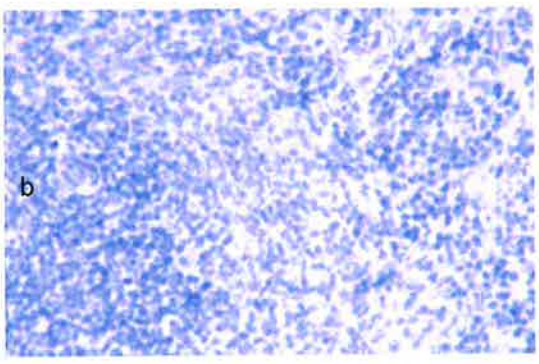


Figure 52. Experiment 4, LM, haematoxylin, magnification x100. Photomicrographs of sections of rat spleen processed via trypsin enzyme (a) or microwave (b) retrieval techniques but omitting ED1 as the primary antibody. No evidence of labelling is apparent.

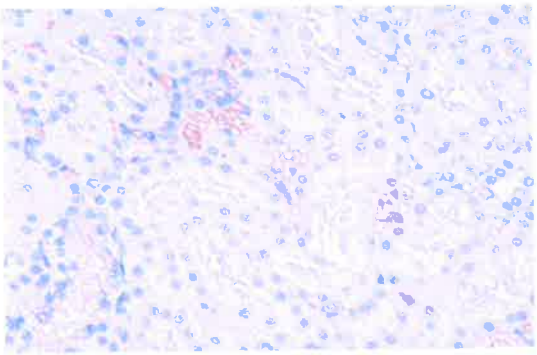


Figure 53. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Photomicrograph of section of rat kidney showing positive reaction to ED1 antibody.

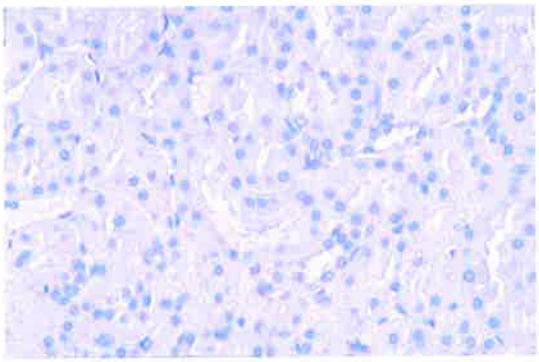


Figure 54. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Photomicrograph of section of rat kidney to which primary antibody had not been added. No label is evident.

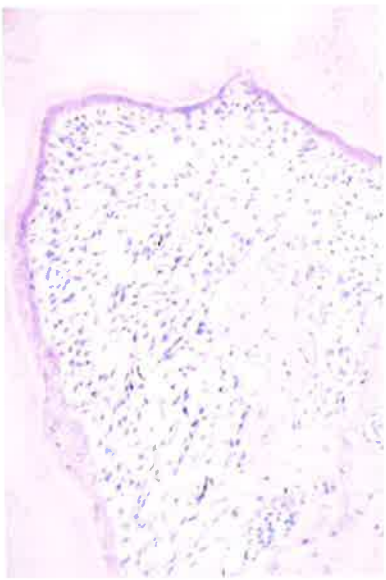


Figure 55. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of an unfrozen rat molar used as an immunologic control. No ED1 antibody was applied and no staining is apparent in the PDL.

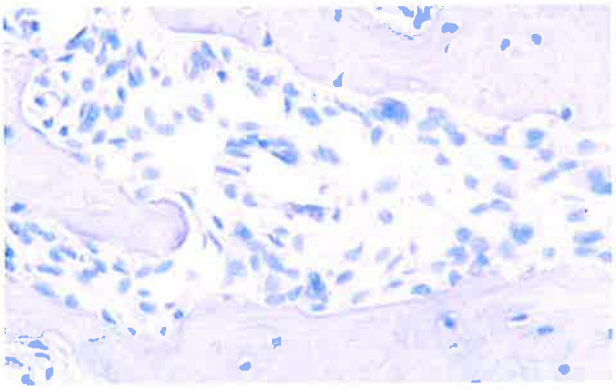


Figure 56. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Immunological ED1 control without antibody applied. No label is evident in bone or in trabecular spaces.

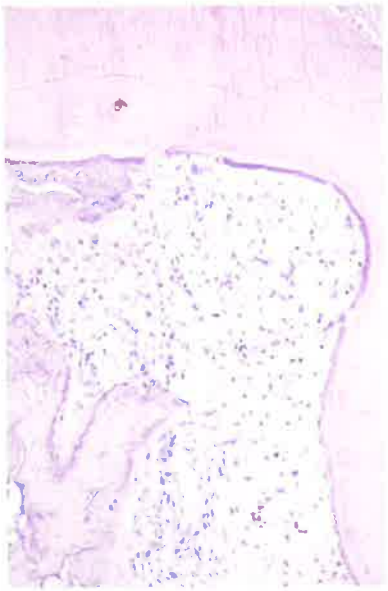


Figure 57. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of negative immunologic control of interradicular area of a frozen molar to which ED1 antibody had not been applied. No ED1 reaction is seen in the PDL nor ankylotic area.

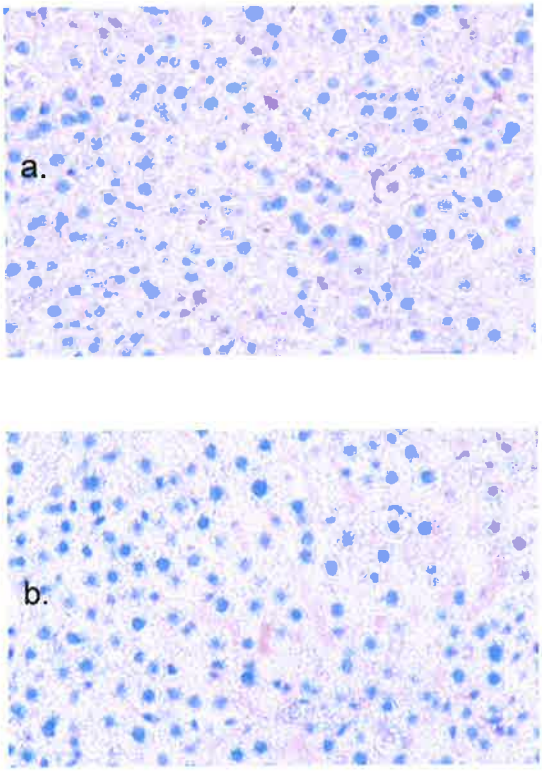


Figure 58. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Photomicrographs of sections of rat liver showing weak positive labelling reactions when primary ED1 antibody is added (a) and also when the primary antibody is omitted as a negative control (b).

B. Control teeth – unfrozen

ED1 labelling, identified by a brown diaminobenzidine (DAB) stain, was apparent in the PDL of unfrozen molars. The label was seen in mononuclear cells that appeared to be sparsely scattered throughout the ligament (Fig. 59). Larger positively-staining cells were seen on hard tissue surfaces that appeared to be undergoing resorptive activity (Fig. 60). Resorptive activity occurring towards the apical region of the tooth also attracted a positive label in a clast-like cell as well as mononuclear cells in the vicinity (Fig. 61). Nuclear identity was preserved in cells with positive reactivity as staining only involved the cytoplasmic components (Fig. 60). ED1 label was detected on interradicular alveolar bone surfaces in addition to marrow endosseous surfaces (Fig. 62).

C. Experimental teeth- frozen

ED1 labelling in the PDL surrounding a frozen rat molar was more widespread and intense compared with control unfrozen teeth. Brown DAB stain was seen on tooth root and bone surfaces as well as within the PDL and bone marrow spaces. Strongly staining mononuclear cells were evident in the periodontal ligament particularly in association with inflammatory change (Fig. 63). Areas of inflammation were surrounded by ED1-positive cells.

ED1-positive cells that appeared to be in intimate contact with hard tissue surfaces were associated with large resorption lacunae. Most label reactivity was noted in the interradicular region (Fig. 63) where strongly staining cells lined lacunae on the interradicular surface of the rat molar (Fig. 64).

Rat PDLs that showed an ankylotic change as a result of the experimental procedures presented a unique distribution of label. Within the ankylotic union little label was seen except in developing marrow spaces (Fig. 65) but there was greater reactivity in adjacent PDL tissue. A strong line of label identified cells on the surface of interradicular alveolar bone at both the crestal level and more apically (Fig. 66). Areas of adjacent inflammation were also seen to incorporate label.

Magnified views of the cells lining the surface of bone revealed both multinucleated as well as mononuclear contributors to the resorptive process (Fig. 67). Similarly, large ED1-positive cells in dental resorptive lacunae displayed a multinucleated appearance (Figs. 68 and 69). Resorption lacunae that were apparently undergoing repair contained no evidence of ED1 activity but

mononuclear cells in the neighbouring PDL exhibited label (Fig. 70). Interradicular alveolar bone marrow spaces contained positively labelled megakaryocytes in abundance (Fig. 71).

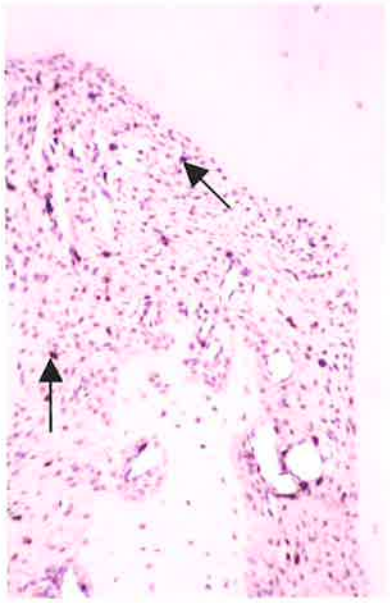


Figure 59. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of an unfrozen rat molar indicating ED1 activity largely involving mononuclear cells (arrows) in the PDL.

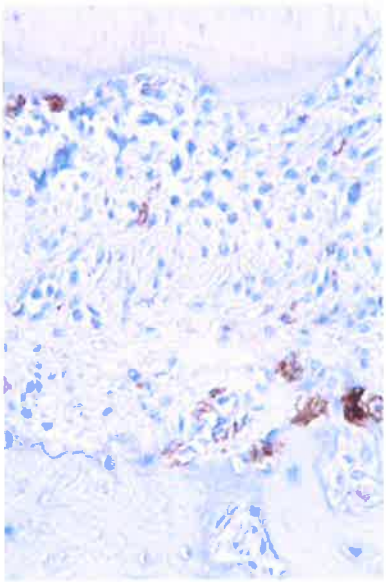


Figure 60. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Photomicrograph of an unfrozen rat molar revealing ED1 activity in the interradicular area. ED1 positive cells are visible on the crestal bone and also on the tooth surface.

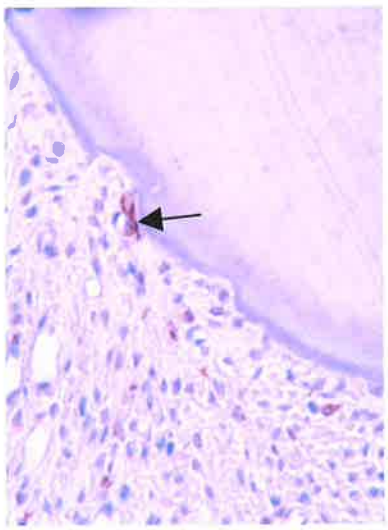


Figure 61. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Photomicrograph of an isolated ED1-positive clast cell (arrow) adjacent to a resorption lacuna towards the apical area of an unfrozen molar. Sparse and weak positive staining is also seen cells in the surrounding PDL.

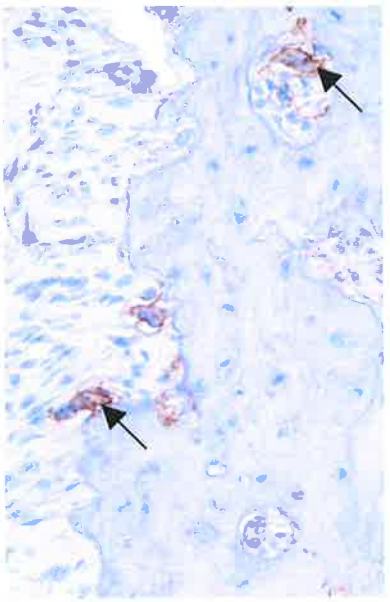


Figure 62. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. ED1 activity in unfrozen alveolar bone. Label is seen in large cells (arrows) located on bone surfaces as well as marrow endosseus surfaces.

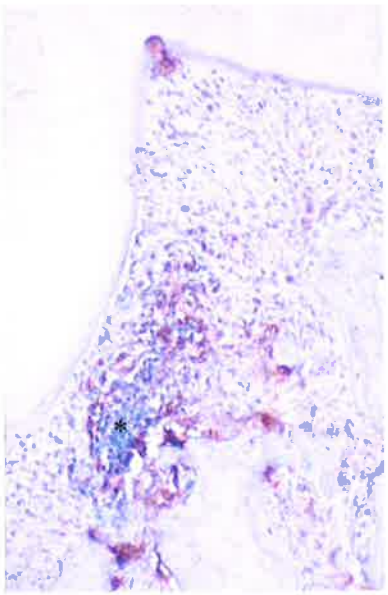


Figure 63. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of frozen molar PDL showing ED1 label. Label is concentrated around an area of inflammation (*) but hard tissue surface cells are also labelled.

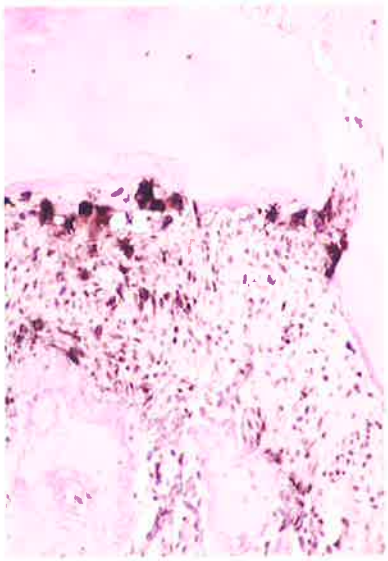


Figure 64. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of interradicular region of a frozen rat molar indicating ED1 activity in dental resorptive lacunae.

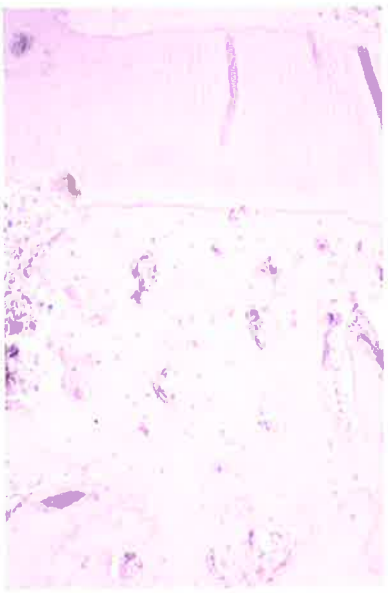


Figure 65. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of frozen molar exhibiting ankylosis and adjacent ED1 label.

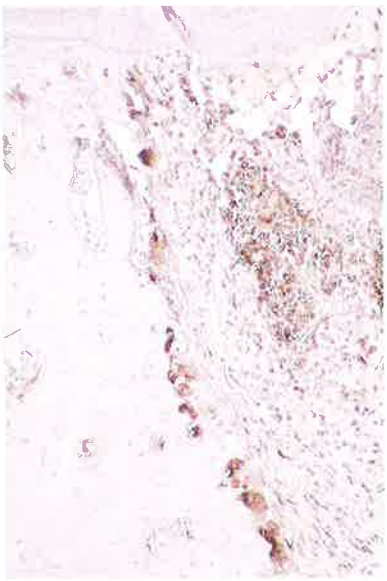


Figure 66. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of a frozen rat molar showing ankylotic union and adjacent PDL. ED1-positive cells line the surface of interradicular bone and also reveal macrophage involvement within the PDL.

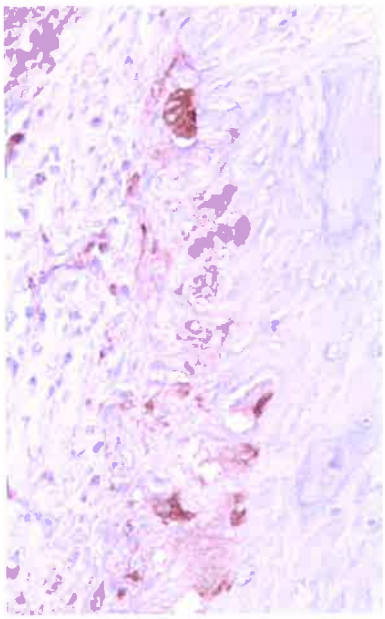


Figure 67. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Photomicrograph of ED1 distribution as a line of activity on the bony interradicular wall of a frozen molar.

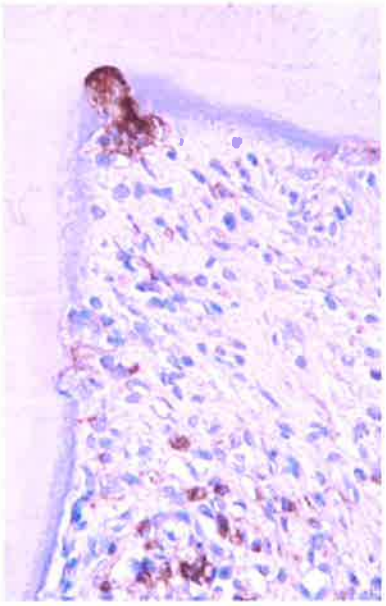


Figure 68. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Higher powered photomicrograph of Fig. 63 showing an ED1-positive multinucleated cell in a resorption lacuna in a frozen rat molar.

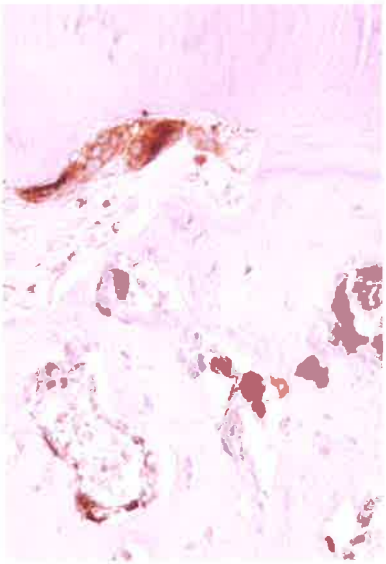


Figure 69. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Photomicrograph of ED1 distribution around ankylotic union of a frozen molar. A large positvely-staining multinucleated cell is present in an adjacent resorption lacuna.

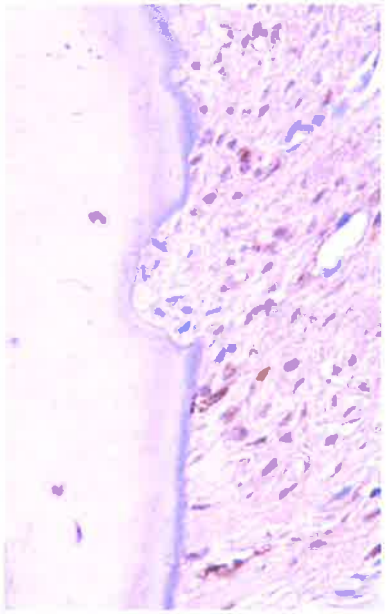


Figure 70. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Photomicrograph of a repairing resorption lacuna in a frozen molar showing no ED1 label except in scattered cells within the adjacent PDL.

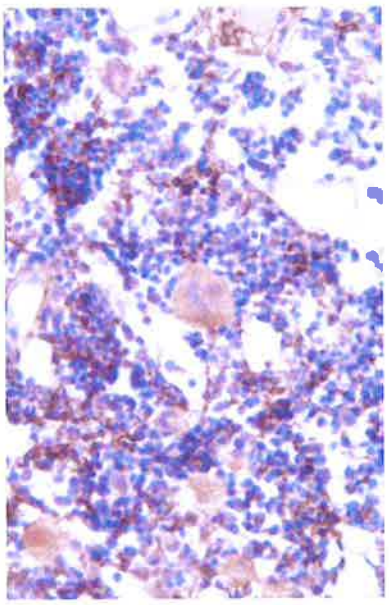


Figure 71. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Photomicrograph of haemopoietic marrow tissue of interradicular alveolar bone of a frozen molar. ED1 label is seen in megakaryocytes.

• Anti-CD45 antibody (Anti-leucocyte common antigen)

A. Immunolabelling control tissue sections

Sections of rat spleen and lymph node (Fig. 72) used as positive control tissues failed to label with anti-LCA antibody irrespective of the antigen retrieval method and the antibody titre employed.

B. Control teeth - unfrozen

In the unfrozen dental tissue an inconsistent pattern of label was seen. The PDL on one side of the molar root displayed label whereas the other side did not. The maxillae were embedded and cut in transverse (coronal) section and it was apparent that the stained ligament areas were associated with resorptive bone surfaces. Periodontal ligament associated with appositional bone surfaces was devoid of LCA label (Fig. 73)

C. Experimental teeth - frozen

Sections of PDL from frozen experimental teeth revealed heavy diffuse label in the cervical and interradicular areas. Label was noted in many cells and also in the extracellular areas in the region of the inflammatory damage. Areas of ankylosis were also surrounded by intensely-labelled tissues and cells. Heavy label was seen associated with the hard tissue surfaces adjacent to the ankylotic areas (Fig. 74).

A high level of background staining was evident in sections from frozen molars (Fig. 73). This, coupled with the failure of the positive lymphoid tissue to react, made the diffuse distribution of the anti-LCA label questionable.

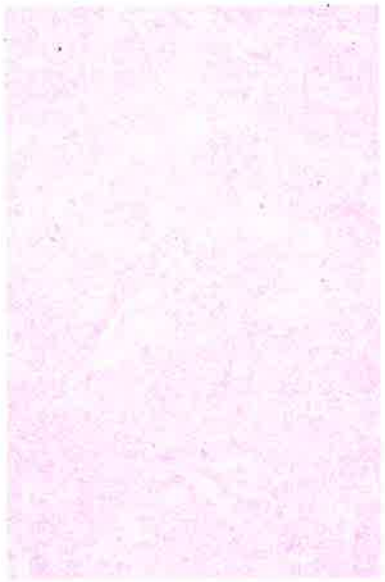


Figure 72. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of a section of rat lymph node used as a positive control tissue for CD45. No label is evident.

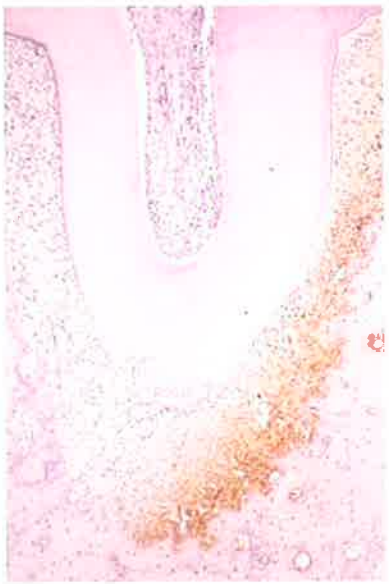


Figure 73. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x25. Photomicrograph of anti-CD45 distribution in unfrozen rat periodontal tissues. Note the unique distribution of the label.

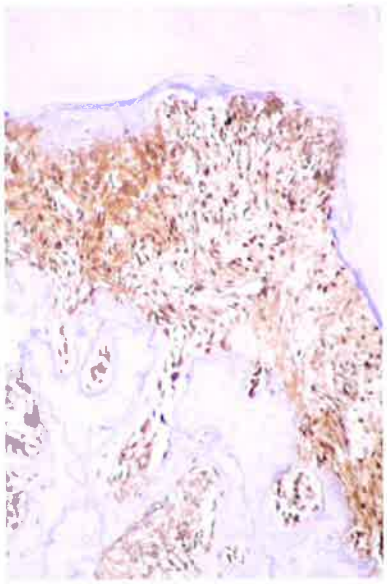


Figure 74. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x50. Photomicrograph of anti-CD45 label distribution in the PDL of a frozen rat molar. Label is diffuse but concentrated around the ankylotic inflammation and in larger resorptive cells on the cementum surface. Considerable background label is evident.

- **Anti-CD13, anti-CD15 antibodies**

Extremely weak labelling of periodontal tissues occurred with anti-CD13 and anti-CD15 antibody as myeloid cells markers (Figs. 75, 76). Mononuclear cells appeared to be labelled at sites of hard tissue resorption but a meaningful interpretation became uncertain due to the unreliable nature of the label. Interpretation was further clouded by the weak labelling of rat spleen sections as positive control tissue.

- **Anti-cathepsin L, anti-CD61 and anti-carbonic anhydrase II antibodies**

Anti-cathepsin L (Fig. 77), anti-CD61 and anti-carbonic anhydrase II antibodies failed to positively react with any of the tissues examined. Antigen retrieval by trypsin enzyme digestion or microwaving techniques in citrate buffer failed to affect visualization. Similarly, altering the titres of the antibodies provided no assistance.

- **AE1/AE3 antibody**

As a pan-cytokeratin marker, AE1/AE3 strongly labelled epithelial tissue in frozen and unfrozen sections of rat molar PDL prepared following the fixation, decalcification, antigen retrieval and labelling protocols employed by this study (Fig. 78, 79). No label of significance was detected in the PDL.

The overall summary of the results of the immunolabelling is presented in Table 4 below.

Antibody	Frozen molar	Control molar	+ve tissue control	-ve control
KP1	-	-	+	+
Anti-Cathepsin L	-	-	-	-
ED1	+++	+++	+++	-
Anti-CD13	+-	+-	+-	-
Anti-CD15	+-	+-	+-	-
Anti-CD45 (Anti-leucocyte common antigen)	+-	+-	-	-
Anti-CD61	-	-	-	-
Anti-Carbonic anhydrase II	-	-	-	-
AE1/AE3	+ (surface epithelium)	+ (surface epithelium)	+ (surface epithelium)	-

Table 4. Tabulated results of the antibodies tested.

Figure 75. Experiment 4, LM, microwave retrieval, haematoxylin, magnification x50. Photomicrograph of weak CD13 label within the frozen rat PDL. Large cells in resorption lacunae revealed some activity but interpretation was inconclusive.

Figure 76. Experiment 4, LM, microwave retrieval, haematoxylin, magnification x100. Photomicrograph of frozen rat periodontium showing weak labelling for CD15.

Figure 77. Experiment 4, LM, microwave retrieval, haematoxylin, magnification x100. Photomicrograph of frozen rat periodontal tissues indicating a lack of labelling with anti-cathepsin L antibody.

Figure 78. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x25. Photomicrograph of section of unfrozen rat molar labelled with AE1/AE3 in order to check labelling protocol and tissue preparation techniques. Strong epithelial label was evident.

Figure 79. Experiment 4, LM, trypsin antigen retrieval, haematoxylin, magnification x100. Higher power photomicrograph of Fig. 78 showing epithelial labelling and also a sparse light staining in PDL cells.

4.3.4.4 Transmission electron microscopy

A. Control teeth ultrastructure - unfrozen

The width of the periodontal ligament in the cervical area appeared to be relatively uniform. Although, its dimensions were difficult to accurately determine due to the differing levels and planes of sectioning they were of the order of approximately 100–150 µm. Tooth dentine was covered by a layer of mineralized cementum, which in turn, was covered by a layer of non-mineralized pre-cementum (Fig. 80). The non-mineralized layer appeared to be less than 1 µm in thickness and was seen to contain bundles of collagen fibrils orientated parallel or at right angles to a lining cellular layer and the cemental surface (Fig. 81).

The cells near the root surface were seen to be relatively flat, and to line the cemental surface in a monolayer (Fig. 81). These cells possessed a large, rounded nucleus with a reduced amount of cytoplasm and fewer organelles (Fig. 82). Few cellular processes were evident and the lining cells were surrounded by collagen fibrils (Fig. 82). Further from the root surface cells were seen to be elongated or spindle shaped with increased cytoplasmic organelle content. Organelles were more readily and clearly visible and mainly comprised mitochondria and endoplasmic reticulum. Multinucleated cells, macrophages and macrophage-like cells were seldom seen near the cervical or interradicular root surfaces. When they were observed, they appeared singly and adjacent to the root surface (Fig. 83). Their nuclear size accounted for the majority of the cell's volume with little cytoplasm and no ruffled borders seen.

Resorption lacunae were not routinely evident, but when encountered, did not appear to be active in nature. Resorption bays contained mononuclear cells and collagen fringes (Figs. 84 and 85).

A little further apically the collagenous precementum layer became more pronounced as its width increased (Fig. 86). Collagen appeared in clumps orientated parallel and transversely to the hard tissue surface. Cells and their nuclei were more elongated (Fig. 87) and at higher magnification showed little cytoplasmic content (Fig. 88).

The pulp tissue of control unfrozen teeth presented an organized ultrastructural appearance. Cuboidal odontoblasts lined the pulp chamber (Fig. 89) and a connective tissue reticulum supported cellular, neural and vascular elements (Fig. 90). Myelinated and unmyelinated nerve fibres were in abundance. Cellular elements within the pulp chamber were round or cuboidal in shape with similarly shaped nuclei.

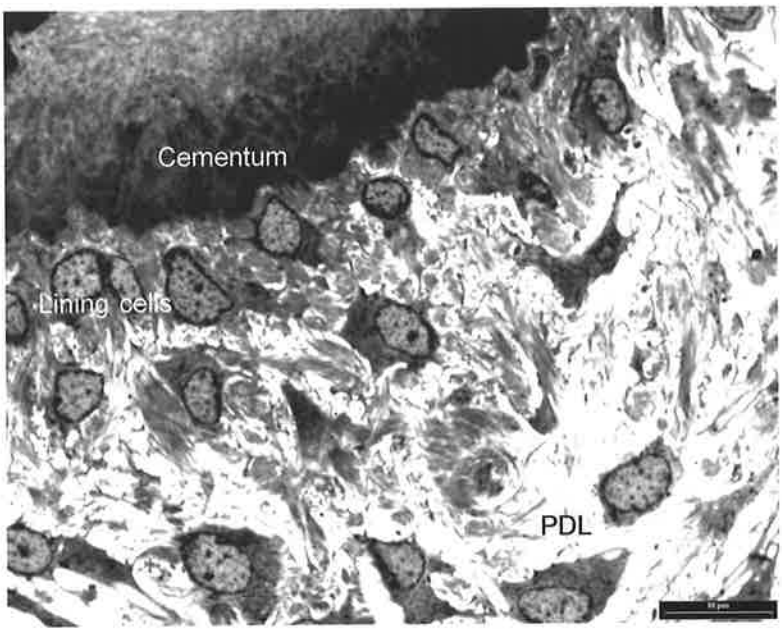


Figure 80. Experiment 4, transmission electron microscopy (TEM), uranyl acetate and Reynold's lead, magnification x1100. Electronmicrograph of interradicular cementum surface of unfrozen molar showing a single layer of lining cells.

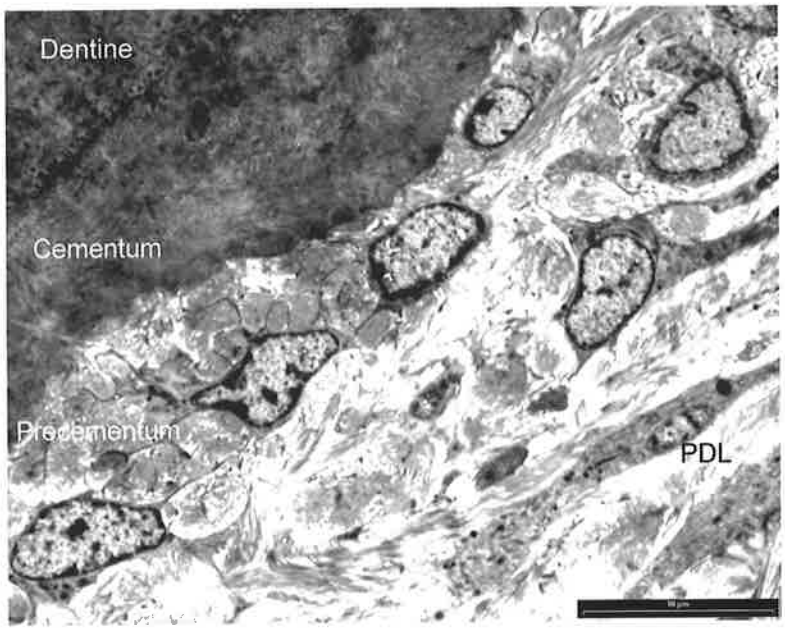


Figure 81. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1950. Electronmicrograph of lining cells (cementoblasts) and collagenous precementum orientated between cells and cementum surface of an unfrozen molar.

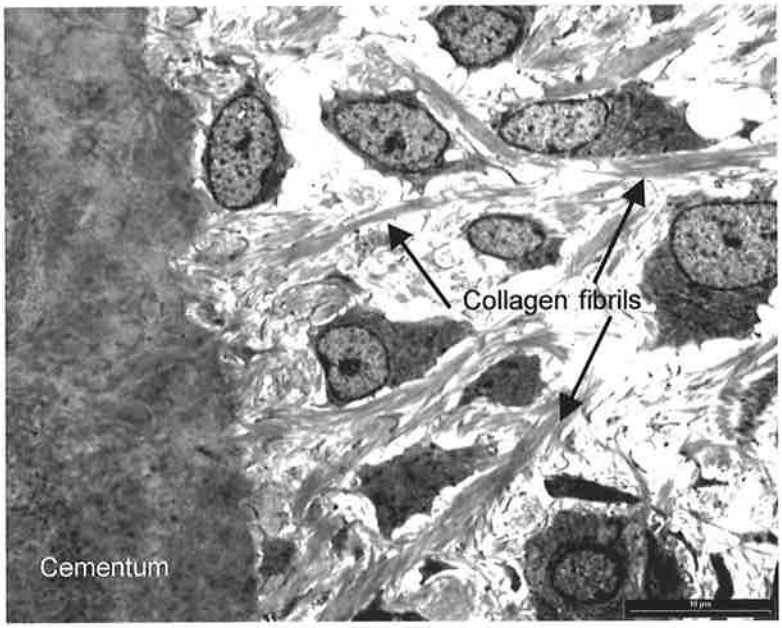


Figure 82. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1450. Electronmicrograph of cementum lining cells on unfrozen molar. Cells show large nuclei, reduced cytoplasm but few cell processes. Collagen fibrils (arrows) separate the cells.

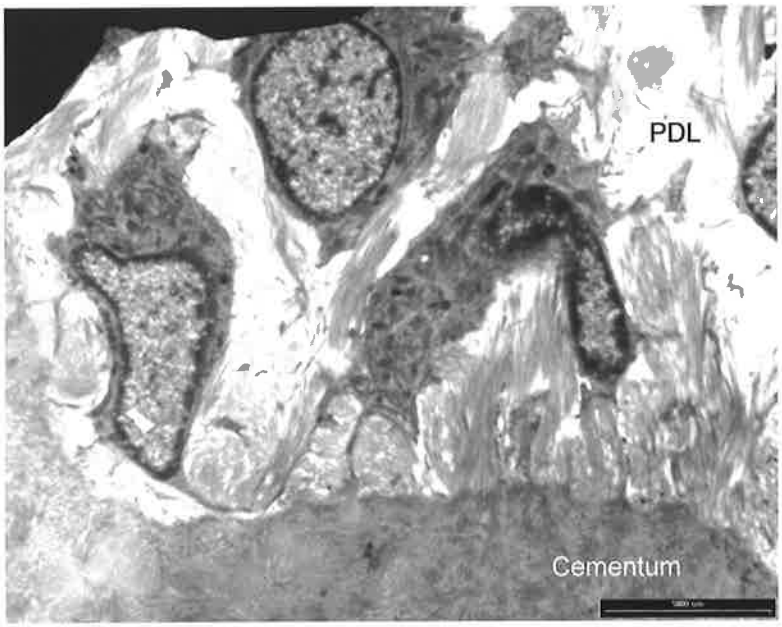


Figure 83. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x3400. Electronmicrograph of multinucleated macrophage-like cell adjacent to cementum surface of an unfrozen molar. Organelle-rich cytoplasm showed no border ruffling.

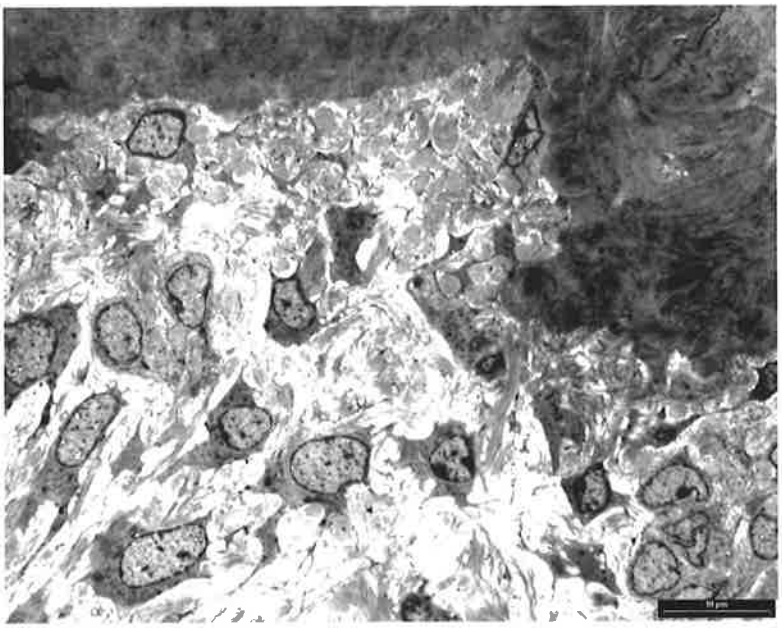


Figure 84. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1100. Electronmicrograph of an unfrozen molar showing an inactive resorption bay apparently undergoing repair. No multinucleated cells are evident but mononuclear blast-like cells are plentiful.

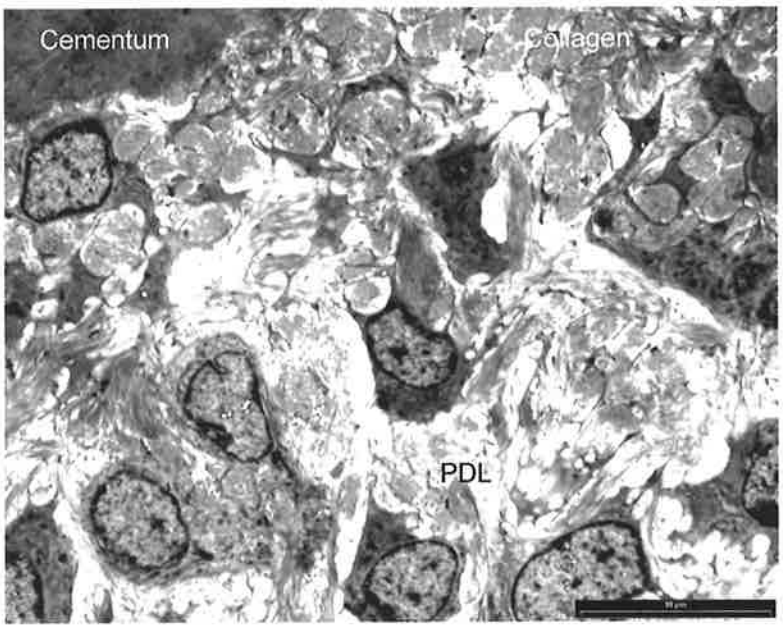


Figure 85. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1950. Higher power electronmicrograph of Fig. 84 showing heavy collagen deposition in the repairing lacuna.

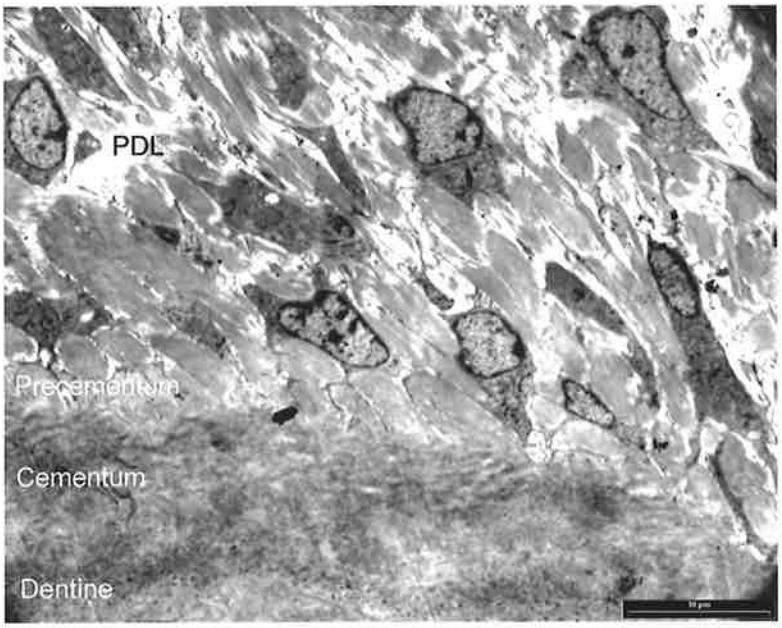


Figure 86. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1450. Electronmicrograph of root surface distant to furcation area of an unfrozen molar showing pronounced precementum layer of collagen fibril bundles.

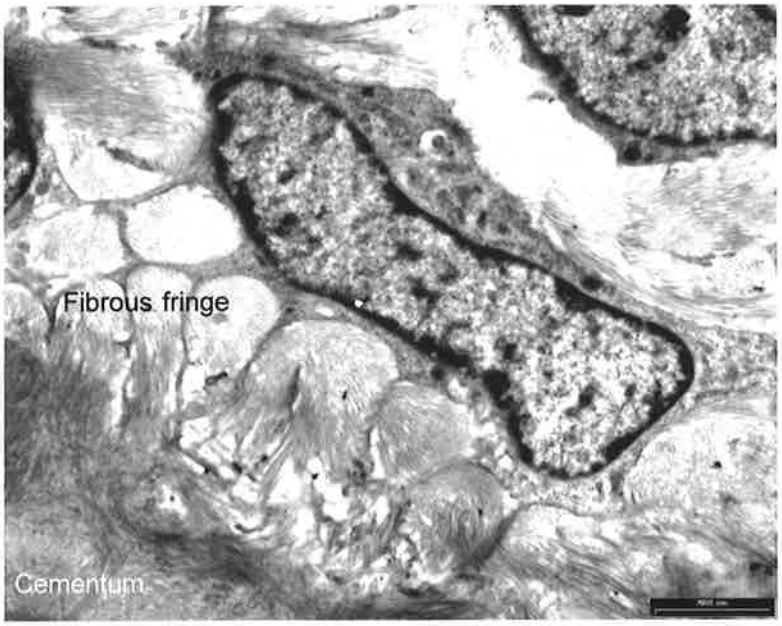


Figure 87. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x5800. Electronmicrograph of an unfrozen molar showing an elongated nucleus of lining cell over collagen fibrils as a fibrous fringe.

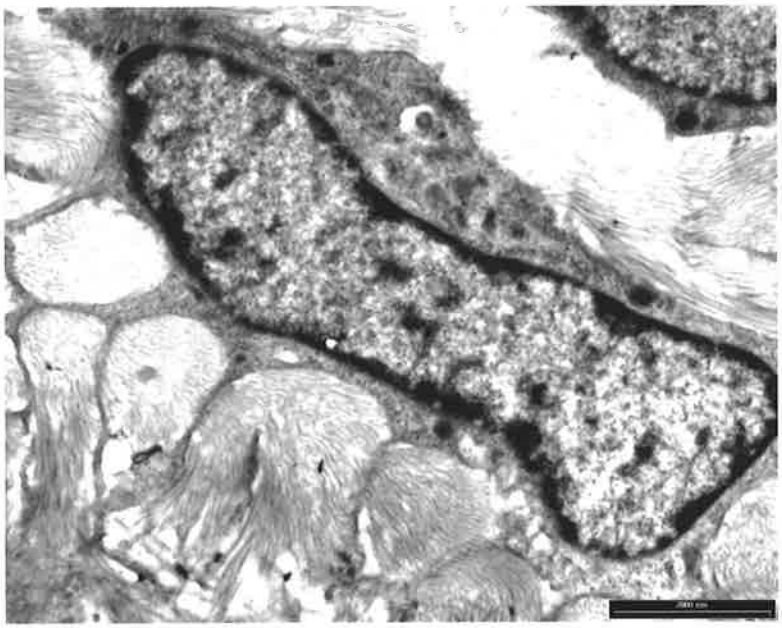


Figure 88. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x7900. Higher powered electronmicrograph of Fig. 87 revealing a reduced cytoplasmic content of the elongated cell. The collagen fringe is evident. Magnification x7900

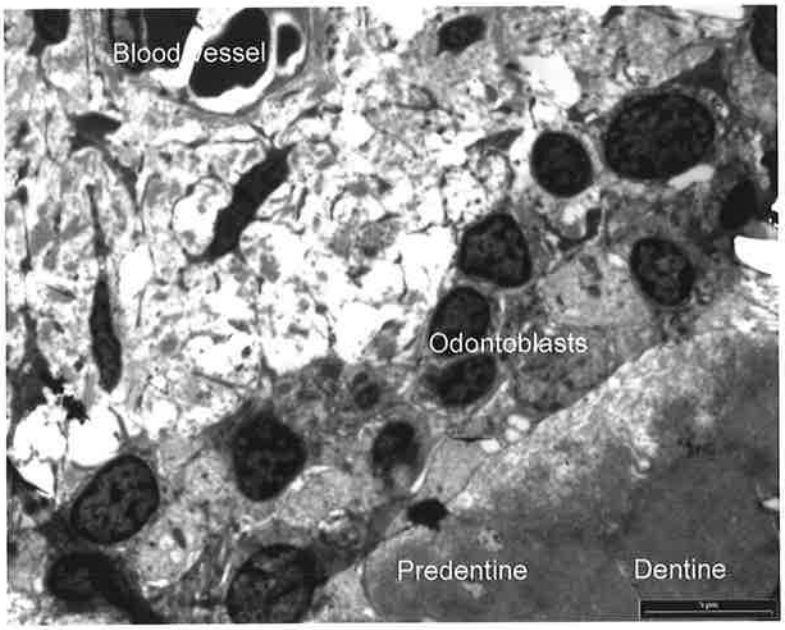


Figure 89. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x2600. Electronmicrograph of pulp tissue in an unfrozen molar indicating the odontoblastic layer and a fine vascular reticulum.

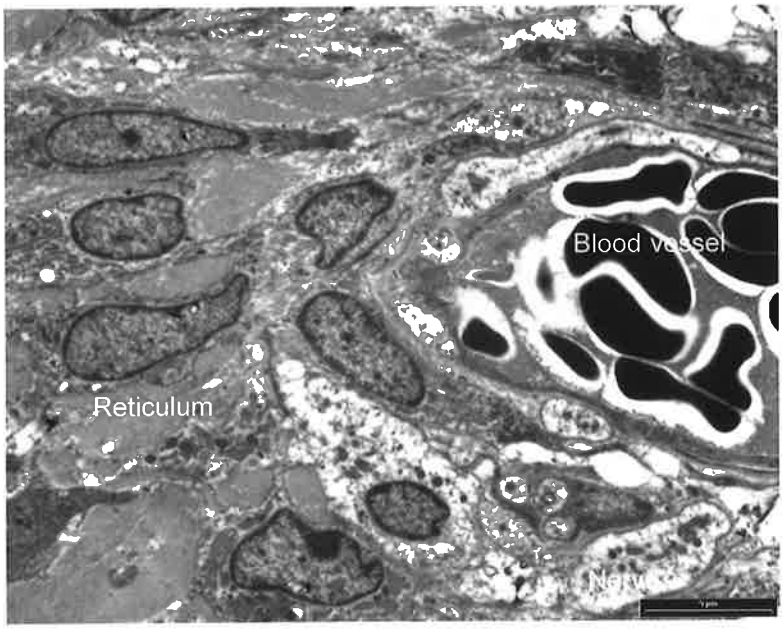


Figure 90. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x2600. Electronmicrograph of unfrozen pulp tissue showing blood vessel and nerves in a connective tissue reticulum.

B. Experimental teeth ultrastructure - frozen

An ultrastructural examination of the periodontal ligament 7 d after the application of the cold thermal stimulus to the rat molar revealed disorganization and disruption (Fig. 91). Periodontal cells appeared fewer in number while cell size and shape were highly irregular. Lining cells were absent from the cemental surface (Fig. 92, 93) and the surrounding collagen fibres appeared disorientated (Fig. 94).

No pre-cementum layer was noted on root surfaces but shallow cemental surface indentations were seen. Many of these scalloped areas did not appear to have any cellular involvement (Fig. 92) and were consistent with the acellular areas of hyalinization. Other resorptive areas contained large cells adjacent to the root surface (Fig. 95). The cells contained numerous vacuoles and organelles but presented an appearance inconsistent with resorptive activity. No ruffled borders or clear cytoplasmic zones were seen in these large cells. Other large vacuole-filled cells were seen at a distance from the cemental surface along with collagen fibre disarray (Fig. 96).

Resorption lacunae contained multinucleated cells adjacent to the cementum surface (Fig. 92, 97). These cells were irregular in shape with numerous cytoplasmic processes. No evidence of ruffled borders was observed in all of the sections examined. Nuclear number varied up to a maximum of six per cell in any section. Often multinucleated cells showed signs of polarization with organelle-free regions apparent but often not orientated towards apparent hard tissue surfaces (Fig. 97, 98). Many cytoplasmic vesicles were seen along with the intracellular organelles.

Other multinucleated cells, in close relationship with cementum, were associated with surface cavitation (Figs. 99, 100). These cells did not appear to be firmly attached to the hard tissue surface or possess a ruffled border in the observed sections. Cytoplasmic contents showed signs of polarization away from the cementum surface. Neighbouring cells of the ligament were either few in number (Fig. 99) or showed signs of degeneration and the loss of cytoplasmic contents into the extracellular spaces (Fig. 98).

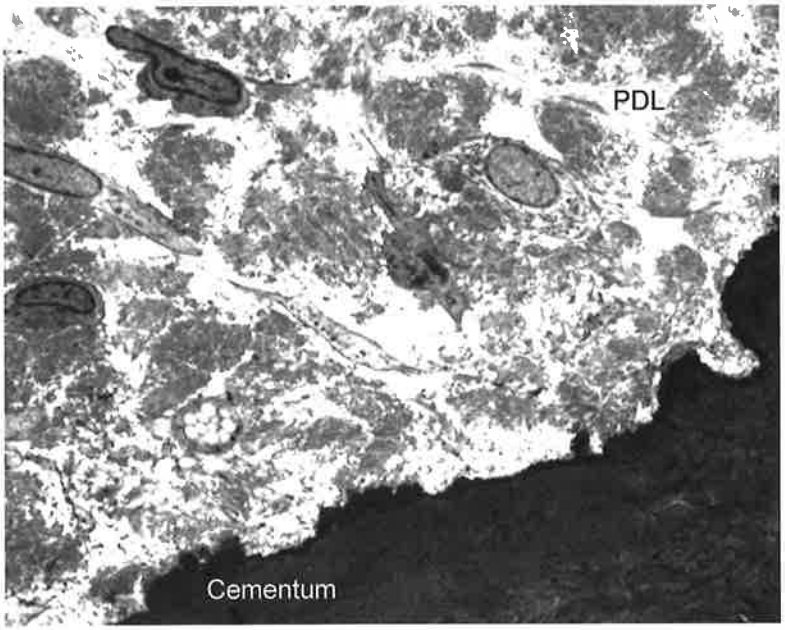


Figure 91. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1450. Electronmicrograph of the PDL of a molar subjected to freezing revealing no lining cell layer and fibre disruption.

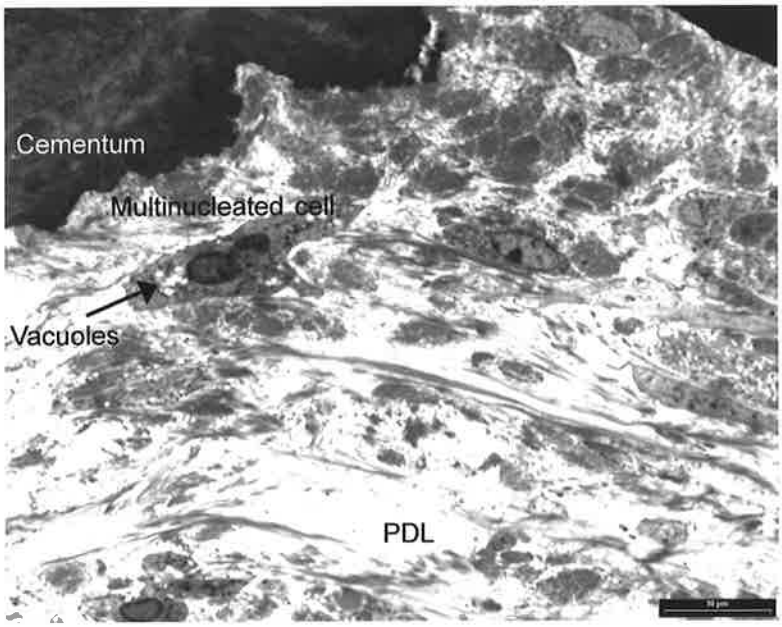


Figure 92. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1100. Electronmicrograph of the PDL surrounding a frozen molar showing no cell lining layer. A multinucleated cell with vacuoles (arrow) is present.

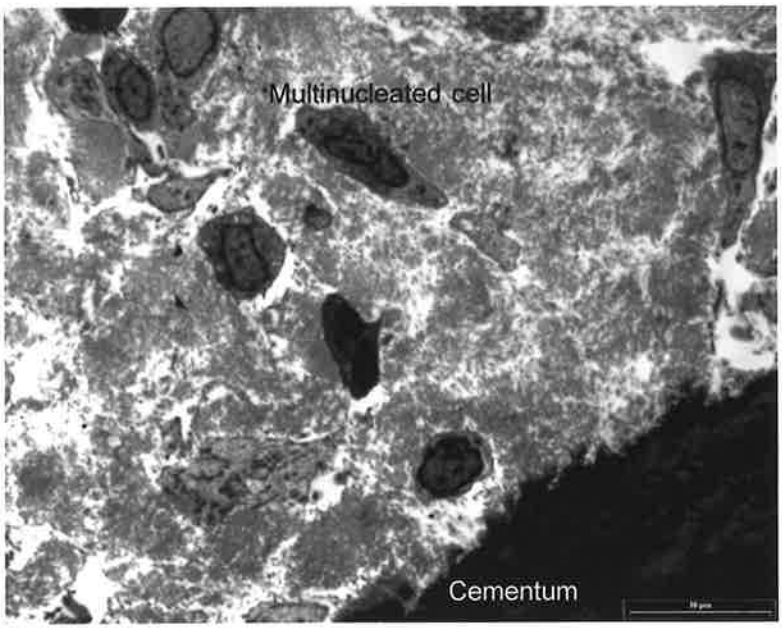


Figure 93. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1450. Electronmicrograph showing periodontal disorganization following molar freezing.

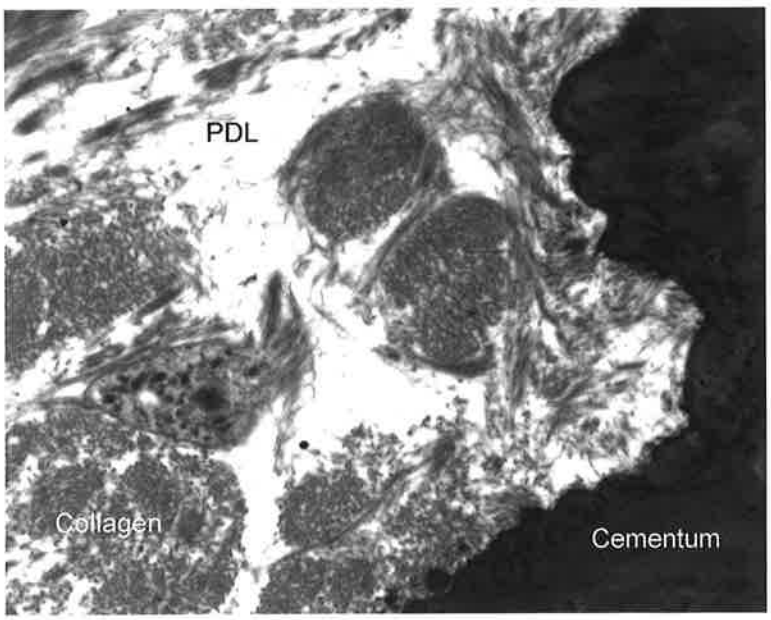


Figure 94. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x3400. Electronmicrograph of disorganized PDL of frozen molar showing relative cellular paucity. A single cell is evident but the overall picture is one of disruption and disorientation.

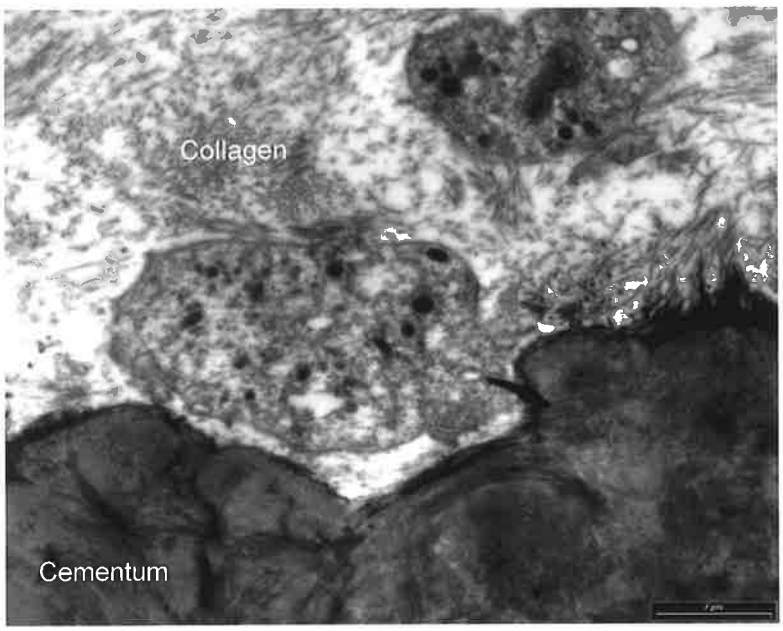


Figure 95. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x5800. Electronmicrograph of large cell adjacent to the cementum surface of a frozen rat molar. The cell contains vacuoles and organelles but morphologically presents few signs of resorptive activity.

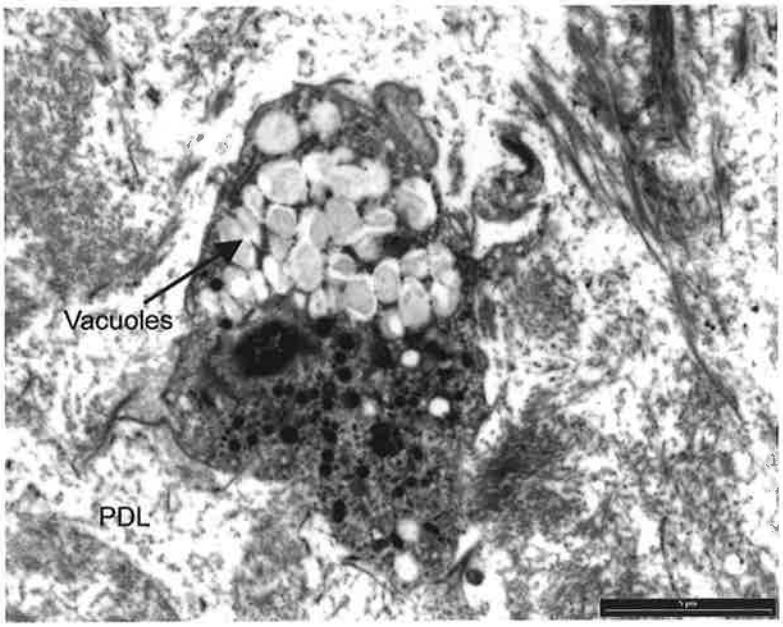


Figure 96. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x3400. Electronmicrograph of a large vacuole-filled cell in the PDL surrounding a frozen rat molar. The cell is some distance from the cementum surface.

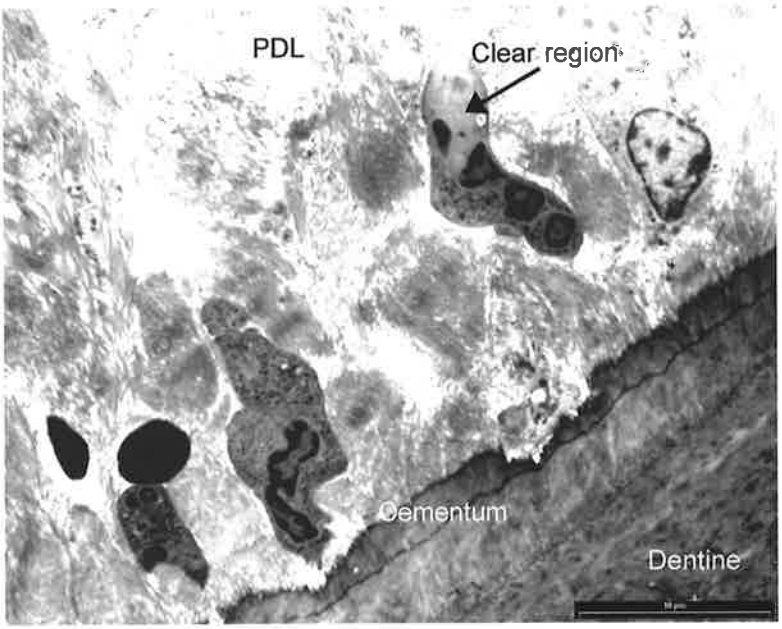


Figure 97. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1950. Electronmicrograph of cementum surface of a frozen rat molar with adjacent multinucleated cells. Organelle-free areas are apparent as clear regions of cytoplasm. Magnification x1950

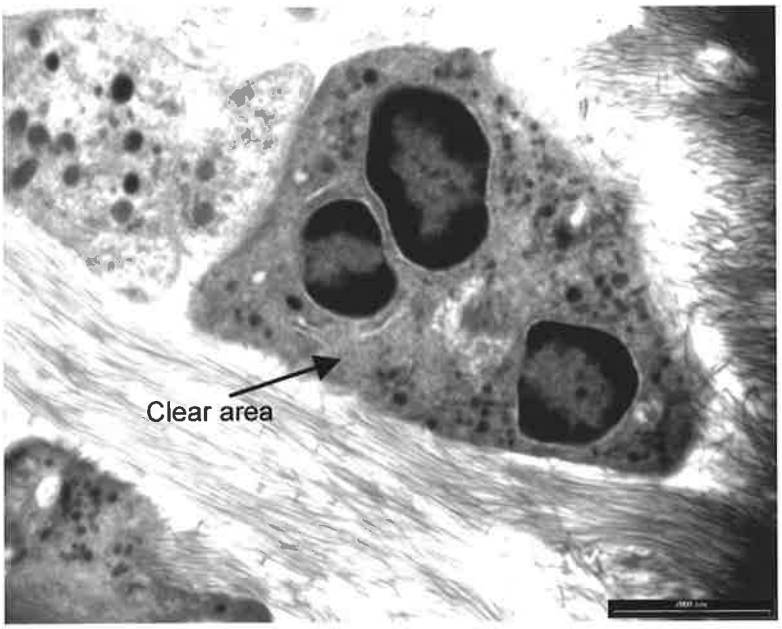


Figure 98. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x7900. Electronmicrograph of multinucleated cell adjacent to cementum surface. The cytoplasm reveals clear areas free of organelles (arrow).

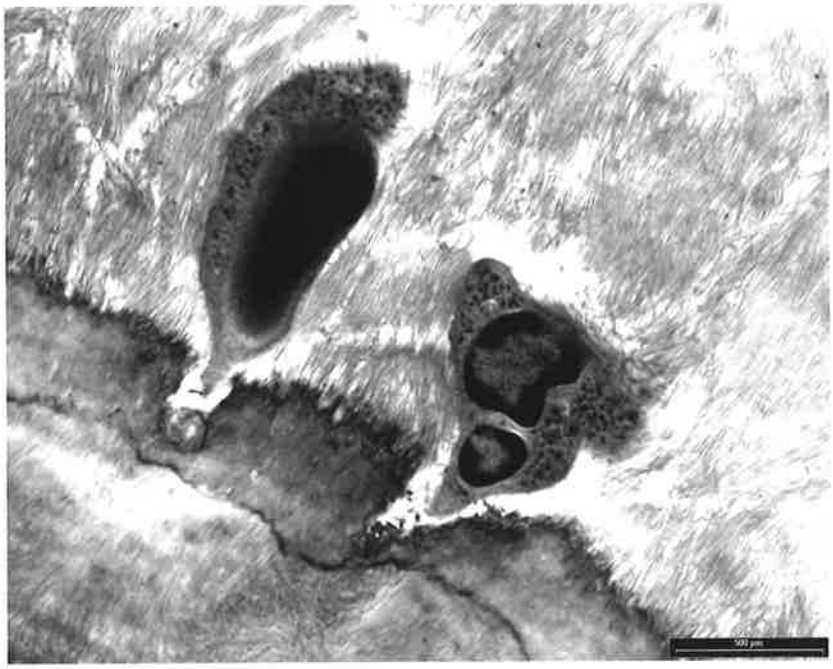


Figure 99. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x4600. Electronmicrograph of large multinucleated cells associated with resorbing cementum surfaces of a frozen rat molar.

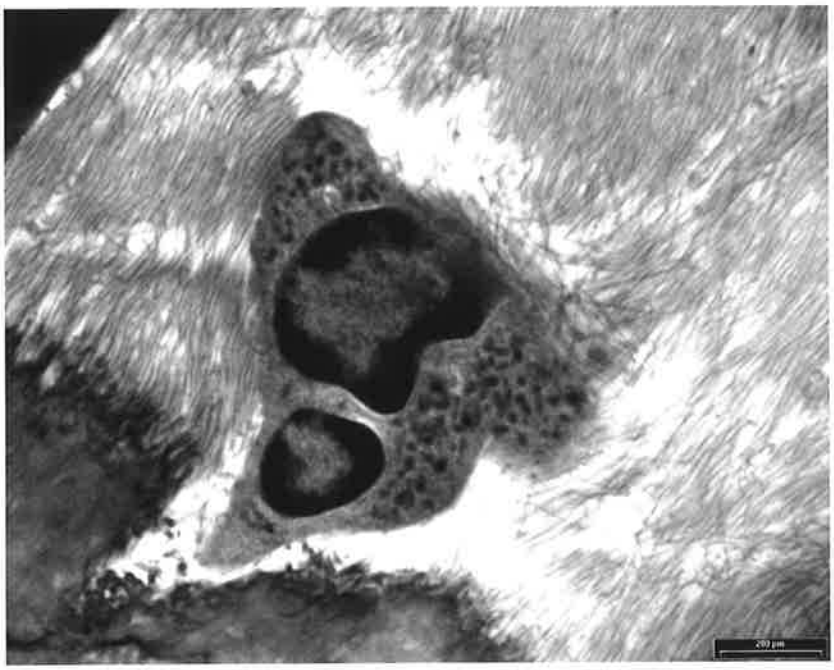


Figure 100. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x7900. Higher power electronmicrograph of Fig. 99 showing the multinucleated cell and cavitation of the cementum surface. The cell's cytoplasmic contents appear polarized away from the tissue surface and no ruffled border is apparent.

- **Bone surface**

The surface of bone was indented with resorption bays presenting two different appearances. Larger areas of resorption containing a variety of cells were seen (Fig. 101). There was evidence of multinucleated cell involvement; however, cells did not appear to be actively resorbing bone (Fig. 102). No ruffled borders were obvious nor polarization seen. Mononuclear cells were present in the resorption bays in close association with the hard tissue surface. Many of the resorption bays were repairing with evidence of bone deposition in association with a reversal line (Fig.103). However, an examination of nearby areas revealed bone resorption and multinucleated cells (Figs. 104 and 105). Cells were in intimate contact with the surface of bone (Fig. 105) and were accompanied by small mononuclear cells. The mononuclear cells appeared to be in contact with the bone-resorbing multinucleated cells by fine membranous processes (Figs. 104 and 105). Cells in the resorption lacunae were surrounded by collagen fibrils and cellular debris (Fig. 106).

Other bone surfaces showed mononuclear osteoblast-like cells in close apposition to one another (Fig. 107). Deposited bone matrix as bundled collagen was seen on the hard tissue surface and surrounded these cells. The occasional multinucleated cell was evident (Fig. 108). Clusters of blast-like cells, some with indented nuclei were in close approximation to newly laid surface osteoid (Figs. 109 and 110).

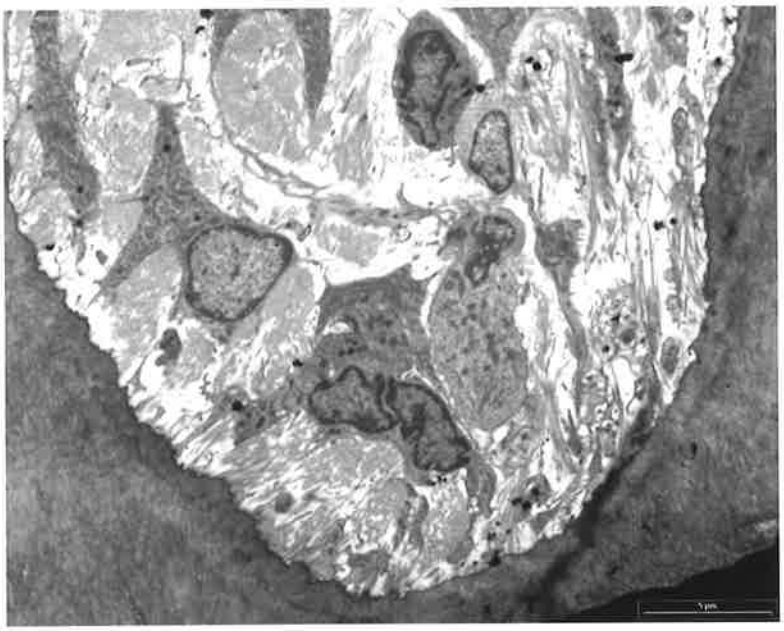


Figure 101. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x2600. Electronmicrograph of a bone resorption lacuna with multinuclear cell profiles present. This section reveals no border ruffling, clear zones nor intimate cell contact with the hard tissue surface.

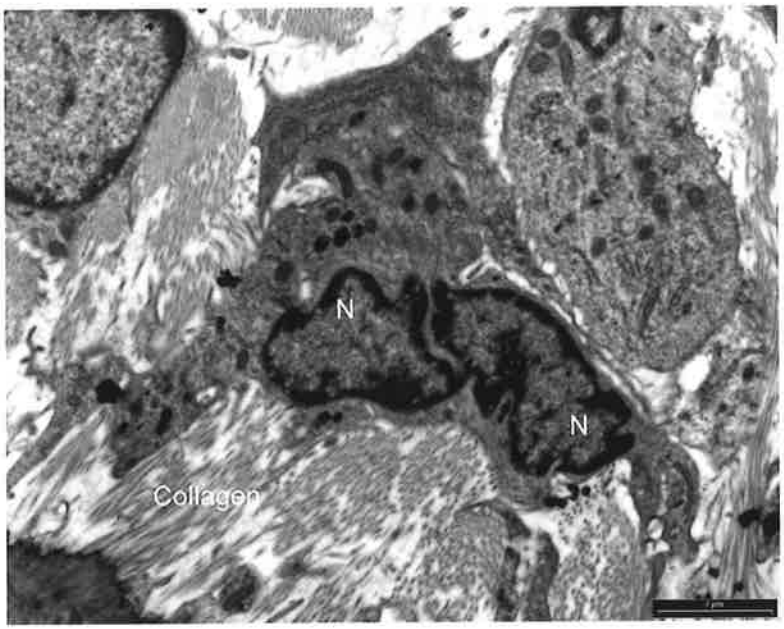


Figure 102. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x5800. A higher power electronmicrograph of cell with multinuclear profile seen in Fig. 101. The cell is largely surrounded by collagen fibrils but is adjacent to another large cell that has a high mitochondrial content. N - nucleus

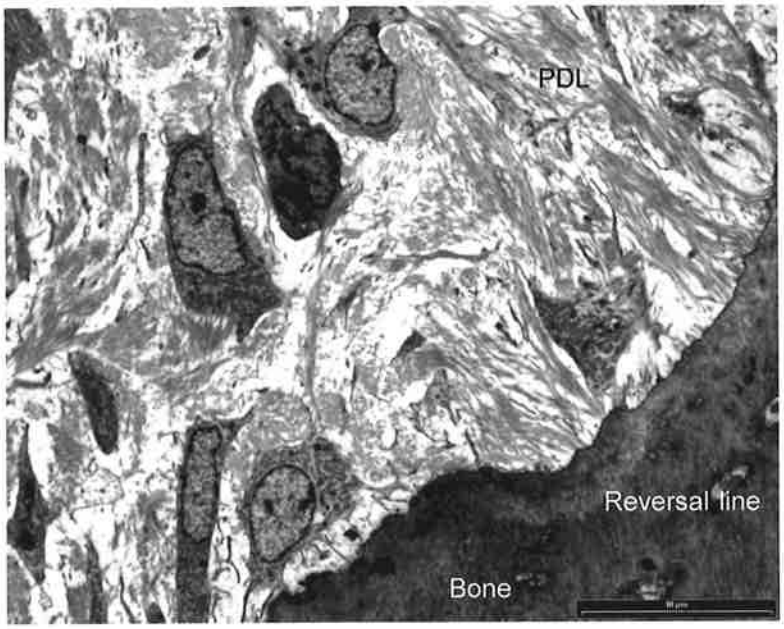


Figure 103. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1950. Electronmicrograph of frozen crestal bone surface indicating past signs of resorption and a reversal line. No multinucleated cells are apparent and collagen arrangement is disrupted. Magnification x1950

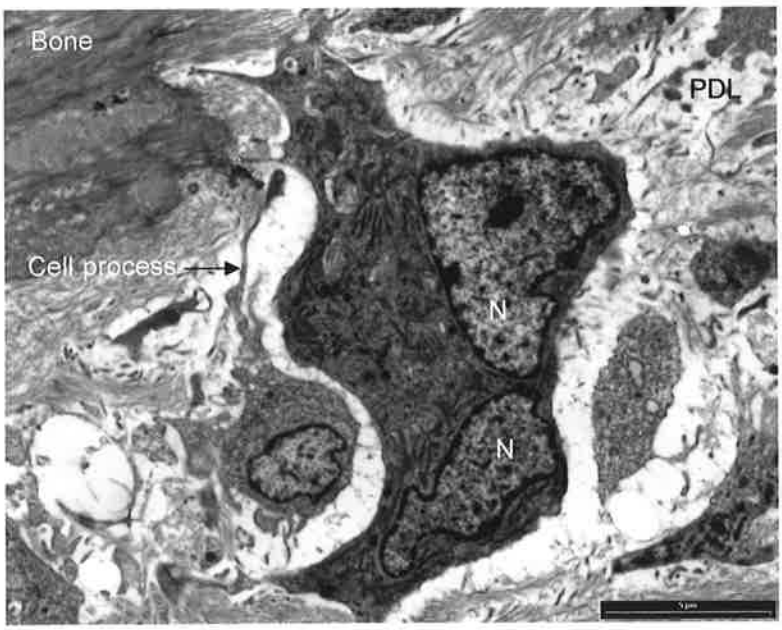


Figure 104. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x3400. Electronmicrograph of a multinucleated cell in bone resorption lacuna. In intimate contact with the large cell are smaller mononuclear cells with extending processes. N - nucleus

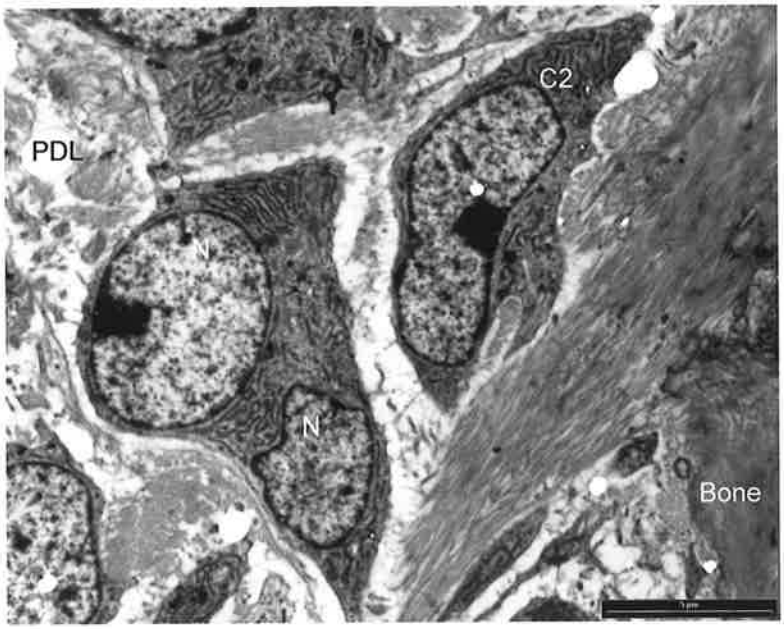


Figure 105. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x3400. Electronmicrograph of multinucleated cell in bone resorption lacuna. An adjacent cell (C2) bears similar morphologic features and is in close approximation with the bone surface. Magnification x3400, N - nucleus

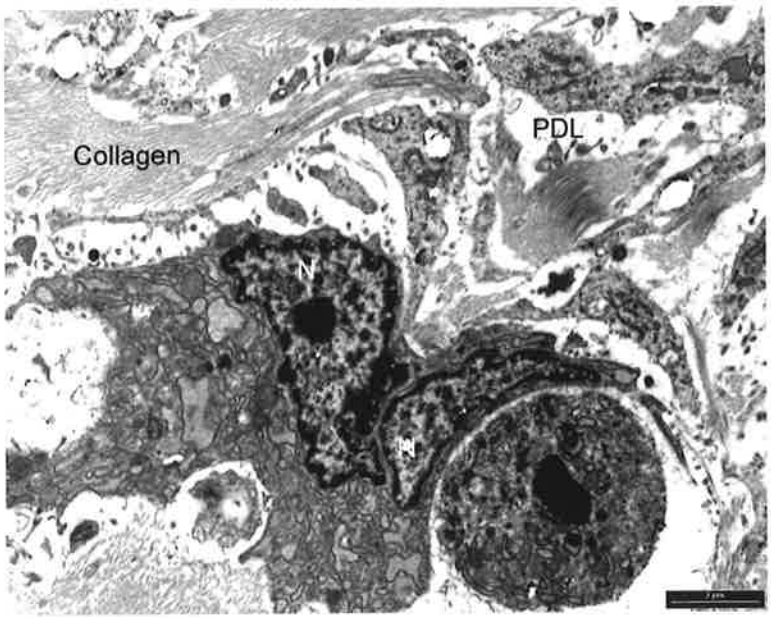


Figure 106. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x4600. Electronmicrograph of frozen molar showing multinucleated cell surrounded by collagen fibrils and cellular debris. N - nucleus

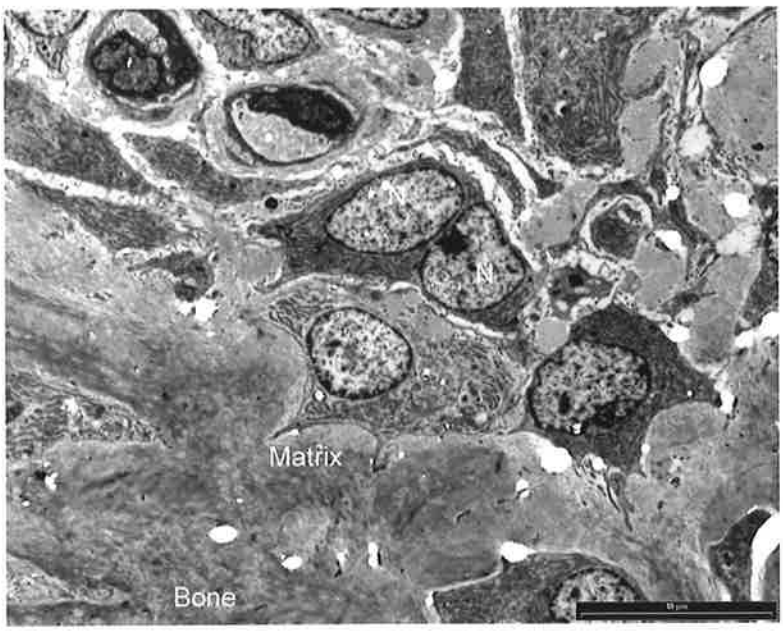


Figure 107. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1950. Electronmicrograph of frozen molar showing a multinucleated cell surrounded by mononuclear cells lining the surface of bone. Bone cells appear to be laying down collagenous matrix. N - nucleus

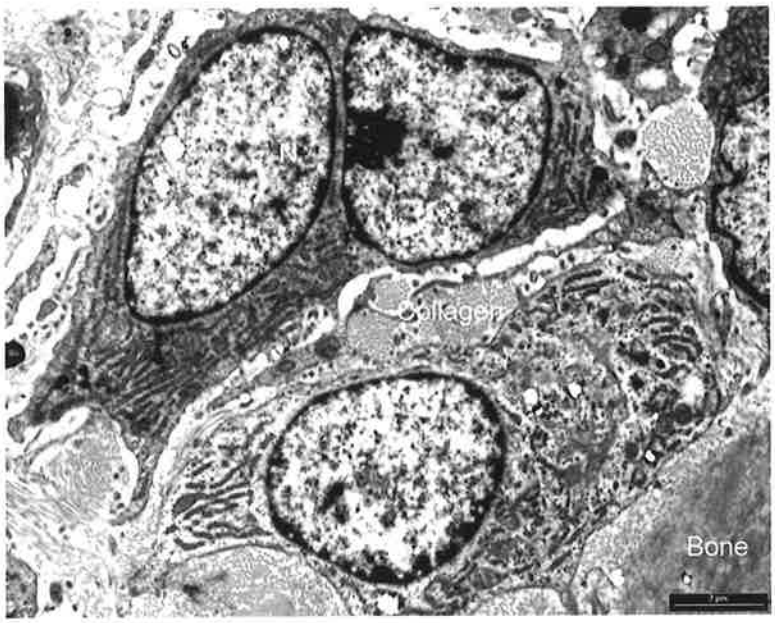


Figure 108. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x4600. Electronmicrograph of frozen molar showing a multinucleated cell in close relation to bone forming mononuclear cell. Different staining characteristics of the cytoplasms are apparent as are differences in intracellular content. Magnification x4600, N - nucleus

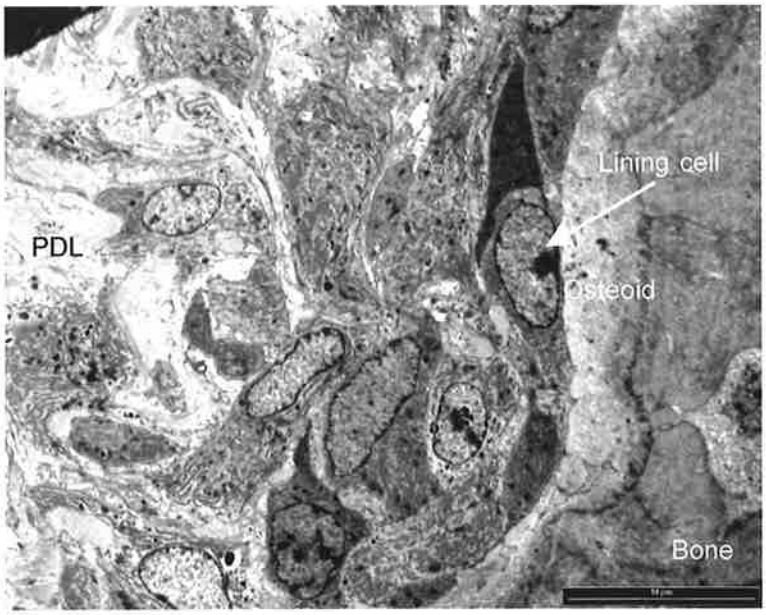


Figure 109. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1950. Electronmicrograph of bone lining cells over an osteoid layer on the surface of bone adjacent to a frozen molar. Magnification x1950

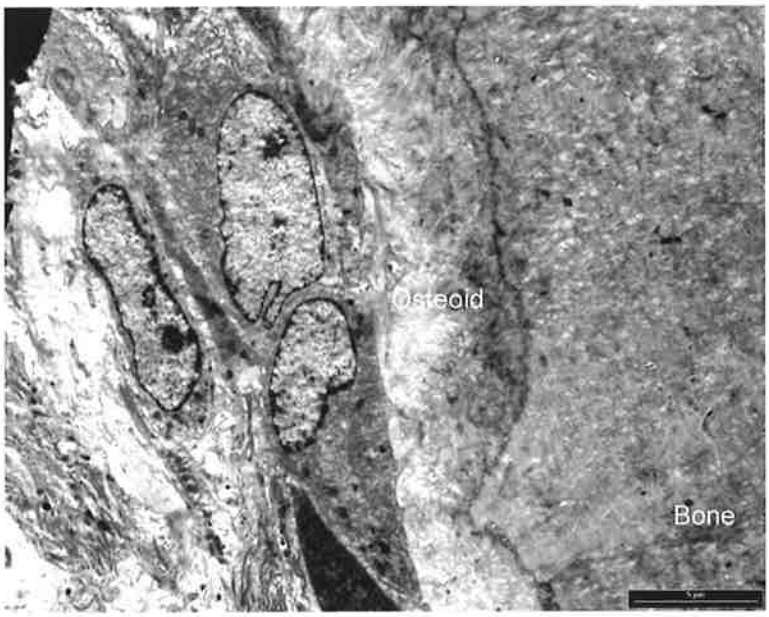


Figure 110. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x2600. Electronmicrograph of blast cells with indented nuclei over osteoid layer on alveolar bone surface adjacent to a frozen molar.

- **Bone marrow**

The haemopoietic tissue contained in alveolar bone marrow spaces was surrounded by large multinucleated cells that lined the endosteal surfaces (Fig. 111). Large cells were also seen interspersed with the haemopoietic elements distant to the bone surfaces.

- **Ankylosis**

Ankylosis was a feature of many specimens examined. There appeared to be almost a seamless union between tooth and bone (Fig. 112). Bone could be distinguished by the entrapment of cells and dentine was identified by the localization of tubules. The entrapped cells appeared to be undergoing degeneration (Fig. 113). The occasional multinucleated cell was seen caught in the ankylotic area (Fig. 114). Collagen of the newly laid bone was not observed in discrete bundles but appeared to be haphazardly arranged.

- **Pulp**

The effects of a cold stimulus on the dental pulp resulted in a highly damaged and disorganized tissue. The odontoblastic layer was obliterated and the other cellular elements exhibited necrosis and lysis (Fig. 115). Connective tissue and vascular structures were also disrupted with the extravascular deposition of blood cells apparent. The tissue contained many spaces that were partly filled with disgorged cellular products.

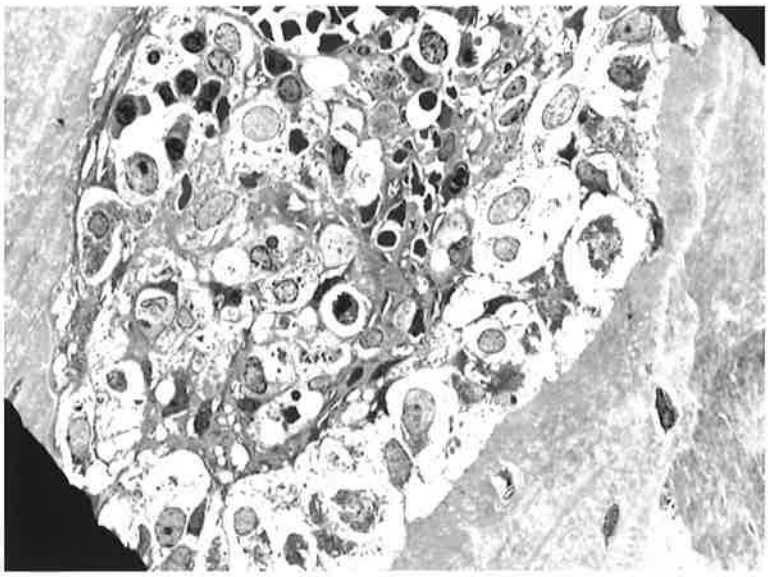


Figure 111. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x620. Electronmicrograph of marrow space in alveolar bone near frozen rat molar revealing a layer of giant cells lining the endosteal surface.

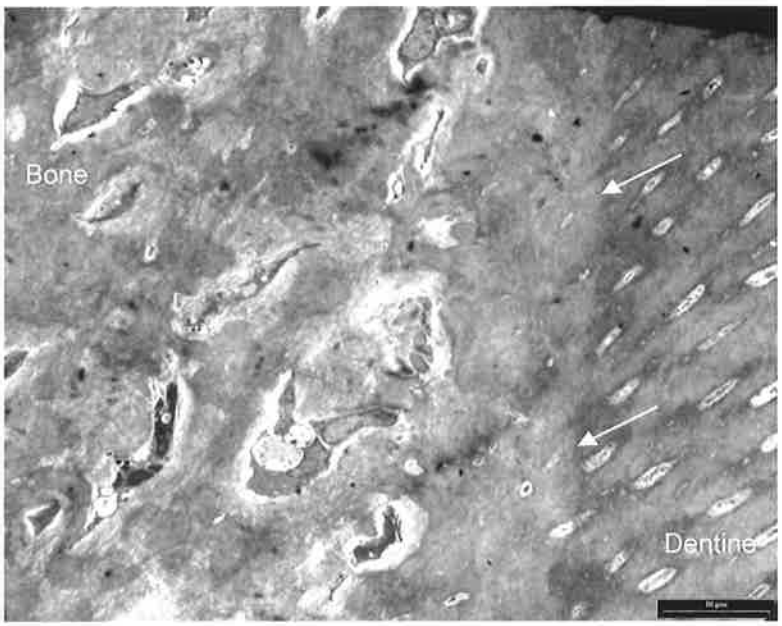


Figure 112. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1100. Electronmicrograph of an interradicular area of ankylosis (arrows) affecting a frozen molar.

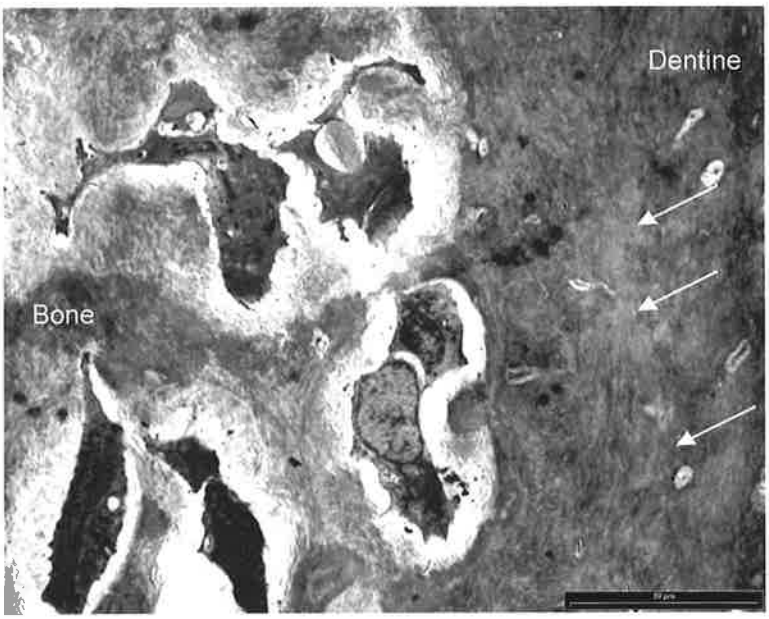


Figure 113. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1950. Higher power electronmicrograph of ankylotic union (arrows) with entrapped degenerating cells.

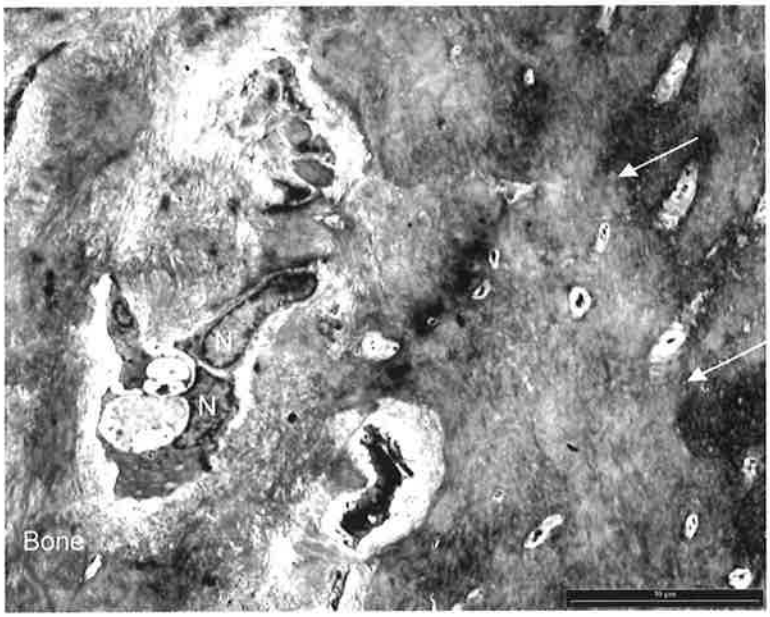


Figure 114. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x1950. Higher power electronmicrograph of interradicular area of ankylosis (arrows) depicted in Fig. 112 showing entrapped cells, possibly multinuclear in type. Bone deposition appeared to be haphazard in nature. N - nucleus

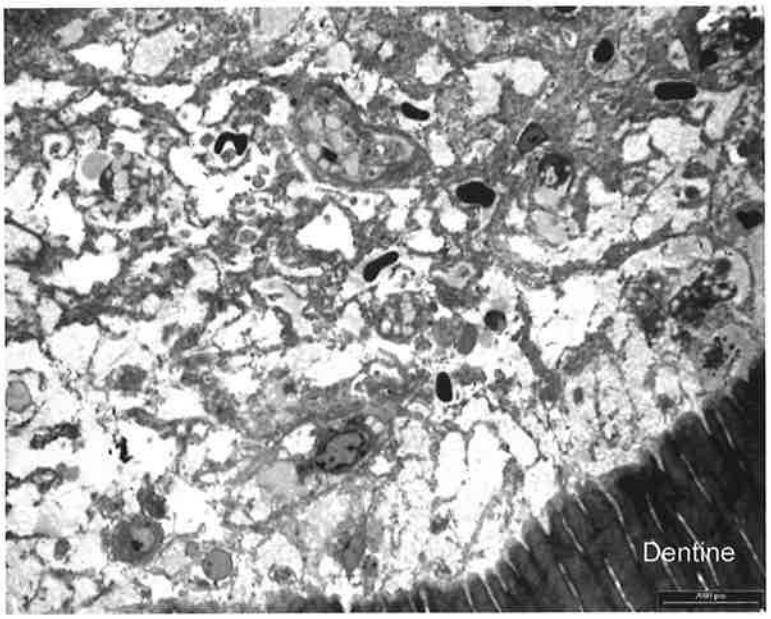


Figure 115. Experiment 4, TEM, uranyl acetate and Reynold's lead, magnification x800. Electronmicrograph of pulp tissue from a molar subjected to freezing. Obliteration of the odontoblast layer has occurred as well as necrosis of the structural elements of the reticulum. Compare with Figs. 89, 90.

4.3.5 Experiment 5 – Single prolonged freezing episode – Sacrifice at 7 d.

To assess the effect of a single prolonged thermal insult using enzyme histochemistry, immunohisto-chemistry and ultrastructural evaluations.

4.3.5.1 Light microscopy

A. Control teeth - unfrozen

Results were similar to those described in experiment 1.

B. Experimental teeth - frozen

A single freezing episode of 20 min produced results similar to those obtained in experiments 2 and 3. Active resorption was evident in the cervical areas of the teeth with no signs of repair. However, unlike shorter applications of dry ice, the longer applications produced ankylosis in the majority of animals (Fig. 116). The ankylosis was unusual in nature insofar as it appeared that bone was generated by cells within the PDL and actively deposited on the root surface. There appeared to be discontinuity between the interradicular crest and the ankylotic bone deposition giving the appearance that bone-like tissue was deposited on the root surface and extended towards crestal bone, rather than bone deposition occurring on the interradicular crest and advancing towards the tooth.

4.3.5.2 TRAP enzyme histochemistry

A. Control teeth – unfrozen

Results for TRAP distribution in control unfrozen molars were similar to those presented in experiment 4.

B. Experimental teeth – frozen

Results for TRAP distribution in molars experimentally frozen for an extended period were similar to those presented in experiment 4.

4.3.5.3 Immunolabelling

- **ED1**

A. Control teeth – unfrozen

Results for ED1 immunolabelling on control unfrozen molars were similar to those presented in experiment 4.

B. Experimental teeth – frozen

Results for ED1 immunolabelling on experimentally frozen molars were similar to those presented in experiment 4.

4.3.5.4 Transmission electron microscopy

A. Control teeth – unfrozen

Ultrastructural results for control unfrozen teeth were similar to those presented in experiment 4.

B. Experimental teeth – frozen

Ultrastructural results for experimentally frozen molars were similar to those presented in experiment 4.

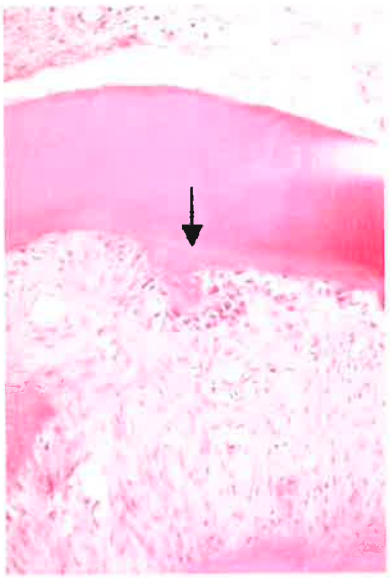


Figure 116. Experiment 5, LM, H&E, magnification x50. Photomicrograph of interradicular area of a rat molar frozen for 20 min and sacrificed after 7 d. PDL disruption and hard tissue deposition is evident, producing an ankylosis (arrow).

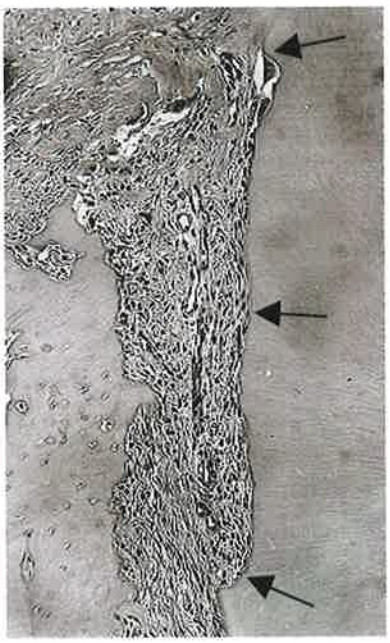


Figure 117. Experiment 6, LM, H&E, magnification x50. Photomicrograph of cervical region of rat molar that has been subjected to both freezing and a mechanical insult. An extensive longitudinal area of resorption is seen (arrows) along with clast cell activity.

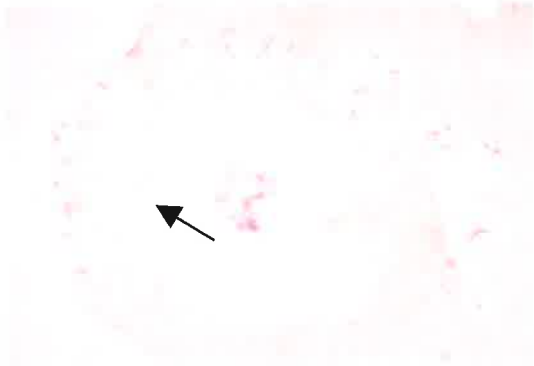


Figure 118. Experiment 7, LM, magnification x25. Photomicrograph of an obliquely cut control section showing a dental resorption cavity (arrow) without obvious succinic dehydrogenase presence but a visible line of activity in the surrounding alveolar bone surface.

4.3.6 Experiment 6 – Single freezing episode plus mechanical insult. To determine the combined effects of mechanical and thermal insults to the periodontium.

4.3.6.1 Light microscopy

A. Control teeth - unfrozen

The histological appearance of the PDL of control teeth for experiment 6 was similar to those described in experiment 1.

B. Experimental teeth - frozen plus mechanical insult

The addition of mechanical trauma produced more extensive resorption, but in two distinct patterns. As well as interradicular and cervical resorption, long resorptive defects were visible running longitudinally down the cervical region of the molar. These longitudinal resorption zones were filled with clast cells (Fig. 117).

4.3.6.2 TRAP enzyme histochemistry

A. Control teeth – unfrozen

Results for TRAP distribution in control unfrozen molars were similar to those presented in experiment 4.

B. Experimental teeth – frozen plus mechanical insult

While the distribution of TRAP stain in the interradicular and cervical areas was similar to that previously described in experiment 4, the addition of the mechanical insult produced TRAP activity in cells associated with the longitudinal cementum damage. Large multinucleated cells present in the long resorption lacunae stained heavily as did mononuclear cells in the adjacent PDL.

4.3.6.3 Immunolabelling

- **ED1**

A. Control teeth – unfrozen

Results of ED1 immunolabel distribution in control unfrozen molars were similar to those presented in experiment 4.

B. Experimental teeth – frozen plus mechanical insult

The distribution of ED1 immunolabel was similar to that previously described in experiment 4 except for extra label evident in the longitudinal mechanically damaged area. Label was seen in multinucleated cells in the resorption lacunae in addition to numerous mononuclear cells in the nearby PDL.

4.3.7 Experiment 7 - Succinic dehydrogenase enzyme histochemistry.

To determine succinic dehydrogenase activity in the periodontium after cold thermal insult.

In the presence of appropriate substrates, ditetrazolium chloride (BT) serves as a hydrogen acceptor and is reduced to a blue water-insoluble pigment (diformazan). The demonstration of succinic dehydrogenase was therefore evident by the granular intracellular deposition of blue pigment in the cryo-sections examined. This corresponded to sites of high enzyme activity but in areas of lower activity a reddish-purple colour was seen as only partial reduction of BT occurred to a monoformazan.

4.3.7.1 Light microscopy

A. Control teeth - unfrozen

Sections of control unfrozen PDL revealed succinic dehydrogenase activity in multinucleated cells lining the alveolar bone walls, bone marrow spaces and occasionally in isolated resorption pits on the tooth root surface (Figs. 118 and 119). The pattern of staining appeared to be consistent with the normal direction of rat molar migration in a distal and buccal direction (Fig. 118).

B. Experimental teeth - frozen

At 2 days after thermal insult to the molar, succinic dehydrogenase activity was seen within the periodontal hard tissues. Blue staining was evident along resorbing bone surfaces but little evidence of staining on tooth root surfaces was seen (Fig. 120). Bone marrow spaces revealed activity on endosseous surfaces (Fig. 121) but no activity was seen within the periodontal ligament itself.

At 7 days, blue staining succinic dehydrogenase activity appeared to be more widespread. Pigment was still evident along alveolar bone surfaces but also in dental resorption cavities (Fig.122). The staining followed a consistent pattern around the cervical and interradicular areas of the tooth and PDL. Blue staining in dental resorptive lacunae appeared of similar intensity compared with that noted in bone resorption cavities (Fig. 123). However, no activity was seen in the PDL and other soft tissues surrounding the teeth.

At 14 days, the amount of active dental resorption appeared to be reduced as revealed by the scarcity of clastic cells and a decrease in their staining intensity adjacent to the root surface. Occasional pigmented cells were seen but the

majority of the dental resorption bays were devoid of succinic dehydrogenase activity (Figs. 124). However, resorption activity was evident in the surrounding alveolar bone. At all time periods, it was clear that cells exhibiting staining reaction were large in size and showed multinuclearity (Figs. 121 and 123). No small mononuclear cells showed evidence of blue coloration.

Figure 119. Experiment 7, LM, magnification x50. Photomicrograph of control unfrozen rat molar revealing succinic dehydrogenase activity in multinucleated cells on hard tissue surfaces (arrows).

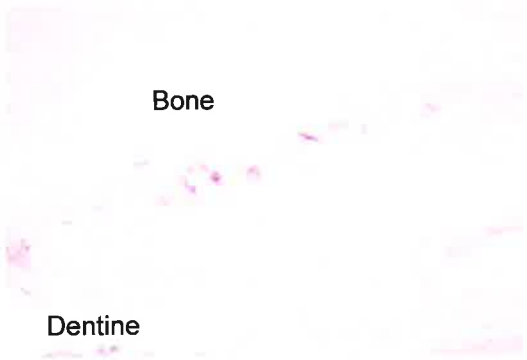


Figure 120. Experiment 7, LM, magnification x50. Photomicrograph of a 20 μm thick cryo-section of rat molar 2 d after freezing showing succinic dehydrogenase activity on bone surface but little on tooth surface.

Figure 121. Experiment 7, LM, magnification x100. Photomicrograph of cryo-section of bone marrow showing succinic dehydrogenase activity on endosseous surfaces. Cells appear large and multinucleated.

Figure 122. Experiment 7, LM, magnification x50. Photomicromicrograph of cryo-section of rat molar PDL 7 d after freezing showing succinic dehydrogenase activity. Biformazan staining is evident in resorption lacunae on tooth and bone.

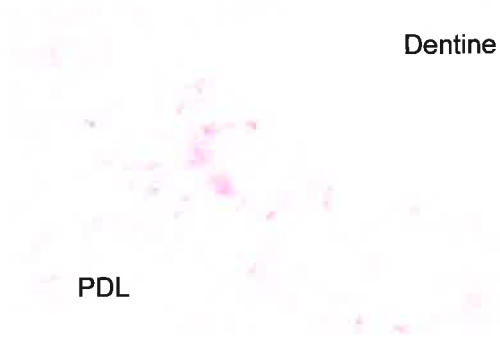


Figure 123. Experiment 7, LM, magnification x100. Higher power photomicrograph of Fig. 122 revealing succinic dehydrogenase activity in large cells associated with root resorption lacunae.

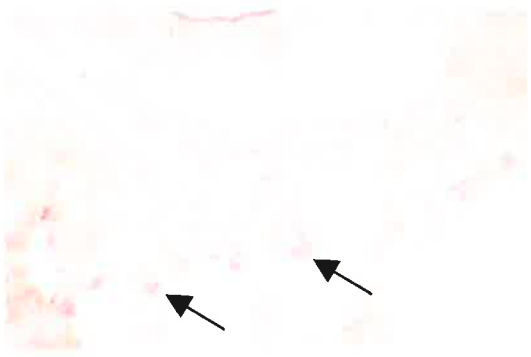


Figure 124. Experiment 7, LM, magnification x100. Photomicrograph of rat molar PDL 14 d after freezing insult revealing little dental resorption activity but succinic dehydrogenase activity was evident in surrounding alveolar bone (arrows).

4.3.8 Experiment 8 – Osteoprotegerin (OPG).

To determine the effects of osteoprotegerin on the resorption model.

4.3.8.1 Light microscopy

A. Control teeth – unfrozen, no OPG

Histological results for the OPG-unaffected PDL were similar to those described for control teeth in experiment 1.

B. Control teeth – unfrozen, OPG - treated

Haematoxylin and eosin stained sections of unfrozen molars of OPG-affected rats revealed altered histological features compared with those of untreated animals. Shallow resorption lacunae were occasionally present on root and bone surfaces (Fig. 125). Most dental lacunae were located towards the root apex region and appeared to be inactive as no obvious large multinucleated cells were seen in relation to the lacunae. In addition, no overt sign of hard tissue repair was evident (Fig. 126). Alveolar bone marrow spaces adjacent to the PDL appeared devoid of megakaryocytes (Fig. 127), while many haemopoietic cells possessed cytoplasmic signs suggestive of cellular apoptosis.

Hard tissue surfaces were covered by a layer of lining cells. Periodontal fibres attached to cementum surfaces interrupting the layer of cementoblasts (Fig. 128). Bone lining cells were interspersed by principal fibres of the PDL which entered bone as Sharpey fibres (Fig. 129). The opposite side of the interradicular bone also revealed the attachment of principal PDL fibres into old resorption lacunae, suggesting that active resorption had ceased on previously resorbing surfaces possibly due to cessation of physiological tooth drift.

C. Long bone – OPG untreated

Although not high in number, the presence of large multinucleated clast cells was noted in resorption lacunae on endosseous surfaces of long bone sections. Similar to sections of alveolar bone, multinucleated megakaryocytes were observed in marrow haemopoietic tissue.

D. Long bone – OPG treated

Clast cell activity and resorption in sections of long bone were not observed in any of the experimental (frozen molar) or control OPG-treated animals (Fig. 130).

Figure 125. Experiment 8, LM, H&E, magnification x25. Photomicrograph of an unfrozen control molar from an OPG-affected rat. Periodontal structures reveal resorption cavities but no obvious histological signs of clast cells.

Figure 126. Experiment 8, LM, H&E, magnification x50. Higher power photomicrograph of PDL seen in Fig. 125 revealing resorption cavities in the apical region but no obvious clast cells or signs of repair.

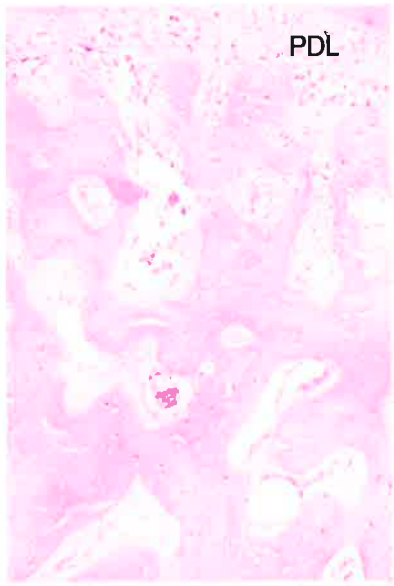


Figure 127. Experiment 8, LM, H&E, magnification x100. Photomicrograph of alveolar bone from an unfrozen control molar of an OPG-affected rat revealed a scarcity of marrow megakaryocytes.

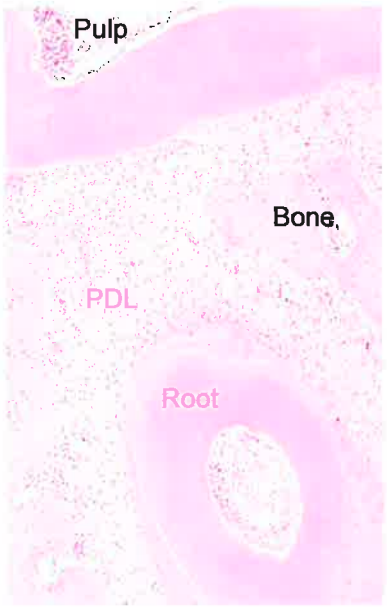


Figure 128. Experiment 8, LM, H&E, magnification x50. Photomicrograph of the interradicular area of an unfrozen control molar of an OPG-affected rat showing cementum lining cells and PDL fibre attachment into the root surface.

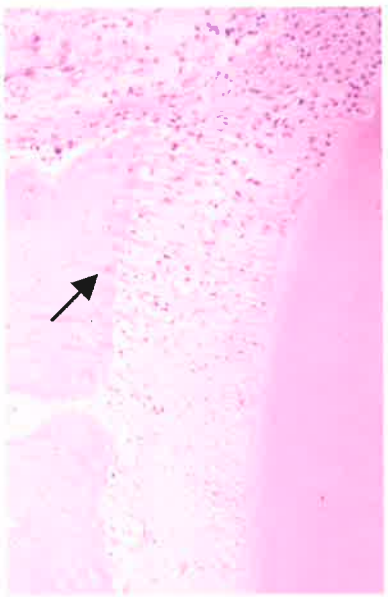


Figure 129. Experiment 8, LM, H&E, magnification x100. Photomicrograph of unfrozen molar periodontium of an OPG-affected rat showing Sharpey fibre insertion (arrow) into appositional alveolar crestal bone.

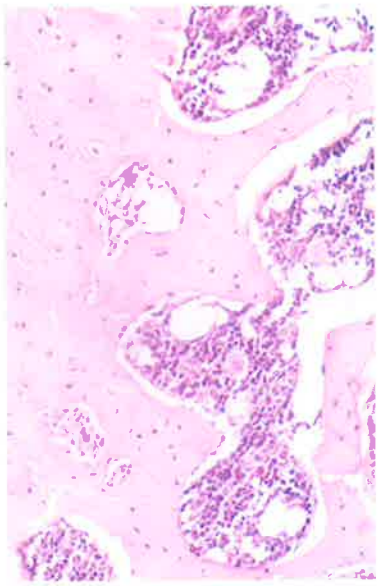


Figure 130. Experiment 8, LM, H&E, magnification x50. Photomicrograph of long bone (tibia) from OPG-affected rat indicating a lack of clast cell activity on endosseal surfaces.

E. Experimental teeth – frozen, OPG treated

Sections examined from teeth subjected to freezing insult showed a markedly disrupted appearance. In the interradicular area, lining cells on both tooth root and bone surfaces were patchy and PDL fibre orientation was disrupted (Fig. 131). Areas of old and apparently inactive resorption lacunae were evident in the cervical regions of the molar (Fig. 132). However, the most striking feature was the presence of ankylosis in all animals subjected to the combined effects of thermal insult and OPG administration (Fig. 133). The ankylosis did not appear to be of the replacement type but rather, appositional deposition occurred on the root surface exclusively in the interradicular region (Fig. 134). Bone deposition did not appear to be an aggregational process from the crestal bone surface towards the tooth. The ankylosis appeared to progress from the root surface towards the crestal bone (Fig. 134) and appeared to stain like bone rather than dentine. Appositional cells became entrapped as deposition occurred. Large multinucleated cells were not obvious in the ankylotic areas where mononuclear cells predominated.

Root resorption and multinucleated cells were seen in interradicular areas adjacent to the regions of ankylosis (Figs. 135 and 136). Resorption lacunae were multiple and mostly shallow but appeared to be active in nature (Figs. 135 and 136)

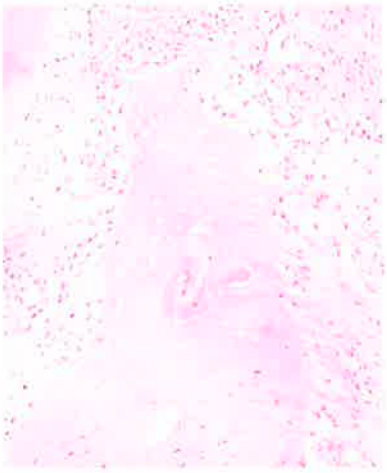


Figure 131. Experiment 8, LM, H&E, magnification x100. Photomicrograph of interradicular bone and associated PDL of a frozen molar in an OPG-affected rat. The PDL fibres are disorganized but principal and Sharpey fibres are still evident.

Figure 132. Experiment 8, LM, H&E, magnification x100. Photomicrograph of periodontal cervical and crestal structures of a frozen molar in an OPG-affected rat showing extensive resorption lacunae (arrows) that appear to be inactive. No sign of clast cell presence is seen.

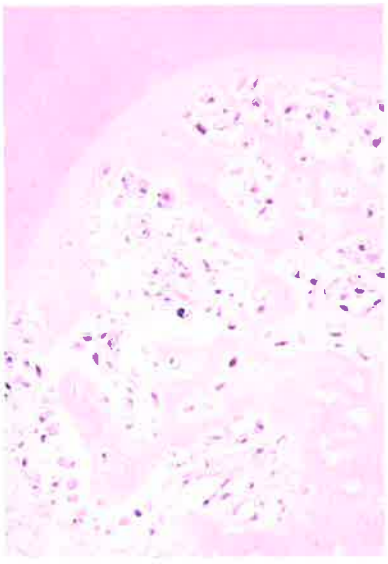


Figure 133. Experiment 8, LM, H&E, magnification x100. Interradicular area of a frozen molar in an OPG-affected rat revealing ankylosis of the appositional type.

Figure 134. Experiment 8, LM, H&E, magnification x50. Photomicrograph of interradicular region of an OPG-affected frozen rat molar showing ankylotic union.

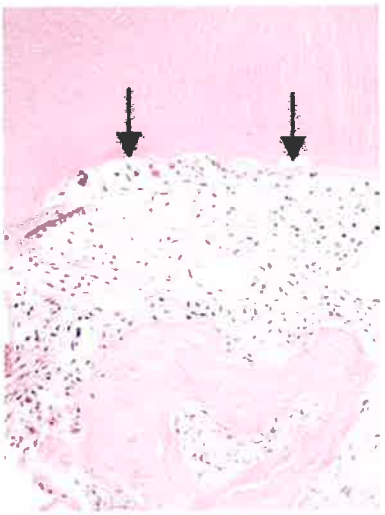


Figure 135. Experiment 8, LM, H&E, magnification x50. Photomicrograph of the interradicular area of a frozen molar in an OPG-affected rat showing root resorption lacunae (arrows).

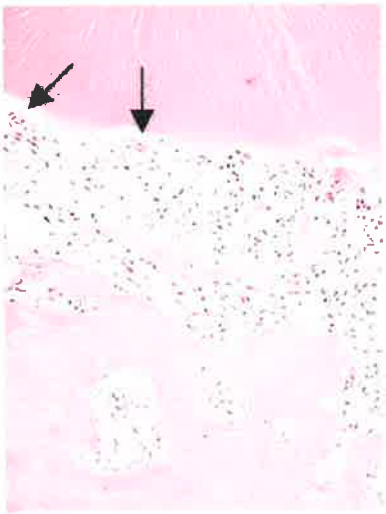


Figure 136. Experiment 8, LM, H&E, magnification x50. Photomicrograph of frozen molar PDL in an OPG-affected animal revealing disorganization in the ligament. Areas of hyalinization are present as are root resorption lacunae and clast cells (arrows).

4.3.8.2 TRAP enzyme histochemistry

A. Control teeth – unfrozen, OPG untreated

Results for TRAP distribution in control unfrozen molars were similar to those presented in experiment 4.

B. Control teeth – unfrozen, OPG treated

The PDL and surrounding structures of OPG-affected animals showed little TRAP staining (Fig. 137). Although hard tissue surface defects resembling resorption lacunae were clearly evident on both tooth and bone, none appeared to be active. No red TRAP stain was visible in control sections of unfrozen PDL (Fig. 138) nor on stain-control sections to which the hexazotized basic fuchsin substrate had not been added. TRAP stained sections of alveolar and long bone also revealed no staining reaction (Figs. 139 and140).

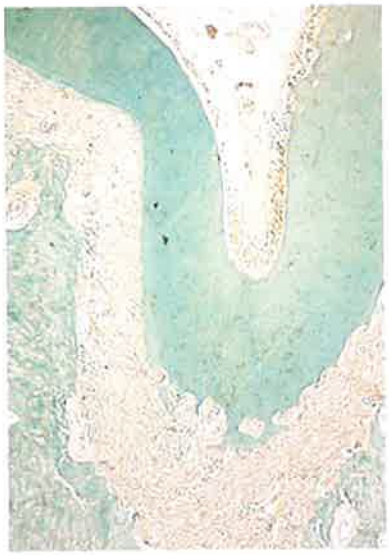


Figure 137. Experiment 8, LM, Fast Green, magnification x25. Photomicrograph of PDL of unfrozen control molar of an OPG-affected rat showing no TRAP stain is evident.

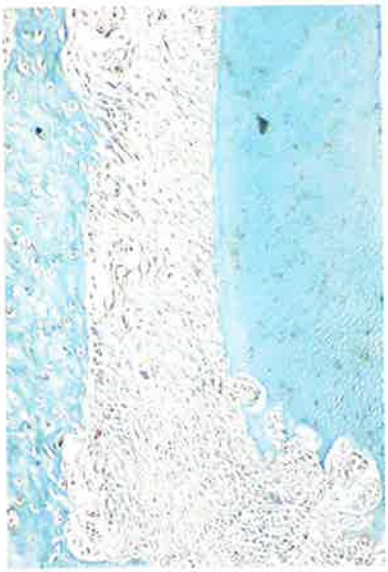


Figure 138. Experiment 8, LM, Fast Green, magnification x50. Higher power photomicrograph of PDL shown in Fig. 137. No TRAP activity is evident in tooth and bone resorption cavities.

Figure 139. Experiment 8, LM, Fast Green, magnification x50. Photomicrograph of TRAP-stained section of unfrozen control molar PDL of OPG-affected rat. No TRAP activity is evident.

Figure 140. Experiment 8, LM, Fast Green, magnification x50. Photomicrograph of long bone (tibia) from an OPG-affected rat showing no TRAP activity.

Figure 141. Experiment 8, LM, Fast Green, magnification x50. Photomicrograph of interradicular area of a frozen molar in an OPG-affected rat showing ankylosis but no TRAP staining.

Figure 142. Experiment 8, LM, Fast Green, magnification x100. Higher powered photomicrograph of frozen molar PDL of an OPG-affected rat indicating a lack of TRAP staining in the ankylotic area.

C. Experimental teeth – frozen, OPG treated

All sections of the experimentally frozen molar of OPG-affected rats displayed ankylosis in the interradicular region. The ankylotic regions appeared to be lacking in enzyme activity as no definitive TRAP stain was evident (Figs. 141 and 142). A rare sighting of TRAP-positive cells was seen within the ankylotic area adjacent to the tooth (Fig. 143). However, at the periphery of the hard tissue union, TRAP-positive cells were encountered and seen on the crest of the interradicular bone that had not yet been involved in the ankylosis (Fig. 144). In addition, strong resorption activity was seen in the cervical area of the tooth (Fig. 145) where lines of intensely TRAP-positive cells appeared to be actively engaged in the resorption of tooth structure. This contrasted with the decrease in resorption apparent in other regions of OPG-affected animals. Adjacent alveolar bone also revealed heavy osteoclast activity (Fig. 146).

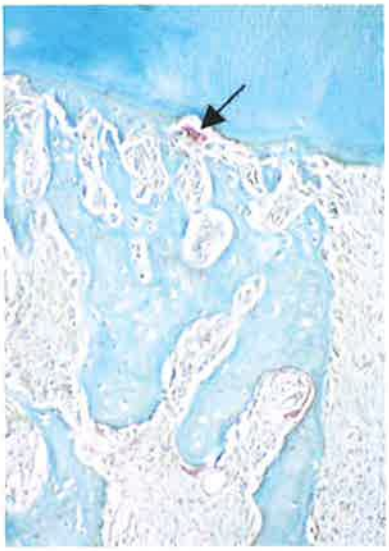


Figure 143. Experiment 8, LM, Fast Green, magnification x100. TRAP stained section of ankylotic interradicular bone of a frozen molar from an OPG-affected rat revealing little enzyme activity except for an isolated cell embedded in bone (arrow).

Figure 144. Experiment 8, LM, Fast Green, magnification x50. Photomicrograph of TRAP stained section of ankylotic interradicular bone of a frozen molar from an OPG-affected rat revealing little enzyme activity except for the crestal bone (arrow). Ankylosis is present in the furcation area but no positive TRAP staining is evident.

Figure 145. Experiment 8, LM, Fast Green, magnification x100. Photomicrograph of the cervical area of a frozen molar of an OPG-affected rat revealing heavy TRAP activity.

Figure 146. Experiment 8, LM, Fast Green, magnification x100. Photomicrograph of OPG-affected rat showing TRAP activity in osteoclasts in alveolar crestal bone near frozen molar.

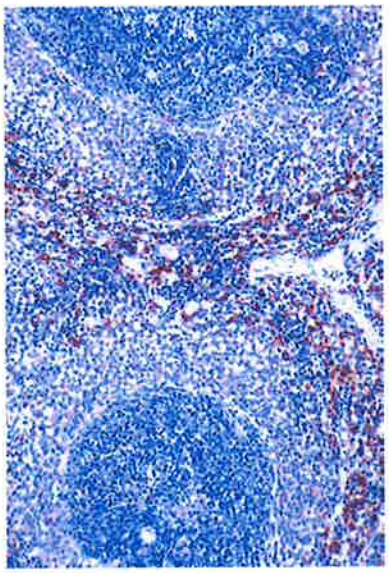


Figure 147. Experiment 8, LM, haematoxylin, magnification x50. Photomicrograph of ED1 antibody distribution in the spleen of an OPG-affected rat.

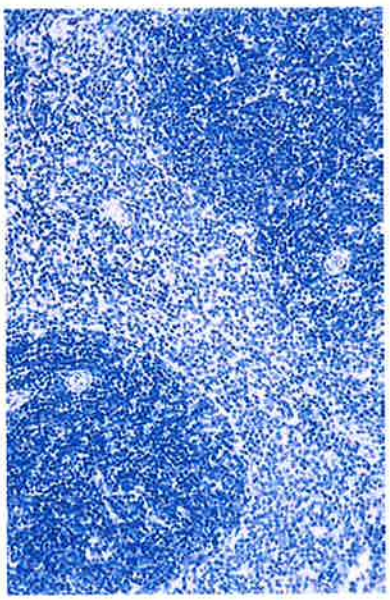


Figure 148. Experiment 8, LM, haematoxylin, magnification x50. Photomicrograph of positive immunologic control tissue in an OPG-affected rat. Primary antibody was omitted from the labelling protocol.

4.3.8.3 Immunolabelling

Because of the eqivocal results with the other antibodies trialled in experiment 4, only ED1 was used during the experiments involving OPG administration.

- **ED1**

A. Immunolabelling control tissue – OPG untreated

Results for ED1 immunolabelling on control spleen tissue in animals unaffected by OPG were similar to those presented in experiment 4.

B. Immunolabelling control tissue – OPG treated

ED1 distribution in sections of spleen tissue of OPG-affected rats was similar to that seen in unaffected animals. Label was evident in haemopoietic tissue that surrounded lymphoid germinal centres (Fig. 147). No label was observed in immunologic control sections in which the primary antibody was omitted during the staining process (Fig. 148).

C. Control teeth – unfrozen, OPG untreated

Results for ED1 immunolabelling on control unfrozen molars in OPG untreated animals were similar to those presented for control animals in experiment 4.

D. Control teeth – unfrozen, OPG treated

ED1 label was evident in OPG-affected rat unfrozen molars. However, the stain was not widespread and largely occupied the apical regions of the tooth and surrounding alveolar bone (Fig. 149). The interradicular area revealed little ED1 activity (Figs. 150 and 151). Occasional isolated cells were encountered (Fig. 151) but the unfrozen molar PDL predominately stained negative for ED1. Faint

ED1 label was evident on endosseous surfaces of long bones and within bone marrow megakaryocytes (Fig. 152).

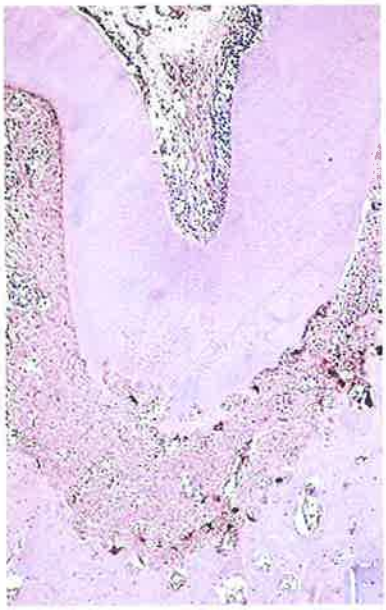


Figure 149. Experiment 8, LM, haematoxylin, magnification x25. Photomicrograph of an unfrozen molar of an OPG-affected rat revealing ED1 labelling of small mononuclear cells in the apical region adjacent to tooth and bone. The pattern reflects the direction of buccal tooth drift.

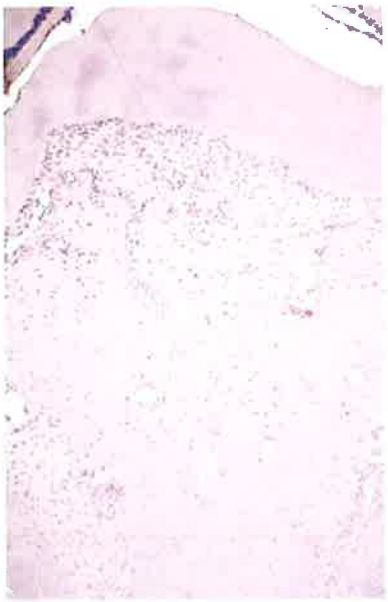


Figure 150. Experiment 8, LM, haematoxylin, magnification x50. Photomicrograph of unfrozen molar of an OPG-affected rat revealing little ED1 label in the interradicular PDL and bone.

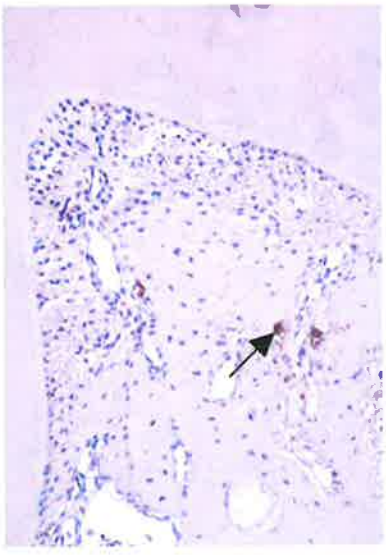


Figure 151. Experiment 8, LM, haematoxylin, magnification x100. Higher power photomicrograph of Fig. 150 showing the paucity of ED1 label in the interradicular PDL and bone. Isolated cells (arrow) contain label.

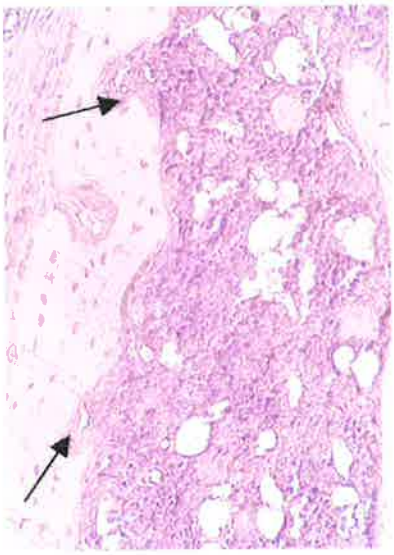


Figure 152. Experiment 8, LM, haematoxylin, magnification x50. Photomicrograph of long bone (tibia) from OPG-affected rat showing weak ED1 activity on endosseous surfaces (arrows).

E. Experimental teeth – frozen, OPG treated

Immunolabelling of the frozen upper right molar in OPG-affected rats displayed a distinctive pattern of ED1 in the surrounding periodontal structures. Label was evident on the hard tissue surfaces (Fig. 153) as well as within the cells of the PDL. Staining was intense as shown by the line of positively labelled osteoclasts on the surface of the interradicular crestal bone (Fig. 154) and odontoclasts on interradicular root surfaces (Fig. 155). The intensity of staining suggested that OPG had apparently failed to inhibit clast cell activity when stimulated by the cold thermal insult.

In areas of interradicular ankylosis, universally created in the OPG-affected animals subjected to molar freezing, ED1 label was visible (Fig. 156). Label was localized in cells on hard tissue surfaces particularly on marrow space endosteal surfaces (Fig. 157). Cells trapped within the ankylotic areas also exhibited a positive reaction (Fig. 158) suggesting an ongoing inflammatory process requiring macrophage-like activity.

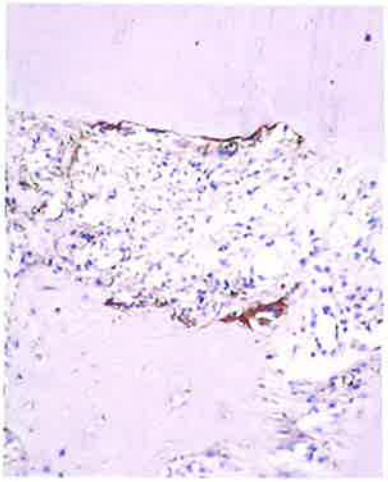


Figure 153. Experiment 8, LM, haematoxylin, magnification x50. Photomicrograph of frozen molar PDL in an OPG-affected rat showing ED1 activity. Label is associated with resorption cavities on tooth and bone.

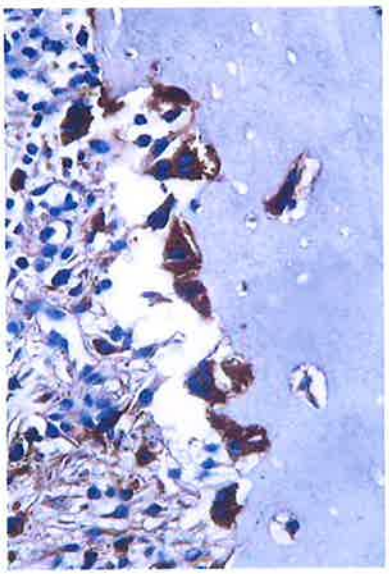


Figure 154. Experiment 8, LM, haematoxylin, magnification x50. Photomicrograph of large multinucleated cells in bone resorption lacunae in interradicular bone in a frozen rat molar of an OPG-affected rat. Intense ED1 label is evident in these cells.

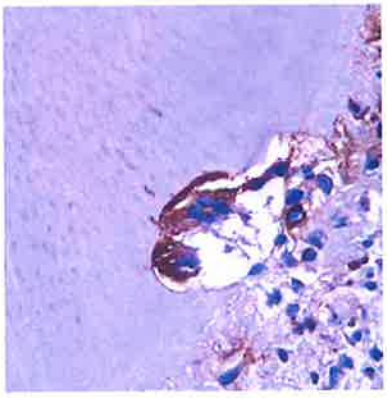


Figure 155. Experiment 8, LM, haematoxylin, magnification x100. Photomicrograph of large multinucleated cells in dental resorption lacuna of a frozen molar in an OPG-affected rat. Cytoplasmic ED1 label is strongly evident.

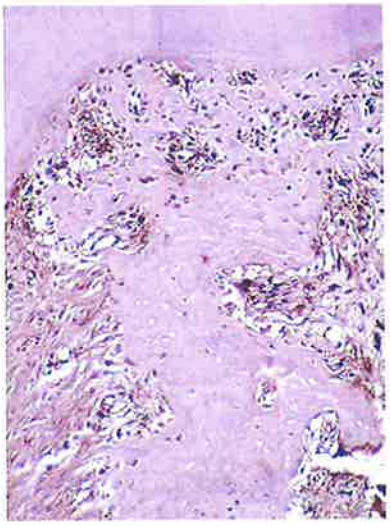


Figure 156. Experiment 8, LM, haematoxylin, magnification x50. Photomicrograph of interradicular ankylosis in a frozen molar of an OPG-affected rat. ED1 label is shown in bone resorption lacunae.

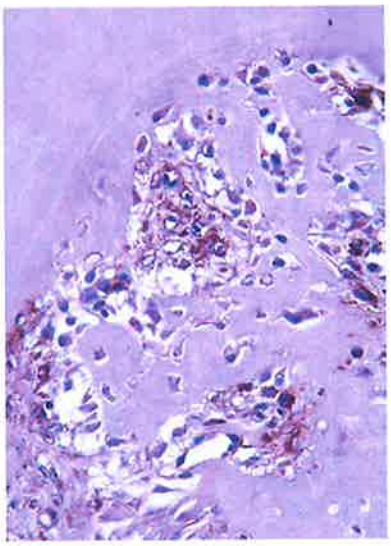


Figure 157. Experiment 8, LM, haematoxylin, magnification x100. Higher power photomicrograph of Fig. 156 revealing ED1 label in interradicular ankylotic area of a frozen molar in an OPG-affected rat.

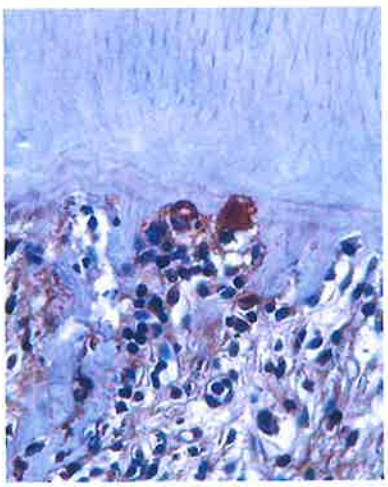


Figure 158. Experiment 8, LM, haematoxylin, magnification x100. Photomicrograph of interradicular area of a frozen molar of an OPG-affected rat showing ED1 label in large cells in dental resorption lacunae adjacent to ankylotic area.

4.3.8.4 Transmission electron microscopy

A. Control teeth – unfrozen, OPG untreated

The ultrastructural results of control unfrozen teeth in OPG unaffected animals is the same as for normal rat PDL ultrastructure presented in experiment 4.

B. Control teeth – unfrozen, OPG treated

The periodontal ligament of the unfrozen molars of OPG-treated animals revealed no signs of active root resorption although ultrastructural signs of previous resorption lacunae were visible. The ligament contained mononuclear cells consistent with fibroblasts interspersed with collagen fibrils (Fig. 159). Cell nuclei were regular in outline with no apparent indentations or invaginations of the nuclear envelope. Blood vessels and nerves were also freely seen intermingling between the cells and fibrils (Fig. 160).

Cementoblasts further down the root surface produced a lining layer and exhibited a fringe of collagen matrix (Fig. 161, 162).

Ambiguous signs of resorptive activity were seen in the adjacent alveolar bone (Fig. 163). The alveolar wall was indented with the occasional lacunae that contained mononuclear cells with abundant cytoplasm. Some cells exhibited small areas of cytoplasm that were free of organelle content (Fig. 164). These organelle free areas were located adjacent to the hard tissue surface whereas elsewhere, intracellular vacuoles and endoplasmic reticulum filled the cell (Fig. 164). Cytoplasmic processes appeared to extend into the hard tissue surface to surround bone spicules (Fig. 165).

Despite this, most bone surfaces appeared to be appositional in nature (Fig. 166). Osteoblast-like cells amongst deposited collagen matrix lined the hard tissue surface. Matrix appeared to embed the occasional cell producing osteocytes (Fig. 167). In addition, collagen became organized and penetrated the surface of bone (Fig. 168).

- **Bone Marrow**

Bone marrow contained a range of haemopoietic cells with blood vessels containing erythrocytes (Fig. 169). No marked multinucleated cell activity was apparent although large mononuclear cells were seen adjacent to the hard tissue surface (Fig. 170). These cells possessed ruptured cell membranes that allowed the disgorgement of intracellular contents into the extracellular spaces.

- **Pulp**

An intact layer of odontoblasts was clearly visible on a layer of predentine lining the pulp chamber (Fig. 171). The stroma of the pulp tissue contained a connective tissue reticulum supporting mononuclear fibroblast-like cells with blood vessels and nerves. Nerves appeared to be mainly of a myelinated type although unmyelinated fibres were visible. Nerve fibres were seen in close association with small blood vessels with walls of one endothelial cell thickness (Fig. 172).

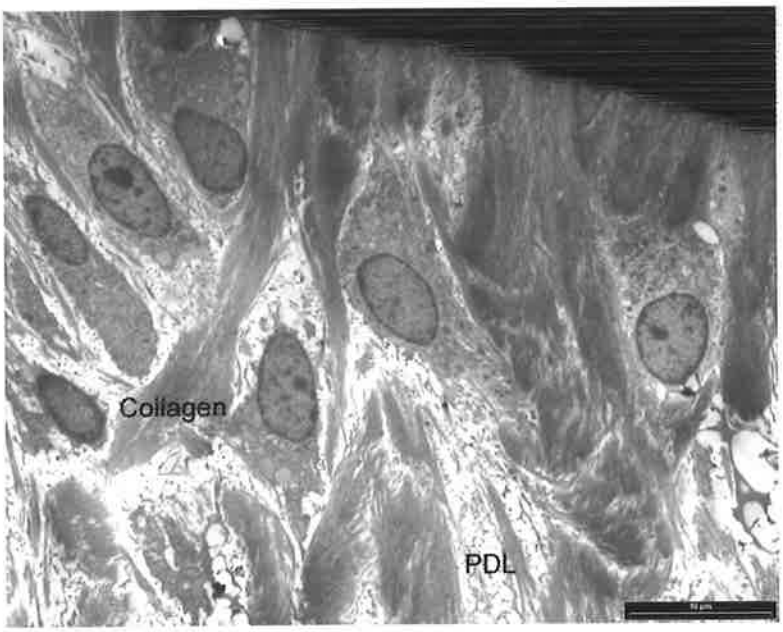


Figure 159. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1450. Electronmicrograph of control unfrozen molar PDL of a rat treated with OPG showing blast cells intermingled with collagen fibrils.

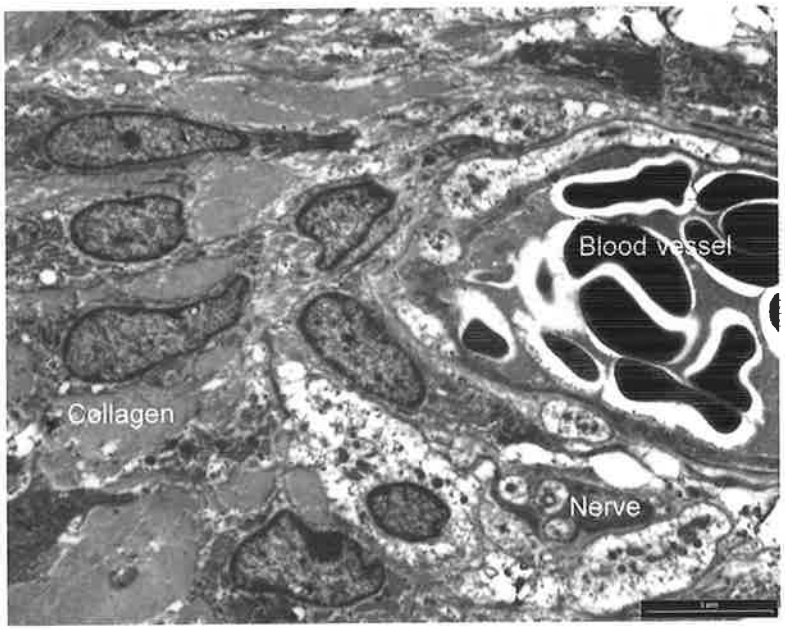


Figure 160. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x2600. Electronmicrograph of control unfrozen molar PDL of an OPG-affected rat revealing normal cellular features and blood vessels.

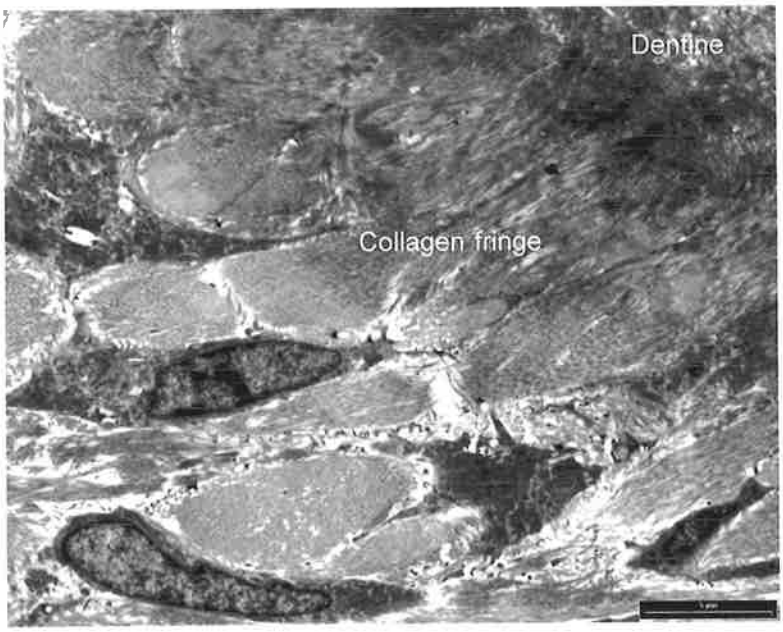


Figure 161. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x2600. Electronmicrograph of cementoblasts lining the unfrozen root surface of a rat administered OPG. Cells appear to be actively depositing collagen matrix.

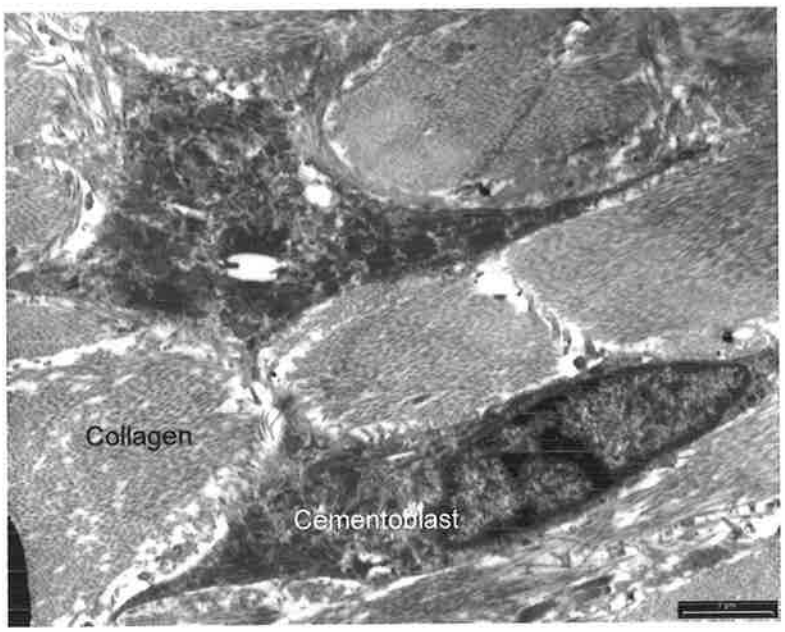


Figure 162. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x2600. A higher power view of Fig.161 revealing matrix deposition by cementum lining cells.

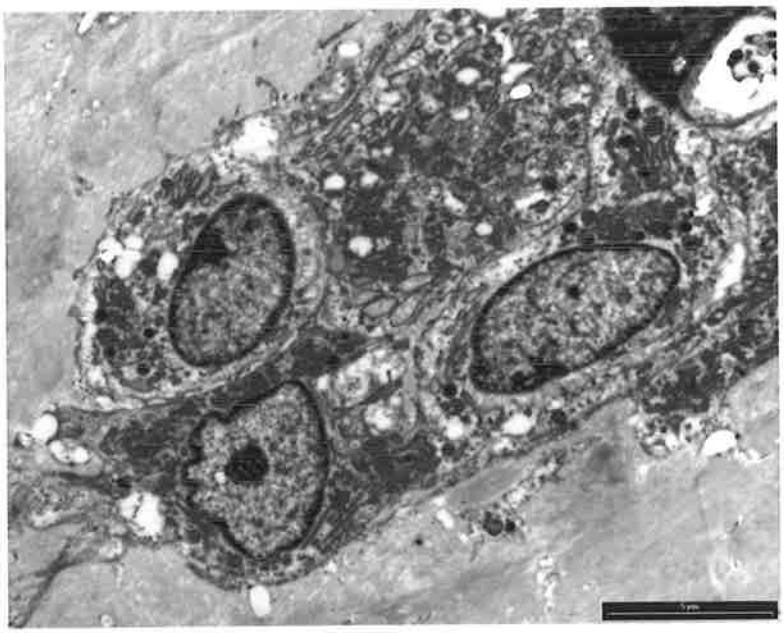


Figure 163. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x3400. Electronmicrograph of resorption lacuna in alveolar bone of an OPG-affected rat showing a group of mononuclear cells containing mitochondria in intimate contact with hard tissue.

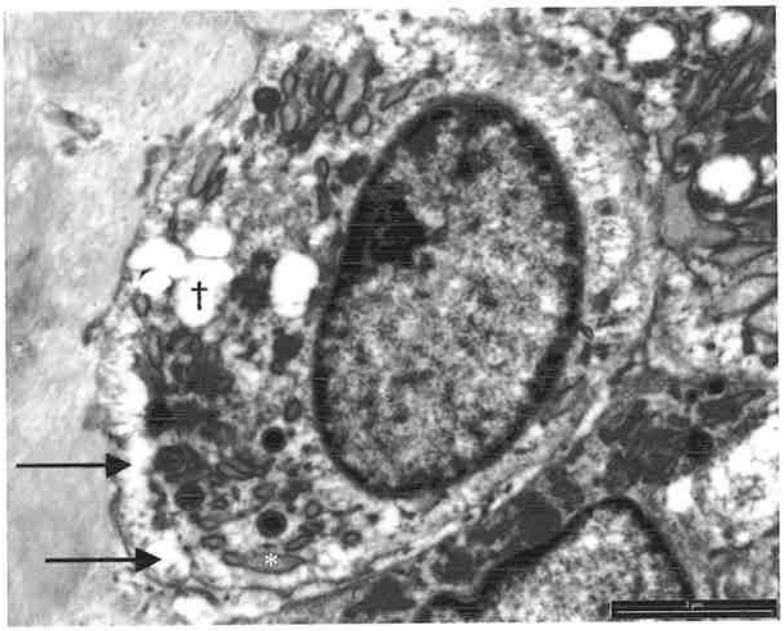


Figure 164. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x7900. Higher power electronmicrograph of a cell from Fig. 163 showing cytoplasmic clear areas (arrows) and also the presence of vacuoles (†) and endoplasmic reticulum (*).

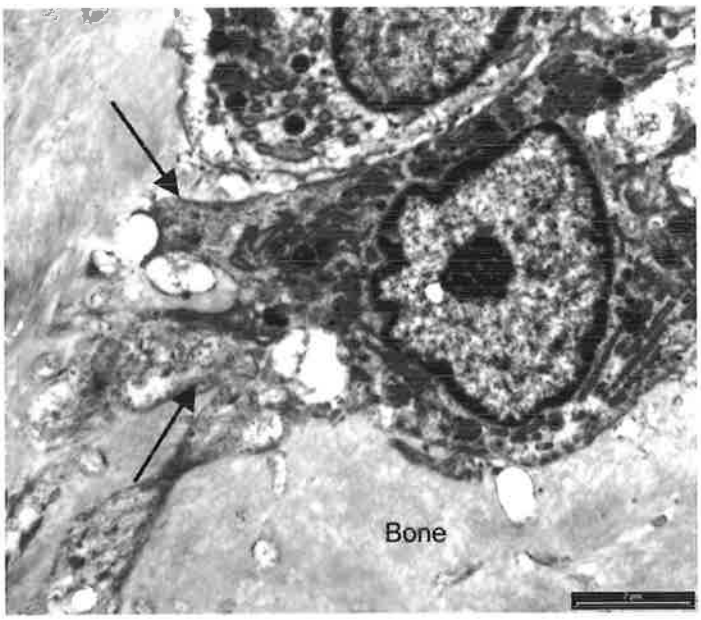


Figure 165. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x7900. Higher power electronmicrograph of cell depicted in Fig.163 revealing cytoplasmic processes (arrows) extending into the hard tissue surface surrounding spicules of bone.

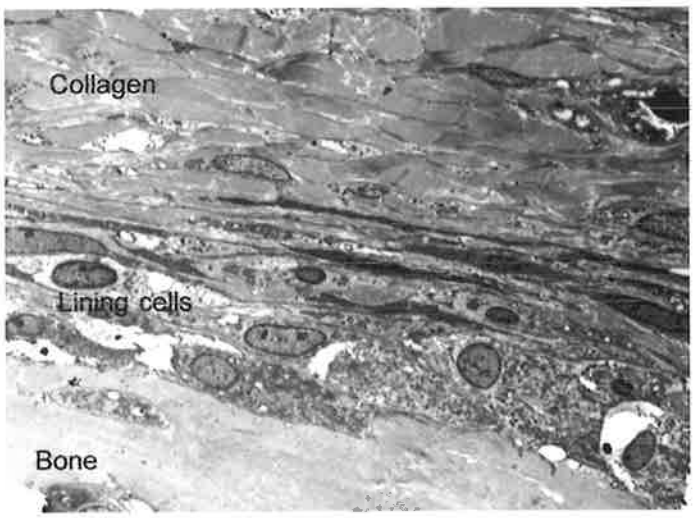


Figure 166. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1100. Electronmicrograph of OPG-affected alveolar bone surface showing an appositional layer of lining cells and collagen bundles.

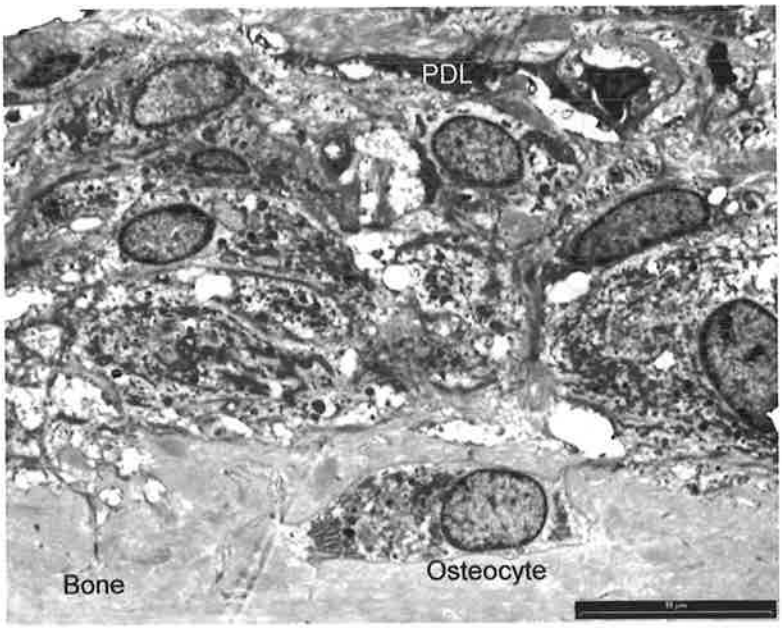


Figure 167. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1950. Electronmicrograph of an appositional alveolar bone surface in an OPG-affected rat revealing matrix formation embedding a lining cell to become an osteocyte.

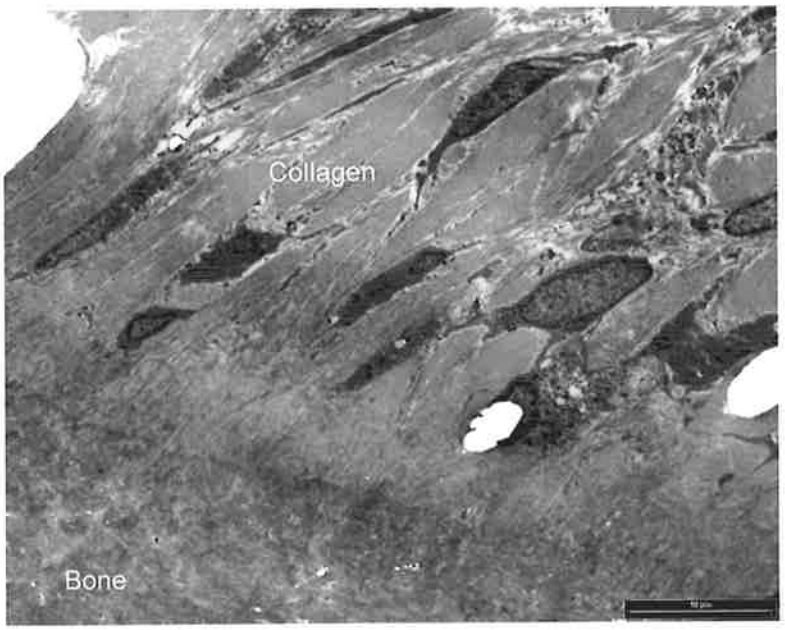


Figure 168. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1450. Electronmicrograph of an appositional alveolar bone surface in an OPG-affected rat showing the embedding of deposited collagen fibrils.

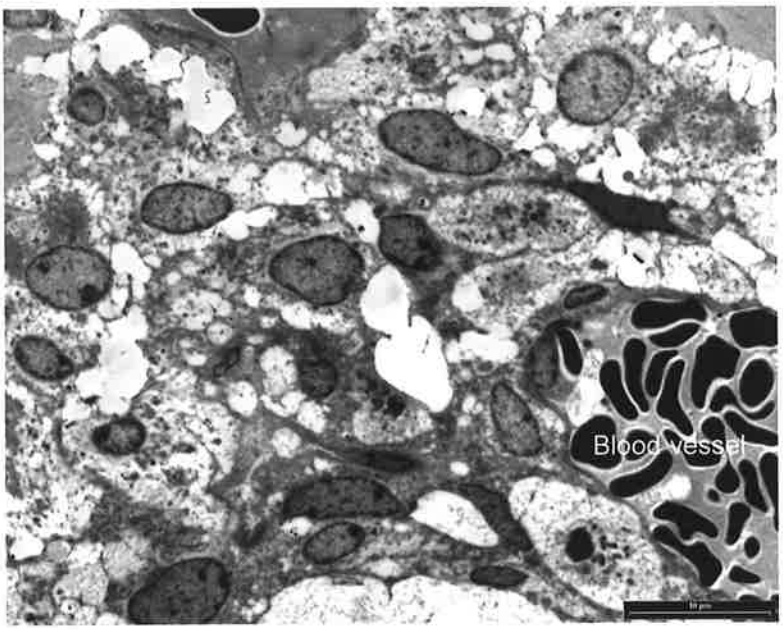


Figure 169. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1450. Electronmicrograph of alveolar bone marrow in an OPG-affected rat revealing blood vessels and an array of haemopoietic cells but no significant megakaryocyte activity.

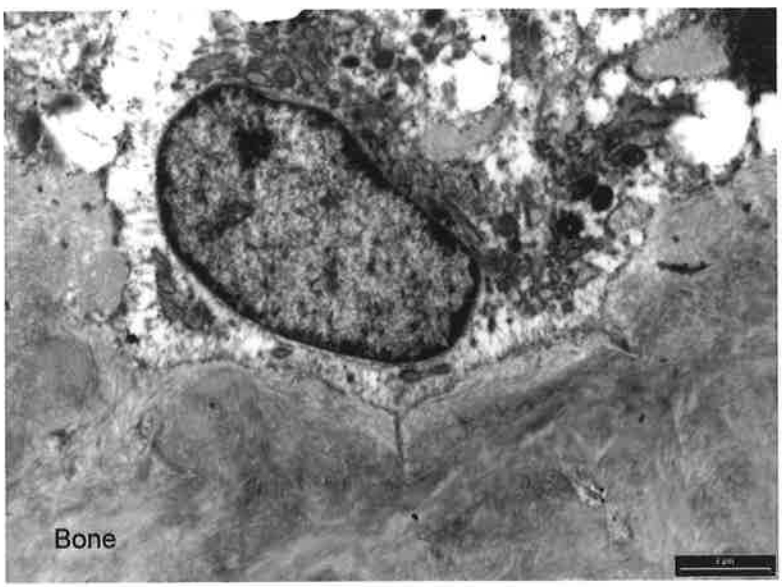


Figure 170. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x4600. Electronmicrograph of alveolar bone resorption lacuna of an OPG-affected rat revealing a large mononuclear cell in an apparent state of degeneration.

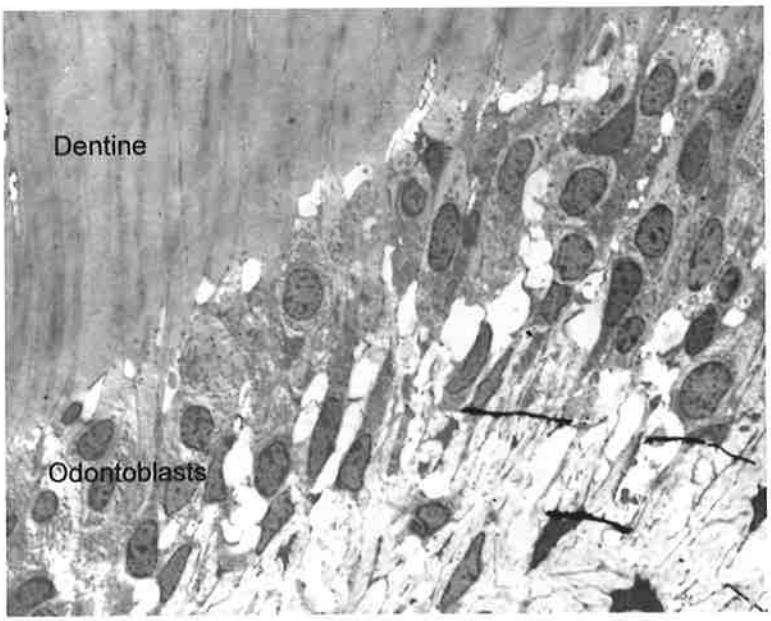


Figure 171. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1100. Electronmicrograph of pulp tissue in an unfrozen molar in an OPG-affected rat revealing a normal odontoblastic layer and reticulum.

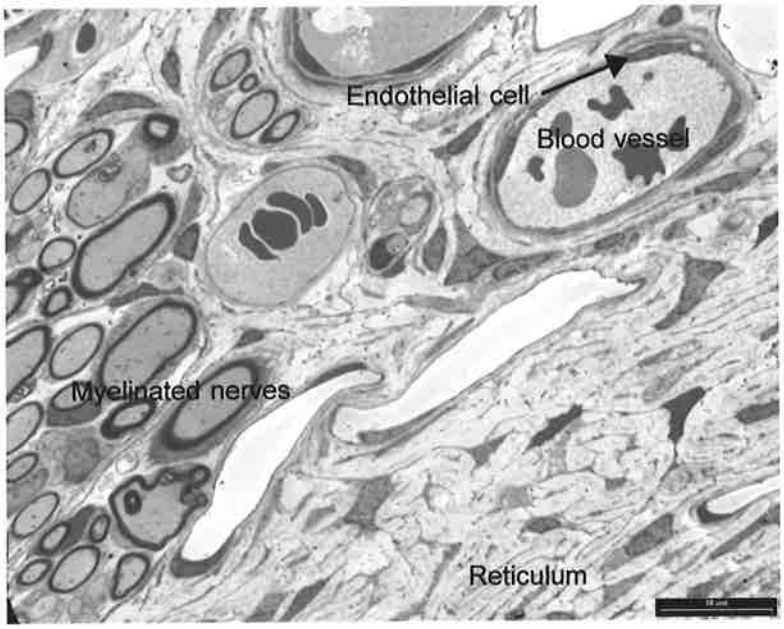


Figure 172. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1100. Electronmicrograph of pulp tissue in an unfrozen molar of an OPG-affected rat revealing normal neural and vascular elements.

C. Experimental teeth – frozen, OPG treated

Active resorption was noted in the roots of frozen molars of OPG-affected rats. Large multinucleated cells with extensive ruffled borders were seen lining the hard tissue surfaces in the interradicular area (Figs. 173 and 174). However, multinucleated cells without ruffled borders were also apparent on root surfaces (Fig. 175). Cells appeared to be actively competing for root surface contact as clusters of cells were seen in a single resorption bay (Fig. 176).

The clast cells on root surfaces excavated substantial resorption cavities and showed no signs of being inhibited by the OPG. Cells exhibited a variety of shapes and sizes and ranged in diameter from approximately 20 to 100 μm. The number of nuclei varied depending on the clast cell's activity and the plane of section but typically there were 2 to 6 nuclei. Often nuclei were irregular in outline with indentations and invaginations affecting their outer membrane (Fig. 175). At high magnification, active clast cells were replete with cytoplasmic organelles consisting mainly of lysosomes, vacuoles and mitochondria (Fig.177).

The prominent ultrastructural feature of the active clast cells was the ruffled border found adjacent to the hard tissue surface. The cell membrane infoldings and convolutions varied in size and extent but were clearly seen in OPG- and thermally-affected rat molar PDL (Figs. 178 and 179).

Intracellular clear zones were often not readily apparent in association with the ruffled borders. When present, these organelle-free regions were small in area and adjacent to the border ruffling (Fig. 174). However, small vesicles and fine

fibrils were often evident in these relatively clear areas while vacuoles were plentiful within the active cell's cytoplasm (Fig. 178).

The morphology of the resorption lacuna appeared to be related to the morphology of the resorbing cell. Circular cells were seen in rounded resorption cavities (Fig. 179) while flattened cells were related to shallow lacunae (Fig. 173). Clast cells, full of cytoplasmic granules characteristic of apoptosis, were seen on adjacent tooth surfaces (Fig. 180). Correspondingly, the resorption lacuna contained cellular debris as well as cementum matrix and collagen remnants of the resorption process.

In juxtaposition to the clast cells, the PDL contained mononuclear cells in close approximation to the cellular resorption activity (Fig. 173). These neighbouring cells were elongated with spindle-shaped nuclei, and presented an ultrastructural appearance characteristic of blast cells.

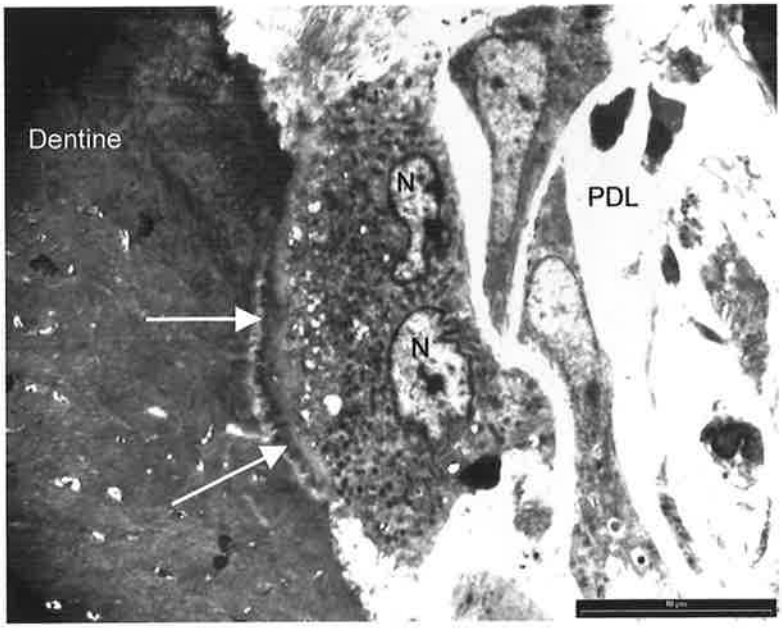


Figure 173. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1950. Electronmicrograph of multinucleated clast cell on the frozen root surface of an OPG-affected rat. A ruffled border (arrows) is seen in close contact with the tooth surface. N - nucleus

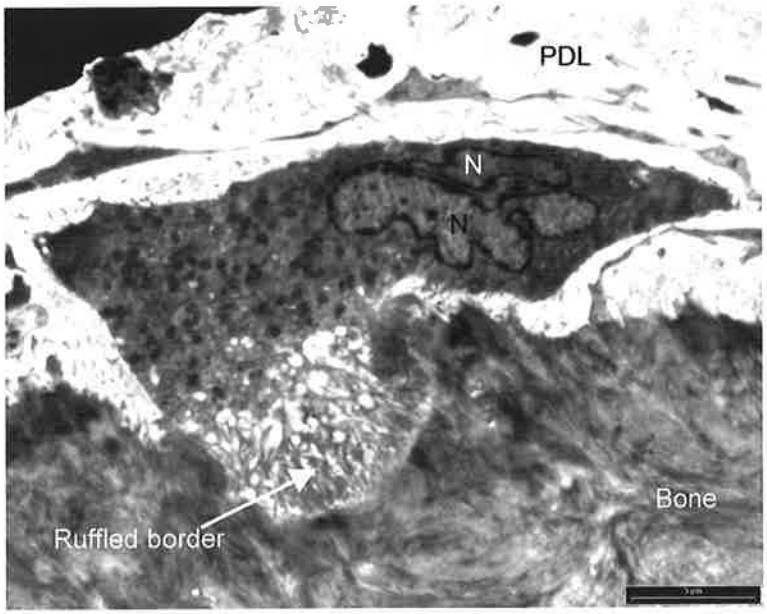


Figure 174. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x2600. Electronmicrograph of a multinucleated clast cell with an extensive ruffled border on the frozen alveolar bone surface of an OPG-affected rat. N - nucleus

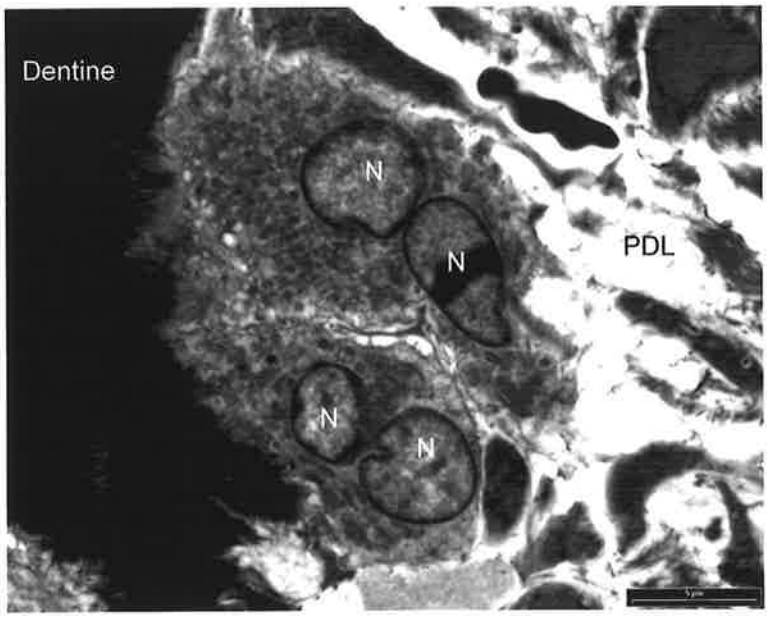


Figure 175. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x2600. Electronmicrograph of multinucleated clast-like cells in close contact with the root surface of a frozen molar in an OPG-affected rat. N - nucleus

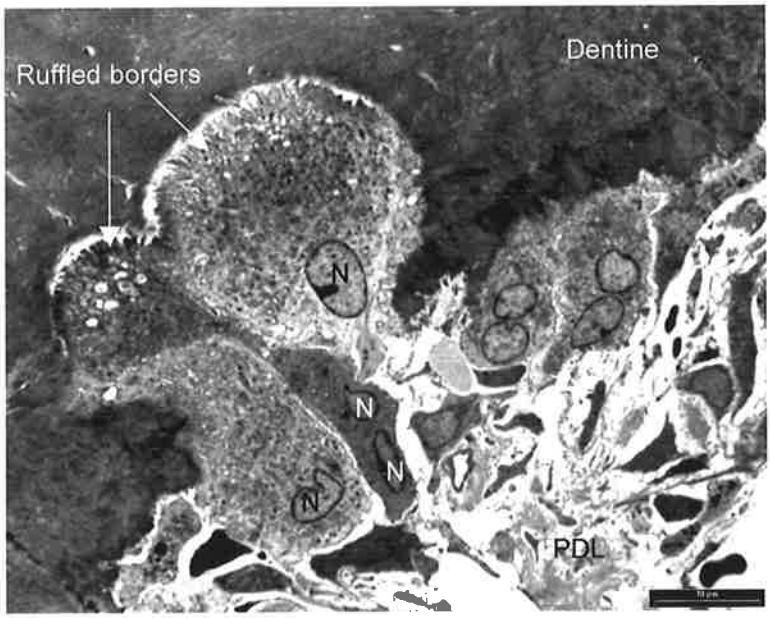


Figure 176. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1100. Electronmicrograph of several multinucleated cells with ruffled borders actively resorbing the root surface of a frozen molar in an OPG-affected rat. The cells appear to be aggressively competing for surface space. N - nucleus

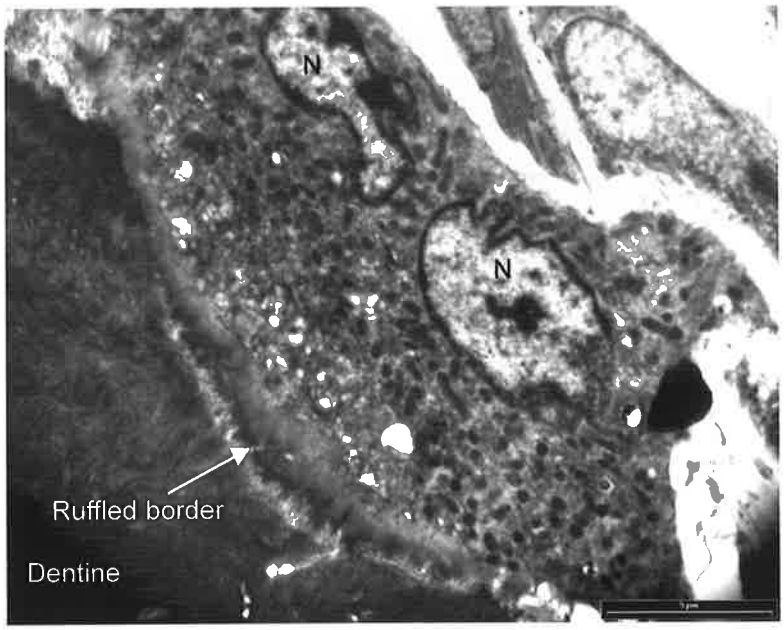


Figure 177. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x3400. Higher power electronmicrograph of the clast cell seen in Fig. 173. The cell is full of organelles and displays nuclei of irregular outline. N - nucleus

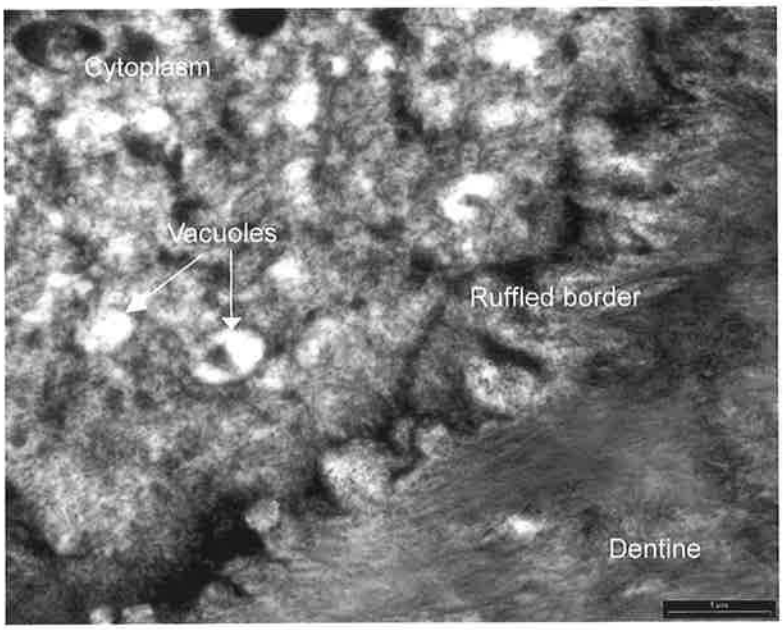


Figure 178. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x10500. Electronmicrograph of intracellular vacuoles and a ruffled border adjacent to cementum of a frozen molar in an OPG-affected rat.

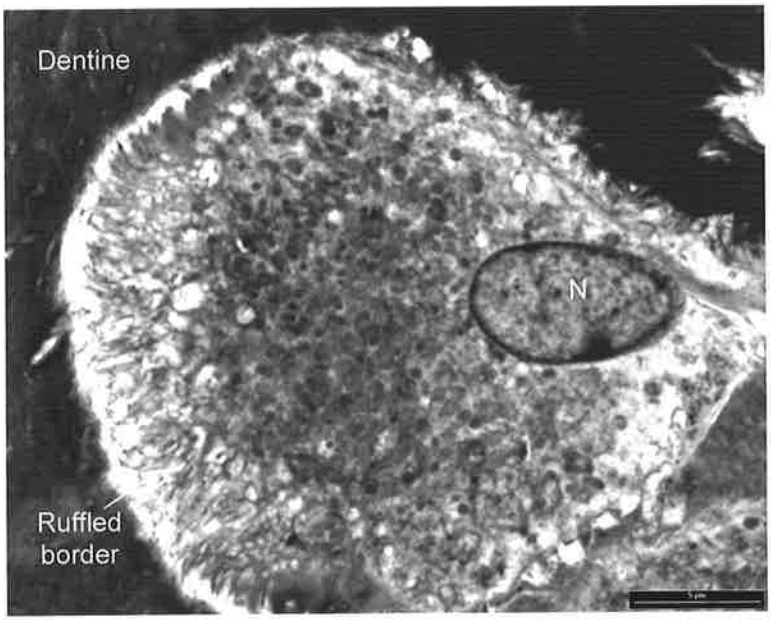


Figure 179. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x2600.
Electronmicrograph of a rounded clast cell in a similarly shaped resorption lacuna in a frozen molar of an OPG-affected rat. N - nucleus

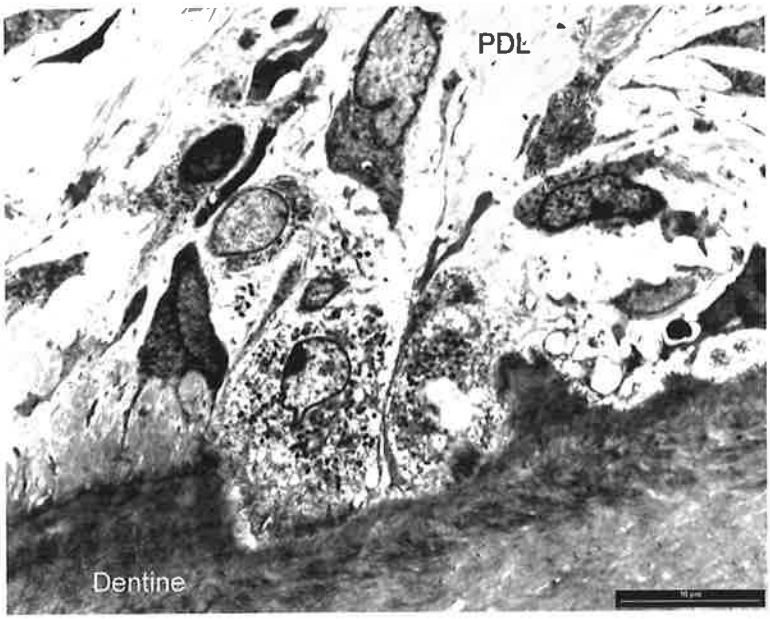


Figure 180. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1450.
Electronmicrograph of resorption lacuna in a frozen molar of an OPG-affected rat indicating cellular, collagenous and hard tissue debris.

- **Bone**

Active osteoclasts displaying ruffled borders and multinucleatedity were seen on bone surfaces and in marrow spaces adjacent to areas of dental resorption (Figs. 181 and 182). Ruffled border disruption and cell breakdown indicative of osteoclast apoptosis was noted in many instances (Fig. 183). No osteoclast activity was observed at a distance from the interrradicular area where other inflammatory cells were also absent.

Moreover, no definitive clast cell activity was seen in the long bones of rats administered OPG (Fig. 184). Previous resorption lacunae were seen on tibial endosseous surfaces but these were filled with osteoblast-like cells high in endoplasmic reticulum content (Fig. 185). The osteoblast-like cells and bone lining cells were adjacent to bone surfaces (Fig. 186). Multinucleated cells were also seen in marrow near bone surfaces (Fig. 187) but these cells bore no other morphological similarities to resorptive clast cells.

- **Ankylosis**

Ankylosis was a prevalent feature in sections of the frozen PDL of OPG-affected rats (Fig. 188). The ankylosis was of the appositional variety rather than bony replacement of previous resorptive areas. Cells became entrapped in the appositional bone. The disorganized appearance of the latter gave way to the organized structure of dentine at a line of often indistinct demarcation.

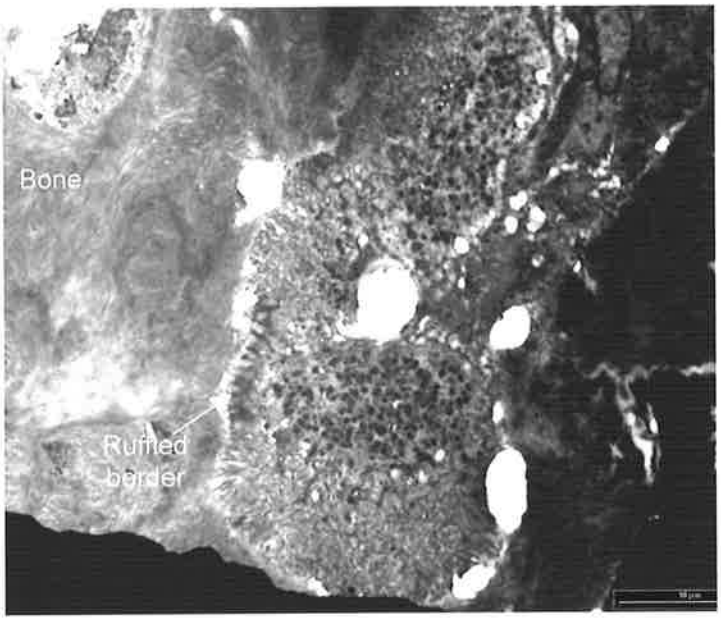


Figure 181. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1450. Electronmicrograph of osteoclasts in crestal alveolar bone resorption lacuna in an OPG-affected rat. Border ruffling is evident along with numerous mitochondria but no nuclei are seen in this view.

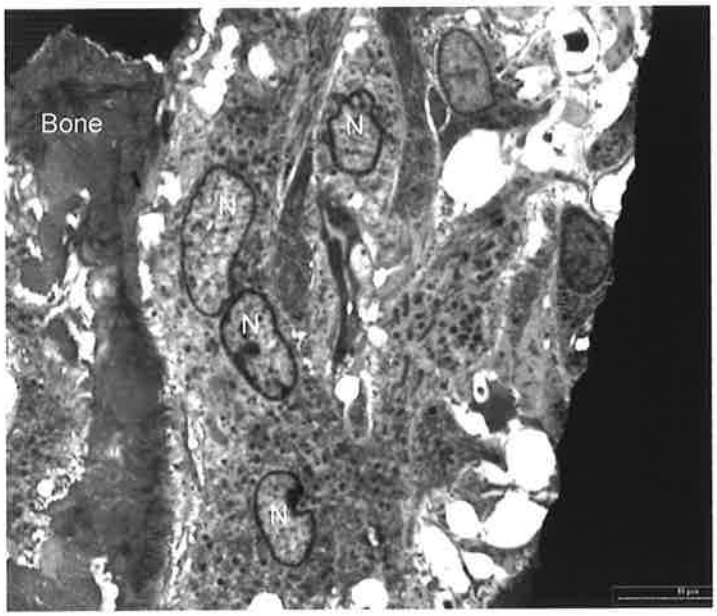


Figure 182. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1450. Electronmicrograph of megakaryocytes in frozen alveolar bone marrow of OPG-affected rat. N - nucleus

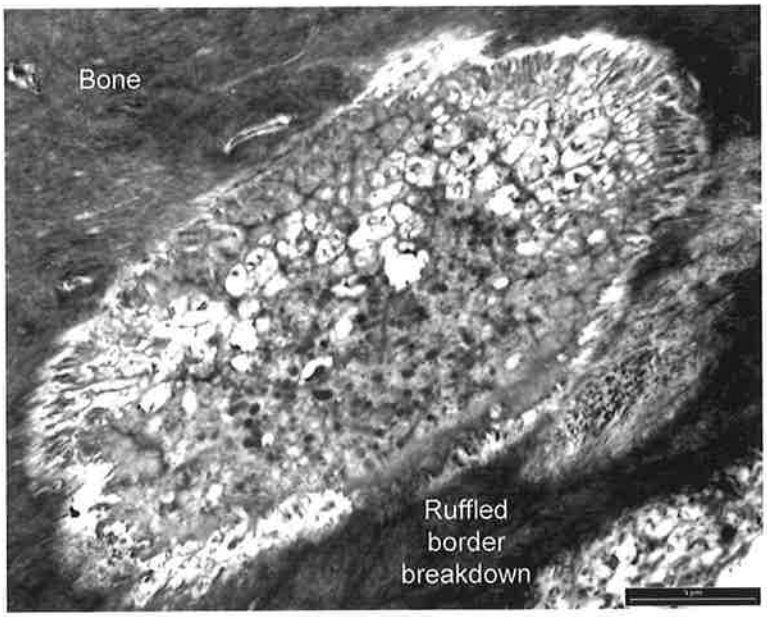


Figure 183. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x2600. Electronmicrograph of a clast cell in frozen alveolar bone resorption lacuna of OPG-affected rat. Vacuole disruption and ruffled border breakdown indicates that cell degeneration appears to be occurring.

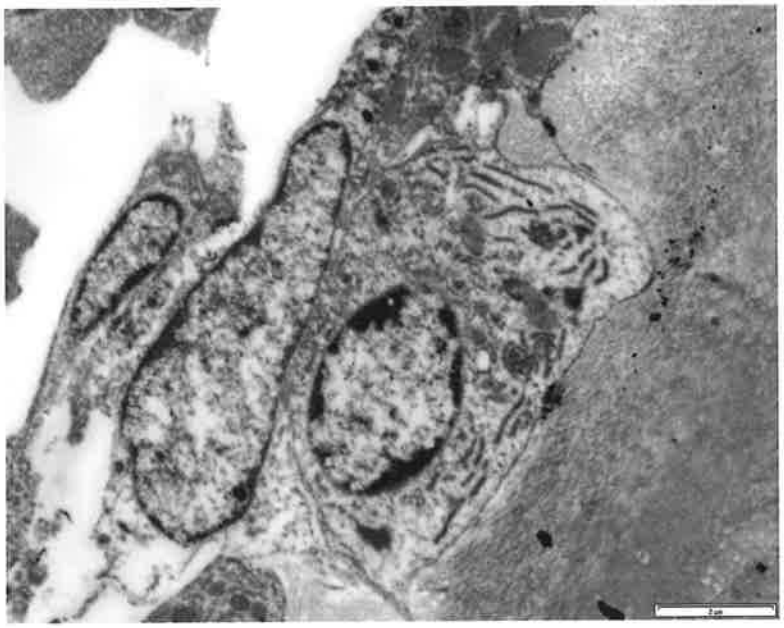


Figure 184. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x5800. Electronmicrograph of tibial endosseous surface of an OPG-affected rat revealing a bony lacuna containing an osteoblast-like cell filled with endoplasmic reticulum.

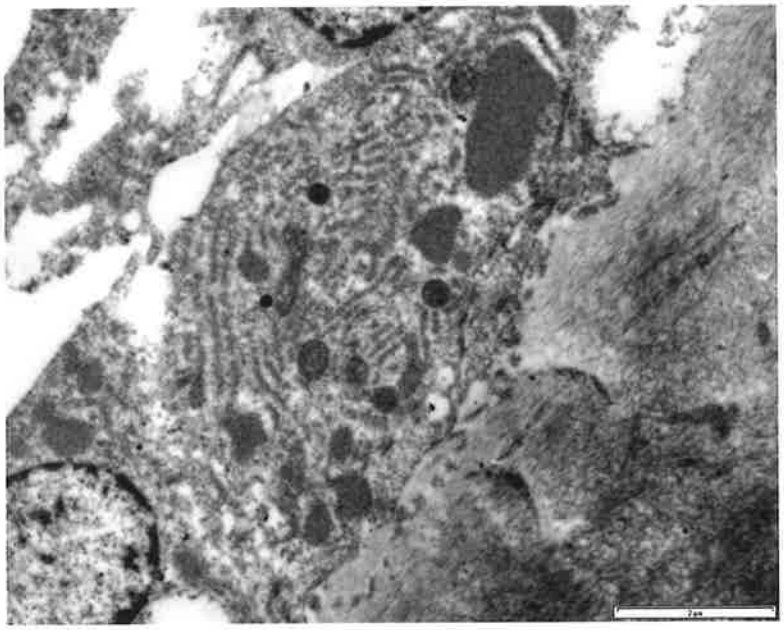


Figure 185. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x7900. Electronmicrograph of a cell rich in endoplasmic reticulum adjacent to an endosseous surface of an OPG-affected rat.

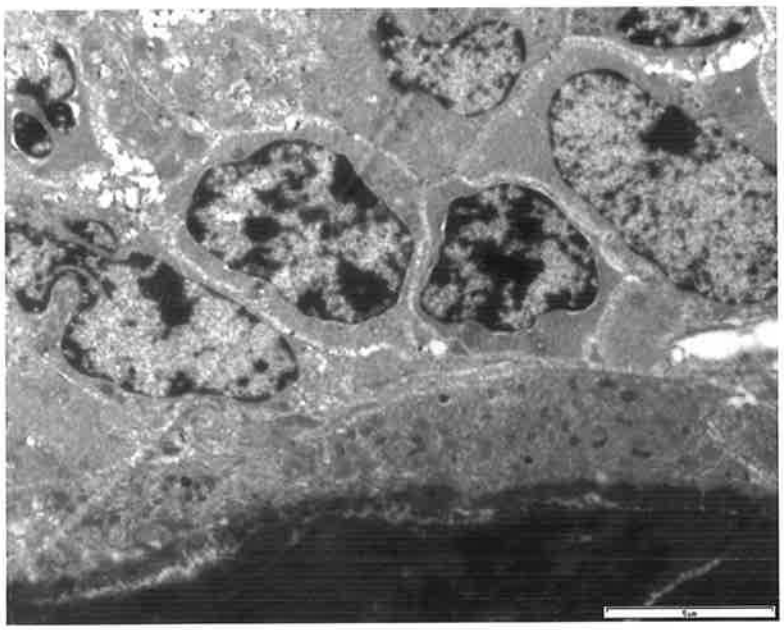


Figure 186. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x4600. Electronmicrograph of lining cells on the surface of endosteal bone of an OPG-affected rat.

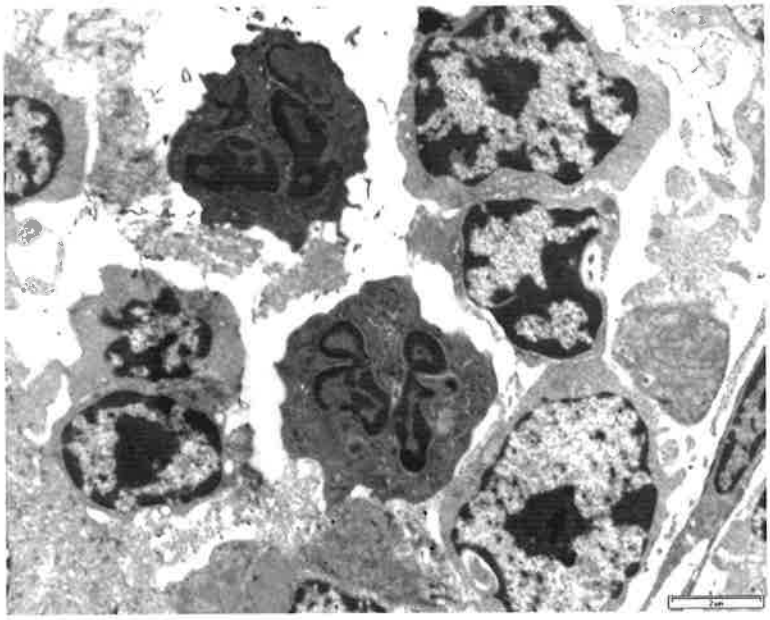


Figure 187. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x4600. Electromicrograph of multinucleated cells in marrow spaces of tibial bone in an OPG-affected rat.

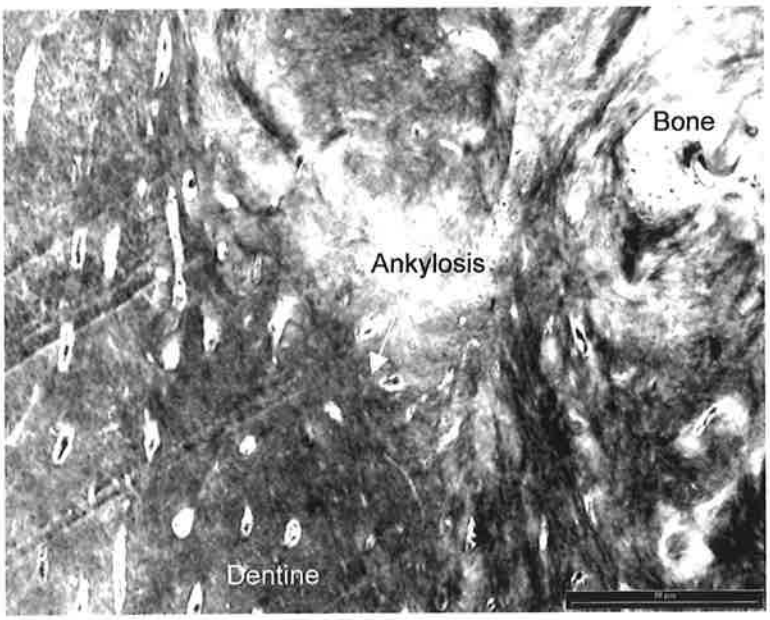


Figure 188. Experiment 8, TEM, uranyl acetate and Reynold's lead, magnification x1950. Electronmicrograph of ankylotic union in a frozen molar of an OPG-affected rat.

A summary of the results of the freezing procedures is presented in Table 5.

Procedure	Sacrifice at	Resorption	Repair	Ankylosis
Unfrozen control		+-	+-	-
10 min freeze Experiment 1	2 d	+-	-	-
	7 d	+	-	-
	14 d	+	+	-
	28 d	-	+	-
20 min freeze Experiments 2,5	2 d	+-	-	-
	7 d	+	-	+
	14 d	+	+	+
	28 d	-	+	+
20 min freeze x 3 Experiment 3	2 d	+-	-	-
	7 d	+	-	+
	14 d	+	+	+
	28 d	-	+	+
10 min freeze x 3 Experiment 4	7 d	+	-	+
	14 d	+	+	+
10 min freeze + mechanical insult Experiment 6	7 d	+	-	-
10 min freeze + succinic dehydrogenase Experiment 7	2 d	+-	-	-
	7 d	+	-	-
	14 d	+-	+	-
10 min freeze + OPG Experiment 8	7 d	+	+	+

Table 5. A summary of the experimental procedures indicating those that were successful in producing hard tissue resorption. Repair of resorption lacunae and experiments resulting in ankylosis are also presented.

CHAPTER 5

DISCUSSION

5.1 Anaesthesia

Trialled as a rat anaesthetic during this study, fentanyl-fluanisone (Hypnorm®) proved to be safe and reliable. Its availability is restricted by Australian legislation and its importation currently occurs only under license. Nevertheless, the value of the anaesthetic is worth the import expense as all animals used survived the experimental procedures and subsequently thrived. The anaesthesia produced at the recommended dosage was profound and sufficient to maintain the comfort of the animals both when the cold thermal stimulus was being applied and during the subsequent recovery period. This profound anaesthesia was previously shown by Clowry and Flecknell (2000) who performed intracranial surgery on rats without ill-effects.

The safety and inert nature of Hypnorm® was shown by Hertsens *et al.* (1984) who compared its effects with those of chloralosane and pentobarbital on mitochondrial ultrastructure. While differences were seen in mitochondrial intramembrane particles with the other anaesthetics, no structural changes were caused by Hypnorm® which provided confidence in the ultrastructural images of the resorption cells generated by the aforementioned study.

Cells of the haemopoietic system need to escape from blood vessels into the tissues In order to perform their anti-inflammatory functions. Some anaesthetics have been shown to have an inhibitory effect on this process but Hypnorm® was found to have a facilitatory role (Janssen *et al.*, 1997). Hence, the migration of clast cell precursors to sites of thermal inflammatory damage was considered to be unaffected, or perhaps assisted by the anaesthetic.

The sedative effects of midazolam (Hypnovel®) complimented the anaesthetic effects of Hypnorm®. The muscle relaxation produced in the oral region assisted in the retraction of tissues and allowed easy access to the upper right first molar. Compared with related sedatives such as climazolam, midazolam has the additional benefit of potentiating the effects of the anaesthetic (West and Green, 1987). In conclusion, the benefits of Hypnorm®/Hypnovel®, and particularly its efficacy and safety, make it the animal anaesthetic of choice, even though its current cost in Australia would make routine use prohibitive.

5.2 The frozen tooth model

5.2.1 Usefulness of model

One of the aims of the current study was to produce a model of aseptic root resorption which mimics orthodontic root resorption. The latter is a common adverse treatment sequela, the pathogenesis of which is poorly understood. While previously employed model systems used to generate aseptic resorption in laboratory animals yielded useful results, most have been handicapped with manufacturing, implementation or management problems (Clark *et al.*, 1991;

Rygh, 1992; Brudvik and Rygh, 1993a;1993b). The majority of these systems were based upon the movement of teeth by orthodontic elastomerics or fixed appliances, the longevity of which, in the oral environment, has been variable and somewhat unpredictable (Waldo and Rothblatt, 1954; Davidovitch *et al.*, 1980; 1988; Yen *et al.*, 1992). Animal models utilizing primates, dogs, cats and rodents have been reported, but rats remain the most widely used animal for examination at the light (Reitan and Kvam, 1971; Storey, 1973) and electron (Rygh, 1976; Roberts and Chamberlain, 1978) microscopic levels. Hence, the aim of the current study was to produce a more simple and efficient method of generating aseptic resorption in the periodontium to assist in the investigation of the processes and cells involved.

Earlier studies applied cryotherapy to the alveolar bone and periodontal ligament of rats via a surgical approach (Wesselink *et al.*, 1986; Tal and Stahl, 1986). As these studies reported resorption of periodontal hard tissues, it was considered that a cold thermal insult applied directly to the tooth crown might be an effective and reliable method for initiating root resorption and subsequently examining clast cell function.

The cold thermal insult delivered to the occlusal surface of the rat molars produced PDL disruption and changes to hard tissue which are discussed below. Multinucleated cell activity was evident on PDL hard tissue surfaces in areas radially affected by the freezing process, and it was concluded that the application of a cold stimulus is an effective way of producing experimental PDL

hard tissue resorption. Furthermore, the healing response following the thermal insult permitted an investigation of the subsequent repair mechanisms and produced an additional model for the study of ankylosis.

5.2.2 Cryotherapy

It is an interesting observation that extreme cold may be used in a preservative or in a destructive way. For example, the preservative properties of cryotherapy are utilized in the long-term storage of developing teeth (Bartlett and Reade, 1972), haemopoietic cells (Lazzari *et al.,* 2001), of reproductive cells for eventual use in *in vitro* fertilization programs (Porcu, 2001; Donnelly *et al.*, 2001), and of entire organs for surgical transplantation (Jacobsen and Pegg, 1984; Fahy and Ali, 1997). In contrast, the destructive properties of cryotherapy are utilized in the surgical management and removal of pathological lesions such as tumours (Malafosse *et al.*, 2001; Schmidt and Pogrel, 2001) or warts (Connolly *et al.*, 2001). The destructive injury caused by freezing has been variously argued to result from electrolytic gradients across cell membranes (Lovelock, 1953), cellular dehydration leading to a reduction in cell volume and denaturation of intracellular proteins (Levitt, 1962; Meryman, 1970), or the freezing of intracellular water (Mazur, 1963). According to Mazur (1984) the survival of cells is known to be highly dependent on the rate of cooling. This author reported that rapid cooling results in intracellular freezing because there is insufficient time for the osmotic removal of cellular water thereby preventing supercooling. Importantly, however, the cellular changes are also dependent on the distance from the freezing source and the time of application.

The targeted and controlled delivery of a cold thermal stimulus has routinely been performed using a cryoprobe (Maroon and Bailes, 1995; Rabin and Shitzer, 1996; Ishida and Ramos-e-Silva, 1998). While a carbon dioxide cryoprobe was initially tried in this project, it was found to be unsuitable because of access problems to the small rat oral cavity and, specifically, to the rat molar. The available size of the probe tips made delivery of the insult to the molar crown, without affecting the neighbouring tissues, difficult. Dry ice was supplied as pellets which were able to be quartered, trimmed and customized to the size of the rat molar which facilitated handling and application to the tooth. The application of the customized pellets to the molar crown proved to be an effective way of delivering a thermal insult through the tooth to the periodontium. The procedure was facilitated by the use of the animal holding rack and the mouth prop that permitted a single operator to carry out the retraction of the soft tissues and the administration of the dry ice unaided.

According to Cooper and Dawber (2001), carbon dioxide applied directly to skin is unable to reduce the surface temperature below -79°C and is therefore unlikely to cause the deeper freezing of tissues. If deeper freezing is required liquid air, oxygen or nitrogen should be used. However, in the present study carbon dioxide, as dry ice, proved effective in eliciting necrotic destruction of tissues. This was evident in the tissue ablation seen in the pulp chamber of the frozen teeth (Figs. 16 and 115) and in the changes evoked in the periodontium (Figs. 15, 93 and 99).

5.2.3 Thermal conductivity and radial effects

Past studies examining the effects of low temperatures applied to periodontal structures described thermal injuries and resorption of adjacent root surfaces (Wesselink *et al*, 1986; Tal and Stahl, 1986). Cryoprobe and liquid nitrogen application to the external gingival tissues and alveolar bone caused periodontal cell death and bared root surfaces to clastic attack. It has been reported that exposure of mineralized tissue is a prerequisite in the initiation of resorption by clastic cells (Vaes, 1988; Zaidi *et al.*, 1993; Martin *et al.,* 1993; Orlandini *et al.,* 1995). However, Wesselink *et al.* (1986) reported an influx of reparative cells in the form of fibroblasts and macrophages and the deposition, after 3 d, of mineral crystals on the root surface. Seven to 12 d after freezing, this mineral was phagocytosed by mononuclear cells, as root resorption and ankylosis occurred.

In the present study, cell death was caused by the occlusally applied cold thermal stimulus as revealed by the lysis of odontoblasts and other cellular elements within the pulp chamber. It was likely that the thermal effects were conducted through the tooth to the pulp chamber and thence to the external surfaces of the root to similarly affect the adjacent cells in the PDL. The subsequent degeneration of the adjacent structures of the PDL exposed the root surfaces to resorption attack. There was evidence of hyalinization and inflammatory cell infiltration into the periodontal ligament of experimental teeth (Fig. 15), but the major response was interradicular and cervical aseptic resorption (Fig. 23).

Craig and Peyton (1961) indicated that dental tissues were good thermal insulators but reported lower thermal conductivity in dentine compared with enamel which was considered to be a reflection of a higher amount of organic matrix present in the former structure. Since thermal conduction by dental tissues is reportedly low, sufficient time is necessary for a thermal change to affect surrounding tissues. This was shown in the current study, in which a minimal application time of 10 min was adequate to induce a PDL change with resulting resorption.

Further work by Brown *et al.* (1970) on dental tissue heat diffusivity indicated that transient heat conduction occurs more readily in enamel than in dentine. Additionally, it was ascertained that a difference existed in thermal conductivity when the stimulus was applied parallel, compared with transversely, to the dental tubules. This may explain the increase in the severity of the cold thermal effects seen in the interradicular and cervical areas of teeth in the current study where the alignment of the dentinal tubules follows a radial direction away from the pulp chamber (Schour and Massler, 1971).

The effects of the cold thermal stimulus to the crown of the rat molar extended, but were radially confined to the surrounding PDL in the interradicular and cervical areas (Diagram 5). In this respect the tissue effects were directional in nature. The inflammatory and resorption changes involving interradicular bone and tooth root facilitated the investigation of the clast cells involved in the resorption processes.

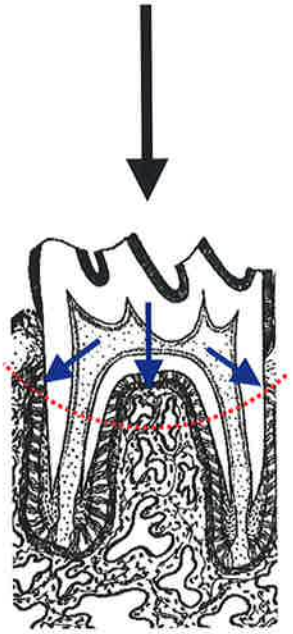


Diagram 5. Dry ice application to the molar crown and the proposed radial dissemination of the thermal effects to involve the interradicular and cervical areas of the PDL.

5.2.4 Effects of different experimental protocols on the resorption model

In the current study, the number of freezing episodes and the duration of application of the dry ice was varied in order to find the optimal combination to yield overt resorption. Fraser and Gill (1967) reported that cell death in uncalcified tissues was achieved after one episode of tissue freezing but that repetitive freezing only served to extend the boundary of thermal effect. The minimal application time of 10 min employed in the current study was sufficient and effectively generated active resorption in dental tissues and bone 7 d after injury, and provided a suitable resorption model for the investigation of the cells involved.

However, all experimental application times employed in this study resulted in localized tissue necrosis and hard tissue effects. It was clear that a single, prolonged dry ice application time of 20 min increased the area of tissue injury while the superimposition of mechanical trauma extended the area of injury further (Fig. 117).

Mechanical injury to the cementum surface was introduced into the current study in order to augment clastic cell attachment and activity. Adhering root surface soft tissue was likely damaged by the trauma and facilitated the resorption process (Nakane and Kameyama, 1987). However, the physical process of scraping the tooth surface also pierced the gingival attachment allowing ingress of microflora from the oral cavity. The possibility of a subsequent septic inflammatory resorption in the neighbouring hard tissues could not be

discounted. Because of the uncertainty of the aetiology of the observed cementum resorption, mechanical insult to the tooth root was discontinued as a reliable method of initiating aseptic resorption.

While multiple delivery of the cold thermal insult did not appear to extend the tissue damage and hard tissue resorption significantly, multiple freezing episodes placed the resorptive effects of the insult open to question. Sloughing of the gingival tissues was a feature of those animals with molars undergoing repeated freezing, and an invasion by oral bacteria was again possible due to attachment tissue breakdown. Although no histomorphometry was performed, the efficacy of the multiple insults did not appear to be significantly different from a single freezing episode. This was attributed to the likelihood of the additional insults causing repeated necrosis of all cells repopulating the area during the time of the sequential freezing and therefore preventing an increase in resorption. An inhibition of early repair to the frozen damage was also considered likely. It was, therefore, concluded that a single freezing insult of 10 min duration would initiate resorption and permit its investigation, while longer and multiple freezing times, increased the soft tissue disruption with minimal effects on the resulting area of resorption. Furthermore, longer and multiple freezing times were more likely to initiate an ankylotic union. Interestingly, this also produced a model for the study of ankylosis.

Thus, a reproducible and reliable method for initiating aseptic root resorption in rats resulted following a single 10 min application of dry ice to the molar crown

surface. Furthermore, consistent ankylosis resulted with longer and multiple freezing times.

5.2.5 Simulation of orthodontic tooth movement and subsequent root resorption

Because the tissue destructive characteristics of cryotherapy were used to initiate an aseptic inflammatory response within the PDL, it was considered that the model might permit investigation of the cellular aspects of orthodontic tooth movement and avoid the need for intricate orthodontic mechanical systems. Orthodontic tooth movement has been described as a force-induced inflammatory change within the periodontal ligament from which the tissues ultimately recover (Davidovitch, 1991). The applied orthodontic force, reportedly detected by strain changes in osteoblasts and osteocytes (Pavlin and Gluhak-Heinrich, 2001), evokes a complex series of transductive cellular responses in the PDL and surrounding tissues leading to the release of inflammatory chemical mediators. The cellular recognition of strain has been hypothesized to come from any one of a number of molecular, electrical and cellular cytoskeletal events that, once received, alter the biochemistry of the cell (Dolce *et al.,* 2002). A signalling cascade involving receptor activation, initiates secondary messenger generation and release (Herrlich and Ponta, 1989) and many cytokines, neurotransmitters and growth factors have been implicated as primary and secondary messengers (Chow *et al.,* 1998). Identified as being of importance are the prostaglandins (Sandy *et al.*, 1993), the interleukins (Davidovitch *et al.*, 1988), tumour necrosis factors (Bertolini *et al.*, 1986), and cyclic AMP (Lowik *et al.*, 1985). By definition,

forces applied to teeth in order to produce movement have a directional property (Davidovitch, 1991). The subsequent pressure effects initiated in the PDL would therefore reflect the direction and magnitude of the applied force and result in a localized and characteristic pattern of inflammatory change (Linge and Linge, 1991).

Williams (1984), and Brudvik and Rygh (1992; 1993a,b), in comprehensive examinations of the effects of orthodontic force on the periodontal ligament and root resorption, described a specific PDL sequence of events. Forced compression of the PDL initiates resorption of the alveolar bone adjacent to amorphous acellular areas of hyalinization. Root resorption was apparent in their rat model, 3 d after the application of force, and hyalinization occurred 1 or 2 d before root resorption became evident. The results of the present study are in agreement with the aforementioned chronology in so far as resorption was initiated between 2 and 7 d after freezing injury and further, was associated with prior hyalinization within the ligament.

In addition, results of the present study showed a similar chronology and sequence of events associated with progression and repair of the resorptive lesion after 14 days. However, Williams (1984) and Brudvik and Rygh (1993a,b) described resorptive effects in the apical and mid-root areas of the teeth, whereas resorptive changes were observed in the interradicular and cervical areas of experimental teeth in animals used in the present study. This could be explained by the differences in application and directional nature of the insult.

The periodontal effects of orthodontic forces are dependent on an array of factors but most commonly on the magnitude and direction of the applied forces and the resultant compression of the PDL (Rygh, 1973). A pattern of bone resorption and possibly dental resorption is initiated that reflects the type and direction of tooth movement (Davidovitch, 1991). Although a somewhat different pattern of resorption was revealed in the present study, the overall hard tissue responses of the periodontal structures to thermal insult is recommended as a valuable method of studying the cells involved while providing a particular advantage in its ease of application.

5.3 The chronology of PDL change

The cold thermal insult leading to necrotic and hyaline changes within the PDL followed a chronology of events. Hard tissue resorption and clast cells were not immediately apparent in large numbers and at 2 d after cold application no significant tooth resorption was observed. However, by 7 d, resorption and clast cell presence was seen in the tooth root areas most affected by the thermal insult. Clast cell activity therefore, commenced between these time periods as suggested by Williams (1984) and Moroney *et al.* (1999). After initial response by precursors in the nearby tissues, clast cells required time to differentiate from haemopoietic sources and migrate via the vascular system to the area of the stimulus (Chambers, 2000). Once present, additional time was required for contact and attachment to the hard tissue surface prior to the production of proteolytic enzymes and H^+ ions (Baron, 1989a; 1995). Time scales of 4-5 d were suggested by Uy *et al.* (1995) and Moroney *et al.* (1999) in *in vivo* model systems, and lesser periods for clast cell activation in *in vitro* model systems (Lambrecht and Marks, 1996; Quinn *et al.*, 1996).

Tal *et al.* (1991) reported that by 30 d following injury, the frozen periodontal ligament did not differ significantly from surrounding tissues and neither bone resorption nor ankylosis was seen in the experimental sites. By 14 d in the present study, clast cell activity was reduced and had all but disappeared by 28 d when no differences could be seen between the PDLs of control and frozen molars except for the repaired resorption bays. This indicated that the PDL has the capacity to recover after an insult, but if the insult is persistent [for example,

as seen in infected teeth in several experimental animals (Fig. 28)], clast cell activity appears to continue and hard tissue resorption increases (Andreason, 1981b; Lindskog *et al.,* 1985; 1988a). This has repercussions in areas of dentistry such as endodontics and orthodontics. Endodontic therapy requires the complete removal of any noxious stimuli emanating from diseased pulp chambers to avoid the likely continuation of periapical tissue damage (Andreasen and Kristerson, 1981a). Similarly, the continuous nature of force application during orthodontic treatment has the potential to cause tooth root damage due to the ongoing inflammation in the periodontal ligament. Although this inflammation is sterile in nature, Linge and Linge (1991) and Rygh (1992) have shown that the continuity of the tooth-moving forces and the induced inflammatory state reduces the likelihood of PDL repair and increases the potential for significant hard tissue resorption.

The ability of the tooth to repair areas of resorption appeared commensurate with the level of thermal insult. The short duration, single frequency episodes saw more rapid repair of resorption lacunae by 14 d, compared with longer and multiple exposures but tooth repair appeared to have been completed by 28 d, irrespective of the level of insult. Of significance is the fact that the tooth and PDL apparently retain an ability to repair themselves provided the insult is no longer being applied. This was seen in rats 14 d after thermal injury, at which time resorption activity had diminished or was absent and signs of reparative cementum deposition were apparent in the lacunae. It would further indicate that the cells lining the root either recovered or were replaced following the injury. In

two animals where the pulp chamber had succumbed to the freezing injury resulting in abscess formation and a pulpal influx of inflammatory cells, heavy osteoclast activity persisted on the root surface and aggressive resorption remained. The stimulus for the resorption process had likely changed to an infective one and could no longer be regarded as aseptic. It indicated, however, that continuing insult and injury is necessary for the perpetuation of the resorption process. This is seen in the orthodontic induction of root resorption in which root damage occurs under the influence of orthodontic loading. Cessation of orthodontic pressure permits recovery and limited repair of root form (Barber and Sims, 1981; Langford and Sims, 1982).

5.4 Ankylosis

Ankylosis was a feature observed in many of the experimental animals (Figs. 21, 26, 40 and 112). Its appearance indicated a breakdown in cellular or organic layers protecting hard tissue surfaces. The action of osteoclasts on bone is dependent on the resorptive cell's ability to contact and adhere to the mineralized substrate (Vaes, 1988; Baron *et al.*, 1993; Baron, 1995; Suda *et al.,* 1997). Osteoid and bone lining cells must be removed from the bony surface prior to resorptive attack (Baron, 1989a; Zaidi *et al.*, 1993). It is conceivable that a similar mechanism might be applicable to the dental tissues in so far as cementoblasts and precementum must allow resorptive cell access to dental hard tissue surface (Sasaki *et al.,* 1990). The freezing of the molar crowns and the transmission of the thermal insult through the tooth resulted in lining cell and adjacent periodontal tissue necrosis, followed by resorption effects in hard tissue surfaces. The observed ankylotic union may therefore be attributed to an effusive reparative response by unaffected cells and blood vessels in adjacent tissues and alveolar bone (Andreasen, 1980a).

In his time-related study of periodontal healing and root resorption in reimplanted monkey teeth Andreasen (1980a) described two types of ankylosis. The first occurs in old resorption lacunae where the ankylosis is preceeded by resorption of cementum and dentine and ultimately results in alveolar bony union with root structure. The second type of ankylosis is created by the replacement of periodontal membrane by bone without the prior resorption of cementum. Alveolar bone is in close contact with an apparently intact cemental surface and

the respective hard tissues either fuse or are in close approximation, possibly separated by a thin amorphous layer.

The ankylosis generated in the present study was of the appositional rather than the replacement type. No histological signs of preceeding or ongoing resorption were seen in the ankylotic areas although resorption activity was often seen in neighbouring and adjacent sites. Furthermore, the ankylosis was only sporadically seen in the animals used in the development of the resorption model. It was noted that in animals with teeth frozen for frequent or longer periods, ankylosis was more likely. However, all animals treated with OPG displayed ankylotic union of alveolar bone and cementum even though the dry ice application times were shorter.

It could, therefore, be concluded that ankylosis was dependent upon the severity of the insult to the tissues. The longer dry ice application times of up to 20 min, in addition to the multiple applications in many animals, were likely to cause more widespread damage to the cells and tissues. The following enthusiastic healing response by the periodontal ligament and surrounding alveolar bone resulted in hard tissue overgrowth and subsequent fusion but relied heavily on intact osteoblasts or a bone cell lining layer on the alveolar bone surface (Lindskog *et al.*, 1985) or the rapid differentiation of osteoblast precursors. If intact bone lining cells are necessary for bone deposition and ankylotic healing, it might indicate that the effects of freezing did not penetrate across the periodontal ligament space and cause necrosis of these cells. Further support for this premise is the

fact that soft tissues are poor thermal conductors (Craig and Peyton, 1961; Brown *et al.*, 1970) and that the thermal effects may not have penetrated to affect alveolar bone and its accompanying cells. The lack of cold penetration was unlikely given the uncharacteristic experimental pattern of resorption on the cervical and interradicular bone surfaces and thus it is more likely that bone-forming cells differentiated and migrated from adjacent unaffected areas.

Previous studies employing a cold thermal stimulus to the periodontal structures also created ankylosis of a variable nature possibly due to different application approaches (Wesselink *et al.,* 1986; Tal and Stahl, 1986). The ankylosis was not a dominant feature in these studies and was exceeded by the generated resorption as the long-term effect. Wesselink and Beertsen (1994) suggested that masticatory function was important in the pathogenesis of resorption and ankylosis. Although the animals in the present study showed no outward signs of distress, the frozen molar might have been a source of discomfort and its participation in mastication avoided. According to Wesselink and Beertsen (1994) hypofunction would have resulted in less root resorption and a slower repair of the PDL. The time scales in these studies ranged up to 8 wk during which time root resorption and ankylosis were continuing. After 4 wk in the present study, repair of previous resorption cavities had been completed which may be related to the lesser intensity of dry ice application compared with liquid nitrogen or a carbon dioxide gas cryoprobe placed on the soft tissues (Wesselink *et al.*, 1986; Tal and Stahl, 1986). It is likely that the direct cryoprobe application

to the periodontium likely ensured thermal necrosis of the various tissue elements and increased the severity of resorption and ankylotic effects.

5.5 The identification of clast cells

5.5.1 Microscopy

5.5.1.1 Light microscopy

Osteoclasts were identified under the light microscope in haematoxylin and eosin stained sections by their large size, multinucleated appearance and their location in resorption lacunae on bone surfaces (Fig. 14). The association of these cells with bone has been the accepted and traditional way of identification (Arey, 1920; Suda *et al.*, 1992a,b; Baron *et al.*, 1993; Athanasou, 1996). Osteoclast precursors (reported to exist as mononuclear cells) were extremely difficult, if not impossible, to identify under routine histology. Mononuclear cells were seen to accompany and almost surround osteoclasts in their resorption cavities but rather than precursors, these cells had the characteristics of mesenchymal fibroblasts or osteoblasts. Multinucleated cells classified as osteoclasts were also seen on endosteal surfaces of marrow spaces in company with haemopoietic elements while additional large cells were identified within haemopoietic marrow tissue and classified as megakaryocytes. The distinction and precise identification of these large multinucleated cells based on their light microscopic characteristics was difficult and identification was largely made by their relationship with surrounding tissues.

Similarly, odontoclasts were identified by their histological relationship with the tooth root surface. Under light microscopy in haematoxylin and eosin

stained sections, these cells also appeared as large multinucleated cells located in resorption lacunae on root surfaces (Fig. 13). Often there were accompanying and surrounding mononuclear cells. However, under haematoxylin and eosin staining, no particular distinguishing features could be detected between osteoclasts, odontoclasts and megakaryocytes.

5.5.1.2 Transmission electron microscopy

Accepted ultrastructural descriptions of clast cells depict multinucleation, cytoplasmic organelle-free zones, and areas of high organelle mitochondria-rich content polarized with respect to hard tissue surfaces (Holtrop, 1992; Brudvik and Rygh, 1995b). However, the most characteristic morphological feature of the active cell is a ruffled membranous border (Lucht, 1972a).

Multinucleated cells were in abundance in the haemopoietic marrow tissue where they were classified as megakaryocytes (Figs. 71 and 187). Vast numbers of multinucleated cells were also observed in the frozen PDL adjacent to hard tissue surfaces but few contained all of the ultrastructural features associated with fully active clast cells (Figs. 92 and 101).

While multinucleated, cells associated with experimental resorption lacunae also possessed high mitochondrial and vacuole content and organelle-free areas in their cytoplasm (Figs. 97 and 99). However, ruffled border presence adjacent to hard tissue surfaces was rarely seen in control and frozen PDL and perhaps indicated that the multinucleated cells associated with resorption were not clast

cells or were clast cells that had not completely differentiated to a full level of activity.

While Holtrop (1992) indicated that the plane of sectioning had a significant role to play in the ultrastructural appearance of the cell, it was unexpected to identify only a few ruffled borders in control and experimental tissues.

5.5.2 Enzyme histochemistry

5.5.2.1 Succinic dehydrogenase

The blue positive staining reaction seen in tissue sections is a consequence of the complete chemical reduction of nitro blue tetrazolium (BT) to a diformazan at sites of highest enzymatic activity (Seligman and Rutenburg, 1951). If the reduction potential is low a purple colour is seen as the BT is incompletely reduced to a monoformazan. However, it is worthy to note that other factors are involved in the demonstration of succinic dehydrogenase in tissues. Fixation of tissues, particularly with formaldehyde, completely destroys enzymatic activity and so unfixed cryopreserved tissue is essential (Puchtler and Meloan, 1985). The current study followed the guidelines established by Takimoto *et al.* (1966) who examined the presence of succinic dehydrogenase in the periodontal ligament of rats undergoing orthodontic treatment. Thick frozen sections were employed to preserve enzyme integrity after decalcification in EDTA at neutral pH.

As well as fixation, the temperature of enzymatic activity has been shown to be important. Succinic dehydrogenase activity is enhanced at 37°C but according to Seligman and Rutenburg (1951) is destroyed at 50°C or reduced if tissues are stored at 4°C for lengthy periods. The conventional processing of tissues for histological examination is therefore inappropriate as the heat generated by paraffin embedding denatures the enzyme (Pearse, 1972). In addition, it was expected that the thermal insult to the dental tissues might have an influence on enzyme activity but perhaps in a beneficial way. The positive staining results

indicated that succinic dehydrogenase activity was preserved by the experimental procedures and processing employed.

It has previously been shown that osteoclasts are involved in the process of orthodontic tooth movement (Rygh, 1992) and they also exhibited succinic dehydrogenase activity (Burstone, 1960; Balogh, 1963; Fullmer, 1964). Subsequently, Takimoto *et al.* (1966) established that enzyme histochemistry utilizing tetrazolium, rather than routine histological haematoxylin-eosin staining, more clearly distinguishes osteoclasts from other tissue elements.

The current study confirmed the findings of Takimoto *et al.* (1966), as large blue-purple staining cells were evident along resorbing bone surfaces. In addition, positive staining was also seen in large cells along dental resorption surfaces induced by the thermal insult. It was further evident that only large cells were stained which implied that the functional status (involving succinic dehydrogenase activity in the citric acid cycle) was possibly not achieved until clast cell maturation was advanced.

Whereas multinucleation has been suggested as an indicator of osteoclastic activity, Jones and Boyde (1988) considered that the hard tissue resorbing capability of osteoclasts was enhanced by an increase in nuclear numbers. Although nucleation was not clearly visible with the staining protocol employed, the present study revealed succinic dehydrogenase activity was associated with large cells on hard tissue surfaces. A chronology of events indicated that

succinic dehydrogenase activity relating to root resorption was maximal at the 7 d time frame and was not apparent at the 2 d period. At 14 d after the insult, succinic dehydrogenase activity was diminished on tooth surfaces as the resorption cavities were undergoing repair.

It is likely that the succinic dehydrogenase staining in the present study was seen in active clast cells due to their increase in mitochondrial content. Lucht (1972b) showed ultramicroscopically that clast cells contained high numbers of mitochondria to fulfil their energy requirements and hence it would be expected that succinic dehydrogenase staining would be intense. It is suggested that succinic dehydrogenase label is a reliable morphological marker for clast cell activity provided that cryo-preparation techniques are adopted. As no differences in staining properties were noted, osteoclasts and odontoclasts were identified as similar cells as far as succinic dehydrogenase activity was concerned.

5.5.2.2 TRAP labelling

TRAP labelling enabled the detection of acid phosphatase in large osteoclasts and verified their presence along resorptive bony surfaces (Figs. 32 and 33). All of the TRAP-positive multinucleated cells were in association with bony resorption lacunae and appeared to match the histological distribution. Mononuclear cells exhibiting TRAP staining characteristics were also associated with bony surfaces but were additionally identified in the PDL and neighbouring connective tissue (Fig. 35). Positive TRAP staining was also associated with the multinucleated cells in root resorption bays (Fig. 34).

The enzyme histochemical staining of TRAP has been reported to be a reliable marker for clast cell activity (Minkin, 1982; Andersson *et al.*, 1989; Athanasou, 1996; Suda *et al.,* 2001). However, Hattersley and Chambers (1989b) doubted TRAP's usefulness and Modderman *et al.* (1991) indicated that macrophages also attained TRAP properties indicating that the reliability of TRAP labelling in osteoclasts is not absolute. TRAP specificity has been further questioned by Halleen *et al.* (2000) who indicated that two forms exist. Dependent on the presence of sialic acid, the "5b" form of TRAP is localized to clast cells while the "5a" form is derived from other sources. Bonucci and Nanci (2001) indicated that TRAP is a typical non-specific marker for osteoclasts that could also be found in osteoblasts and macrophages.

The present study identified TRAP activity in cells involved in root resorption caused by sterile inflammatory degeneration, using naphthol AS-BI phosphoric acid as a substrate and basic fuchsin as a coupler. Cells that were tartrate sensitive did not form precipitates, while those that were tartrate resistant coupled with the substrate to form a red precipitate against a background stain of Fast Green. Both TRAP-positive mono- and multi- nucleated cells were observed in the resorption lacunae on root surfaces, alveolar bone and in the bone marrow as well as in the PDL. It was evident that large multinucleated osteoclasts and odontoclasts in resorption lacunae stained similarly.

Cole and Walters (1987) reported that the use of 50mM L(+) tartaric acid as an inhibitor and a fixative solution containing formaldehyde would suppress cells

containing tartrate-sensitive acid phosphatase such as leukocytes, red blood cells and monocyte-macrophages. This led to the assumption that only clast cells and their precursors would stain positively for TRAP (Brudvik and Rygh, 1993a). The TRAP-positive mononuclear cells found in bone marrow and dental resorption bays were believed to be members of the mononuclear phagocyte system and therefore probable precursors of osteoclasts (Baron *et al.*, 1986; Athanasou, 1996).

TRAP-positive cells were found in the PDLs of both control and experimentally frozen teeth. The freezing of teeth may have caused cell death of osteoclast mononuclear precursors that arise from within the periodontal ligament. Consequently, a second population of cells could have arisen from neighbouring healthy tissue and claimed responsibility for the resorption observed. A possible lineage may include monocyte-macrophages, as this cell line could represent a population of TRAP-positive mononuclear cells found in root resorption lacunae. The macrophage has been shown to degrade bone matrix under some circumstances (Mundy *et al.,* 1977; Fallon *et al.*, 1983). Furthermore, Brudvik and Rygh (1993a) observed mononuclear cells near the cementum 1-2 d after experimental tooth movement, suggesting that these were fibroblast-like cells which may have a role in non-clastic root resorption. Alternatively, the relatively small percentage of lacunae containing TRAP-positive cells may be partly explained by the post-freezing observation periods selected for this study.

Considering that ankylosis and reparative cementum were often observed within lacunae at the 7 d post-insult period but more particularly at 14 d post-insult, it is feasible that resorption activity had ceased, and clastic cells had been replaced by reparative cementoblasts and fibroblasts. This is further supported by the finding that TRAP activity was not seen in areas of ankylosis where bone deposition rather than resorption was occurring.

The observation of TRAP activity in resorption bays of control unfrozen molars was an occasional finding. It is likely that this surface resorption was physiologic in nature and normal for these animals. It may also be possible that the experimental teeth were sensitive after the application of cold, and that the contralateral side was preferred for mastication. The stressed animal may therefore have placed higher loads on control teeth, resulting in the observed resorption pattern.

TRAP reactivity appears to be a relatively late occurrence in the differentiation pathway and mononuclear cells reportedly stain just prior to multinucleation. Hence acid phosphatase production is apparently a late event in clast cell development in preparation for their involvement in the resorption process. This may account for the small number of TRAP-positive mononuclear cells in the frozen PDL adjacent to resorption lacunae containing multinucleated TRAP-positive cells. It is suggested that TRAP labelling as a cell marker would appear more reliable during the later stages of clast cell differentiation when maturation and resorptive capabilities had been acquired.

Takahashi *et al.* (1999), in a discussion of osteoclast differentiation and function, indicated that resistance to tartrate was acquired prior to fusion and multinucleation. This took place after OPGL/OPG interaction and the stimulation of NF-kappa β via a cJun N-terminal kinase pathway within the osteoclast (Collin-Osdoby *et al.*, 2001). The stimulation of NF-kappa β plays an important transcriptional role in the development of clast cell characteristics such as multinucleation, size increase and the appearance of a ruffled border (Ghosh *et al.*, 1998).

The present study confirms that TRAP staining occurs in mono- and multinucleated cells associated with hard tissue resorption lacunae. The finding that few mononuclear cells showed activity supports previous evidence that TRAP activity is a late characteristic in clast cell differentiation. It is further concluded that TRAP activity is less likely when bone deposition is occurring, such as in areas of hard tissue repair and ankylosis.

5.5.3 Immunolabelling studies

The immunolabelling process relies on the specific attraction of (manufactured) antibodies to cellular antigens (Beesley, 1993). The antigens (haptens) must be exposed and available (unmasked) for reaction and the antibody must be in an optimal concentration to prevent unnecessary and unwanted background stain (Mighell *et al.*, 1998). Experience and mastery of the complexities and subtleties of the technique overcomes many problems and limitations that often diminish the full potential of this powerful research tool. Mighell *et al.* (1998), and McNichol and Richmond (1998) reported that particular attention should be given to tissue fixation, antigen retrieval, appropriate controls, the considered interpretation of staining patterns and the correct selection of the primary antibody as factors governing success. However, any and all procedures in the tissue processing and labelling protocols have the potential to deny labelling success (Boenisch, 1989).

The immunolabelling protocols employed in the present study were identified as fastidious. Initial uncertain and equivocal results produced by a number of the antibodies placed doubt on the entire protocol. In order to test immunolabelling methods, AE1/AE3 antibody was used. AE1/AE3 is known to label the cytokeratin of epithelial cells (Tseng *et al.*, 1982) and to be revealed with a streptavidin-biotin peroxidase detection method (Leedham, 1990). The success of AE1/AE3 label (Figs. 78 and 79) provided confidence that, for at least this antibody, the described protocols were appropriate and suggested that the protocols might also be suitable for the other antibodies under consideration.

Despite these precautions, not all antibodies used to identify clast cells in the present study were successful even though past reports had indicated the likelihood of success. Anti-cathepsin L, anti-carbonic anhydrase II and anti-CD61 antibodies did not label their respective clast cell antigens even though all had been previously shown to label routinely-fixed human tissue (Athanasou, 1996). Results of CD13, CD15 and CD45 labelling provided equivocal results and no meaningful conclusions could be drawn.

5.5.3.1 Effects of tissue processing on antigenicity

- **Fixation**

A vital prerequisite of all histological and cytological techniques is the preservation of cells and tissues in as reproducible and life-like state as possible (Farmilo and Stead, 1989). Fixation prevents tissue autolysis by inhibiting lysosomal enzymes and further, prevents the growth of micro-organisms (Hopwood, 1969). In order to achieve this goal tissues are either immersed in a fixative fluid or, if rapid fixation is required, tissues are perfused via the animal's circulation. Because adequate fixation is essential and immersion of specimens in fixative solution depends on tissue permeability, thickness and time for success (Hopwood, 1969), perfusion fixation was selected for the present study.

Fixation of the rat's head and neck via carotid artery cannulation was challenging. Fixation was almost immediate once entry and injection into the artery was achieved; however, the time limit was short before vasospasm of the partially severed vessel made cannulation extremely difficult. Intracardiac

perfusion was a comparatively simpler procedure if the entire animal needed to be fixed, and was routinely employed as carotid cannulation became increasingly more demanding.

In performing their preserving and protective role, fixatives stabilize proteins by coagulation, by forming additive compounds, or by a combination of the two processes (Werner *et al.*, 2000). Following these reactions, conformational changes are likely in the structure of proteins which may cause the inactivation of enzymes. The resulting complexes may therefore differ from the unfixed proteins and the dilemma of fixation remains the necessity to introduce an artifact in order to produce a protective effect (Fox *et al.,* 1985; Fox and Benton, 1987). By so doing, the chemical and the antigenic nature of the tissues under examination may be altered (Berod *et al.*, 1981).

In addition to altering the chemical nature of the cells and tissues to which they are applied, fixatives also cause physical changes to cellular and extracellular elements (Hopwood, 1969; 1985). Viable cells are encased in an impermeable membrane; however, fixation breaks down this barrier and allows relatively large molecules to penetrate and escape (Beesley, 1993). Furthermore, the cytoplasm undergoes essentially a sol-gel transformation and forms a proteinaceous network sufficiently porous to allow further flow of large molecules. Importantly, different fixatives cause different levels of porosity that then have a bearing on immunochemical demonstration of all but the most superficial cellular antigens (Farmilo and Stead, 1989).

Tissue preservation for immunolabelling is therefore a compromise, as good fixation and adequate antibody penetration are often opposed to each other (Rainbow, 1994). However, many immunolabels have been designed for use in tissues that have been fixed with formaldehyde due to the widespread use of this fixative in pathology (Fox *et al.,* 1985; Athanasou, 1996; Mighell *et al.*, 1998). Also, much archival material is then available for examination. Formaldehyde fixes by reacting primarily with basic amino acids to form cross-linking methylene bridges (Helander, 2000). Subsequently there is relatively low permeability to macromolecules and the structures of intracytoplasmic proteins are not significantly altered (Werner *et al.*, 2000).

Routinely prepared human pathology specimens that used formaldehyde as the primary fixative have permitted an immunological examination of the antigenic profiles of osteoclasts (Athanasou *et al.,* 1986; 1991; Athanasou, 1996). Fixation in the current study employed Zamboni's solution (Zamboni and De Martino, 1967) which has paraformaldehyde as its base. According to Glauert (1975), paraformaldehyde is a weaker fixative compared with formaldehyde as it produces fewer cross-links and is less likely to mask tissue antigens in comparison with other aldehydes. However, formaldehyde is derived from paraformaldehyde which has therefore become a widely used immunohistochemical fixative (Robertson *et al.*, 1963; Fox and Benton, 1987).

Some antigens are not well demonstrated after formaldehyde fixation but may be revealed after enzyme digestion (MacIntyre, 2001). Proteolytic digestion

compensates for the impermeable nature of non-coagulant fixatives by "etching" the tissue and allowing hidden epitopes to be exposed (Cattoretti *et al.*, 1993). Trypsin cleaves amino acid chains adjacent to arginine and lysine and was the enzyme of choice for the present study because of its high reactivity with formaldehyde (Battifora and Kopinski, 1986). In addition, enzyme digestion of the tissues prior to immunolabelling was the retrieval technique recommended by the manufacturers of many of the antibodies employed.

The success of immunolabelling with monoclonal antibodies applied to formalin-fixed paraffin-embedded tissues depends on three considerations. It is imperative to know whether formaldehyde reacts with the epitope under investigation or whether it reacts with adjacent amino acids causing a conformational change. Thirdly, the temperature of histological processing in paraffin wax may destroy the epitope being examined (Mighell *et al.*, 1998). Failure in any of these areas may be one of a number of possible reasons why negative results were produced by the some of the antibodies employed in the current study.

- **Decalcification**

Decalcification involves the removal of calcium salts from specimens and in the present study EDTA was chosen for this purpose. EDTA acts via a chelation process that is reported to preserve tissue antigenicity even after long periods of decalcification (Mukai *et al.*, 1986). Matthews (1982) and Matthews and Mason (1984) indicated that neutral EDTA or 10% formic acid produced optimal immunostaining after trypsinization of sections. EDTA decalcification did not

interfere with cytokeratin immunolabelling from inner ear tissue (Tonnaer *et al.*, 1990), lymphoid cell immunolabelling from inflammatory tissue (Jonsson *et al.*, 1986) or neuropeptide staining in spinal cord tissue fixed with Zamboni's solution (Wakisaka *et al.*, 1986). Athanasou *et al.* (1987b) and Mullink *et al.* (1985) compared the usefulness of various decalcifying agents, excluding EDTA, prior to immunohistochemical staining. All decalcifying methods employed preserved antigenicity but some were better than others. However, Bjurholm *et al.* (1989) indicated that EDTA is preferable compared with other methods, particularly after fixation with formaldehyde and picric acid. It was therefore with confidence than EDTA was currently used for the gentle removal of calcium salts from dental and neighbouring bony hard tissues.

- **Tissue processing**

It has been reported that tissue processing has little effect on the immunoreactivity of most tissue antigens (Rainbow, 1994). Dehydration and clearing of the specimens leaves the tissues unaffected although Matthews (1981) reported that labelling intensity is increased with less background if chloroform instead of xylene is used as the clearing agent. Antigenicity has reportedly been reduced by embedding tissue in hot paraffin wax which forced Sheibani and Tubbs (1984) to recommend that high temperatures be avoided. Low melting point waxes were used in the present study but it is imperative that processing and embedding techniques are standardized and consistent. Automatic processors were employed whenever possible and reagents changed

regularly in order to achieve this aim. Once labelling success was realized, as depicted with AE1/AE3 and ED1 staining, protocols were then rigidly followed.

5.5.3.2 The avidin-biotin-peroxidase complex (ABC) method

An immunoperoxidase procedure for localizing a variety of tissue antigens was established by Nakane and Pierce (1967). Visualization of the peroxidase was achieved by the use of substrate to produce a coloured reaction product (Nakane, 1968). The immunolabelling method employed a biotinylated secondary antibody and an avidin-biotin enzyme complex and has therefore been termed the "ABC" technique. Because avidin has an extraordinarily high affinity for biotin, the binding of these two proteins is deemed to be irreversible (Hsu *et al.*, 1981). In addition, avidin has four binding sites for biotin and most proteins including enzymes may be conjugated with several molecules of biotin to produce macromolecules (Jackson and Blythe, 1993). The form and number of active enzyme molecules associated with the complex and the rapid, irreversible interaction of the complex with the biotinylated antibody is thought to account for the high sensitivity of the technique (Wilchek and Bayer, 1989). For this reason, as well as the method's efficiency and low background stain production, it was the immunolabelling protocol selected for the current study.

Successful labelling was identified as a positive brown diaminobenzidine (DAB) stain over tissues under examination. The DAB acted as a substrate for the horseradish peroxidase enzyme that was linked to the streptavidin (Boenisch, 1989). This was readily identified from a light counterstain of blue haematoxylin.

The procedural steps in the ABC technique involve the incubation of the section with the primary antibody raised against the antigen of interest. A biotin-labelled secondary antibody is added which has been raised against the first antibody. The avidin-biotin enzyme complex is then introduced which binds to the biotinylated secondary antibody. The final step localizes the tissue antigen by the incubation with a substrate (DAB) for the enzyme. To eliminate any non-specific adsorption of the primary antibody, a blocking procedure is considered necessary. This could take the form of dilute serum from the same species as the biotinylated secondary antibody but in the present study involved the use of hydrogen peroxide in methanol to block endogenous peroxidase. Endogenous peroxidases produce reaction products with DAB and routine blocking of these enzymes is recommended when using peroxidase detection methods (Fahimi, 1979; Strauss, 1979).

5.5.3.3 KP1 antibody

KP1 (CD68) was selected and currently used in an attempt to identify macrophages and osteoclasts in the experimentally frozen periodontium that had been fixed in formalin and embedded in paraffin. Dental tissues were not reactive with the antibody although liver, which was being used as a positive control tissue, labelled under a low antibody concentration (Figs. 41 and 42). Unfortunately, negative control liver tissue also labelled (Fig. 44) even when the primary antibody was omitted from the staining protocol. While it was considered that the anti-human KP1 was not reactive in the rat tissues, it was likely that the avidin-biotin complex detection technique labelled endogenous biotin found in

liver. Liver contains a high level of biotin (Van Noorden, 1993) and the biotinylated secondary antibody in the detection system produced disappointingly false labelling results. No anti-rat KP1 monoclonal antibody was commercially available and, consequently, this immunolabel was discarded as a viable and useful marker for rat macrophage/clast cell activity under the conditions of this study. It was concluded that KP1 antibody is inappropriate with the ABC technique, and that other detection systems need to be tried.

5.5.3.4 Anti-CD13 antibody

The anti-CD13 antibody, as a myeloid cell marker, reacts with a variety of cell types possibly explaining why the label present in the experimentally frozen PDL appeared widespread and poorly localized (Fig. 75). While it was intended that this antibody identify clast cells, the proximity of other types of cells within the PDL resulted in the poor and therefore unreliable specificity of the label.

Microwave antigen retrieval in citrate buffer failed to improve labelling even though it is recommended by Taylor *et al.* (1996). Koch *et al.* (1990) determined that positive staining was possible using methanol-Carnoy's fluid fixation with paraffin tissue embedding. Formalin-fixation and paraffin embedding are not suitable and provided a likely reason for the uncertainties of labelling. In addition, the anti-human CD13 antibody used in the present study is reported by the manufacturers as unreactive in rat tissues (Serotec, 1999). No positive information could be deduced regarding clast cells from the current results.

Therefore the usefulness of anti-CD13 antibody as a clast cell marker was diminished and considered unsuitable for routine clast cell identification.

5.5.3.5 Anti-CD15 antibody

The anti-CD15 antibody as an immunolabel is useful for the identification of promyelocytes, monocytes and mature granulocytes (Nakayama *et al.*, 2001). In the present study, weak staining was achieved in rat tissues even though the antibody was prepared against human tissues. An extremely light anti-CD15 immunolabel was evident in large cells that were in close relationship with resorption lacunae on root surfaces. Macrophages were strongly and positively labelled in human skin which was processed as a positive control tissue at the same time as experimental tissues were labelled. It would appear that there is some species cross-reactivity between human and rat CD15.

Cellular morphology under anti-CD15 immunolabelling was unclear and identification proved difficult. However, cells on hard tissue surfaces were large and of clast cell dimensions which provided weak morphological evidence relating monocytes, macrophages, clast cells and resorption. While the CD15 antigen was weakly labelled in cells on hard tissue surfaces (Fig. 76), so too were adjacent cells within the PDL. Labelled cells were most likely attributed to the influx of inflammatory cells as a result of the cold thermal stimulus. The reliability of anti-CD15 antibody as a marker for clast cells was therefore considered not absolute.

5.5.3.6 Anti-cathepsin L, anti-CD61, and anti-carbonic anhydrase II antibodies

Anti-cathepsin L, anti-CD61, and anti-carbonic anhydrase II antibodies failed to immunoreact with the dental resorbing tissues (Fig. 77). All had been designed against specific functional antigens related to clast cell activity. Anti-cathepsin L antibody was directed against a secreted enzyme reported to be essential for the dissolution of collagen and elastin at an acidic pH (Chapman *et al.*, 1997). Anti-CD61 antibody was intended to react against a cell adhesion molecule termed the β_3 integrin (Nesbitt *et al.*, 1993), while the carbonic anhydrase II antibody was directed at the intracellular, mitochondrial enzyme involved in the dissociation of carbonic acid and the production of H^+ ions (Gay and Mueller, 1974; Blair *et al.*, 1989; Lehenkari *et al.*, 1998). Because of their involvement with the physiology of the osteoclast these antibodies were selected as specific markers of this unique cell (Athanasou, 1996).

Tissue processing steps may have interfered with the antigenic profile of clast cells and rendered antigenic sites unavailable to these antibodies. Even with antigenic unmasking procedures involving either the microwaving of sections in citrate buffer or the trypsin digestion of tissues, the control and experimental tissued remained unreactive to the three antibodies. Since the labelling procedures had previously been shown to work in humans (Athanasou, 1991; 1996), it was possible that a lack of antibody species cross-reactivity played a potential role in the negative results. The anti-cathepsin L antibody was raised in goat against mouse antigens and although some species tolerance exists

between mouse and the rats used in the current study, labelling is not assured (Rainbow, 1994). Recently, however, Drake *et al.* (1996) reported that cathepsin K was more abundantly expressed in osteoclasts compared with cathepsins B, L or S and therefore might be a more suitable marker.

The available carbonic anhydrase II antibody employed in the present study was raised in mice against human antigens which could produce cross-species incompatibilty problems and explain the failure of the label (Boenisch, 1989).

The anti-CD61 antibody was raised in mouse against rat antigens and was considered the most likely to be successful. Despite using many different antibody titres, no labelling was seen and no specific reason could be advanced.

5.5.3.7 ED1 antibody

ED1 is one of a family of monoclonal antibodies that recognizes different but specific antigens on lysosomal membranes of mononuclear phagocytic cells (Damoiseaux *et al.*, 1994). These authors further reported that the immunolabel is strongly specific for macrophages rather than other cell types containing lysosomes. ED1 antibody was assessed as being a useful marker for the identification of cells from the monocyte/macrophage lineage provided that tissue antigenic properties remained unaltered throughout cell differentiation and also during the later histological processing of tissues. It was further considered that osteoclasts and odontoclasts, as more highly differentiated cells along the monocyte-phagocyte pathway, would also label with this antibody.

Results indicated that formalin-fixed paraffin-embedded rat tissues positively reacted with ED1 antibody after antigen unmasking using enzyme digestion of tissues with trypsin. Strong positive labelling, with minimal background stain, was found to occur in control (Fig. 60) and experimental molar periodontal tissues (Fig. 64) as well as spleen tissue as an immunological control (Fig. 51). In addition, immunological control tissue to which primary antibody was omitted in the labelling protocol, failed to react (Fig. 52) and hence the ED1 labelling was viewed with confidence.

The cytoplasm of large multinucleated cells was identified along resorptive bone and root surfaces using ED1 label (Figs. 67 and 69). In addition, heavily staining mononuclear cells were seen in the PDL apparently unassociated with bone surfaces (Fig. 64). The distribution of the positively labelled cells in the PDL was not uniform as clusters of cells gathered in areas of inflammation (Fig. 63). It was clear that the number of cells labelling with ED1 was high in the PDL and neighbouring hard tissues indicating that cytoplasmic lysosomal content in these cells was also high. This confirms the results of (Lucht, 1972b) and others who have shown ultrastructurally that clast cells possess a high vacuole and lysosomal content.

The pattern of ED1 label indicated that macrophage-related cells were widely distributed throughout the experimentally-frozen periodontium and surrounding alveolar bone. Mononuclear cells were located within the PDL, while larger, positively-staining multinuclear cells were found adjacent to hard tissue surfaces

undergoing resorption. In addition, the intense labelling of cells in the marrow spaces (Fig. 71) strongly suggested a haemopoietic association between these and the large hard tissue-related cells in resorption zones. It is possible, and even likely, that the clastic cells resorbing bone and tooth originated from the same source. Proof that osteoclasts and odontoclasts are the same cells has always resided in their similar morphological appearances (Jones and Boyde, 1988) and functional attributes (Lucht, 1972a,b) as well as their positive tartrate-resistant acid phosphatase staining (Andersson and Marks, 1989). The positive ED1 staining in the periodontal tissues indicated that haemopoietic cells, osteoclasts and odontoclasts possessed the ED1 antigen thereby accentuating the strong, positive link between these cell types, and further supporting the concept that they emanate from the same differentiation pathway.

Vandevska-Radunovic *et al.* (1997) evaluated the number and distribution of immunocompetent cells in the normal and orthodontically-affected periodontium. Examination of serial cryostat sections to which a variety of monoclonal antibodies directed against macrophages, dendritic cells and other cell members of the immune system had been applied revealed that experimental tooth movement attracted cells of the mononuclear phagocyte system rather than other immunological cell types. An additional study by Jäger *et al.* (1993), utilizing ED1 and ED2 on the periodontium of orthodontically-moved teeth, revealed a positive reaction to the ED1 antibody on both test and control specimens with macrophages, osteoclasts and odontoclasts identified in the stressed PDL.

ED1 has also been used to examine the pathogenesis of rat periapical lesions. By using immunohistochemical techniques, Akamine *et al.* (1994a,b) investigated the localization of macrophages and plasma cells around infection-induced periapical lesions and reported that macrophages had a close relationship with bone destruction. This association was also indicated by Sminia and Dijkstra (1986) following an examination of mononuclear phagocytes in embryonic rat bone. Furthermore, hard tissue resorption was shown to be directly mediated by inflammatory cells (Sabokbar *et al.*, 1997) based on the release of osteolytic cytokines by heavy macrophage response to biomechanical wear in cases of failed hip replacements. The ED1 labelling of mononuclear cells responding to cold thermal necrosis of the cervical and interradicular PDL implicates macrophages and provides further evidence of their intimate association in the processes of bone resorption and remodelling or at least in the associated inflammatory mechanisms.

Although ED1 identified mononuclear inflammatory cells in the present study, it had been previously shown that there are cytochemically distinct macrophage populations related to the state of inflammation induced (Beelen and Walker, 1983). Beelen *et al.* (1987) further reported that ED1 is a general rat macrophage marker but that other ED labels exist for specific types of macrophages related to other tissues. Since ED1 is a lysosomal antibody, it is therefore possible that specific macrophages, with varying functions depending on their lysosomal activity and content, are attracted to the dental resorption site.

Additionally, it has been suggested that marrow derived giant macrophage polykaryons migrate to nearby hard tissue surfaces to initiate resorption (Stutzmann *et al.*, 1980). Moreover, the large number of immunopositive mononuclear cells identified within the periodontal ligament might continue to differentiate into large multinuclear clastic cells for the same purpose (Sabokbar *et al.*, 1997). Although both mechanisms for clast cell derivation within the periodontal ligament are possible, more highly specific differentiation labels would be required in order to provide conclusive evidence.

The high number of ED1-labelled cells suggested that it is likely that labelling occurs early in the differentiation pathway and persists until multinucleation and resorption activity commences. It has been considered that ED1 is suitable for the study of PDL remodelling because it demonstrates a clear positive reaction with macrophages, osteoclasts and odontoclasts (Jäger *et al.,* 1993). TRAP labelling occurring later in the differentiation cycle and involving mono- and multi-nucleated cells was promoted as a sign of maturity (Oddie *et al.,* 2000) and governed by the secretion of RANKL and M-CSF (Rani and MacDougall, 2000). Succinic dehydrogenase reactivity appears to be a later event which involves large cells actively engaged in resorption and attached to hard tissue surfaces. A schematic representation of the likely sites of label influence appears in Diagram 6 below.

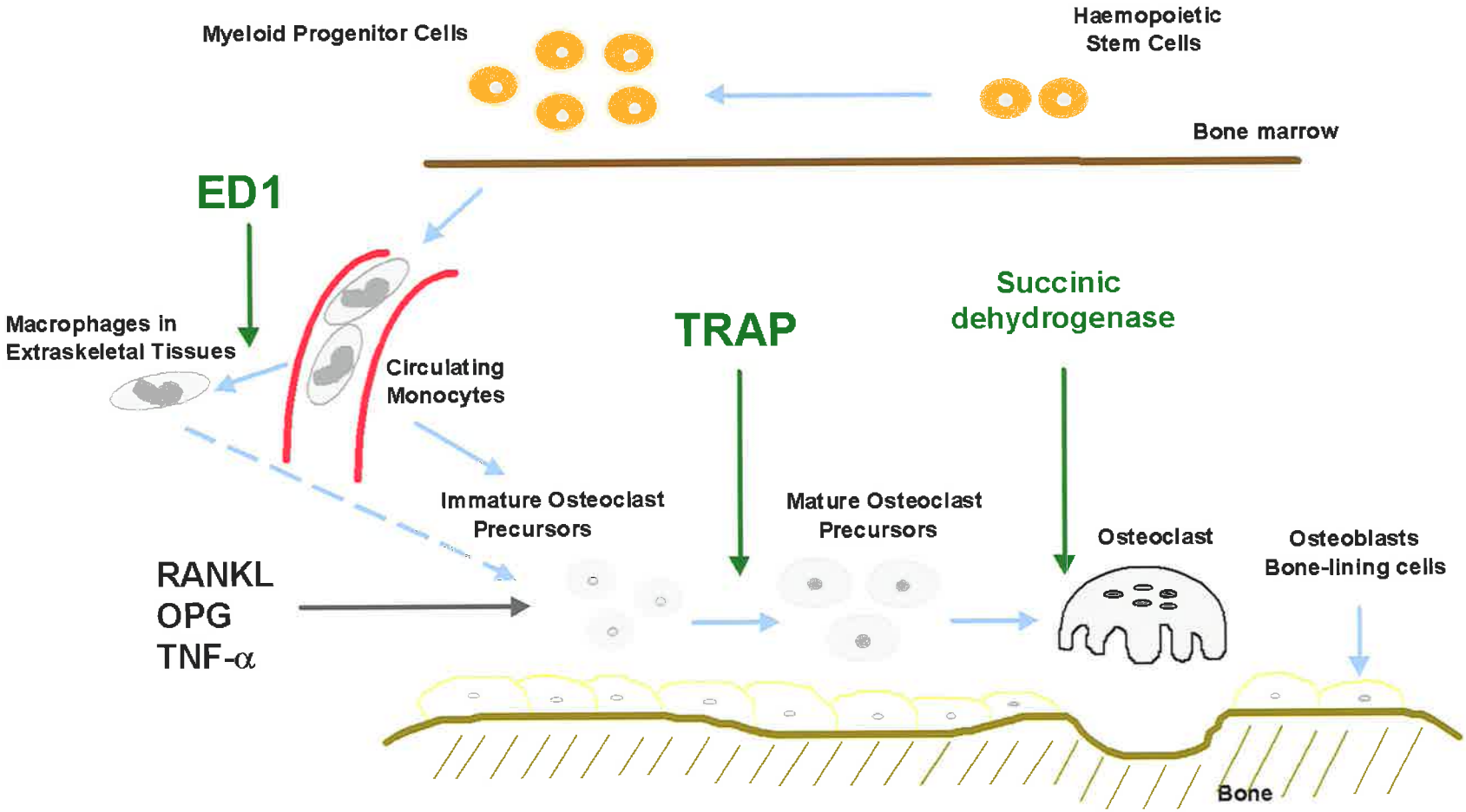


Diagram 6. Schematic representation of osteoclast development from haemopoietic stem cell origin to the fully functioning cell. ED1 immunolabel, TRAP staining and succinic dehydrogenase staining are suggested to occur at different stages in the differentiation pathway. (Adapted from Athanasou, 1996).

5.5.4 Are osteoclasts and odontoclasts the same cells?

It is generally accepted that both odontoclasts and osteoclasts are similar cells and are derived from the same mononuclear progenitor haemopoietic stem cells (Chambers, 1980; 1988; 2000; Mundy and Roodman, 1987; Quinn *et al.*, 1996). However, controversy over the identity of these cells has reigned for some time with the consensus being that they are the same (Jones and Boyde, 1988). Their names are derived from the substrate to which they are associated and the biochemical make-up of their associated hard tissues might realize a possible difference between the two cell types. Organic matrix and inorganic calcium salt content differs between tooth and bone and may promote a functional dissimilarity between the two cells (Slavkin, 1988; Sodek and McKee, 2000,).

Odontoclasts have been reported to be insensitive to the effects of parathyroid hormone whereas osteoclasts are stimulated into resorbing bone (Freilich, 1971). Takahashi *et al.* (1988) developed a mouse marrow culture model that induced mononuclear precursors to differentiate into odontoclast- and/or osteoclast-like multinucleated cells that resorbed hard tissue. Cell differentiation was facilitated by the addition of calcium-regulating hormones (parathyroid hormone, 1,25,dihydroxyvitamin D_3) into the culture media which attempted to simulate physiological mechanisms of calcium homeostasis.

Sasaki *et al.* (1988a,b) showed that odontoclasts developed a ruffled border only when they were in direct contact with mineralized tooth surfaces *in vivo* and *in vitro*. After multinucleation, ruffled borders were not seen in cells out of contact

with root surface. To a degree, this finding was supported by the results of the present study. Many multinucleated cells were in evidence near root surfaces. These cells often presented a polarized cytoplasmic appearance as well as clear areas (Figs. 97 and 98). However, other multinucleated cells were clearly associated with resorption lacunae on root surfaces but showed no signs of a ruffled border (Fig. 100). This finding may be explained by a timing sequence in which ruffled borders had not yet been able to form but does not explain the fact that the detection of odontoclast ruffled borders ultrastructurally was the exception rather than the rule.

Sahara (1998) indicated that odontoclasts, in distinction to osteoclasts, became multinucleated after adherence to tooth surfaces. Their studies of exfoliating deciduous teeth revealed a different pattern of morphologic change compared with osteoclast differentiation. Although Baron *et al.* (1986) reported otherwise, most osteoclast research indicates that these cells became multinucleated prior to attachment to bone surfaces (Väänänen, 1996; Roodman, 1999) after which ruffled border formation occurs. Sahara *et al.* (1996) indicated that odontoclasts behave differently and undergo morphologic changes after hard tissue attachment.

This view could not be supported by the findings of the current study. Multinucleation was a feature of the unattached cells in the vicinity of root and alveolar bone surfaces of thermally-insulted molars. This observation invites speculation as to the origin and identity of these cells. One concept suggests that

all cells in the inflammatory area might be osteoclastic in nature, and that they arise from vital haemopoietic tissue in bone marrow spaces. Supportive evidence for this theory is the high number of multinucleated cells found within the bone marrow spaces adjacent to the zone of inflammation. These cells may have migrated via the vascular route of reparatory blood vessels into the hyalinized tissue preparatory to the initiation of resorption. TRAP label revealed staining in marrow spaces and along both hard tissue surfaces (Fig. 32) while ED1 label was heavy in the three areas (Figs. 69). If the source of the multinucleated cells located in the PDL is of bone marrow tissue origin, there would be no expected differences between the cell types. The multinucleated cells associated with both bone and tooth would therefore be similar and capable of resorbing both tissues, and variations might be explained by substrate composition.

Examination of the ultrastructure of the cells resorbing hard tissues in the frozen-tooth model indicated that few attain all of the morphological features characteristic of the fully-functioning clast cell. In none of the sections and electronmicrographs examined was there any evidence of a ruffled border on the mono- and multi- nucleated cells associated with the resorption lacunae. Ruffled borders are considered a plasma membrane specialization associated with active clast cells (Lucht, 1972a). Clear zones are also found in active clast cells but are also reported to exist in macrophages in contact with bone or cartilage (Rifkin *et al.*, 1979). As clear zones are believed to be an attachment mechanism they are not unique to a specific cell type. According to Rivkin *et al.* (1979), a correlation between macrophage-mediated bone resorption and the

ultrastructural appearance of macrophages showed that these cells apparently engulfed mineralized matrix without the assistance of a ruffled border thus providing evidence supporting non-clast cell mediated resorption of calcified tissue.

The concept that macrophages are involved in bone resorption is not new. Mundy *et al.* (1977), Kahn *et al.* (1978) and Mundy (1983) provided evidence that linked macrophages with the release of bone mineral and matrix from long bones of foetal rats *in vitro.* These effects were inhibited by cortisol as an anti-inflammatory agent, but were not altered by hormones that normally stimulate osteoclastic bone resorption. In addition, macrophages may be important participants in alveolar bone remodelling by digesting released matrix molecules (Dorey and Bick, 1977a,b). It was further proposed that hard tissue resorption requires two different types of cells (Heersche, 1978). The osteoclast was reportedly responsible for the removal of bone mineral while a mononuclear cell, presumed to be a macrophage, was responsible for the removal of bone matrix. In addition, macrophages were identified in apposition to resorbing alveolar bone in experimental periodontitis (Rifkin and Heijl, 1979) suggesting that these mononuclear cells complimented the action of osteoclasts by ingesting and degrading matrix molecules. However, no evidence of morphologic differentiation of the macrophages into osteoclasts during bone resorption was provided.

Evidence supporting the differentiation of macrophages into osteoclastic bone resorbing cells was provided by Sabokbar *et al.* (1997), and Chun *et al.* (1999)

who examined arthroplasty-derived macrophages with TRAP and antibody labelling. The failure of joint replacements was attributed to osteoclastic change in macrophages attracted to the site by foreign body wear particles from the prosthesis. In contrast with other macrophage populations M-CSF was not required for the osteoclastic change to occur but the resulting osteolysis caused prosthesis failure. This study highlighted the fact that areas of pathology adjacent to bone have the potential to cause osteolysis due to an osteoclastic change in the macrophages involved.

Brudvik and Rygh (1993a) were the first to indicate that macrophages were involved in the resorption of teeth undergoing orthodontic movement. In their study, removal of the area of PDL hyalinization by peripheral macrophage attack preceded root resorption. In addition, macrophages have been reported as major constituents of periapical lesions (Metzger, 2000; Lin *et al.,* 2000)) and a likely source of bone-resorbing mediators. Pulp exposures in rat molars produced periapical granulomas and the presence of ED1-positive macrophages. Macrophages also produced transforming growth factor β-1, which is known to be active in bone resorption (Bonewald and Mundy, 1990). The possibility of macrophages being involved or responsible for dental resorption was overlooked by Metzger (2000) and Lin *et al.* (2000), but suggested by the results of the present study. Multinucleation and cytoplasmic polarization were evident without the formation of a ruffled border and other expected characteristics of hard tissue resorbing cells.

Identifying markers indicated that cells resorbing cementum are ED1 positive, TRAP positive and possibly positive for succinic dehydrogenase activity. The light microscopic appearance painted by their multinucleated nature and their close association with the root surface creates the impression of an active clast cell. While these cells are apparently capable of removing hard dental tissue they do not represent the classical picture of an odontoclast described by Sahara *et al.* (1994) and Sahara *et al.* (1996) in which ruffled borders and multinucleation were features of cells resorbing deciduous tooth roots.

Whereas Brudvik and Rygh (1993a) contended that macrophages are important cells in the initiation of root resorption, the current study has supported this premise and extended it to suggest that multinucleated macrophages may be directly involved in the removal of dental hard tissue. This raises the question regarding the origin of the dental resorbing cells. If these macrophage-like dental resorbing cells continue to differentiate into clast cells with ruffled borders, their differentiation pathway may be different to that followed by clast cells derived under physiological conditions involved in bone and calcium maintenance.

Clast cells have been identified as originating from haemopoietic stem cells along a monocyte-phagocyte differentiation pathway under the influence of numerous cytokines and growth factors (Burger *et al.,* 1984; Horton *et al.,* 1984; 1985c; Chambers, 2000). It has further been suggested that clast cells arise from a macrophage lineage and show macrophage and clast cell characteristics (Athanasou *et al.,* 1986; Athanasou and Quinn, 1990; Takeshita *et al.* 2000).

Growth and stimulating factors derived from pathological conditions are considered responsible for the clast cell change in macrophages in a number of medical conditions including failed arthroplasties and tumours (Sabokbar *et al.*, 1997; Quinn *et al.,* 1998a,b; Itonaga *et al.,* 2000; Haynes *et al.*, 2001).

The present study has shown that the cells involved in dental resorption are possibly more related to macrophages or at least differentiate along a macrophage pathway. It appears that the PDL pathology created by the thermal challenge attracted ED1-labelled macrophages to the area of inflammation and that differentiation of these cells continued until TRAP reactivity was attained and succinic dehydrogenase staining reflected a functioning intracellular Krebs cycle.

It is, therefore, hypothesized that odontoclasts actively engaged in the resorption of permanent tooth root structure differentiate from a secondary macrophage pathway rather than from a primary pathway involving monocytes/phagocytes. They arise as a response to pathological conditions around the tooth root (necrotic tissue) rather than under normal physiological circumstances (excluding deciduous root resorption). Normal skeletal physiology does not involve teeth as part of the calcium homeostatic mechanism and so the calcium regulating hormones have been shown to have little effect. Recent evidence suggests that OPG is more likely to have an inhibitory effect on clast cells under physiologic conditions rather than pathologic states (Simonet *et al.*, 1997; Aubin and Bonnelye, 2000). The effects of OPG in pathologic situations such as arthritis appear equivocal and inconclusive possibly due to the involvement of different

cell populations (Gravallese *et al.,* 1998) or to the interference of other inflammatory mediators (Atkins *et al.,* 2000; Fazzalari *et al.,* 2001). The difference between odontoclasts and osteoclasts may therefore be in their respective cells of origin and the environment in which they differentiate and function.

5.6 Osteoprotegerin (OPG)

As a new member of the tumour necrosis factor receptor family, OPG is reported to play a key role in the physiological regulation of osteoclastic bone resorption (Simonet *et al.*, 1997). This protein, produced by osteoblasts and other stromal cells, has no signalling capability but acts as a secreted decoy receptor by binding to its natural ligand (RANKL). This binding prevents RANKL from activating its cognate receptor RANK, which is an osteoclast receptor vital for clast cell differentiation, activation and survival (Collin-Osdoby *et al.*, 2001). The secretion of OPG and RANKL from osteoblasts and stromal cells is regulated by numerous hormones and cytokines to levels that govern bone homeostasis. However, excess RANKL increases bone resorption whereas excess OPG inhibits resorption by blocking the effects of factors that stimulate clast cell differentiation. Kosteniuk and Shalhoub (2001) considered that since OPG also inhibits bone resorption in a variety of animal disease models, it might be an effective therapeutic agent for diseases associated with excessive clast cell activity.

It was in this inhibitory capacity that OPG was expected to act in the dental model of resorption produced by the cold thermal stimulus. The administration of OPG at a dose of 2.5 mg/kg was predicted to prevent clast cell activity and retard or prevent resorption of dental and surrounding bony tissues. It was therefore unexpected to observe cells exhibiting positive TRAP and ED1 staining and electron microscopically presenting with ruffled borders in intimate contact with resorption lacunae (Figs. 173 and 174). It was even more surprising to see

numbers of clast cells apparently competing for access to resorbing sites (Fig. 176). TRAP activity was noted at the cervical region of the experimentally frozen teeth (Fig. 145) while ED1 labelling also occurred in the surrounding PDL (Fig. 154).

Initial communication with Amgen Inc. ascertained that a dose of 2.5 mg/kg/d would be sufficient to provide profound inhibition of osteoclastic activity in rodents. This was based on a study by Morony *et al.* (1999) who challenged mice with various cytokines and hormones that are known to stimulate bone resorption. Affected mice became hypercalcaemic in 3 d and showed elevated osteoclast numbers in 5 d. However, mice also injected with OPG at a dose of 2.5 mg/kg/d showed no hypercalcaemia and maintained osteoclast numbers within a normal range. The OPG negated the effects of the administered bone-resorption cytokines but did not prevent resorptive cell differentiation entirely. Previously, Simonet *et al.* (1997) administered a dose of 10 mg/kg/d for 7 d in mice and reported a 3-fold increase in bone mass, while a dose of 5 mg/kg/d for 14 d completely prevented ovariectomy-induced bone loss in rats. Additionally, Yasuda *et al.* (1998a) noted a 1.8-fold increase in bone mass in rats after OPG administration at a dose of 24 mg/kg/d. Importantly, OPG was shown to inhibit increased bone resorption during normal linear bone growth as well as after challenges with interleukin-1β, tumour necrosis factor-α, parathyroid hormone, parathyroid hormone-related peptide and vitamin D_3 (Dunstan *et al.*, 1997; Moroney *et al.*, 1999).

A single 3 mg/kg dose of OPG was administered to postmenopausal women in order to biochemically determine the effects on bone resorption in this susceptible group (Bekker *et al.*, 2001). The urinary secretion of collagen breakdown product, N-telopeptide, was found to be markedly reduced within 12 hours of administration and still low after 6 wk. Bekker *et al.* (2001) concluded that a single injection of OPG was effective in rapidly and profoundly reducing bone turnover for a sustained period.

A study by Shalhoub *et al.* (1999) determined that OPG, used at a concentration of 5-250 ng/ml, antagonized the effects of RANKL on the morphology of osteoclast-like cells in experimental culture conditions. Simonet *et al.* (1997) further showed that *in vitro* osteoclast differentiation from precursor cells was blocked by OPG in a dose-dependent manner. Later research revealed that OPG at low concentrations had the ability to interfere with the physiological development and function of osteoclasts (Hofbauer *et al.*, 2000). Because all previous studies supported the inhibition of osteoclastogenesis at varying dosages of OPG, it was expected that the concentration of 2.5 mg/kg on alternate days as delivered by the protocol of the present study would be adequate to prevent clast cell differentiation and hence resorption.

The results of the current study indicated that OPG at a concentration of 2.5 mg/kg was effective in stopping clast cell activity. This was revealed by a lack of resorption activity in unfrozen control specimens (Fig. 125), coupled with the lack of TRAP reactivity (Fig.137) and the minimal ED1 response (Figs. 149 and 150)

in those animals. In addition, ultrastructurally, no morphological sign of clast cell activity was seen in the periodontium of unfrozen teeth (Figs. 159 and 161), and particularly in alveolar bone (Figs. 166 and 168), of OPG-affected rats. More specifically, no osteoclastic activity was evident on endosseal surfaces of long bones. Bone surfaces appeared to be covered by osteoblast-like cells or bone lining cells and were therefore regarded as appositional in nature.

Despite these observations, the present results indicated that OPG failed to inhibit clast cell activity in the localized periodontal structures affected by the experimental freezing of the rat molars (Figs. 173 and 179). As this was unpredicted and unexpected, a number of issues were raised.

While the dilutions and doses of the provided samples of OPG were routinely checked at the time of application, it may be that alternate day administration was insufficient for inhibition of the inflammatory stimulus created by the molar freeze. Even though there appeared to be long term benefits from OPG administration in the human (Bekker *et al.*, 2001), this might not apply to other animal species.

Lee and Lorenzo (1999) showed that hormones and inflammatory cytokines have an effect on the function of RANKL and OPG. In particular, parathyroid hormone stimulates RANKL and clast cell formation in a dose dependent manner, while parathyroid hormone effect on OPG is inhibitory after a short initial phase of stimulation. Similarly, Sakata *et al.* (1999) reported that IL-1β and TNF-α affect

OPG activity in cultures of periodontal mesenchyme which suggests that the local production of OPG regulates the resorption of dental hard tissues. Importantly, Collin-Osdoby *et al.* (2001) demonstrated that pro-inflammatory cytokines, TNF-α and IL-1α, induce increased levels of RANKL and OPG *m*RNA in vascular endothelial cells according to different expression profiles. TNF-α and IL-1α were shown to promote the development and activation of bone resorbing osteoclasts that form in cultures with bone marrow-derived stromal cells. Furthermore, they were also shown to be potent mediators of bone resorption (Sabatini *et al.*, 1988; Bertolini *et al.*, 1986; Dunstan *et al.*, 1997) and have the potential to influence resorption in areas of inflammation.

5.6.1 Tumour necrosis factor

Tumour necrosis factor alpha (TNF-α) is synthesized by monocytes and macrophages as cells of the haemopoietic lineage (Shalaby *et al.*, 1985). Formally known as cachectin (Carswell, 1975), TNF-α is elevated in ischaemic conditions (Yao *et al.*, 1995) and is also a known regulator of bone resorption, particularly in association with inflammatory processes such as periodontal disease (Quintero *et al.*, 1995), dental periapical lesions (Tani-Ishii *et al.*, 1995) and rheumatoid arthritis (Shiozawa and Kuroki, 1994). Furthermore, TNF-α has been associated with postmenopausal osteoporosis (Kitazawa *et al.*, 1994).

Evidence implicating TNF-α in clast cell activity has recently been reported by Chambers (2000), Zernecke *et al.* (2001), Hatano and Brenner (2001), Komine *et al.* (2001) and Kamata *et al.* (2001) who associated this cytokine with the

transcription factor NF-kappa β. Haynes *et al.* (2001) showed that OPG and RANKL also regulate osteoclast formation via the receptor activator of NF-kappa β located on osteoclast precursors. In addition, Kon *et al.* (2001) reported that OPG and RANKL as well as the pro-inflammatory cytokines, TNF-α and IL-1α were crucial in the early and late phases of fracture healing and acted by stimulating NF-kappa β. It is therefore recognized that NF-kappa β stimulation may occur from a variety of sources all of which lead to clast cell activity. It is possible that an alternative activation of NF-kappa β by an inflammatory cytokine may have occurred in the present study to generate the observed osteoclastic response to the cold thermal stimulus.

As TNF-α has been identified in inflammatory conditions, it is likely that it was present as a by-product of the inflammatory process created by the experimental freezing of the rat molar. The local concentration of TNF-α may have exceeded the inhibitory concentration of OPG administered to the rats and therefore stimulation of clast cell precursors into active clast cells was possible. Evidence supporting this concept has been recently provided by Romas *et al.* (2002) who indicated that TNF-α acted directly to stimulate macrophages and myeloid progenitor cells as well as promoting the differentiation of osteoclasts in rheumatoid arthritis sufferers. Acting in concert with RANKL, TNF-α and IL-1 powerfully promoted osteoclast recruitment, activation and osteolysis and it was considered that modulation of the RANKL/OPG equilibrium provides an answer for the control of this debilitating disease. The potent activation of osteoclasts by

TNF-α was also reported by Fuller *et al.* (2002) who believe that this cytokine is pivotal for the pathogenesis of inflammatory osteolysis. Importantly, Fuller *et al.* (2002) determined that the activation of TNF-α is unaffected by OPG and so bone resorption is considered directly and potently stimulated by TNF-α in a RANKL-independent manner. Previously, Suda *et al.* (2001) were able to show that TNF-α stimulates osteoclast differentiation through a mechanism independent of the RANKL–RANK interaction which therefore suggests that TNF-α plays an important role in pathological resorption due to inflammation.

The release of TNF-α and other cytokines during the inflammatory process (Kacena *et al.*, 2001; Urra *et al.*, 2001) to affect bone resorption and formation, has caused TNF-α to be linked with orthodontic tooth movement (Uematsu *et al.*, 1996). Vitouladitis *et al.* (2000) identified paradental cells containing TNF-α and examined the short- and long-term effects of orthodontic forces on these cells. TNF-α was unexpectedly found in sites of PDL compression and tension where it was hypothesized to perform different, but unidentified, functions under the influence of force-induced tissue remodelling.

The argument that TNF-α, in inflammatory areas, induces clast cell activity is countered by the fact that additional cytokines are released. Inflammatory cytokines such as GM-CSF, IL-4 and IFN-γ are also released and these have been shown to have an inhibitory effect on clast cells (Gowan *et al.*, 1986; Hattersley and Chambers, 1990; Lacey *et al.*, 1995; Udagawa *et al.*, 1997).

Moreover, TGF-β, as another anti-inflammatory cytokine, has been revealed to deactivate or negate the effects of TNF-α (Letterio and Roberts, 1998).

It is conceivable that the effects of the TNF-α or other inflammatory mediators released by the cellular destruction caused by the freezing process have exceeded the ability of OPG to inhibit clast cell differentiation and function. This is likely to be a local effect as the results of the present study and others (Simonet *et al.*, 1997; Hsu *et al.*, 1999) have shown that elsewhere in the skeleton resorption and osteoclastic activity are inhibited. Recently, TNF-α-mediated joint destruction in transgenic mice suffering arthritis was inhibited by OPG which reduced the number of bone-resorbing cells but did not eliminate them entirely (Redlich *et al.,* 2002). OPG effects were enhanced in combination with the inhibitory resorption effects of bisphosphonates. A new anti-TNF antibody has been identified against rat TNF (Urra *et al.*, 2001; Gawad *et al.*, 2001) and cycloprodigiosin hydrochloride identified as a TNF-α suppressant (Kamata *et al.,* 2001) which provide opportunities for TNF-α involvement in aseptic inflammation to be examined as a fruitful line of continuing research.

5.6.2 Role of RANKL

The lack of OPG inhibition in the frozen molar PDL model of the present study may be due an increase of RANKL expression in the affected area (Gori *et al.,* 2001). Whereas RANKL is produced by osteoblasts and other stromal cells (Simonet *et al.*, 1997) and acts in a competitive way against OPG for binding to

the RANK receptor, an elevated concentration of RANKL may be responsible for the inhibitory effects of the OPG.

In addition, the ankylotic repair evident in the experimentally frozen rat molars is suggestive of a high level of stromal cell, osteoblastic and fibroblastic activity. Accordingly, it is likely that high local levels of RANKL are present (Udagawa *et al.*, 2000) which may act as an osteoclast stimulant and consequently initiate hard tissue resorption as a component of repair remodelling. RANKL, either as a result of the inflammatory process or of the repair process, may be present in a concentration sufficient to negate the inhibitory effects of OPG.

The osseous repair processes have also been shown to express TNF-α and IL-1α (Kon *et al.*, 2001) which are important cytokines in the initiation of repair whilst also playing important functional roles in bone formation and remodelling. Although TNF-α and IL-1α are primarily located in macrophages and inflammatory cells during the early stages of inflammation, in later repair processes, they have been identified in blast cells (Kon *et al.*, 2001). The hypothesized presence of RANKL in addition to TNF-α and IL-1α may provide reasons for the resorption activity evident in the frozen molar PDL.

5.6.3 Penetration of OPG

Because the effects of the cold thermal stimulus caused a local area of hyalinization and necrosis which initiated the resulting inflammatory response, it is conceivable that the OPG was unable to penetrate the necrotic and

inflammatory area due to the size of the molecule. Simonet *et al.* (1997) indicated that OPG is a large molecule of the order of 90 kDa. Molecular size has been shown to be important in facilitating tissue permeability (Lacey *et al.*, 1998) and a large molecule may be hampered in its penetration through the inflammatory site unless wide tissue channels are present (Cooper *et al.*, 1990). Moreover, frozen tissue necrosis would also cause a time delay by delaying the ingress of reparative cells and blood vessels.

While the inflammatory area might create permeability and therefore access problems for large molecules, it is hypothesized that the change of tissue biochemistry and the release of many inflammatory products may moderate the efficacy of OPG. Even though some studies have reported the inhibition of bone resorption by OPG in areas of inflammatory challenge (Atkins *et al.,* 2001; Haynes *et al.,* 2001), there have been species and dose related differences in the experimental protocols. It is concluded that the precise nature of OPG effects in inflammation is an area that requires future assessment and research.

5.7 Future research directions

The present study has generated many additional questions regarding the identification and activity of clast cells and their precursors. While the immunolocalization of clast cells initially produced disappointing results, the commercial availability of additional anti-rat antibodies against CD15, CD45 and the cathepsins since the completion of this study provides further expectation of success. The employment of a different immunolabel detection system may also prove fruitful. Furthermore, enhanced detection may also facilitate histomorphometric quantitation which was not possible in the current study.

The hypothesis that TNF-α has a role to play in the activation of clast cells in areas of inflammation may be assessed by the administration of granulocyte-macrophage colony-stimulating factor. GM-CSF has recently be shown to inhibit TNF-α in culture systems (Miyamoto *et al.*, 2001). By reducing the expression of transcription factors c-fos and Fra-1, GM-CSF and IL-3 have been shown to inhibit osteoclastogenesis leading Miyamoto *et al.* (2001) to conclude that GM-CSF treatment functions through the regulation of c-fos expression. The addition of GM-CSF to the frozen tooth resorption model may arrest the resorption seen in areas of inflammation.

In addition, TNF-α has been shown by Kaji *et al.* (2001) to require tumor necrosis factor receptor-associated factor 6 in order to stimulate RANK signalling for osteoclasts. The removal of this factor by the use of TRAF6 deficient mice, may also prevent osteoclastogenesis and resorption in the frozen PDL.

To date no studies have been published regarding the orthodontic use of OPG. As an inhibitor of osteoclastogenesis and hence hard tissue resorption, OPG might be predicted to arrest orthodontic tooth movement. However, since orthodontic treatment invokes an inflammatory response within the PDL and therefore a change in local tissue biochemistry, the inhibitory effects of OPG are not assured. Should OPG inhibit force-induced tooth movement an orthodontic anchorage mechanism might be produced to facilitate treatment. Successful OPG-generated anchorage would only be possible if administration could be applied locally rather than systemically. The local chemical mediation of tooth movement via the administration of bisphosphonate (Igarashi *et al.,* 1994; Sato *et al.*, 2000) and prostaglandin (Kawaguchi *et al.,* 1995; Boekenoogen *et al.,* 1996; Quinn et al., 1997) have been reported with mixed results and have not found widespread use. The local delivery of OPG is currently not possible and while its effects remain systemic, its use would be inappropriate in young growing patients. Nevertheless, continuing research into the effects of OPG on the response of rat molars to an orthodontic force is in progress.

CHAPTER 6

CONCLUSIONS

Major conclusions of the current study were:

1. A simple, reproducible and reliable method for producing aseptic root resorption in rats resulted following the application of a single 10 min administration of dry ice to the molar crown surface. Furthermore, consistent ankylosis resulted with longer and multiple freezing times.

2. Of the experimental time periods examined by this study, root resorption was at its maximum 7 days after molar freezing and was principally located in the interradicular and cervical regions of the tooth. At 14 days after freezing, repair of the resorption lacunae was taking place, and this was completed by 28 days.

3. ED1, as a macrophage lysosomal marker, proved to be the most reliable immunolabel for both mononuclear cells within the PDL and clast cells on hard tissue surfaces. Clear labelling of multinucleated clast cells indicated a functional relationship between the macrophages involved in the inflammatory response in the PDL and the clast cells which were actively resorbing hard tissue.

4. Electron microscopy of the frozen PDL indicated that clast cells were only observed infrequently in the resorbing hard tissue cavities. No ruffled

borders were seen on resorbing cells which had a multinucleated and often polarized appearance. It was concluded that these cells might have been macrophages capable of hard tissue resorption or osteoclasts that had not reached full operational/activation status. Confirmation of the identity of these cells was provided by their TRAP and ED1 labelling.

5. Macrophages are important cells in the initiation of root resorption. The current study has supported this premise and extended it to suggest that multinucleated macrophages may be directly involved in the removal of dental hard tissue.

6. Osteoprotegerin at a dose of 2.5 mg/kg of body weight effectively prevented clast cell activity and hence resorption under physiological circumstances. In both the skeleton (long bones) and periodontium of contralateral unfrozen molars, OPG appears to be an effective pharmacological agent that inhibits osteoclastogenesis, and, therefore, hard tissue resorption.

7. At the 2.5 mg/kg dosage, OPG failed to prevent hard tissue resorption in the local area of aseptic inflammation in the PDL of molars challenged by the cold thermal stimulus. Clast cells with well-formed ruffled borders were actively and competitively resorbing tooth root and bone surfaces.

8. These results indicate that inflammatory cytokine pathways distinct from those initiated by OPG may also be operative in osteoclastogenesis. Alternatively, the effects of OPG were possibly dose related.

9. The use of OPG as a moderator of orthodontic tooth movement is unlikely to be successful due to the vagaries of its activity in inflammatory states. The current inability to administer the systemically-acting OPG at a local level make its orthodontic application uncertain but research efforts are being directed to this area.

Other conclusions included the following:

10. Hypnorm®/Hypnovel® administration provided excellent anaesthesia throughout the experimental procedures. It proved to be safe, effective and of sufficient duration to allow the experiments to be performed with minimal stress to the animals.

11. The healing response following thermal insult permitted an investigation of the subsequent repair mechanisms and produced an additional model for the study of ankylosis.

12. TRAP histochemistry using hexazotised basic fuchsin as the coupler provided the most clear and consistent labelling of clast cells.

13. TRAP labelling was evident in large cells located on hard tissue surfaces but also, to a much lesser extent, in small mononuclear cells located in the PDL. Nevertheless, whereas TRAP labelling has been commonly used as a clast cell marker, its specificity is not absolute.

14. Immunocytochemistry is a fastidious process that relies on strict adherence to a successful protocol. Mislabelling can occur at any step on the staining pathway and, hence positive and negative controls are mandatory, particularly when species cross reactivity is not definitively established.

15. Electron microscopy remains the most reliable method of revealing clast cell activity. The unique morphological characteristics of multinucleation, cytoplasmic polarization, high mitochondrial content and the formation of ruffled borders makes these cells readily identifiable.

16. Succinic dehydrogenase appears to be a reliable marker for clast cell activity provided that cryo-preparation techniques are adopted. The similarity of labelling between osteoclasts and odontoclasts provided further evidence of the close relationship between the cells.

17. Mechanical insult to the tooth root succeeded in producing a degree of resorption commensurate with the pattern of injury. The mechanically induced resorption could be clearly distinguished from the resorption initiated by the freezing injury, but the possibility of bacterial invasion and its influence could not be discounted due to the forced break in the gingival attachment and a likely communication with the oral cavity.

CHAPTER 7

APPENDICES

APPENDIX 1

ANTICOAGULANT - HEPARIN

Heparin Injection B.P. (containing no antiseptic) was supplied in 1 ml plastic ampoules (David Bull Laboratories, Mulgrave, Australia).

Contained 1000 units (IU) per 1 ml.

Dosage: 0.02 ml of heparin sodium per 100 g of body weight

Route: Intravenous injection via femoral vein

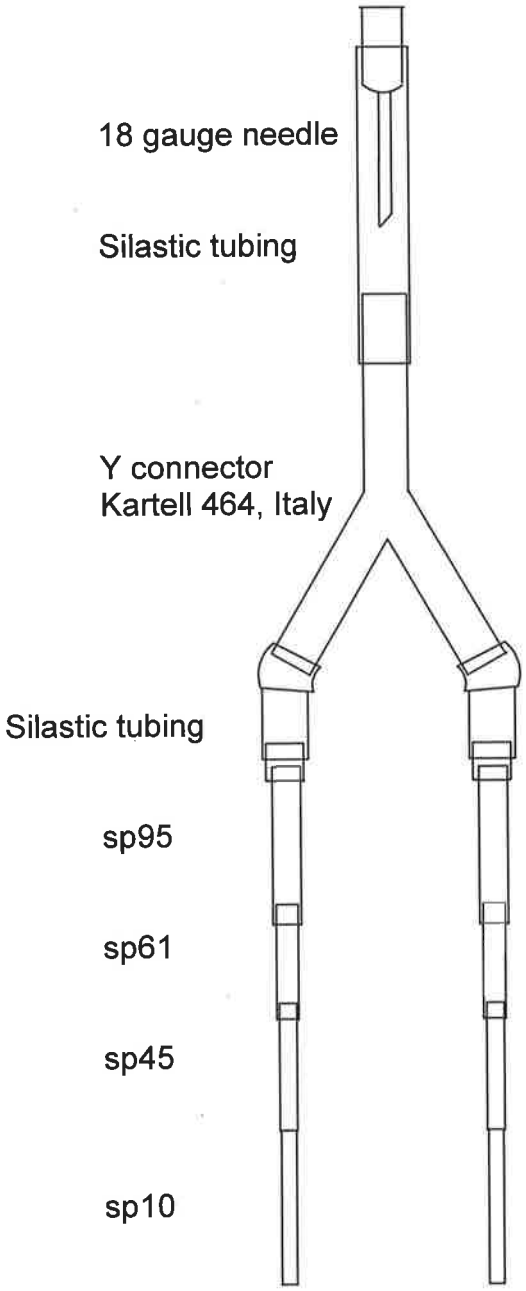
Shelf life: Discard unused heparin after vial seal is broken

Storage: Below 25°C

APPENDIX 2

TUBING FOR CAROTID CANNULATION

Polyethylene Medical Grade tubing (Dural Plastics and Engineering, Dural, Australia)



APPENDIX 3

FIXATIVES AND BUFFERS

I. Phosphate Buffer

Part A
31.2 g $NaH_2PO_4.2H_2O$ in 1 L of distilled water (0.2M)

Part B
28.39 g Na_2HPO_4 in 1 L distilled water (0.2M)

Mix 240 ml of Part A and 760 ml of Part B to make 1 L

II. Neutral buffered formalin

Use: Fixation of tissue for light microscopy

Formula: 4% buffered neutral formal-saline

Preparation: 100 ml of stock 40% formaldehyde solution
900 ml of phosphate buffer

Comments: Used for preserving and temporary storage of tissues for histology and immunohistochemistry

---

III. Karnovsky's (1965) fixative

Use: Fixation of tissue for light or electron microscopy

Formula: 4% paraformaldehyde
5% glutaraldehyde
0.05% $CaCl_2$
0.2M cacodylate or phosphate buffer
1N sodium hydroxide

Preparation:
1. Dissolve 2 g of paraformaldehyde in 25 ml of distilled water heated to 60-70°C.
2. Add 1 to 2 drops of 1N NaOH and stir until the solution clears. Cool and add 10 ml of 25% glutaraldehyde.
3. Make up the volume to 50 ml with 0.2M cacodylate or phosphate buffer.
4. If using cacodylate buffer, add 25 mg anhydrous $CaCl_2$.
5. The final pH is 7.2.

Comments: It is believed that formaldehyde penetrates more quickly and temporarily stabilizes structures which are then stabilized more permanently by glutaraldehyde. It may be used at half strength.

IV. Zamboni's fixative

2% formaldehyde in 0.1M phosphate buffer with 15% picric acid (Zamboni and De Martino, 1967)

For 1 L of fixative, add:
200 ml of Part A phosphate buffer
300 ml of Part B phosphate buffer
200 ml of 10% formaldehyde solution
150 ml of saturated picric acid (filtered)

Filter saturated picric acid through funnel using Whatman No. 1 filter paper before use.

Procedure:
1. Add 20 g of paraformaldehyde to 200 ml of buffer solution
2. Heat to 55°C in a fume cupboard – DO NOT EXCEED 60°C
3. Add several drops of 2.5% NaOH to clear and allow to cool
4. Add 150 ml of filtered picric acid
5. Make up to 1 L with buffer solution
6. Store in refrigerator at 4°C

V. 0.6 M cacodylate buffer (sodium cacodylate)

Formula: $Na(CH_3)_2AsO_2.3H_2O$

Preparation: For 0.6 M solution add 25.69 g sodium cacodylate to 2 L of double distilled water
Add 1N HCl to adjust pH to 7.4 at 20°C

Shelf life: 7 d at 4°C

Comments: The solution is stable and does not support microorganisms. High toxicity to humans due to arsenic content and so gloves, mask and a fume cupboard should be used in its preparation

For **0.2 M** solution add 4.28 g to 100 ml of doubled distilled water

---

VI. Phosphate buffered saline

10 mM sodium phosphate in 0.15 M sodium chloride

Saline solution:
8.79 g $NaCl_2$ in 1 L of distilled water (0.879%)

Part A phosphate buffer

Part B phosphate buffer

Procedure: to 16 ml of Part A and 84 ml of Part B add 100 ml of saline solution to make 200 ml with a pH of 7.5

APPENDIX 4

DECALCIFYING SOLUTIONS

"DECAL" (Institute of Medical and Veterinary Science, Adelaide)

For the rapid decalcification of histological specimens.

Reagents:
950 ml hydrochloric acid (36% concentration)
100 g sodium acetate MW 82.04 dissolved in 1 L of distilled water
100 g EDTA (ethylenediaminetetra-acetic acid,
$[CH_2.N(CH_3.COOH).CH_2COONa]_2.2H_2O$) in 1 L of distilled water
7050 ml of distilled water
The 1% solution of EDTA in water precipitates out to give EDTA in excess on the bottom of the container. EDTA is a chelating agent which binds free calcium ions in the solution.

Method for 10 L:
1. Weigh sodium acetate and dissolve in 1 L of warmed distilled water
2. Add to a large container
3. Weigh 100 g of EDTA and dissolve in 1 L of warmed distilled water
4. Add to container
5. Add 7050 ml of distilled water to container
6. Add with care, 950 ml of hydrochloric acid

WARNING: Acid is both exothermic and explosive when mixed with small quantities of water. Always add the acid to water.

4% EDTA in phosphate buffer

Reagents:
Phosphate buffered saline – Parts A and B (Appendix 3)
EDTA - 80 gm

Procedure:
To 280 ml of part A and 720 ml of part B add 1 L of distilled water and EDTA to give a pH of 7

For **10% EDTA** in phosphate buffer, 200 g of EDTA was added to the 2 L of phosphate solution.

4% EDTA in cacodylate buffer with 2.5% glutaraldehyde

EDTA	80 gm
Cacodylate buffer (0.06M)	1800 ml (Appendix 3)
25% glutaraldehyde	200 ml

Dissolve EDTA in cacodylate buffer by gently heating.
When cool, add the glutaraldehyde
pH to 6 at 4°C using a pH meter
Shelf life is 7 d at 4°C

APPENDIX 5

TISSUE DEHYDRATION AND PARAFFIN EMBEDDING

Shandon Citadel 2000 automatic processor (Shandon Industries, Pittsburgh, Pennsylvania)

The following automatic procedure was used for the impregnation of tissues with paraffin wax prior to embedding:

1.	70% alcohol	1 h
2.	70% alcohol	1 h
3.	80% alcohol	3 h
4.	90% alcohol	3 h
5.	100% alcohol	4 h
6.	100% alcohol	4 h
7.	100% alcohol	4 h
8.	70% xylene/alcohol	4 h
9.	100% xylene	5 h
10.	100% xylene	5 h
11.	paraffin	7 h under vacuum
12.	paraffin	7 h under vacuum

Tissues were then embedded in paraffin using a Reichert Jung Histostat.

APPENDIX 6

SLIDE COATING PROCEDURES FOR IMMUNOCYTOCHEMISTRY

Slides for antibody staining were coated according to the following procedure using 3-aminopropyltriethoxysilane (APT, Sigma code 3648).

1. Place slides in racks.
2. Clean slides in 1% detergent (Decon) solution overnight.
3. Wash in running tap water overnight.
4. Pre-rinse slides in 100% ethanol for 30 s, twice.
5. Dip in 2% APT in ethanol for 10 s.
6. Rinse in 100% ethanol for 30 s twice.
   The final rinses in ethanol were found to be too long and were reduced to 10 s each.

Warning: APT is classified as a harmful substance and should be used with suitable protective clothing in a fume cupboard.

APPENDIX 7

STAINS FOR LIGHT MICROSCOPY

I. Mayer's haematoxylin and eosin

1.	Remove wax	
	Xylol	2 x 2 min
	Absolute alcohol	2 x 2 min
2.	Dip in water to check for complete wax removal	
3.	Stain in haematoxylin	10 min
4.	Wash in running water	1 min
5.	Differentiate in 0.5% HCl	one dip
6.	Wash in running water	10 min
7.	Blue in dilute alkali (NH_4OH in alcohol)	2 dips
8.	Counterstain in eosin	30 s
9.	Differentiate in 70% alcohol	3 dips
10.	Dehydrate and clear	
	Absolute alcohol	2 x 2 min
	Xylol	2 x 2 min
11.	Mount and coverslip using DePex	

Haematoxylin

Formula:

Haematoxylin	5 g
Ammonium alum	50 g
Glycerol	300 ml
Distilled water	700 ml
Sodium iodate	1 g
Glacial acetic acid	20 ml

Eosin

Formula:

Eosin	10 g
Potassium dichromate	5 g
Saturated picric acid	100 ml
Absolute alcohol	100 ml
Distilled water	800 ml

II. 0.05% toluidine blue (for resin embedded sections)

Solution: 0.05% toluidine blue in double distilled water

Preparation: 0.05 g toluidine blue
100 ml double distilled water
Dissolve by stirring

Shelf life: 6 months at room temperature

Precautions: Avoid contact with skin

III. 1% borax

Solution: 1% borax in double distilled water

Preparation: 1 g sodium borate (tetraborate)
100 ml double distilled water
Dissolve by stirring

Shelf life: 6 months at room temperature

Precautions: Avoid contact with skin

Protocol:
1. Mix equal parts A and B just before staining
2. Stain for 30 s on a hot plate at 60°C
3. Rinse with millipored double distilled water
4. Dry on a hot plate
5. Mount in DePex if required

APPENDIX 8

TISSUE PROCESSING FOR ELECTRON MICROSCOPY

1. After decalcification wash specimens in phosphate buffered saline to which 4% glucose has been added - 2 changes of 30 min each

2. Post fix in osmium tetroxide for 1-2 h on a rotator

3. Dehydrate:
   70% ethanol – 2 changes of 30 min each
   90% ethanol - 2 changes of 30 min each
   95% ethanol - 2 changes of 30 min each
   100% ethanol - 2 changes of 30 min each plus 1 change of 1 h
   50%/ethanol/50% resin – 1 change of 8 h or overnight
   100% resin – 3 changes of 8 h each at 4°C (change morning, evening and following morning)

4. Embed specimens in resin following the third resin stage

5. Polymerize in oven at 60°C overnight

APPENDIX 9

STAINING FOR TRANSMISSION ELECTRON MICROSCOPY

Uranyl acetate and Reynolds' lead

Uranyl acetate
0.125 g of uranyl acetate
7 ml of ethanol made up to 25 ml with millipored water

Dissolve uranyl acetate in alcohol solution
Cover with aluminium foil to exclude light
Shelf life: approximately 4 months at room temperature

Reynolds' lead
1.33 g lead nitrate
1.76 g sodium citrate
30 ml millipored water
4 pellets of sodium hydroxide
Millipored water

Procedure:
1. In a 50 ml vessel place lead nitrate and sodium citrate with 30 ml of water
2. Shake continuously for 1 min, then let stand for 30 min but with intermittent shaking
3. Dissolve 4 pellets of sodium hydroxide in 8 ml of millipored water. Add 2 ml of millipored water. After 30 min add 8 ml of sodium hydroxide solution to other solution and shake well.
4. Add millipored water to final solution to make up 50 ml.
5. Final solution should be clear with a pH less than 14. If not, repeat.

Shelf life: 7 d at 4°C.

APPENDIX 10

MONOCLONAL MOUSE ANTI-HUMAN MACROPHAGE (CD68) KP1
(Zymed Laboratories, San Francisco, USA)

Form: Liquid. Ready to use undiluted. This antibody is presented stored in PBS containing Bovine Serum Albumin and 0.05% sodium azide as a preservative. It is made from culture supernatant.

Specificity: Reacts with the CD68 antigen, a glycoprotein with a molecular weight of approximately 110kD. Expressed primarily as an intracytoplasmic molecule, associated with lysosomal granules.

Clone: KP1

Isotype: IgG1, kappa

Application: Detects macrophages in a wide variety of human tissues including Kupffer cells and macrophages in the spleen, in lamina propria of the gut, in lung alveoli and in bone marrow. Peripheral blood monocytes also stain positively. Reacts with myeloid precursors and peripheral blood granulocytes. May be useful for the identification of myeloid leukaemias.

Use: Suitable for the immunohistochemical staining of formalin-fixed paraffin-embedded or frozen tissues. Trypsin or pepsin digestion is required for specific staining of formalin-fixed paraffin-embedded tissue sections. To stain, incubate for 30-60 min at room temperature or at 4°C overnight.

Storage: Store at 2-8°C.

APPENDIX 11

ANTI-CATHEPSIN–L ANTIBODY (M-19) sc-6502
Santa Cruz Biotechnology, Santa Cruz, USA

Background: The cathepsin family of proteolytic enzymes contains several diverse classes of proteases. The cysteine protease class comprises cathepsins B, L, H, K, S and O. The aspartyl protease class is composed of Cathepsins D and E. Cathepsin G is in the serine protease class. Most cathepsins are lysosomal and each is involved in cellular metabolism, participating in various events such as peptide biosynthesis and protein degradation. Cathepsin L has been identified as a 38kDa protein that is most closely related to cathepsin H.

Source: Anti-cathepsin L is an affinity-purified goat monoclonal antibody raised against a peptide mapping at the carboxy terminus of the light chain of cathepsin L of mouse origin.

Product: Each vial contains 200 µg IgG in 1.0 ml of PBS containing 0.1% sodium azide and 0.2% gelatin. Blocking peptide is available for competition studies (sc-6502 P) (100 µg peptide in 0.5ml PBS with 0.1% sodium azide and 100 µg BSA.

Specificity: Anti-cathepsin L (M-19) reacts with cathepsin L of mouse and rat origin by western blotting and immunohistochemistry.
Recommended dilution for Western blot analysis: 1:100 - 1:1000

Storage: Store at 4°C, do not freeze, stable for 1 y from date of shipment.

APPENDIX 12

MOUSE ANTI-RAT ED1 ANTIBODY (MCA341R)
Serotec, Oxford, United Kingdom

Clone number: ED

Volume/quantity: 0.25 mg/0.25 ml

Product form: Purified IgG – liquid

Preparation: Purified IgG prepared from tissue culture supernatant

Buffer: Phosphate buffered saline

Preservatives/stabilizers: 0.1% sodium azide (NaN_3)

Protein concentration: Approximately 1.0 mg/ml IgG

Immunogen: Rat spleen cells

Fusion partners: Spleen cells from immunized BALB/c mice were fused with cells of the SP2/0-Ag14 mouse myeloma cell line

Isotype: IgG1 (mouse)

Specificity: ED1 recognizes a single chain glycoprotein of 90-100kD that is expressed predominantly on the lysosomal membrane of myeloid cells. Weak cell surface expression also occurs. The antigen is expressed by the majority of tissue macrophages and weakly by peripheral blood granulocytes. Studies have shown that the antigen recognized by ED1 has many characteristics in common with macrosiallin in the mouse and CD68 in the human.

Applications: Immunohistology using paraffin sections

Recommended secondary reagents:
Goat anti-mouse IgG:HRP conjugate (non-cross reacting with rat IgG) – STAR77

Recommended negative controls:
Mouse IgG1 negative control – MCA1209

Storage:	Store at 4°C for one month or at –20°C for longer Avoid repeated freezing and thawing as this may denature the antibody Should the product contain a precipitate, microcentrifugation is recommended before use.
Shelf life:	12 months from the date of despatch
Health and safety:	Contains sodium azide: a poisonous and hazardous substance which should be handled by trained staff

APPENDIX 13

ANTI-CD13 MONOCLONAL MOUSE ANTI-HUMAN MYELOID CELL ANTIBODY
Dako, Carpinteria, USA

Presentation: Monoclonal mouse antibody in liquid form as tissue culture supernatant (RPMI 1640 medium containing fetal calf serum) dialysed against 0.05 M Tris/HCl, pH 7.2 containing 15 mM NaN_3.

Mouse Ig concentration: 135 mg/L

Isotype: IgG1, kappa

Total protein concentration: 7.5 g/L

Storage: 2-8°C

Clone: WM-47

Immunogen: Leukaemic cells from a case of chronic myeloid leukaemia

Specificity/reactivity: The DAKO antibody reacts with a 150kDa cell surface glycoprotein (CD13, gp150) that is expressed by a fraction of the committed granulocyte-monocyte progenitors (CFU-GM) and by cells of the granulocyte and monocyte lineages. The CD13 antigen is identical to aminopeptidase N. The specificity of the antibody is similar to the CD13 antibody WM-15 and My7 as indicated by immuno-precipitation, immuno-histochemical analysis, competetive binding assays and reactivity with transfected murine cell lines expressing gp150.

The antibody labels monocytes, granulocytes in peripheral blood, and myeloblasts, promyelocytes and myelocytes in normal bone marrow. Lymphocytes, platelets and red blood cells are not stained by the antibody. The majority of acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML) in myeloid blast crisis and myeloid cell lines, are positively stained.

Non-haemopoietic reactivity is observed in neuroblastoma and certain gynaecological carcinoma lines.

By immunochemistry, myeloid and epithelial layer in tonsil, smooth muscle cells and connective tissue in

spleen are weakly positive. The antibody is negative on liver, cerebellum and kidney.

Staining procedures:

Formalin-fixed, paraffin-embedded tissues
Not suitable for labelling

Frozen sections and cell smears
Can be used for labelling acetone-fixed cryostat sections or fixed cell smears. For the latter type of preparation, the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique is recommended.
The antibody may be used at a dilution of 1:50 – 1:100 in the 3-stage immunoperoxidase technique when tested on acetone-fixed sections of normal tonsil.
It may be used at a dilution of 1:50 – 1:100 in the APAAP technique on fixed cell smears.

Flow cytometry
The antibody is well-suited for flow cytometry (indirect method) using DAKO Rabbit Anti-Mouse Immunoglobulins/FITC, code No. F 0313, or using Rabbit Anti-mouse Immunoglobulins/RPE, code No. R 0439

APPENDIX 14

ANTI-CD15 MONOCLONAL MOUSE ANTI-HUMAN GRANULOCYTE ASSOCIATED ANTIBODY
Dako, Carpinteria, USA

Synonyms: X hapten, Lewis X (Le^x), FAL (3-fucosyl-N-acetyl-lactosamine)

Introduction: CD15 is an oligosaccharide near related to blood groups. CD15 is found on mature granulocytes and on Hodgkins and Reed-Sternberg cells.
In Hodgkins disease CD15 is strongly expressed on Hodgkins and Reed-Sternberg cells except in the lymphocyte-predominant type of the disease, in which only a minority of cases are positive. The majority of non-Hodgkins lymphomas do not generally express the CD15 antigen

Presentation: Monoclonal mouse antibody supplied in liquid form as tissue culture supernatant (RPMI 1640 medium containing fetal calf serum) dialysed against 0.05 mol/L Tris/HCl, pH 7.2 containing 15 mM/L NaN_3

Mouse Ig concentration: 175 mg/L

Isotype: IgM, kappa

Total protein concentration: 4.5 g/L

Stroage: 2-8°C

Clone: C3D-1

Immunogen: Purified neutrophils from normal human peripheral blood

Specificity/reactivity: Anti-CD15, C3D-1 was submitted to the Fifth International Workshop and Conference on Human Leucocute Differentiation Antigens (Boston 1993) and clustered in the CD15 group.
Anti-CD15, C3D-1 labels neutrophils in peripheral blood. Monocytes are weakly stained, whereas lymphocytes and platelets are consistently negative.
Anti-CD15, C3D-1 also reacts with dendritic reticulum cells

Staining procedures:

Formalin-fixed paraffin-embedded sections

Can be used on formalin-fixed paraffin-embedded sections.

To improve the staining pattern, antigen retrieval such as heating in 10 mM citrate buffer, pH 6.0 or in DAKO Target Retrieval Solution, code No. S 1700 can be used. The slides should not dry out during this treatment or during the following immuno-histochemical procedure.

For tissue sections a variety of sensitive staining techniques are suitable including immunoperoxidase procedures, the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique and avidin-biotin methods such as LSAB methods.

The antibody may be used at a dilution of 1:25 – 1:50 with the StreptABComplex/HRP duet, mouse/rabbit (K 0492) method when tested on formalin-fixed paraffin-embedded sections of normal tonsil. Optimal results are obtained when Biotinylated Rabbit Anti-Mouse IgM (code No. E 0465) is used as a link antibody.

Frozen sections and cell smears

Can be used for labelling acetone-fixed cryostat sections or fixed cell smears.

For staining of cell smears, the APAAP technique is recommended.

The antibody may be used at a dilution at 1:25 – 1:50 in the APAAP technique and the StreptABComplex/HRP duet, mouse/rabbit (K 049) methods, when tested on acetone-fixed cryostat sections of normal tonsil. Optimal results are obtained when Biotinylated Rabbit Anti-Mouse IgM (code No. E 0465) is used as a link antibody.

Automation: The antibody may be used in automated immunostaining systems.

Flow cytometry

The antibody is well-suited for flow cytometry (indirect technique) using DAKO Rabbit Anti-Mouse Immunoglobulins/FITC, code No. F0313 or Rabbit Anti-Mouse Immunoglobulins/RPE, code No. R0439

APPENDIX 15

MOUSE ANTI-RAT ANTI-CD45RC ANTIBODY – Anti-Leucocyte common antigen
Serotec, Oxford, United Kingdom

Clone number: MRC OX-22

Volume: 0.25 ml

CD number: CD45RC

Product form: Ascitic fluid – liquid

Preservative: 0.1% Sodium azide (NaN_3)

Immunogen: PHA stimulated rat lymphocytes

Fusion partners: Spleeen cells from immunized BALB/c mice were fused with cells from the NS1 mouse myeloma cell line.

Isotype: IgG1 (Mouse)

Specificity: Reacts with high molecular weight form of the leucocyte common antigen. This antigen is found on B cells, approximately 50% of bone marrow cells, all CD8+ve T cells but splits CD4+ve T cells into two populations differing in their helper activities.

Immunohistology: This product requires retrieval using heat treatment prior to staining of paraffin sections.

Recommended secondary reagents: Goat anti-mouse IgG: HRP conjugate (non-cross reacting with rat IgG).

Storage conditions: Store at +4°C or at –20°C if preferred. Avoid repeated freezing and thawing as this may denature antibody. Should the product contain a precipitate, microcentrifuge before use.

Shelf life: 12 months.

Health and Safety: This product contains sodium azide: a poisonous and hazardous substance which should be handled using appropriate precautions.

APPENDIX 16

MOUSE ANTI-RAT ANTI-CD61 ANTIBODY
Serotec, Oxford, USA

Catalogue number: MCA 1773

Clone number: F11

Volume/quantity: 0.25 mg

CD number: CD61

Product form: Purified IgG – liquid

Buffer: Phosphate buffered saline pH 7.4

Preservatives/stabilizers: 0.1% sodium azide (NaN_3)

Approximate protein concentration: IgG concentration 1.0 mg/ml

Immunogen: Bone cell suspension from SD rats

Fusion partners: Spleen cells from immunized Balb/c mice were fused with the cells of the mouse X63-Ag8 myeloma cell line

Isotype: IgG1 (mouse)

Specificity: MCA 1773 recognizes the rat CD61 cell surface antigen, also known as the integrin beta 3 chain. CD61 is expressed by platelets, megakaryocytes and osteoclasts.

Species reactivity: Weak reactivity with human CD61 is reported

Recommended secondary reagents: Goat anti-mouse IgG FITC conjugate (non-cross-reacting with rat IgG)
Goat anti-mouse IgG RPE conjugate (non-cross reacting with rat IgG)

Recommended secondary controls: Mouse IgG1 Negative Control

Storage conditions: Store at +4°C for 1 month or at –20°C for longer
Avoid repeated freezing and thawing as this may denture the antibody
Should this product contain a precipitate micro-centrifugation before use is recommended

Shelf life:	12 months from the date of despatch
Health and safety:	This product contains sodium azide: a poisonous and hazardous substance which should be handled by trained staff

APPENDIX 17

SHEEP ANTI-HUMAN ANTI-CARBONIC ANHYDRASE II ANTIBODY
Serotec, Oxford, United Kingdom

Batch No:	261199
Volume:	1 ml
Preparation:	Antisera to human carbonic anhydrase II were raised by repeated immunization of sheep with highly purified antigen. Purified IgG was prepared from whole serum by ion exchange chromatography.
Buffer:	Glycine buffered saline pH 7.4
Preservatives:	0.1% Sodium Azide (NaN_3) 0.1% EACA 1 mM EDTA 0.01% Benzamidine
Protein concentrations:	IgG at 15.5 mg/ml
Immunogen:	Purified human carbonic anhydrase II (CA II) prepared from erythrocytes.
Specificity:	Product identity is confirmed by double diffusion versus human CA II. No reactivity is seen in immunodiffusion against CA1.
Species Cross reactivity:	Not tested
Storage:	Store at 4°C or at –20°C if preferred. Storage in frost-free freezers is not recommended. Avoid repeated freezing and thawing as this may denature the antibody
Shelf life:	12 months

APPENDIX 18

CYTOKERATIN (AE1/AE3) ANTIBODY
Cell Marque, Austin, USA

Presentation:	Cytokeratin AE1/AE3 is a cocktail of mouse monoclonal antibodies from ascites fliud diluted in phosphate buffered saline, pH 7.6, with 1% bovine serum albumin, and preserves with sodium azide.
Applications:	Cytokeratin, AE1/AE3 is well suited to distinguish epithelial carcinoma from non-epithelial malignancies and is used to aid epithelial tumour classification. This antibody has been used to characterize the source of various neoplasms and to study the distribution of keratin containing cells in epithelia during normal development and during the development of epithelial neoplasms. This antibody stains cytokeratins present in normal and abnormal human tissues. This antibody has shown high sensitivity and specificity in recognizing epithelial cells, especially neoplastic ones, by immunohistological methods.
Reactivity:	Paraffin or frozen sections
Control:	Prostate tissue
Visualization:	Cytoplasmic
Stability:	24 months; store at 2-8°C

APPENDIX 19

AVIDIN-BIOTIN (ABC) PEROXIDASE IMMUNOLABELLING TECHNIQUE FOR PARAFFIN SECTIONS

1. Mount cut sections on silane-coated slides.

2. Dewax in xylene and take sections to absolute alcohol — 2 x 3 min

3. Block endogenous peroxidase with 0.5% hydrogen peroxide in methanol at room temperature for 30 min.
   (H_2O_2 vol 30% w/v
   8.3 ml in 500 ml of methanol)

4. Rinse in PBS buffer — 2 x 3 min

5. Pretreatment antigen retrieval
   Enzyme digestion with trypsin
   0.0625 g of trypsin II in 250 ml PBS
   Prewarm 250 ml PBS in 37°C water bath
   Dissolve enzyme in PBS
   Incubate slides in plastic racks in plastic microwave containers at 37°C for 3 min

6. Rinse in PBS buffer — 2 x 3 min

7. Circle sections with a PAP pen and incubate in 3% normal (horse) serum — 30 min

8. Drain normal horse serum and incubate with primary antibody overnight at 4°C

9. Rinse in PBS — 2 x 3 min

10. Incubate with biotinylated linking antibody — 30 min

11. Rinse in PBS — 2 x 3 min

12. Incubate with streptavidin peroxidase — 60 min

13. Rinse in PBS — 2 x 3 min

14. Apply peroxidase substrate solution
    Vial A - DAB stabilizing buffer
    Vial B – Substrate buffer H_2O_2 in Tris HCl buffer
    In a separate vial mix 1 drop of vial A to 1 ml of vial B
    Incubate sections for 5-10 min at room temperature
    Monitor reaction on positive control sections at 3 min

15. Rinse in PBS — 2 x 3 min

16. Lightly stain with Mayer's haematoxylin

17. Blue with ammonia solution
    1.4 ml ammonia in 250 ml distilled water — 2 dips

18. Mount and cover slip in permanent aqueous mounting medium – aquatex.

APPENDIX 20

ANTIGEN RETRIEVAL FOR IMMUNOLABELLING

I. Enzyme digestion

Enzyme used: TRYPSIN
Trypsin type II (Sigma Cat. No. T-8128)
0.0625 g in 250 ml of phosphate buffered saline

Pre-warm 250 ml of PBS in a 37°C waterbath.
Dissolve enzyme in the buffer.
Incubate slides in plastic racks in plastic microwave containers at 37°C for 3 min.
The enzyme solutions may be used for more than 1 rack of slides, but must be made fresh each day.

II. Microwave retrieval

Citrate buffer
10mM sodium citrate pH 6.0

Distilled water	1000 ml
5N NaOH	25 ml
Citric acid	10.5 g

Place sections into plastic racks and load into 250 ml of citrate solution in a microwave pot. Use the HIGH setting (Toshiba 1000W microwave oven) until solution starts to boil. Then place racks in an NEC 750W microwave oven and set for 10 min on power level 2 (for 2 pots - use a ballast pot of distilled water if necessary), or power level of 3 for three pots. Observe solution for bubbling when magnatron cuts in and subsides as it cuts out. Remove pots when time completed, and allow to cool to 50°C then discard solution.

APPENDIX 21

TECHNICAL DETAILS OF DETECTION SYSTEM
Signet DAB Chromogen System

The DAB Chromogen Systems are intended for use in for immunohistochemistry and immunoblotting procedures. They produce a golden brown precipitate localized to the specific site of antigen as detected by direct or indirect immunochemical staining procedures with horseradish peroxidase.

Reagents:

Vial A (Chromogen) – Liquid stable 3,3´diaminobenzidine, (DAB) chromogen in stabilizing buffer
Vial B (Substrate buffer) – H_2O_2 in Tris HCl buffer

Contents:

300 Test DAB Chromogen System (Signet CAT#1040) contains:
Vial A: 1 x 4 ml DAB Chromogen
Vial B: 6 x 6 ml Substrate buffer

1000 Test Signet Chromogen System (Signet Cat#1042) contains:
Vial A: 1 x 6 ml DAB Chromogen
Vial B: 2 x 50 ml Substrate buffer

Immunohistochemical staining procedure:

1. Combine reagents:
   300 Test System: Remove dropper plug from 6 ml DAB Substrate Buffer – Vial B, add 6 drops of DAB Chromogen from Vial A, replace dropper and mix gently
   1000 Test System: in a separate vial mix 1 drop, ~40µl, of DAB Chromogen from Vial A with every 1 ml DAB Substrate Buffer from Vial B

2. Apply solution to tissue sections within 6 hours of combining reagents from Vial A and Vial B

3. Incubate tissue sections for 5-10 min at room temperature or 2-5 min at 37°C on a Stainplate™ Staining Accelerator (Signet Cat# 1060 or 1080). Begin to monitor the development of the reaction on the positive control tissue at 3 min (room temperature) or 2 min (37°C Stainplate).

4. Rinse well with distilled water

5. Counterstain with Mayer's haematoxylin for 5 min.

6. Blue haematoxylin with ammonia water for 10 s (Ammonia water: add 1.4 ml concentrated ammonium hydroxide to 250 ml distilled water).

7. Mount and coverslip with permanent aqueous mounting medium (Signet Cat# 1010 or 1012)

Caution: DAB has been classified as a carcinogen and can cause irritation upon exposure to skin. Avoid skin contact with the DAB when preparing solutions or when cleaning equipment. Wash hands thoroughly after exposure. Solutions containing DAB should be disposed of according to local regulations.

APPENDIX 22

Chequerboard approach to antibody titrations.

Antibodies were diluted according to the concentrations listed in the vertical column and applied for time periods listed in the horizontal row.

Time (hours) →	1	2	4	8	12	24
Dilution (in PBS)↓						
1:1						
1:2						
1:5						
1:10						
1:50						
1:100						
1:500						
1:1000						
1:5000						
1:10000						

APPENDIX 23

SUCCINIC DEHYDROGENASE STAINING (Takimoto et al., 1966)

Solutions:
5 ml phosphate buffer 0.2M (Appendix 3)
5 ml sodium succinate 0.2M
6 ml nitro BT at (5 mg/ml)

Combine the solutions and incubate slides at 37°C for 45 min
Fix in 10% formalin (Appendix 3) for 30 min

APPENDIX 24

Datasheet for Osteoprotegerin FcOPG
Amgen, California, USA

Batch number:	35000F8
Concentration:	10 mg/ml
Presentation:	1 ml vials
Strorage:	-70°C
Administration:	Subcutaneously

Each 1 ml vial of OPG was diluted with 3 ml of sterile water for injection to give a final concentration of 2.5 mg/ml. Before subcutaneous administration, each animal was weighed and a volume for injection calculated. For most animals weighing in the range of 250–300 g, this involved a dose of 0.25-0.30 ml.

CHAPTER 8

BIBLIOGRAPHY

Abu'l-Fadl, M.A.M. and King, E.J. (1949). Properties of the acid phosphatases of erythrocytes and of the human prostate gland. *Biochemical Journal,* 45: 51-60.

Abu-Amer, Y., Erdmann, J., Alexopoulou, L., Kollias, G., Ross, F.P. and Teitelbaum, S.L. (2000). Tumor necrosis factor receptors types 1 and 2 differentially regulate osteoclastogenesis. *Journal of Biological Chemistry,* 275: 27307-27310.

Addison, W.C. (1976). Properties and Functions of Odontoclasts. A Comparative Study. PhD Thesis, University of Birmingham.

Addison, W.C. (1978a). The distribution of nuclei in human odontoclasts in whole cell preparations. *Archives of Oral Pathology,* 23: 1167-1171.

Addison, W.C. (1978b). β-Hydroxybutyrate dehydrogenase activity in human and kitten odontoclasts. *Histochemical Journal,* 10: 731-737.

Addison, W.C. (1979). Enzyme histochemical characteristics of human and kitten odontoclasts and kitten osteoclasts: a comparative study using whole cells. *Histochemical Journal,* 11: 719-735.

Addison, W.C. (1980). The effect of parathyroid hormone on the numbers of nuclei in feline odontoclasts in vivo. *Journal of Periodontal Research,* 15: 536-543.

Akamine, A., Tsukuba, T., Kimura, R., Maeda, K., Tanaka, Y., Kato, K. and Yamamoto, K. (1993). Increased synthesis and specific localization of a major lysosomal membrane sialoglycoprotein (LGP107) at the ruffled border membrane of active osteoclasts. *Histochemistry,* 100: 101-108.

Akamine, A., Anan, H., Hamachi, T. and Maeda, K. (1994a). A histochemical study of the behaviour of macrophages during experimental apical periodontitis in rats. *Journal of Endodontology,* 20: 474-478.

Akamine, A., Hashiguchi, I., Toriya, Y. and Maeda, K. (1994b). Immunohistochemical examination on the localization of macrophages and plasma cells in induced rat periapical lesions. *Endodontics and Dental Traumatology,* 10: 121-128.

Akatsu, T., Takahashi, N., Udagawa, N., Imamura, K., Yamaguchi, A., Sato, K., Nagata, N. and Suda, T. (1991). Role of prostaglandins in interleukin-1 induced bone resorption in mice *in vitro. Journal of Bone and Mineral Research,* 6: 183-190.

Alam, A.S., Moonga, B., Bevis, P.J.R., Huang, C.L.H. and Zaidi, M. (1991). Selective antagonism of calcitonin-induced osteoclastic quiescence (Q effect) by human calcitonin gene-related peptide (Val8Phe37). *Biochemical and Biophysical Research Communications,* 179: 134-139.

Alvarez, J.I., Teitelbaum, S.L., Blair, H.C., Greenfield, E.M., Athanasou, N.A. and Ross, F.P. (1991). Generation of Avian Cells Resembling Osteoclasts from Mononuclear Phagocytes. *Endocrinology,* 128: 2324-2335.

Anderson, D.M., Maraskovsky, E., Billingsley, W.L., Dougall, W.C., Tometsko, M.E., Roux, E.R., Teepe, M.C., DuBose, R.F., Cosman, D. and Galibert, L. (1997). A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature,* 390: 175-179.

Anderson, R.E. and Toverud, S.U. (1977). Chromatographic Separation of Two Acid Phosphatases from Rat Bone. *Calcified Tissue Research,* 24: 187-190.

Anderson, R.E., Schraer, H. and Gay, C.V. (1982). Ultrastructural immunocytochemical localization of carbonic anhydrase in normal and calcitonin-treated chick osteoclasts. *The Anatomical Record,* 204: 9-20.

Andersson, G.N. and Marks, S.C. (1989). Tartrate-resistant acid ATPase as a cytological marker for osteoclasts. *Journal of Histochemistry and Cytochemistry,* 37: 115-117.

Andersson, G.N., Ek-Rylander, B., Hammarström, L., Lindskog, S. and Toverud, S.U. (1986). Immunocytochemical localization of a tartrate-resistant and vanadate-sensitive acid nucleotide tri- and diphosphatase. *Journal of Histochemistry and Cytochemistry,* 34: 293-298.

Andersson, L., Jonsson, B.G., Hammarström, L., Blomlöf, L., Andreasen, J.O. and Lindskog, S. (1987). Evaluation of statistics and desirable experimental design of a histomorphometrical method for studies of root resorption. *Endodontics and Dental Traumatology,* 3: 288-295.

Andersson, G.N., Lindunger, A. and Ek-Rylander, B. (1989). Isolation and characterization of skeletal acid ATPase: a new osteoclast marker? *Connective Tissue Research,* 20: 151-158.

Andreasen, J.O. (1975). The effect of splinting upon periodontal healing after replantation of permanent incisors in monkeys. *Acta Odontologica Scandinavica,* 33: 313-323.

Andreasen, J.O. (1980a). A time-related study of periodontal healing and root resorption activity after replantation of mature permanent incisors in monkeys. *Swedish Dental Journal,* 4: 101-110.

Andreasen, J.O. (1980b). Analysis of topography of surface and inflammatory root resorption after replantation of mature permanent incisors in monkeys. *Swedish Dental Journal,* 4: 135-144.

Andreasen, J.O. (1981a). The effect of pulp extirpation or root canal treatment on periodontal healing after replantation of permanent incisors in monkeys. *Journal of Endodontology,* 7: 245-252.

Andreasen, J.O. (1981b). The effect of extra-alveolar period and storage media upon periodontal and pulpal healing after replantation of mature permanent incisors in monkeys. *International Journal of Oral Surgery,* 10: 43-53.

Andreasen, J.O. (1985). External root resorption: its implication in dental traumatology, paedodontics, periodontics, orthodontics and endodontics. *International Endodontic Journal,* 18: 109-118.

Andreasen, J.O. (1987). Experimental dental traumatology: development of a model for external root resorption. *Endodontics and Dental Traumatology,* 3: 269-287.

Andreasen, J.O. (1988). Review of root resorption systems and models. Etiology of root resorption and the homeostatic mechanisms of the periodontal ligament. *In: The Biological*

Mechanisms of Tooth Eruption and Root Resorption. Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, p. 9-21.

Andreasen, J.O. and Andreasen, F.M. (1992). Root resorption following traumatic dental injuries. *Proceedings of the Finnish Dental Society,* 88: 95-114.

Andreasen, J.O. and Kristerson, L. (1981a). The effect of extra-alveolar root filling with calcium hydroxide on periodontal healing after replantation of permanent incisors in monkeys. *Journal of Endodontology,* 7: 349-354.

Andreasen, J.O. and Kristerson, L. (1981b). The effect of limited drying or removal of the periodontal ligament. Periodontal healing after replantation of mature incisors in monkeys. *Acta Odontologica Scandinavica,* 39: 1-13.

Andreasen, J.O., Paulsen, H.U., Yu, Z. and Schwartz, O. (1990). A long-term study of 370 autotransplanted premolars. Part III. Periodontal healing subsequent to transplantation. *European Journal of Orthodontics,* 12: 25-37.

Antanaitis, B.C. and Aisen, P. (1983). Uteroferrin and the purple acid phosphatases. *Advances in Inorganic Biochemistry,* 5: 111-136.

Arey, L.B. (1920). The Origin, Growth and Fate of Osteoclasts and Their Relation to Bone Resorption. *The American Journal of Anatomy,* 26: 315-345.

Ash, P., Loutit, J.F. and Townsend, K.M.S. (1980). Osteoclasts derived from haemopoietic stem cells. *Nature,* 283: 669-670.

Assuma, R., Oates, T., Cochran, D., Amar, S. and Graves, D.T. (1998). IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. *Journal of Immunology,* 160: 403-409.

Athanasou, N.A. (1996). Cellular biology of bone-resorbing cells. *Journal of Bone and Joint Surgery,* 78A: 1096-1112.

Athanasou, N.A. and Quinn, J. (1990). Immunophenotypic differences between osteoclasts and macrophage polykaryons: Immunohistochemical distinction and implications for osteoclast ontogeny and function. *Journal of Clinical Pathology,* 43: 997-1003.

Athanasou, N.A., Bliss, E., Gatter, K.C. and Heryet, A. (1985). An Immunohistological Study of Giant-Cell Tumor of Bone: Evidence for an Osteoclast Origin of the Giant Cells. *Journal of Pathology,* 147: 153-158.

Athanasou, N.A., Heryet, A., Quinn, J., Gatter, K.C., Mason, D.Y. and McGee, J.O. (1986). Osteoclasts contain macrophage and megakaryocyte antigens. *Journal of Pathology,* 150: 239-246.

Athanasou, N.A., Quinn, J. and McGee, J.O. (1987a). Leucocyte common antigen is present on osteoclasts. *Journal of Pathology,* 153: 121-126.

Athanasou, N.A., Quinn, J., Heryet, A., Woods, C.G. and McGee, J.O. (1987b). Effect of decalcifying agents on immunoreactivity of cellular antigens. *Journal of Clinical Pathology,* 40: 874-878.

Athanasou, N.A., Hall, P.A., d'Ardenne, A.J., Quinn, J. and McGee, J.O. (1988a). A monoclonal antibody (anti-L-35) which reacts with human osteoclasts and cells of the mononuclear phagocyte system. *British Journal of Experimental Pathology,* 69: 309-319.

Athanasou, N.A., Quinn, J., Heryet, A. and McGee, J.O. (1988b). Localization of platelet antigens and fibrinogen on osteoclasts. *Journal of Cell Science,* 89: 115-122.

Athanasou, N.A., Quinn, J. and McGee, J.O. (1988c). Immunocytochemical analysis of the human osteoclast phenotypic relationship to other marrow-derived cells. *Bone and Mineral,* 3: 317-333.

Athanasou, N.A., Quinn, J., Horton, M.A. and McGee, J.O. (1990). New sites of cellular vitronectin receptor immunoreactivity detected with osteoclast-reacting monoclonal antibodies 13C2 and 23C6. *Bone and Mineral,* 8: 7-22.

Athanasou, N.A., Puddle, B., Quinn, J. and Woods, C.G. (1991). Use of monoclonal antibodies to recognise osteoclasts in routinely processed bone biopsy specimens. *Journal of Clinical Pathology,* 44: 664-666.

Atkins, G.J., Haynes, D.R., Geary, S.M., Loric, M., Crotti, T.N. and Findlay, D.M. (2000). Coordinated cytokine expression by stromal and hematopoietic cells during human osteoclastogenesis. *Bone,* 26: 653-661.

Atkins, G.J., Bouralexis, S., Haynes, D.R., Graves, S.E., Geary, S.M., Evdokiou, A., Zannettino, A.C., Hay, S. and Findlay, D.M. (2001). Osteoprotegerin inhibits osteoclast formation and bone resorbing activity in giant cell tumors of bone. *Bone,* 28: 370-377.

Aubin, J.E. and Bonnelye, E. (2000). Osteoprotegerin and its Ligand: A New Paradigm for Regulation of Osteoclastogenesis and Bone Resorption. *Medscape Women's Health,* 5: 5

Averill, B.A., Davis, J.C., Burman, S., Zirino, T., Sanders-Loehr, J., Loehr, T.M., Sage, J.T. and Debrunner, P.G. (1987). Spectroscopic and Magnetic Studies of the Purple Acid Phosphatase from the Bovine Spleen. *Journal of the American Chemical Society,* 109: 3760-3767.

Balogh, K. (1963). Histochemical Study of Oxidative Enzyme Systems in Teeth and Peridental Tissues. *Journal of Dental Research,* 42: 1457-1466.

Bando, Y., Kominami, E. and Katunuma, N. (1986). Purification and tissue distribution of rat cathepsin L. *Journal of Biochemistry Tokyo,* 100: 35-42.

Barber, A.F. and Sims, M.R. (1981). Rapid maxillary expansion and external root resorption in man: A scanning electron microscope study. *American Journal of Orthodontics,* 79: 630-652.

Barka, T. and Anderson, P.J. (1963). Oxidative enzymes. *In: Histochemistry. Theory, Practice, and Bibliography.* Barka, T. and Anderson, P.J., editors. Hoeber Medical Division, New York, p. 296-333.

Barnes, D.M. (1987). Close Encounters with an Osteoclast. *Science,* 236: 914-916.

Baron, R. (1989a). Molecular Mechanisms of Bone Resorption by the Osteoclast. *The Anatomical Record,* 224: 317-324.

Baron, R. (1989b). Polarity and membrane transport in osteoclasts. *Connective Tissue Research,* 20: 109-120.

Baron, R. (1995). Molecular mechanisms of bone resorption - An update. *Acta Orthopedica Scandinavica (Suppl 266),* 66: 66-70.

Baron, R. and Zaidi, M. (1995). Bone and Cell Biology: Osteoclasts. *Bone,* 17: 55S-56S.

Baron, R., Neff, L., Louvard, D. and Courtoy, P.J. (1985). Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. *Journal of Cell Biology,* 101: 2210-2222.

Baron, R., Neff, L., Tran Van, P., Nefussi, J.R. and Vignery, A. (1986). Kinetic and cytochemical identification of osteoclast precursors and their differentiation into multinucleated osteoclasts. *American Journal of Pathology,* 122: 363-378.

Baron, R., Neff, L., Brown, W., Courtoy, P.J., Louvard, D. and Farquhar, M.G. (1988). Polarized secretion of lysosomal enzymes: co-distribution of cation-independent mannose-6-phosphate receptors and lysosomal enzymes along the osteoclast exocytic pathway. *Journal of Cell Biology,* 106: 1863-1872.

Baron, R., Chakraborty, M., Chatterjee, D., Horne, W., Lomri, A. and Ravensloot, J.-H. (1993). Biology of the Osteoclast. *In: Physiology and Pharmacology of Bone.* Mundy, G.R. and Martin, T,J,, editors. Springer-Verlag, p. 111-147.

Bartlett, P.F. and Reade, P.C. (1972). Cryopreservation of Developing Teeth. *Cryobiology,* 9: 205-211.

Bartold, P.M. (1995). Turnover in periodontal connective tissues: dynamic homeostasis of cells, collagen and ground substances. *Oral Diseases,* 1: 238-253.

Bartold, P.M., Miki, Y. and McAllister, B. (1988). Glycosaminoglycans of human cementum. *Journal of Periodontal Research,* 23: 13-17.

Bartold, P.M., Reinboth, B. and Nakae, H. (1990). Proteoglycans of bovine cementum. Isolation and characterization. *Matrix,* 10: 10-19.

Battifora, H. and Kopinski, M. (1986). The influence of protease digestion and duration of fixation on the immunostaining of keratins. A comparison of formalin and ethanol fixation. *Journal of Histochemistry and Cytochemistry,* 34: 1095-1100.

Beelen, R. and Walker, W. (1983). Dynamics of cytochemically distinct subpopulations of macrophages in elicited rat peritoneal exudates. *Cellular Immunology,* 82: 246-257.

Beelen, R., Eestermans, I., Döpp, E. and Dijkstra, C. (1987). Monoclonal Antibodies ED1, ED2 and ED3 Against Macrophages: Expression of Recognized Antigens in Different Stages of Differentiation. *Transplantation Proceedings,* 19: 3166-3170.

Beertsen, W., Everts, V. and van den Hooff, A. (1974). Fine structure of fibroblasts in the periodontal ligament of the rat incisor and their possible role in tooth eruption. *Archives of Oral Biology,* 19: 1087-1098.

Beertsen, W., McCulloch, C.A. and Sodek, J. (1997). The periodontal ligament: a unique, multifunctional connective tissue. *Periodontology 2000,* 13: 20-40.

Beesley, J.E. (1993). Immunocytochemical avenues. *In: Immunocytochemistry. A Practical Approach.* Beesley, J.E., editor. Oxford University Press, New York, p. 7-13.

Bekker, P.J., Holloway, D., Nakanishi, A., Arrighi, M., Leese, P.T. and Dunstan, C.R. (2001). The effect of a single dose of osteoprotegerin in postmenopausal women. *Journal of Bone and Mineral Research,* 16: 348-360.

Bellido, T., Jilka, R.L., Boyce, B.F., Girasole, G., Broxmeyer, H., Dalrymple, S.A., Murray, R. and Manolagas, S.C. (1995). Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor. *Journal of Clinical Investigation,* 95: 2886-2895.

Berkovitz, B.K.B. (1988). Structural observations on the periodontal ligament in relation to the eruptive mechanism. *In: Biological Mechanisms of Tooth Eruption and Root Resorption.* Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, p. 227-291.

Berkovitz, B.K.B. (1990). The structure of the periodontal ligament: an update. *European Journal of Orthodontics,* 12: 51-76.

Berkovitz, B.K.B. and Shore, R.C. (1995). Cells of the Periodontal Ligament. *In: The Periodontal Ligament in Health and Disease.* Berkovitz, B.K.B., Moxham, B.J., Newman, H.N., editors. Mosby-Wolfe, London, p. 9-33.

Berod, A., Hartman, B.K. and Pujol, J.F. (1981). Importance of fixation in immunohistochemistry: use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. *Journal of Histochemistry and Cytochemistry,* 29: 844-850.

Bertolini, D., Nedwin, G., Bringman, T., Smith, D. and Mundy, G. (1986). Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumor necrosis factors. *Nature,* 319: 516-518.

Bianco, P., Ballanti, P. and Bonucci, E. (1988). Tartrate-resistant acid phosphatase activity in rat osteoblasts and osteocytes. *Calcified Tissue International,* 43: 167-171.

Birkedal-Hansen, H. (1973). External root resorption caused by luxation of rat molars. *Scandinavian Journal of Dental Research,* 81: 47-61.

Birkedal-Hansen, H., Butler, W.T. and Taylor, R.E. (1977). Proteins of the periodontium. Characterization of the insoluble collagens of bovine dental cementum. *Calcified Tissue Research,* 23: 39-44.

Bjurholm, A., Kreicbergs, A. and Schultzberg, M. (1989). Fixation and demineralization of bone tissue for immunohistochemical staining of neuropeptides. *Calcified Tissue International,* 45: 227-231.

Blair, H.C., Kahn, A.J., Crouch, E.C., Jeffrey, J.J. and Teitelbaum, S.L. (1986). Isolated osteoclasts resorb the organic and inorganic components of bone. *Journal of Cell Biology,* 102: 1164-1172.

Blair, H.C., Teitelbaum, S.L., Ghiselli, R. and Gluck, S. (1989). Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science,* 245: 855-857.

Blanco, C.E., Sieck, G.C. and Edgerton, V.R. (1988). Quantitative histochemical determination of succinic dehydrogenase activity in skeletal muscle fibres. *Histochemical Journal,* 20: 230-243.

Blavier, L. and Delaissé, J.M. (1995). Matrix metalloproteinases are obligatory for the migration of preosteoclasts to the developing marrow cavity of primitive long bones. *Journal of Cell Science,* 108: 3649-3659.

Blomlöf, L., Lindskog, S., Appelgren, R., Jonsson, B., Weintraub, A. and Hammarström, L. (1987). New attachment in monkeys with experimental periodontitis with and without removal of the cementum. *Journal of Clinical Periodontology,* 14: 136-143.

Boekenoogen, D.I., Sinha, P.K., Nanda, R.S., Ghosh, J., Currier, F. and Howes, R.I. (1996). The effects of exogenous prostaglandin E_2 on root resorption in rats. *Americal Journal of Orthodontics and Dentofacial Orthopedics,* 109: 277-286.

Boenisch, T. (1989). Basic Immunochemistry. *In: Immunochemical Staining Methods.* Naisch, S.J., editor. Dako Corporation, Carpinteria, California, p. 7-8.

Bonewald, L.F. and Mundy, G.R. (1990). Role of Transforming Growth Factor-Beta in Bone Remodeling. *Clinical Orthopaedics and Related Research,* 250: 261-276.

Bonucci, E. and Nanci, A. (2001). Alkaline phosphatase and tartrate-resistant acid phosphatase in osteoblasts of normal and pathologic bone. *Italian Journal of Anatomy and Embryology,* 106: 129-133.

Bosshardt, D.D. and Schroeder, H.E. (1996). Cementogenesis reviewed: A comparison between human premolars and rodent molars. *The Anatomical Record,* 245: 267-292.

Bosshardt, D.D. and Selvig, K.A. (1997). Dental cementum: the dynamic tissue covering of the root. *Periodontology 2000,* 13: 41-75.

Bosshardt, D.D., Masseredjian, V. and Nanci, A. (1998). Root resorption and tissue repair in orthodontically treated human premolars. *In: Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants.* Davidovitch, Z. and Mah, J., editors. EBSCO Media, Birmingham, Alabama, p. 425-437.

Brezniak, N. and Wasserstein, A. (1993a). Root resorption after orthodontic treatment: Part1. Literature review. *American Journal of Orthodontics and Dentofacial Orthopedics,* 103: 62-66.

Brezniak, N. and Wasserstein, A. (1993b). Root resorption after orthodontic treatment: Part 2. Literature review. *Americal Journal of Orthodontics and Dentofacial Orthopedics,* 103: 138-146.

Brezniak, N. and Wasserstein, A. (2002a). Orthodontically induced inflammatory root resorption. Part I: the basic science aspects. *Angle Orthodontist,* 72: 175-179.

Brezniak, N. and Wasserstein, A. (2002b). Orthodontically induced inflammatory root resorption. Part II: the clinical aspects. *Angle Orthodontist,* 72: 180-184.

Brice, G.L., Sampson, W.J. and Sims, M.R. (1991). An ultrastructural evaluation of the relationship between epithelial rests of Malassez and orthodontic root resorption and repair in man. *Australian Orthodontic Journal,* 12: 90-94.

Brown, W.S., Dewey, W.A. and Jacobs, H.R. (1970). Thermal Properties of Teeth. *Journal of Dental Research,* 49: 752-755.

Brudvik, P. and Rygh, P. (1993a). Non-clast cells start orthodontic root resorption in the periphery of hyalinized zones. *European Journal of Orthodontics,* 15: 467-480.

Brudvik, P. and Rygh, P. (1993b). The initial phase of orthodontic root resorption incident to local compression of the periodontal ligament. *European Journal of Orthodontics,* 15: 249-263.

Brudvik, P. and Rygh, P. (1994a). Root resorption beneath the main hyalinized zone. *European Journal of Orthodontics,* 16: 249-263.

Brudvik, P. and Rygh, P. (1994b). Multi-nucleated cells remove the main hyalinized tissues and start resorption of adjacent root surfaces. *European Journal of Orthodontics,* 16: 265-273.

Brudvik, P. and Rygh, P. (1994c). Root resorption associated with combined orthodontic/experimental tooth movement: the initial attack and the resorption - repair sequence on root surfaces exposed to compression. *In: The Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants,* Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, p. 557-575.

Brudvik, P. and Rygh, P. (1995a). Transition and determinants of orthodontic root resorption - repair sequence. *European Journal of Orthodontics,* 17: 177-188.

Brudvik, P. and Rygh, P. (1995b). The repair of orthodontic root resorption: an ultrastructural study. *European Journal of Orthodontics,* 17: 189-198.

Brunette, D.M., Heersche, J.N.M., Purdon, A.D., Sodek, J., Moe, H.K. and Assuras, J.N. (1979). In vitro cultural parameters and protein and prostaglandin secretion of epithelial cells derived from porcine rests of Malassez. *Archives of Oral Biology,* 24: 199-203.

Bucay, N., Sarosi, I., Dunstan, C.R., Morony, S., Tarpley, J., Capparelli, C., Scully, S., Tan, H.L., Xu, W., Lacey, D.L., Boyle, W.J. and Simonet, W.S. (1998). *Osteoprotegerin*-deficient mice develop early onset osteoporosis and arterial calcification. *Genes and Development,* 12: 1260-1268.

Buckwalter, J.A., Glimcher, M.J., Cooper, R.R. and Recker, R. (1995). Bone Biology. *The Journal of Bone and Joint Surgery,* 77-A: 1256-1275.

Burger, E.H. and Nijweide, P.J. (1992). Cellular Origin and Theories of Osteoclast Differentiation. *In: Bone.* Hall, B.K., editor. CRC Press, Boca Raton, Florida, p. 31-60.

Burger, D. and Dayer, J.M. (1995). Inhibitory cytokines and cytokine inhibitors. *Neurology,* 45: S39-S43.

Burger, E.H., van der Meer, J.W.M., van de Gevel, J.S., Gribnan, J.C., Thesingh, C.W. and van Furth, R. (1982). *In vitro* formation of osteoclasts from long term cultures of bone marrow mononuclear phagocytes. *Journal of Experimental Medicine,* 156: 1604-1614.

Burger, E.H., van der Meer, J.W.M. and Nijweide, P.J. (1984). Osteoclast formation from mononuclear phagocytes, role of bone-forming cells. *Journal of Cell Biology,* 99: 1901-1906.

Burleigh, M.C., Barrett, A.J. and Lazarus, G.S. (1974). Cathepsin B1. A lysosomal enzyme that degrades native collagen. *Biochemical Journal,* 137: 387-398.

Burnett, G.J. (1978). An autoradiographic and developmental study of transalveolar fibres in the mouse mandible. M.D.S. Thesis, The University of Adelaide, South Australia.

Burstone, M. (1960). Histochemical demonstration of succinic dehydrogenase activity in osteoclasts. *Nature,* 185: 866

Butler, W., Birkedal-Hansen, H. and Taylor, R. (1975). Proteins of the periodontium. The chain structure of the collagens of bovine cementum and periodontal ligament. *In: Extracellular Matrix Influences on Gene Expression.* Slavkin, H. and Greulick, R., editors. Academic press, New York, p. 371

Butler, W.T. (1998). Dentin matrix proteins. *European Journal of Oral Sciences,* 106(Suppl. 1): 204-210.

Campbell, H.D., Dionysius, D.A., Keough, D.T., Wilson, B.E., de Jersey, J. and Zerner, B. (1978). Iron-containing acid phosphatases: comparison of the enzymes from beef spleen and pig allantoic fluid. *Biochemical and Biophysical Research Communications,* 82: 615-620.

Canalis, E.M., McCarthy, T.L. and Centrella, M. (1989). Effects of platelet-derived growth factor on bone formation *in vitro*. *Journal of Cell Physiology,* 140: 530-537.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. and Williamson, B. (1975). An endotoxin induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Science USA,* 72: 3666-3670.

Cattoretti, G., Pileri, S., Paravicini, C., Becker, M.H., Poggi, S., Bifulco, C., Key, G., D'Amato, L., Sabattini, E. and Feudale, E. (1993). Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *Journal of Pathology,* 171: 83-98.

Cecchini, M.G. and Fleisch, H. (1990). Bisphosphonates in vitro specifically inhibit, among the hematopoietic series, the development of the mouse mononuclear phagocyte series. *Journal of Bone and Mineral Research,* 5: 1019-1027.

Chambers, T.J. (1980). The cellular basis of bone resorption. *Clinical Orthopaedics,* 151: 283-293.

Chambers, T.J. (1985). The pathobiology of the osteoclast. *Journal of Clinical Pathology,* 38: 241-252.

Chambers, T.J. (1988). The regulation of osteoclastic development and function. *In: Cell and Molecular Biology of Vertebrate Hard Tissues.* Evered, D. and Harnett, S., editors. John Wiley and Sons, New York, p. 92-100.

Chambers, T.J. (2000). Regulation of the differentiation and function of osteoclasts. *Journal of Pathology,* 192: 4-13.

Chambers, T.J. and Horton, M.A. (1984). Failure of cells of the mononuclear phagocyte series to resorb bone. *Calcified Tissue International,* 36: 556-568.

Chambers, T.J. and Fuller, K. (1985). Bone cells predispose bone surfaces to resorption by exposure of mineral to osteoclastic contact. *Journal of Cell Science,* 76: 155-165.

Chambers, T.J., Revell, P.A., Fuller, K. and Athanasou, N.A. (1984). Resorption of bone by isolated rabbit osteoclasts. *Journal of Cell Science,* 66: 383-399.

Chambers, T.J., McSheehy, P.M., Thomson, B.M. and Fuller, K. (1985a). The effect of calcium-regulating hormones and prostaglandin on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. *Endocrinology,* 116: 234-239.

Chambers, T.J., Darby, J.A. and Fuller, K. (1985b). Mammalian collagenase predisposes bone surfaces to osteoclastic resorption. *Cell and Tissue Research,* 241: 671-675.

Chang, C.C., Eshoa, C., Kampalath, B., Shidham, V.B. and Perkins, S. (2000). Immunophenotypic profile of myeloid cells in granulocytic sarcoma by immunohistochemistry. Correlation with blast differentiation in bone marrow. *American Journal of Clinical Pathology,* 114: 807-811.

Chantawiboonchai, P., Warita, H., Ohya, K. and Soma, K. (1998). Confocal laser scanning-microscopic observations on the three-dimensional distribution of oxytalan fibres in mouse periodontal ligament. *Archives of Oral Biology,* 43: 811-817.

Chapman, H.A., Riese, R.J. and Shi, G. (1997). Emerging roles for cysteine proteases in human biology. *Annual Reviews in Physiology,* 59: 63-88.

Chavrier, C., Hartmann, D.J., Couble, M.L. and Herbage, C. (1988). Distribution and organization of the elastic system fibres in healthy human gingiva: Ultrastructural and immunohistochemical study. *Histochemistry,* 89: 47-52.

Chen, J., Yam, L.T., Janckila, A.J., Li, C. and Lam, W.K.W. (1979). Significance of "high" acid phosphatase activity in the serum of normal children. *Clinical Chemistry,* 25: 719-722.

Chikazu, D., Katagiri, M., Ogasawara, T., Ogata, N., Shimoaka, T., Takato, T., Nakamura, K. and Kawaguchi, H. (2001). Regulation of osteoclast differentiation by fibroblast growth factor 2: stimulation of receptor activator kappa β ligand/osteoclast differentiation factor expression in osteoblasts and inhibition of macrophage colony-stimulating factor function in osteoclasts. *Journal of Bone and Mineral Research,* 16: 2074-2081.

Choi, S.J., Cruz, J.C., Craig, F., Chung, H., Devlin, R.D., Roodman, G.D. and Alsina, M. (2000). Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. *Blood,* 96: 671-675.

Choi, S.J., Han, J.H. and Roodman, G.D. (2001). ADAM8: a novel osteoclast stumulating factor. *Journal of Bone and Mineral Research,* 16: 814-822.

Chow, J. and Chambers, T.J. (1992). An assessment of the prevalence of organic matter on bone surfaces. *Calcified Tissue International,* 50: 118-122.

Chow, J.W., Fox, S.W., Lean, J.M. and Chambers, T.J. (1998). Role of nitric oxide and prostaglandins in mechanically induced bone resorption. *Journal of Bone and Mineral Research,* 13: 1039-1044.

Christiansen, C. (1991). Consensus Development Conference: prophylaxis and treatment of osteoporosis. *American Journal of Medicine,* 90: 107-110.

Chun, L., Yoon, J., Song, Y., Huie, P., Regula, D. and Goodman, S. (1999). The characterization of macrophages and osteoclasts in tissues harvested from revised total hip prostheses. *Journal of Biomedical Material Research,* 48: 899-903.

Clark, S.A., Ambrose, W.W., Anderson, T.R., Terrell, R.S. and Toverud, S.U. (1989). Ultrastructural localization of tartrate-resistant, purple acid phosphatase in rat osteoclasts by histochemistry and immunocytochemistry. *Journal of Bone and Mineral Research,* 4: 399-405.

Clark, A.B., Sims, M.R. and Leppard, P.I. (1991). An analysis of the effect of tooth intrusion on the microvasculature bed and fenestrae in the apical periodontal ligament of the rat molar. *Americal Journal of Orthodontics and Dentofacial Orthopedics,* 99: 21-29.

Clowry, G.J. and Flecknell, P.A. (2000). The successful use of fentanyl/fluanisone ('Hypnorm') as an anaesthetic for intracranial surgery in neonatal rats. *Laboratory Animals,* 34: 260-264.

Cobbold, S., Hale, G. and Waldmann, H. (1987). Non-lineage, LFA-1 family and leucocyte common antigens: newly and previously defined clusters. *In: Leucocyte Typing III.* McMichael, A.J., editor. Oxford University Press, Oxford, p. 788-803.

Coccia, P.F., Krivit, W., Cervenka, K., Clawson, C., Kersey, J.H., Kim, T.H., Nesbit, M.E., Ramsay, N.K., Warkentin, P.I., Teitelbaum, S.L., Kahn, A.J. and Brown, D.M. (1980). Successful bone-marrow transplantation for infantile malignant osteopetrosis. *New Engand Journal of Medicine,* 302: 701-708.

Coffman, R.L. and Weissman, I.L. (1981). B220: a B cell-specific member of the T200 glycoprotein family. *Nature,* 289: 681-683.

Cohn, S.A. (1972a). A re-examination of Sharpey's fibres in alveolar bone of the mouse. *Archives of Oral Biology,* 17: 255-260.

Cohn, S.A. (1972b). A re-examination of Sharpey's fibres in alveolar bone of the marmoset (Sanguinus fusicollis). *Archives of Oral Biology,* 17: 261-269.

Cole, A.A. and Walters, L.M. (1987). Tartrate-resistant acid phosphatase in bone and cartilage following decalcification and cold-embedding in plastic. *Journal of Histochemistry and Cytochemistry,* 35: 203-206.

Collin-Osdoby, P., Oursler, M.J., Rothe, L., Webber, D., Anderson, F. and Osdoby, P. (1995). Osteoclast 121F antigen expression during osteoblast conditioned medium induction of osteoclast-like cells *in vitro*: relationship to calcitonin responsiveness, tartrate-resistant acid phosphatase levels, and bone resorptive activity. *Journal of Bone and Mineral Research,* 10: 45-58.

Collin-Osdoby, P., Rothe, L., Anderson, F., Nelson, M., Malony, W. and Osdoby, P. (2001). Receptor activator of NF-kappa B and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. *Journal of Biological Chemistry,* 276: 20659-20672.

Connolly, M., Bazmi, K., O'Connell, M., Lyons, J.F. and Bourke, J.F. (2001). Cryotherapy of viral warts: a sustained 10-s freeze is more effective than the traditional method. *British Journal of Dermatology,* 145: 554-547.

Cooper, S.M. and Dawber, R.P.R. (2001). The history of cryosurgery. *Journal of the Royal Society of Medicine,* 94: 196-201.

Cooper, S.M., Sims, M.R., Sampson, W.J. and Dreyer, C.W. (1990). A morphometric analysis of tissue channels by electron microscopy of ionic tracer in normal and tensioned rat molar apical periodontal ligament. *Archives of Oral Biology,* 35: 499-507.

Craig, R.G. and Peyton, F.A. (1961). Thermal Conductivity of Tooth Structure, Dental Cements, and Amalgam. *Journal of Dental Research,* 40: 411-418.

Crowe, P.R. (1989). The marmoset periodontal ligament: A T.E.M. morphometric analysis following incisor crown fracture, root canal therapy, and orthodontic extrusion. MDS Thesis, The University of Adelaide, South Australia.

Cvek, M. and Lindvall, A. (1985). External root resorption following bleaching of pulpless teeth with oxygen peroxide. *Endodontics and Dental Traumatology,* 1: 56-60.

Damoiseaux, J., Dopp, E.A., Beelen, R.H. and Dijkstra, C.D. (1989). Rat bone marrow and monocyte cultures: influence of culture time and lymphokines on the expression of macrophage differentiation antigens. *Journal of Leucocyte Biology,* 46: 246-253.

Damoiseaux, J.G., Döpp, E.A., Calame, W., Chao, D., MacPherson, G.G. and Dijkstra, C.D. (1994). Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. *Immunology,* 83: 140-147.

Dantzig, A.H., Minor, P.L., Garrigus, J.L., Fukuda, D.S. and Mynderse, J.S. (1991). Studies on the Mechanism of Action of A80915A, a Semi- Naphthoquinone Natural Product, as as Inhibitor of Gastric (H^+- K^+)-ATPase. *Biochemical Pharmacology,* 41: 2019-2026.

Darnay, B.G., Haridas, V., NI, J., Moore, P.A. and Aggarwal, B.B. (1998). Characterization of the Intracellular Domain of Receptor Activator of NF-kB (RANK). *The Journal of Biological Chemistry,* 273: 20551-20555.

Davidovitch, Z. (1991). Tooth movement. *Critical Reviews in Oral Biology and Medicine,* 2: 411-450.

Davidovitch, Z. (1996). Etiologic factors in force-induced root resorption. *In: Biological Mechanisms of Tooth Movement and Craniofacial Adaptation.* Davidovitch, Z. and Norton, L.A., editors. EBSCO Media, Birmingham, Alabama, p. 349-356.

Davidovitch, Z., Finkelson, M.D., Steigman, S., Shanfield, J.L., Montgomerey, P.C. and Korostoff, E. (1980). Electric currents, bone remodeling and orthodontic tooth movement. II. Increase in rate of tooth movement and periodontal cyclic nucleotide levels by combined force and electric current. *American Journal of Orthodontics,* 77: 33-47.

Davidovitch, Z., Nicolay, O.F., Ngan, P.W. and Shanfeld, J.L. (1988). Neurotransmitters, cytokines and the control of alveolar bone remodeling in orthodontics. *Dental Clinics of North America,* 32: 411-435.

Davidovitch, Z., Lee, Y.J., Counts, A.L., Park, Y. and Bursac, Z. (2000). The immune system possibly modulates orthodontic root resorption. *In: Biological Mechanisms of Tooth Movement and Craniofacial Adaptation.* Davidovitch, Z. and Mah, J., editors. EBSCO Media, Birmingham, Alabama, p. 207-217.

Davies, J., Warwick, J., Totty, N., Philp, R., Helfrich, M. and Horton, M. (1989). The Osteoclast Functional Antigen, Implicated in the Regulation of Bone Resorption, Is Biochemically Related to the Vitronectin Receptor. *The Journal of Cell Biology,* 109: 1817-1826.

de la Piedra, C., Torres, R., Rapado, A., Curiel, M.D. and Castro, N. (1989). Serum tartrate-resistant acid phosphatase and bone mineral content in postmenopausal osteoporosis. *Calcified Tissue International,* 45: 58-60.

de St Georges, L., Miller, S.C., Bowman, B.M. and Jee, W.S.S. (1989). Ultrastructural features of osteoclasts *in situ*. *Scanning Microscopy,* 3: 1201

Debari, K., Sasaki, T., Udagawa, N. and Rifkin, B.R. (1995). An Ultrastructural Evaluation of the Effects of Cysteine-Proteinase Inhibitors on Osteoclastic Bone Resorption. *Calcified Tissue International,* 56: 566-570.

Delaissé, J.M. and Vaes, L.G. (1992). Mechanism of Mineral Solubilization and Matrix Degradation in Osteoclastic Bone Resorption. *In: Biology and Physiology of the Osteoclast.* Rifkin, B.R. and Gay, C.V., editors. CRC Press, Boca Raton, p. 289-314.

Delaissé, J.M., Eeckhout, Y. and Vaes, G. (1980). Inhibition of bone resorption in culture by inhibitors of thiol proteinases. *Biochemical Journal,* 192: 365-368.

Delaissé, J.M., Eeckhout, Y. and Vaes, G. (1984). *In vivo* and *in vitro* evidence for the involvement of cysteine proteinases in bone resorption. *Biochemical and Biophysical Research Communications,* 125: 441-447.

Delaissé, J.M., Ledent, P. and Vaes, G. (1991). Collagenolytic cysteine proteinases of bone tissue: Cathepsin B, (pro)cathepsin L and a cathepsin L-like 70 kDa proteinase. *Biochemical Journal,* 279: 167-174.

Delaissé, J.M., Eeckhout, Y., Neff, L., Francois-Gillet, C., Henriet, P., Su, Y., Vaes, G. and Baron, R. (1993). (Pro)collagenase (matrix metalloproteinase-1) is present in rodent osteoclasts and in the underlying bone-resorbing compartment. *Journal of Cell Science,* 106: 1071-1082.

Delaissé, J.M., Ensig, M.T., Everts, V., del Carmen Overjero, M., Ferreras, M., Lund, L., Vu, T.H., Werb, Z., Winding, B., Lochter, A., Karsdal, M.A., Troen, T., Kirkegaard, T,. Lenhard, T.,

Heegaard, A.M., Neff, L., Baron, R. and Foged, N.T. (2000). Proteinases in bone resorption: obvious and less obvious roles. *Clinica Chimica Acta,* 291: 223-234.

Delany, A.M., Dong, Y. and Canalis, E. (1994). Mechanisms of glucocorticoid action on bone cells. *Journal of Cellular Biochemistry,* 56: 295-302.

Dersot, J.M., Colombier, M.L., Lafont, J., Baroukh, B., Septier, D. and Saffar, J.L. (1995). Multinucleated Giant Cells Elicited Around Hydroxyapatite Particles Implanted in Craniotomy Defects Are Not Osteoclasts. *The Anatomical Record,* 242: 166-176.

Diekwisch, T.G. (2001). The developmental biology of cementum. *International Journal of Developmental Biology,* 45: 695-706.

Dijkstra, C.D., Döpp, E.A., Joling, P. and Kraal, G. (1985). The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology,* 54: 589-599.

Doi, K., Antanaitis, B.C. and Aisen, P. (1988). The binuclear iron centers of uteroferrin and the purple acid phosphatases. *Structural Bonding,* 70: 1-26.

Dolce, C., Malone, S. and Wheeler, T.T. (2002). Current Concepts in the Biology of Orthodontic Tooth Movement. *Seminars in Orthodontics,* 8: 6-12.

Domon, T., Sugaya, K., Yawaka, Y., Osanai, M., Hanaizumi, Y., Takahashi, S. and Wakita, M. (1994). Electron Microscopic and Histochemical Studies of the Mononuclear Odontoclast of the Human. *The Anatomical Record,* 240: 42-51.

Domon, T., Yasuda, M., Osanai, M., Suzuki, R., Takahashi, S., Yamamoto, T. and Wakita, M. (1998). Increase in odontoclast nuclei number by cell fusion: a three-dimensional reconstruction of cell fusion of human odontoclasts. *The Anatomical Record,* 252: 462-471.

Donnelly, E.T., McClure, N. and Lewis, S.E. (2001). Cryopreservation of human semen and prepared sperm: Effects on motility parameters and DNA integrity. *Fertility and Sterility,* 76: 892-900.

Dorey, C.K. and Bick, K.L. (1977a). Ultrastructural analysis of glycosaminoglycan hydrolysis in the rat periodontal ligament. II. Aryl sulfatase and bone resorption. *Calcified Tissue Research,* 24: 143-149.

Dorey, C.K. and Bick, K.L. (1977b). Ultrastructural analysis of glycosaminoglycan hydrolysis in the rat periodontal ligament. I. Evidence for macrophage involvement in bone remodelling. *Calcified Tissue Research,* 24: 135-141.

Doussis, I.A., Puddle, B. and Athanasou, N.A. (1992). Immunophenotype of multinucleated and mononuclear cells in giant cell lesions of bone and soft tissue. *Journal of Clinical Pathology,* 45: 398-404.

Drake, F.H., Dodds, R.A., James, I.E., Conner, J.R., Debouck, C., Richardson, S., Lee-Rykaczewski, E., Coleman, L., Rieman, D., Barthlow, R., Hastings, G. and Gowen, M. (1996). Cathepsin K, but not cathepsis B, L, or S is abundantly expressed inhuman osteoclasts. *Journal of Biological Chemistry,* 271: 12511-12516.

Dreyer, C.W. and Sampson, W.J. (1984). Effects of lathyrism on mouse molar migration. *Journal of Periodontal Research,* 19: 424-433.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L. and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell,* 89: 747-754.

Ducy, P. (2000). Cbfa1: a molecular switch in osteoblast biology. Developmental Dynamics, 219: 461-471.

Dunstan, C.R., Capparelli, C., Kelley, M., Shimamoto, G., Kaufman, S. and Russell, D. (1997). Osteoprotegerin (OPG), a novel TNF-receptor family member, profoundly inhibits endosteal bone resorption due to growth, humoral hypercalcemia of malignancy, and interleukin 1. [Abstract] *Journal of Bone and Mineral Research,* 12 (Suppl.1):S136

Duong, L.T. and Rodan, G.A. (2001). Regulation of osteoclast formation and function. *Reviews in Endocrinology and Metabolic Disorders,* 2: 95-104.

Ek-Rylander, B., Bergman, T. and Andersson, G. (1991). Characterization of a Tartrate-Resistant Acid Phosphatase (ATPase) from Rat Bone: Hydrodynamic Properties and N-Terminal Amino Acid Sequence. *Journal of Bone and Mineral Research,* 6: 365-373.

Emery, J., MacDonnell, P., Burke, M.B., Deen, K.C., Lyn, S., Silverman, C., Dul, E., Appelbaum, E.R., Eichman, C., DiPrinzio, R., Dodds, R.A., James, I.E., et al. (1998). Osteoprotegerin Is a Receptor for the Cytotoxic Ligand TRAIL. *Journal of Biological Chemistry,* 273: 14363-14367.

Etherington, D.J. (1972). The nature of the collagenolytic cathepsin of rat liver and its distribution in other rat tissues. *Biochemical Journal,* 127: 685-692.

Everts, V., Delaissé, J.M., Korper, W., Niehof, A., Vaes, G. and Beertsen, W. (1992). Degradation of collagen in the bone-resorbing compartment underlying the osteoclast involves both cysteine-proteinases and matrix metalloproteinases. *Journal of Cell Physiology,* 150: 221-231.

Facchetti, F., Bertalot, G. and Grigolato, P.G. (1991). KP1 (CD68) staining of malignant melanomas. *Histopathology,* 19: 141-145.

Fahimi, H.D. (1979). An assessment of the DAB method for cytochemical detection of catalase and peroxidase. *Journal of Histochemistry and Cytochemistry,* 27: 1365-1366.

Fahy, G.M. and Ali, S.E. (1997). Cryopreservation of the mammalian kidney. II. Demonstration of immediate *ex vivo* function after introduction and removal of 7.5 M cryoprotectant. *Cryobiology,* 35: 114-131.

Fallon, M.D. (1984a). Bone resorbing fluid from osteoclasts is acidic. An *in vitro* micropuncture study. *In: Endocrine control of Bone and Calcium Metabolism, vol 4.* Cohn, D.V., Fujita, T., Potts Jr, J.T., Talmage, R.V., editors. Elsevier Science Publisher, Amsterdam, p. 144-146.

Fallon, M.D. (1984b). Alterations in the pH of osteoclast resorbing fluid reflects changes in bone degradative activity. *Calcified Tissue International,* 36: 458

Fallon, M.D., Teitelbaum, S.L. and Kahn, A.J. (1983). Multinucleation Enhances Macrophage-Mediated Bone Resorption. *Laboratory Investigation,* 49: 159-164.

Farmilo, A.J. and Stead, R.H. (1989). Fixation in Immunocytochemistry. *In: Immunochemical Staining Methods.* Naisch, S.J., editors. Dako Corporation, Carpinteria, California, p. 24-29.

Farrell, L.E., Yen, E.H.K., Brudvik, P. and Rygh, P. (1990). Identification of orthodontically induced root resorptive cells using TRAP stain. [Abstract] *Journal of Dental Research,* 69:201

Faust, J., Hunt, P., Scully, S. and Shalhoub, V. (1998). Multiple myeloma cells and cells of the human osteoclast lineage share morphological and cell surface markers. *Journal of Cellular Biochemistry,* 71: 559-568.

Faust, J., Lacey, D.L., Hunt, P., Burgess, T.L., Scully, S., Van, G., Eli, A., Qian, Y. and Shalhoub, V. (1999). Osteoclast markers accumulate on cells developing from human peripheral blood mononuclear precursors. *Journal of Cellular Biochemistry,* 72: 67-80.

Fazzalari, N.L., Kuliwaba, J.S., Atkins, G.J., Forwood, M.R. and Findlay, D.M. (2001). The ratio of messenger RNA levels of receptor activator of nuclear factor kappaB ligand to osteoprotegerin correlates with bone remodeling indices in normal human cancellous bone but not in osteoarthritis. *Journal of Bone and Mineral Research,* 16: 1015-1027.

Femiano, F., Scully, C., Laino, G. and Battista, G. (2001). Benign fibrous histiocytoma (BFH) of the cheek: CD 68-KP1 positivity. *Oral Oncology,* 37: 673-675.

Fisher, J.E., Caufield, M.P., Sato, M., Quartuccio, H.A., Gould, R.J., Garsky, V.M., Rodan, G.A. and Rosenblatt, M. (1993). Inhibition of osteoclastic bone resorption *in vivo* by echistatin, an "arginyl-glycyl-aspartyl" (RGD)-containing protein. *Endocrinology,* 132: 1411-1413.

Fleisch, H., Russell, R.G.G. and Francis, M.D. (1969). Diphosphonates inhibit hydroxyapatite dissolution *in vitro* and bone resorption in tissue culture and *in vivo*. *Science,* 165: 1262-1264.

Fox, C.H. and Benton, C. (1987). Formaldehyde: The Fixative. *The Journal of Histotechnology,* 10: 199-201.

Fox, C.H., Johnson, F.B., Whiting, J. and Roller, P.P. (1985). Formaldehyde fixation. *The Journal of Histochemistry and Cytochemistry,* 33: 845-853.

Fraser, J. and Gill, W. (1967). Observations on ultra-frozen tissue. *British Journal of Surgery,* 54: 770-776.

Freilich, L.S. (1971). Ultrastructure and Acid Phosphatase Cytochemistry of Odontoclasts: Effects of Parathyroid Extract. *Journal of Dental Research Supplement to No. 5,* 50: 1047-1055.

Frost, H.M. (1989). Some ABCs of skeletal pathophysiology. III. Bone balance and the delta B.BMU. *Calcified Tissue International,* 45: 131-133.

Frost, H.M. and Jee, W.S.S. (1992). On the rat model of human osteopenias and osteoporosis. *Bone and Mineral,* 18: 227-236.

Fuller, K. and Chambers, T.J. (1995). Localization of mRNA for collagenase in osteocytic, bone surface and chondrocytic cells but not in osteoclasts. *Journal of Cell Science,* 108: 2221-2230.

Fuller, K., Owens, J. and Chambers, T.J. (1995). Macrophage inflammatory protein-1 alpha and IL-8 stimulate the motility but suppress the resorption of isolated rat osteoclasts. *Journal of Immunology,* 154: 6065-6072.

Fuller, K., Murphy, C., Kirstein, B., Fox, S.W. and Chambers, T.J. (2002). TNF alpha potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL. *Endocrinology,* 143: 1108-1118.

Fullmer, H.M. (1959). Observations on the development of oxytalan fibres in the periodontium of man. *Journal of Dental Research,* 38: 510-516.

Fullmer, H.M. (1964). Dehydrogenases in Developing Bone in the Rat. *Journal of Histochemistry and Cytochemistry,* 12: 210-214.

Furseth, R. (1968). The resorption process of human deciduous teeth studied by light microscopy, microradiography and electron microscopy. *Archives of Oral Biology,* 13: 417-431.

Gage, A.A. (1982). Current Issues in Cryosurgery. *Cryobiology,* 19: 219-222.

Gaikwad, J.S., Cavender, A. and D'Souza, R.N. (2001). Identification of tooth-specific downstream targets of Runx2. *Gene,* 279; 91-97.

Garant, P.R. and Cho, M.I. (1979). Cytoplasmic polarization of periodontal ligament fibroblasts. *Journal of Periodontal Research,* 14: 95-106.

Gawad, K.A., Schneider, C., Brinkin, B., Huflander, A., Diederichs, B., Mack, D., Gutsensohn, K. and Izbicki, J.R. (2001). Anti-TNF antibody treatment has no positive effect on survival in a model of pneumococcal sepsis in pigs. *Journal of Investigative Surgery,* 14: 291-297.

Gay, C.V. (1992). Osteoclast Ultrastructure and Enzyme Histochemistry: Functional Implications. *In: Biology and Physiology of the Osteoclast.* Rifkin, B.R. and Gay, C.V., editors. CRC Press, Boca Raton, p. 129-150.

Gay, C.V. and Mueller, W.J. (1974). Carbonic anhydrase and osteoclasts: Localization by labelled inhibitor autoradiography. *Science,* 183: 432-434.

Gay, S., Viljanto, J. and Raekillio, H. (1978). Collagen types in early phases of wound healing in children. *Acta Chirurgica Scandinavica,* 144: 205-211.

Gay, C.V., Schraer, H., Anderson, R.E. and Cao, H. (1983). Current studies on the localization and function of carbonic anhydrase in osteoclasts. *Annals of the New York Academy of Science,* 429: 473-478.

Ghosh, S., May, M.J. and Kopp, E.B. (1998). NF-kB AND REL PROTEINS: Evolutionarily Conserved Mediators of Immune Responses. *Annual Reviews in Immunology,* 16: 225-260.

Gibson, W.A. and Fullmer, H.M. (1966). Histochemistry of the Periodontal Ligament: 1. The Dehydrogenases. *Periodontics,* 4: 63-70.

Gilles, J.A., Carnes, D.L. and Windeler, A.S. (1994). Development of an *in vitro* culture system for the study of osteoclast activity and function. *Journal of Endodontics,* 20: 327-331.

Ginsberg, M.H., Loftus, J.C. and Plow, E.F. (1988). Cytoadhesions, integrins, and platelets. *Thrombosis and Haemostasis,* 59: 1-6.

Glauert, A.M. (1975). Fixatives. *In: Fixation, Dehydration and Embedding of Biological Specimens.* Glauert, A.M., editor. Elsevier/North-Holland Biomedical Press, Amsterdam, p. 5-72.

Glomset, J.A. (1959). The further purification and properties of a phosphatase from spleen able to hydrolyze completely the phosporous of α-casein. *Biochimica Biophysica Acta,* 32: 349-357.

Goldberg, A.F. and Barka, T. (1962). Acid phosphatase activity in human blood cells. *Nature,* 195: 297

Gori, F., Hofbauer, L.C., Dunstan, C.R., Spelsberg, T.C., Khosla, S. and Riggs, B.L. (2001). The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. *Endocrinology,* 141: 4768-4776.

Goto, T., Tsukuba, T., Kiyoshima, T., Nishimura, Y., Kato, K., Yamamoto, K. and Tanaka, T. (1993). Immunohistochemical localization of cathepsins B, D and L in the rat osteoclast. *Histochemistry,* 99: 411-414.

Goto, T., Kiyoshima, T., Moroi, R., Tsukuba, T., Nishimura, Y., Himeno, M., Yamamoto, K. and Tanaka, T. (1994). Localization of cathepsins B, D and L in the rat osteoclast by immuno-light and -electron microscopy. *Histochemistry,* 101: 33-40.

Gowen, M., Nedwin, G.E. and Mundy, G.R. (1986). Preferential inhibition of cytokine-stimulated bone resorption by recombinant interferon gamma. *Journal of Bone and Mineral Research,* 1: 469-474.

Grant, D.A. and Bernick, S. (1972). The formation of the periodontal ligament. *Journal of Periodontology,* 43: 17-25.

Grant, D.A., Bernick, S., Levy, B.M. and Dreizen, S. (1972). A comparative study of periodontal ligament development in teeth with and without predecessors in marmosets. *Journal of Periodontology,* 43: 162-169.

Gravallese, E.M., Harada, Y., Wang, J.T., Gorn, A.H., Thornhill, T.S. and Goldring, S.R. (1998). Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis. *American Journal of Pathology,* 152: 943-951.

Greene, E.C. (1971). Gross Anatomy. *In: The Rat in Laboratory Investigation.* Farris, E.J. and Griffith, J.J.Q., editors. Hafner Publishing Co. New York, p. 24-51.

Greenfield, E.M., Bi, Y. and Miyauchi, A. (1999). Regulation of Osteoclast Activity. *Life Sciences,* 65: 1087-1102.

Griffen, J.D., Ritz, J., Nadler, L.M. and Schlossman, S.F. (1981). Expression of myeloid differentiation antigens on normal and malignant myeloid cells. *Journal of Clinical Investigation,* 68: 932-941.

Hall, B.K. (1975). The Origin and Fate of Osteoclasts. *The Anatomical Record,* 183: 1-12.

Hall, B.K. (1991). The Osteoclast. *In: Bone Volume 2.* Hall, B.K., editor. CRC Press, Boca Raton,

Hall, G.E. and Kenny, A.D. (1985a). Carbonic anhydrase in bone resorption induced by prostaglandin E_2 *in vitro. Pharmacology,* 30: 339-347.

Hall, G.E. and Kenny, A.D. (1985b). Role of carbonic anhydrase in bone resorption induced by 1,25 dihydroxyvitamin D_3 *in vitro. Calcified Tissue International,* 37: 134-142.

Halleen, J.M., Alatalo, S.L., Suominen, H., Cheng, S., Janckila, A.J. and Väänänen, H.K. (2000). Tartrat-resistant acid phosphatase 5b: a novel marker of bone resorption. *Journal of Bone and Mineral Research,* 15: 1337-1345.

Hamamoto, Y., Suzuki, I., Nakajima, T. and Ozawa, H. (1991). Immunohistochemical localization of laminin in the epithelial rests of Malassez in immature rat molars. *Archives of Oral Biology,* 36: 623-626.

Hammarström, L., Hanker, J.S. and Toverud, S.U. (1971). Cellular Differences in Acid Phosphatase Isoenzymes in Bone and Teeth. *Clinical Orthopaedics and Related Research,* 78: 151-167.

Hammarström, L. and Lindskog, S. (1985). General morphological aspects of resorption of teeth and alveolar bone. *International Endodontic Journal,* 18: 93-108.

Hammarström, L., Alatli, I. and Fong, C.D. (1996). Origins of cementum. *Oral Diseases,* 2: 63-69.

Hancox, N.M. (1972). Osteoclastic Bone Resorption. *In: Biology of Bone.* Hancox, N.M., editor. Cambridge University Press, Cambridge, England, p 113-135

Harms, G., Dijkstra, C.D. and Hardonk, M.J. (1990). Glycosyl receptors in macrophage subpopulations of rat spleen and lymph node. A comparative study using neoglycoproteins and monoclonal antibodies ED1, ED2 and ED3. *Cell and Tissue Research,* 262: 35-40.

Hatano, E. and Brenner, D.A. (2001). Akt protects mouse hepatocytes from TNF-alpha- and Fas-mediated apoptosis through NK-kappaB activation. *American Journal of Physiology. Gastrointestinal and Liver Physiology,* 281: G1357-G1368.

Hattersley, G. and Chambers, T.J. (1989a). Calcitonin Receptors as Markers for Osteoclastic Differentiation: Correlation between Generation of Bone-Resorptive Cells and Cells that Express Calcitonin Receptors in Mouse Bone Marrow Cultures. *Endocrinology,* 125: 1606-1612.

Hattersley, G. and Chambers, T.J. (1989b). Generation of Osteoclastic Function in Mouse Bone Marrow Cultures: Multinuclearity and Tartrate-Resistant Acid Phosphatase Are Unreliable Markers for Osteoclastic Differentiation. *Endocrinology,* 124: 1689-1696.

Hattersley, G. and Chambers, T.J. (1990). Effects of interleukin 3 and of granulocyte-macrophage and macrophage colony stimulating factors on osteoclast differentiation from mouse hemopoietic tissue. *Journal of Cell Physiology,* 142: 201-209.

Hattersley, G. and Chambers, T.J. (1998). Calcitonin receptors as markers for osteoclastic differentiation: correlation between generation of bone-resorptive cells and cells that express calcitonin receptors in mouse bone marrow cultures. *Endocrinology,* 125: 1606-1612.

Hattersley, G., Owens, J., Flanagan, A.M. and Chambers, T.J. (1991a). Macrophage colony stimulating factor (M-CSF) is essential for osteoclast formation *in vitro*. *Biochemical and Biophysical Research Communications,* 177: 526-531.

Hattersley, G., Kerby, J.A. and Chambers, T.J. (1991b). Identification of Osteoclast Precursors in Multilineage Hemopoietic Colonies. *Endocrinology,* 128: 259-262.

Hay, E.D. (1991). Collagen and other matrix glycoproteins in embryogenesis. *In: Cell biology of extracellular matrix in development.* Hay, E.D., editors. Plenum Press, New York, p. 419-462.

Haynes, D.R., Crotti, T.N., Loric, M., Bain, G.I., Atkins, G.J. and Findlay, D.M. (2001). Osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint. *Rheumatology,* 40: 623-630.

Heersche, J.N.M. (1978). Mechanism of osteoclastic bone resorption. A new hypothesis. *Calcified Tissue Research,* 26: 81-84.

Heithersay, G.S. (1999a). Clinical, radiologic, and histopathologic features of invasive cervical root resorption. *Quintessence International,* 30: 27-37.

Heithersay, G.S. (1999b). Invasive cervical resorption: An analysis of potential predisposing factors. *Quintessence International,* 30: 83-95.

Helander, K.G. (2000). Formaldehyde prepared from paraformaldehyde is stable. *Biotechnic and Histochemistry,* 75: 19-22.

Helfrich, M.H., Nesbitt, S.A., Dorey, E.L. and Horton, M.A. (1992). Rat osteoclasts adhere to a wide range of RGD (Asp-Gly-Asp) peptide-containing proteins, including the bone sialoproteins and fibronectin, via a beta 3 integrin. *Journal of Bone and Mineral Research,* 7: 335-343.

Hellden, L. (1972). Periodontal healing following experimental injury to root surfaces of human teeth. *Scandinavian Journal of Dental Research,* 80: 197-205.

Hentunen, T.A., Lakkakorpi, P.T., Tuukkanen, J., Lehenkari, P.P., Sampath, T.K. and Väänänen, H.K. (1995). Effects of recombinant human osteogenic protein-1 on the differentiation of osteoclast-like cells and bone resorption. *Biochemical and Biophysical Research Communications,* 209: 433-443.

Herrlich, P. and Ponta, H. (1989). "Nuclear" oncogenes convert extracellular stimuli into changes in genetic program. *Trends in Genetics,* 5: 112-115.

Hertsens, R., Jacob, W. and Van Bogaert, A. (1984). Effect of hypnorm, chloralosane and pentobarbital on the ultrastructure of the inner membrane of rat heart mitochondria. *Biochimica Biophysica Acta,* 769: 411-418.

Hess, R., Scarpelli, D.G. and Pearse, A.G.E. (1958). Cytochemical Localization of Pyridine Nucleotide-linked Dehydrogenases. *Nature,* 181: 1531-1532.

Heymann, D., Guicheux, J., Gouin, F., Passuti, N. and Daculsi, G. (1998). Cytokines, growth factors and osteoclasts. *Cytokine,* 10: 155-168.

Hofbauer, L.C. and Heufelder, A.E. (2001). Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. *Journal of Molecular Medicine,* 79: 243-253.

Hofbauer, L.C., Gori, F., Riggs, B.L., Lacey, D.L., Dunstan, C.R., Spelsberg, T.C. and Khosla, S. (1999a). Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. *Endocrinology,* 140: 4382-4389.

Hofbauer, L.C., Lacey, D.L., Dunstan, C.R., Spelsberg, T.C., Riggs, B.L. and Khosla, S. (1999b). Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone,* 25: 255-259.

Hofbauer, L.C., Khosla, S., Dunstan, C.R., Lacey, D.L., Boyle, W.J. and Riggs, B.L. (2000). The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *Journal of Bone and Mineral Research,* 15: 2-12.

Hohmann, E.L., Levine, L. and Tashjian, A.H. (1983). Vasoactive intestinal peptide stimulates bone resorption via a cyclic adenosine 3',5'-monophosphate-dependent mechanism. *Endocrinology,* 112: 1233-1239.

Holtrop, M.E. (1990). Light and Electron Microscopic Structure of Bone Forming Cells. *In: Bone Volume 1: The Osteoblast and Osteocyte.* Hall, B.K., editor. The Telford Press, New Jersey, p. 1-39.

Holtrop, M.E. (1992). Light and Electronmicroscopic Structure of Osteoclasts. *In: Bone Volume 2. The Osteoclast.* Hall, B.K., editor. CRC Press, Boca Raton, Florida, p. 1-29.

Holtrop, M.E. and King, G.J. (1977). The ultrastructure of the osteoclast and its functional implications. *Clinical Orthopedics,* 123: 177-196.

Hopwood, D. (1969). Fixatives and fixation: a review. *Histochemical Journal,* 1: 323-360.

Hopwood, D. (1985). Cell and tissue fixation, 1972-1982. *Histochemical Journal,* 17: 389-442.

Horny, H.P., Schaumburg-Lever, G., Bolz, S., Geerts, M.L. and Kaiserling, E. (1990). Use of monoclonal antibody KP1 for identifying normal and neoplastic human mast cells. *Journal of Clinical Pathology,* 45: 89-90.

Horowitz, M.C., Xi, Y., Wilson, K.M. and Kacena, M.A. (2001). Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands. *Cytokine and Growth Factor Reviews,* 12: 9-18.

Horton, M.A. and Chambers, T.J. (1986). Human osteoclast-specific antigens are expressed by osteoclasts in a wide range of non-human species. *British Journal of Experimental Pathology,* 67: 95-104.

Horton, M.A. and Davies, J. (1989). Perspectives: adhesion receptors in bone. *Journal of Bone and Mineral Research,* 4: 803-808.

Horton, M.A., Rimmer, E.F., Lewis, D., Pringle, J.A.S., Fuller, K. and Chambers, T.J. (1984). Cell surface characterization of the human osteoclast: Phenotypic relationship to other bone marrow-derived cell types. *Journal of Pathology,* 144: 281-294.

Horton, M.A., Lewis, D., McNulty, K., Pringle, J.A.S. and Chambers, T.J. (1985a). Monoclonal Antibodies to Osteoclastomas (Giant Cell Bone Tumors): Definition of Osteoclastic-specific Cellular Antigens. *Cancer Research,* 45: 5663-5669.

Horton, M.A., Pringle, J.A.S. and Chambers, T.J. (1985b). Identification of human osteoclasts with monoclonal antibodies. *The New England Journal of Medicine,* 312: 923-924.

Horton, M.A., Rimmer, E.F., Moore, A. and Chambers, T.J. (1985c). On the Origin of the Osteoclast: The Cell Surface Phenotype of Rodent Osteoclasts. *Calcified Tissue International,* 37: 46-50.

Horton, M.A., Taylor, M.L., Arnett, T.R. and Helfrich, M.H. (1991). Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit resorption and cell spreading by osteoclasts. *Experimental Cell Research,* 195: 368-375.

Hsu, S.M. and Hsu, P.L. (1994). The nature of Reed-Sternberg cells: phenotype, genotype, and other properties. *Critical Reviews in Oncogenetics,* 5: 213-245.

Hsu, S.M., Raine, L. and Fanger, H. (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *Journal of Histochemistry and Cytochemistry,* 29: 577-580.

Hsu, H., Lacey, D.L., Dunstan, C.R., Solovyev, I., Colombero, A., Timms, E., Tan, H.L., Elliott, G., Kelley, M.J., Sarosh, I., Wang, L., Xia, X.Z., Elliott, R., Chiu, L., Black, T., Capparelli, C., Moroney, S., Shimamoto, G., Bass, M.B. and Boyle, W.J. (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proceedings of the National Academy of Science USA,* 96: 3540-3545.

Hughes, D.E., Wright, K.R., Uy, H.L., Sasaki, A., Yoneda, T., Roodman, G.D., Mundy, G.R. and Boyce, B.F. (1995). Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo.*. *Journal of Bone and Mineral Research,* 10: 1478-1487.

Hynes, R.O. (1987). Integrins: a family of cell surface receptors. *Cell,* 48: 549-554.

Igarashi, K., Mitani, H., Adachi, H. and Shinoda, H. (1994). Anchorage and retentive effects of a bisphosphonate (AHBuBP) on tooth movements in rats. *Americal Journal of Orthodontics and Dentofacial Orthopedics,* 106: 279-289.

Ii, K., Hizawa, K., Kominami, E., Bando, Y. and Katunuma, N. (1985). Different immunolocalizations of cathepsins B, H, and L in the liver. *Journal of Histochemistry and Cytochemistry,* 33: 1173-1175.

Inaoka, T., Bilbe, G., Ishibashi, O., Tezuka, K., Kumegawa, M. and Kokubo, T. (1995). Molecular cloning of human cDNA for cathepsin K: Novel cysteine proteinase predominantly expressed in bone. *Biochemical and Biophysical Research Communications,* 206: 89-96.

Inoue, H., Tanaka, N. and Uchiyama, C. (1995). Parathyroid Hormone Increases the Number of Tartrate-Resistant Acid Phosphatase-Positive Cells through Prostaglandin E_2 Synthesis in Adherent Cell Culture of Neonatal Rat Bones. *Endocrinology,* 136: 3648-3656.

Inui, T., Ishibashi, O., Inaoka, T., Irigane, Y., Kumegawa, M., Kokubo, T. and Yamamura, T. (1997). Cathepsin K Antisense Oligodeoxynucleotide Inhibits Osteoclastic Bone Resorption. *The Journal of Biological Chemistry,* 272: 8109-8112.

Ishida, C.E. and Ramos-e-Silva, M. (1998). Cryosurgery in oral lesions. *International Journal of Dermatology,* 37: 283-285.

Itonaga, I., Sabokbar, A., Murray, D.W. and Athanasou, N.A. (2000). Effect of osteoprotegerin and osteoprotegerin ligand on osteoclast formation by arthroplasty membrane derived macrophages. *Annals of the Rheumatic Diseases,* 59: 26-31.

Jackson, P. and Blythe, D. (1993). Immunolabelling techniques for light microscopy. *In: Immunocytochemistry: A Practical Approach.* Beesley, J.E., editor. Oxford University Press, New York, p. 15-41.

Jacobsen, I.A. and Pegg, D.E. (1984). Cryopreservation of Organs: A Review. *Cryobiology,* 21: 377-384.

Jäger, A., Radlanski, R. and Götz, W. (1993). Demonstration of cells of the monocyte-phagocyte lineage in the periodontium following experimental tooth movement in the rat. *Histochemistry,* 100: 161-166.

Janckila, A.J., Li, C., Lam, K. and Yam, L.T. (1978). The cytochemistry of tartrate-resistant acid phosphatase. Technical considerations. *American Journal of Clinical Pathology,* 70: 45-55.

Janssen, G.H., Tangelder, G.J., oude Egbrink, M.G. and Reneman, R.S. (1997). Different effects of anaesthetics on spontaneous leukocyte rolling in rat skin. *International Journal of Microcirculation, Clinical and Experimental,* 17: 305-313.

Jee, W.S.S., Tang, L., Ke, H.Z., Sterberg, R.B. and Kimmel, D.B. (1993). Maintaining restored bone with bisphosphonate in the ovariectomized rat skeleton: Dynamic histomorphometry of changes in bone mass. *Bone,* 14: 493-498.

Jilka, R.L. (1989). Stimulation of collagenolytic enzyme release from cultured bone cells of normal and osteopetrotic (mi/mi) mice by parathyroid hormone and lipopolysaccharide. *Bone and Mineral,* 6: 277-287.

Johnson, R.B. and Pylypas, S.P. (1992). A re-evaluation of the distribution of the elastic meshwork within the periodontal ligament of the mouse. *Journal of Periodontal Research,* 27: 239-249.

Jones, S.J. and Boyde, A. (1977). Some morphological observations on osteoclasts. *Cell and Tissue Research,* 185: 387-397.

Jones, S.J. and Boyde, A. (1988). The resorption of dentine and cementum *in vivo* and *in vitro*. *In: The Biological Mechanisms of Tooth Eruption and Root Resorption.* Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, p. 335-354.

Jones, S.J., Piper, K., Vesely, C., Gray, C. and Boyde, A. (1994). The significance of size of an osteoclast. *In: Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants.* Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, p. 55-71.

Jonsson, R., Tarkowski, A. and Klareskog, L. (1986). A demineralization procedure for immunohistopathological use. EDTA treatment preserves lymphoid cell surface antigens. *Journal of Immunological Methods,* 88: 109-114.

Kacena, M.A., Merrel, G.A., Konda, S.R., Wilson, K.M., Xi, Y. and Horowitz, M.C. (2001). Inflammation and bony changes in the temporomandibular joint. *Cells Tissues Organs,* 169: 257-264.

Kadoya, Y., Al-Saffar, N., Kobayashi, A. and Revell, P.A. (1994). The expression of osteoclast markers on foreign body giant cells. *Bone and Mineral,* 27: 85-96.

Kahn, A.J. and Simmons, D.J. (1975). Investigation of cell lineage in bone using a chimera of chick and quail embryonic tissue. *Nature,* 258: 325-327.

Kahn, A.J., Stewart, C.C. and Teitelbaum, S.L. (1978). Contact-mediated bone resorption by human monocytes in vitro. *Science,* 199: 988-990.

Kaji, K., Katogi, R., Azuma, Y., Naito, A., Inoue, J.L. and Kudo, A. (2001). Tumor necrosis factor alpha-induced osteoclastogenesis requires tumor necrosis factor receptor-associated factor 6. *Journal of Bone and Mineral Research,* 16: 1593-1599.

Kakegawa, H., Nikawa, T., Tagami, K., Kamioka, H., Sumitani, K., Kawata, T., Drobnic-Kosorok, M., Lenarcic, B., Turk, V. and Katunuma, N. (1993). Participation of cathepsin L on bone resorption. *FEBS Letters,* 321: 247-250.

Kalu, D.N. (1991). The Ovariectomized Rat Model of Postmenopausal Bone Loss. *Bone and Mineral,* 15: 175-192.

Kalu, D.N. (1995). Evolution of the Pathogenesis of Postmenopausal Bone Loss. *Bone,* 17: 135S-144S.

Kamata, K., Okamoto, S., Oka, S., Kamata, H., Yagisawa, H. and Hirata, H. (2001). Cycloprodigiosin hydrochloride suppresses tumor necrosis factor (TNF) alpha-induced transcriptional activation by NF-kappaB. *FEBS Letters,* 507: 74-80.

Kameda, T., Ishikawa, H. and Tsutsui, T. (1995). Detection and characterization of apoptosis in osteoclasts *in vitro*. *Biochemical and Biophysical Research Communications,* 207: 753-760.

Kaneda, T., Nojima, T., Nakagawa, M., Ogasawara, A., Kaneko, H., Sato, T., Mano, H., Kumegawa, M. and Hakeda, Y. (2000). Endogenous production of TGF-beta is essential for osteoclastogenesis induced by a combination of receptor activator of NF-kappa B ligand and macrophage colony-stimulating factor. *Journal of Immunology,* 165: 4254-4263.

Kanehisa, J. and Heersche, J.N.M. (1988). Osteoclastic Bone Resorption: In Vitro Analysis of the Rate of Resorption and Migration of Individual Osteoclasts. *Bone,* 9: 73-79.

Kanehisa, J., Yamanaka, T., Doi, S., Turksen, K., Heersche, J.N.M., Aubin, J.E. and Takeuchi, H. (1990). A band of F-actin containing podosomes is involved in bone resorption by osteoclasts. *Bone,* 11: 287-293.

Karnovsky, M.J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *Journal of Cell Biology,* 27: 137A

Kawaguchi, H., Pilbeam, C.C., Harrison, J.R. and Raisz, L.G. (1995). The Role of Prostaglandins in the Regulation of Bone Metabolism. *Clinical Orthopaedics and Related Research,* 313: 36-46.

Ketcham, A.H. (1927). A preliminary report of an investigation of apical root resorption of vital permanent tetth. *International Journal of Orthodontics,* 13: 97-127.

Khosla, S. (2001). Minireview: the OPG/RANKL/RANK system. *Endocrinology,* 142: 5050-5055.

Kindlova, M. (1965). The blood supply of the marginal periodontium in the Macaca rhesus. *Archives of Oral Biology,* 10: 869-874.

Kirschke, H. and Barrett, A.J. (1985). Cathepsin L - A Lysosomal Cysteine Proteinase. *Progress in Clinical and Biological Research,* 180: 61-69.

Kirschke, H., Langer, J., Weideranders, B., Ansorge, S. and Bohley, P. (1977). Cathepsin L - A New Proteinase from Rat-Liver Lysosomes. *European Journal of Biochemistry,* 74: 293-301.

Kirschke, H., Kembhavi, A.A., Bohley, P. and Barrett, A.J. (1982). Action of rat liver cathepsin L on collagen and other substrates. *Biochemical Journal,* 201: 367-372.

Kitazawa, R., Kimble, R.B., Vannice, J.L., Kung, V.T. and Pacifici, R. (1994). Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. *Journal of Clinical Investigation,* 94: 2397-2406.

Kobayashi, K., Takahashi, N., Jimi, E., Udagawa, N., Takami, M., Kotake, S., Nakagawa, N., Kinosaki, M., Yamaguchi, K., Shima, N., Yasuda, H., Morinaga, T., et al. (2000). Tumor necrosis factor α stimulates osteoclastic differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *Journal of Experimental Medicine,* 191: 275-286.

Koch, A.E., Burrows, J.C., Marder, R., Domer, P.H. and Leibovich, S.J. (1990). Reactivity of human tissues with monoclonal antibodies to myeloid activation and differentiation antigens. An immunohistochemical study. *Pathobiology,* 58: 241-248.

Koch, A.E., Burrows, J.C., Skoutelis, A., Marder, R., Domer, P.H., Anderson, B. and Leibovich, S.J. (1991). Monoclonal antibodies detect monocyte/macrophage activation and differentiation antigens and identify functionally distinct subpopulations of human rheumatoid synovial tissue macrophages. *American Journal of Pathology,* 138: 165-173.

Kölliker, A. (1873). Die normale Resorption der Knochengewebes und ihre Bedeutung fur die Entstehung der typischen Knochenformen. *Leipzig, GDR, Vogel,*

Komine, M., Kukita, A., Kukita, T., Ogata, T., Hotokebuchi, T. and Kohashi, O. (2001). Tumor Necrosis Factor-α Cooperates With Receptor Activator of Nuclear Factor kB Ligand in Generation of Osteoclasts in Stromal Cell-depleted Rat Bone Marrow Cell Culture. *Bone,* 28: 474-483.

Komuro, K., Itakura, K., Boyse, E.A. and John, M. (1975). Ly-5: a new T-lymphocyte antigen system. *Immunogenetics,* 1: 452-456.

Kon, T., Cho, T.J., Aizawa, T., Yamazaki, M., Nooh, N., Graves, D., Gerstenfeld, L.C. and Einhorn, T.A. (2001). Expression of osteoprotegerin, receptor activator of NF-kappaB ligand

(osteoprotegerin ligand) and related proinflammatory cytokines during healing. *Journal of Bone and Mineral Research,* 16: 1004-1014.

Kong, Y. and Penninger, J.M. (2000). Molecular control of bone remodeling and osteoporosis. *Experimental Gerontology,* 35: 947-956.

Kong, Y., Yoshida, H., Sarosi, I., Tan, H., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A.J., Van, G., Itie, A., Khoo, W., Wakeham, A., et al. (1999). OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature,* 397: 315-323.

Kosteniuk, P.J. and Shalhoub, V. (2001). Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption. *Current Pharmaceutical Design,* 7: 613-635.

Kukita, T. and Roodman, G.D. (1989). Development of a Monoclonal Antibody to Osteoclasts Formed *in Vitro* which Recognizes Mononuclear Osteoclasts Precursors in the Marrow. *Endocrinology,* 125: 630-637.

Kukita, T., McManus, L.M., Miller, M., Civin, C. and Roodman, G.D. (1989). Osteoclast-like cells formed in long-term human bone marrow cultures express a similar surface phenotype as authentic osteoclasts. *Laboratory Investigation,* 60: 532-538.

Kukita, T., Nomiyama, H., Ohmoto, Y., Kukita, A., Shuto, A., Hotokebuchi, T., Sugioka, Y., Miura, R. and Iijima, T. (1997). Macrophage inflammatory protein-1 alpha (LD78) expressed in human bone marrow: its role in regulation of hematopoiesis and osteoclast recruitment. *Laboratory Investigation,* 76: 399-406.

Kukita, T., Kukita, A., Xu, L., Watanabe, T. and Iijima, T. (2001). Kat1-antigen -- a reliable immunological marker for identifying osteoclast precursors of rats: detection of subpopulations among precursors and initiation of osteoclastogenesis. *Histochemistry and Cell Biology,* 115: 215-222.

Kurihara, S. (1988). 16mm cinematographic observation on behavior of a multinucleated cell cultured with a dentine slice. *In: The Biological Mechanisms of Tooth Eruption and Root Resorption.* Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, p. 371-377.

Kurihara, H., Suda, T., Miura, Y., Nakauchi, H., Kodama, H., Hiura, K., Hakeda, Y. and Kumegawa, M. (1989). Generation of osteoclasts from isolated hematopoietic progenitor cells. *Blood,* 74: 1295-1302.

Kurtin, P.J. and Pinkus, G.S. (1985). Leucocyte Common Antigen - A Diagnostic Discriminant Between Hemopoietic and Nonhemopoietic Neoplasms in Paraffin Sections Using Monoclonal Antibodies. *Human Pathology,* 16: 353-365.

Kutscher, W. and Wolbergs, H. (1935). Prostaphosphatase. *Zeitschrift fur Physiologie und Chemistrie,* 236: 237-243

Lacey, D.L., Erdmann, J.M., Teitelbaum, S.L., Tan, H., Ohara, J. and Shioi, A. (1995). Interleukin 4, interferon-gamma, and prostaglandin E impact the osteoclast cell-forming potential of murine bone marrow macrophages. *Endocrinology,* 136: 2367-2376.

Lacey, D.L., Timms, E., Tan, H., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., et al. (1998). Osteoprotegerin Ligand Is a Cytokine that Regulates Osteoclast Differentiation and Activation. *Cell,* 93: 165-176.

Lakkakorpi, P.T. and Väänänen, H.K. (1991). Kinetics of the osteoclast cytoskeleton during the resorption cycle in vitro. *Journal of Bone and Mineral Research,* 6: 817-826.

Lakkakorpi, P.T. and Väänänen, H.K. (1996). Cytoskeletal Changes in Osteoclasts During the Resorption Cycle. *Microscopy Research and Technique,* 33: 171-181.

Lakkakorpi, P.T., Tuukkanen, J., Hentunen, T., Jarvelin, K. and Väänänen, H.K. (1989). Organization of osteoclast microfilaments during the attachment to bone surface in vitro. *Journal of Bone and Mineral Research,* 4: 817-825.

Lakkakorpi, P.T., Horton, M.A., Helfrich, M.H., Karhukorpi, E.K. and Väänänen, H.K. (1991). Vitronectin receptor has a role in bone resorption but does not mediate tight sealing zone attachment of osteoclasts to the bone surface. *Journal of Cell Biology,* 115: 1179-1186.

Lam, K., Li, O., Li, C. and Yam, L.T. (1973). Biochemical properties of human prostatic acid phosphatase. *Clinical Chemistry,* 19: 483-487.

Lam, K. and Yam, L.T. (1977). Biochemical characterization of the tartrate-resistant acid phosphatase of human spleen with leukemic reticuloendotheliosis as a pyrophosphatase. *Clinical Chemistry,* 23: 89-94.

Lambrecht, J.T. and Marks, S.C. (1996). Human osteoclast-like cells in primary culture. *Clinical Anatomy,* 9: 41-45.

Lang, H., Schuler, N., Arnhold, S., Nolden, R. and Mertens, T. (1995). Formation of differentiated tissues *in vivo* by periodontal cell populations cultured *in vitro*. *Journal of Dental Research,* 74: 1219-1225.

Langford, S.R. and Sims, M.R. (1981). Upper molar root resorption because of distal movement. *American Journal of Orthodontics,* 79: 669-679.

Langford, S.R. and Sims, M.R. (1982). Root surface resorption, repair, and periodontal attachment following rapid maxillary expansion in man. *American Journal of Orthodontics,* 81: 108-115.

Lassus, J., Salo, J., Jiranek, W.A., Santavirta, S., Nevalainen, J., Matucci-Cerinic, M., Horak, P. and Konttinen, Y. (1998). Macrophage activation results in bone resorption. *Clinical Orthopaedics and Related Research,* 352: 7-15.

Lau, K.W., Onishi, T., Wergedal, J.E., Singer, F.R. and Baylink, D.J. (1987). Characterization and assay of tartrate-resistant acid phoshatase activity in serum: Potential use to assess bone resorption. *Clinical Chemistry,* 33: 458-462.

Lazzari, L., Lucchi, S., Montemurro, T., Porretti, L., Lopa, R., Rebulla, P. and Sirchia, G. (2001). Evaluation of the effect of cryopreservation on *ex vivo* expansion of hematopoietic progenitors from cord blood. *Bone Marrow Transplant,* 28: 693-698.

Lee, D., Sims, M.R., Sampson, W.J. and Dreyer, C.W. (1990). Stereo-pair three-dimensional imaging of microvascular architecture in primate dental tissues. *Australian Orthodontic Journal,* 11: 251-255.

Lee, D., Sims, M.R., Dreyer, C.W. and Sampson, W.J. (1991). A scanning electron microscope study of microcorrosion casts of the microvasculature of the marmoset palate, gingiva and periodontal ligament. *Archives of Oral Biology,* 36: 211-220.

Lee, S.K. and Lorenzo, J.A. (1999). Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. *Endocrinology,* 140: 3552-3561.

Leedham, M.D. (1990). The relationship between epithelial cell rests of Malassez and experimental root resorption and repair in Macaca Fascicularis. B. Sci. Dent. (Hons) Thesis, The University of Adelaide.

Lehenkari, P., Hentunen, T.A., Laitala-Leinonen, T., Tuukkanen, J. and Väänänen, H.K. (2001). Carbonic anhydrase II plays a major role in osteoclast differentiation and bone resorption by effecting the steady state intracellular pH and Ca^{++}. *Experimental Cell Research,* 242: 128-137.

Leong, A.S.-Y. and Gilham, P.N. (1989). The effects of progressive formaldehyde fixation on the preservation of tissue antigens. *Pathology,* 21: 266-268.

Lerner, U.H. (2000). Osteoclast formation and resorption. *Matrix Biology,* 19: 107-120.

Lerner, U.H. and Ohlin, A. (1993). Tumor necrosis factor alpha and beta can stimulate bone resorption in cultured mouse calvariae by a prostaglandin-independent mechanism. *Journal of Bone and Mineral Research,* 8: 147-155.

Lerner, U.H., Jones, I.L. and Gustafson, G.T. (1987). Bradykinin, a new potential mediator of inflammatory-induced bone resorption. *Arthritis and Rheumatology,* 30: 530-540.

Letterio, J.J. and Roberts, A.B. (1998). Regulation of immune responses by TGF-β. *Annual Reviews in Immunology,* 16: 137-161.

Levitt, J.A. (1962). A sulfhydryl-disulfide hypothesis of frost injury and resistance in plants. *Journal of Theoretical Biology,* 3: 355-391.

Levrat, C., Larrick, J.W. and Wright, S.C. (1991). Tumor necrosis factor induces activation of mitochondrial succinic dehydrogenase. *Life Sciences,* 49: 1731-1737.

Li, C.Y., Chuda, R.A., Lam, K. and Yam, L.T. (1973). Acid phosphatases in human plasma. *Journal of Laboratory and Clinical Medicine,* 82: 446-460.

Lilja, E., Lindskog, S. and Hammarström, L. (1983). Histochemistry of enzymes associated with tissue degradation incident to orthodontic tooth movement. *Americal Journal of Orthodontics,* 83: 62-75.

Lin, S.K., Hong, C.Y., Chang, H.H., Chiang, C.P., Chen, C.S., Jeng, J.H. and Kuo, M.Y. (2000). Immunolocalization of macrophages and transforming growth factor-beta 1 in induced rat periapical lesions. *Journal of Endodontology,* 26: 335-340.

Lindskog, S. and Hammarström, L. (1980). Evidence in favor of an anti-invasion factor in cementum or periodontal membrane of human teeth. *Scandinavian Journal of Dental Research,* 88: 161-163.

Lindskog, S. and Pierce, A.M. (1988). Spreading of dentinoclasts on various substrata *in vitro. Scandinavian Journal of Dental Research,* 96: 310-316.

Lindskog, S. and Blomlöf, L. (1994). Quality of periodontal healing. IV: Enzyme histochemical evidence for an osteoblast origin of reparative cementum. *Swedish Dental Journal,* 18: 181-189.

Lindskog, S., Blomlöf, L. and Hammarström, L. (1983). Repair of periodontal tissues *in vivo* and *in vitro.*. *Journal of Clinical Periodontology,* 10: 188-205.

Lindskog, S., Pierce, A.M., Blomlöf, L. and Hammarström, L. (1985). The role of necrotic periodontal membrane in cementum resorption and ankylosis. *Endodontics and Dental Traumatology,* 1: 96-101.

Lindskog, S., Blomlöf, L. and Hammarström, L. (1987a). Cellular colonization of denuded root surfaces in vivo: cell morphology in dentin resorption and cementum repair. *Journal of Clinical Periodontology,* 14: 390-395.

Lindskog, S., Blomlöf, L. and Hammarström, L. (1987b). Comparative effects of parathyroid hormone on osteoblasts and cementoblasts. *Journal of Clinical Periodontology,* 14: 386-389.

Lindskog, S., Blomlöf, L. and Hammarström, L. (1988a). Dentin resorption in replanted monkey incisors. Morphology of dentinoclast spreading *in vivo*. *Journal of Clinical Periodontology,* 15: 365-370.

Lindskog, S., Blomlöf, L. and Hammarström, L. (1988b). Evidence for a role of odontogenic epithelium in maintaining the periodontal space. *Journal of Clinical Periodontology,* 10: 188-205.

Linge, L. and Linge, B.O. (1991). Patient characteristics and treatment variables associated with apical root resorption during orthodontic treatment. *Americal Journal of Orthodontics and Dentofacial Orthopedics,* 99: 35-43.

Litvan, G.G. (1972). Mechanism of Cryoinjury In Biological Systems. *Cryobiology,* 9: 182-191.

Lorenzo, J.A., Sousa, S.L., Alander, C. and Raisz, L.G. (1987). Comparison of the bone-resorbing activity in the supernatants from phyohemagglutin-stimulated human peripheral blood mononuclear cells with that of cytokines through the use of an antiserum to interleukin 1. *Endocrinology,* 121: 1164-1170.

Lories, R., J. and Luyten, F.P. (2001). Osteoprotegerin and osteoprotegerin-ligand balance: a new paradigm in bone metabolism providing new therapeutic targets. *Clinical Rheumatology,* 20: 3-9.

Lotz, E., Vaughan, J.H. and Carson, D.A. (1988). Effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science,* 241: 1218-1220.

Loutit, J.F. and Nisbet, N.W. (1979). Resorption of bone. *Lancet,* 2: 26-27.

Loutit, J.F. and Nisbet, N.W. (1982). The Origin of Osteoclasts. *Immunobiology,* 161: 193-203.

Lovelock, J.E. (1953). The haemolysis of human red blood cells by freezing and thawing. *Biochimica Biophysica Acta,* 10: 414-426.

Lowik, C.W.G.M., Van Leeuwen, J.P.T.M., Van Der Meer, J.M., Van Zeeland, J.K., Scheven, B.A.A. and Herrmann-Erlee, M.P.M. (1985). A two-receptor model for the action of parathyroid hormone on osteoblasts: a role for intracellular calcium and cAMP. *Cell Calcium,* 6: 311-326.

Lucht, U. (1971). Acid phosphatase of osteoclasts demonstrated by electron microscopic histochemistry. *Histochemie,* 28: 103-117.

Lucht, U. (1972a). Osteoclasts and Their Relationship to Bone as Studied by Electron Microscopy. *Zeitschrift fur Zellforschung und Mikroskopische Anatomie,* 135: 211-228.

Lucht, U. (1972b). Cytoplasmic vacuoles and bodies of the osteoclast. An electron microscope study. *Zeitschrift fur Zellforschung und Mikroskopische Anatomie,* 135: 229-244.

Lucht, U. (1980). Osteoclasts: ultrastructure and function. *In: The Reticuloendothelial System. A Comprehensive Treatise. Volume 1 Morphology.* Carr, I. and Daems, W.T., editors. Plenum Press, New York, p. 705-734.

Lundy, M.L., Lau, K.H., Blair, H.C. and Baylink, D.J. (1988). Chick osteoblasts contain fluoride-sensitive acid phosphatase activity. *Journal of Histochemistry and Cytochemistry,* 36: 1175-1180.

MacIntyre, I., Zaidi, M., Towhidul Alam, A.S.M., Datta, H.K., Moonga, B.S., Lidbury, P.S., Hecker, M. and Vane, J.M. (1991). Osteoclast inhibition: An action of nitric oxide not mediated by cGMP. *Proceedings of the National Academy of Science USA,* 88: 2936-2940.

MacIntyre, N. (2001). Unmasking antigens for immunohistochemistry. *British Journal of Biomedical Sciences,* 58: 190-196.

Mah, J., Carvalho, R. and Bumann, A. (2000). Current status of root resorption. *In: Biological Mechanisms of Tooth Movement and Craniofacial Adaptation.* Davidovitch, Z. and Mah, J., editors. EBSCO Media, Birmingham, Alabama, p. 195-200.

Malafosse, R., Penna, C., Sa Cunha, A. and Nordlinger, B. (2001). Surgical management of hepatic metastases from colorectal malignancies. *Annals of Oncology,* 12: 887-894.

Mangham, D.C., Scoones, D.J. and Drayson, M.T. (1993). Complement and the recruitment of mononuclear osteoclasts. *Journal of Clinical Pathology,* 46: 517-521.

Manolagas, S.C. (2000). Birth and Death of Bone Cells: Basic Regulatory Mechanisms and Indications for the Pathogenesis and Treatment of Osteoporosis. *Endocrine Reviews,* 21: 115-137.

Manolagas, S.C. and Jilka, R.L. (1995). Bone marrow, cytokines and bone remodeling - emerging insights into the pathophysiology of osteoporosis. *New Engand Journal of Medicine,* 332: 305-311.

Marchisio, P.C., Cirillo, D., Naldini, L., Primavera, M.V., Teti, A. and Zambonin-Zallone, A. (1984). Cell-substratum interaction of cultured avian osteoclasts is mediated by specific adhesion structures. *Journal of Cell Biology,* 99: 1696-1705.

Marchisio, P.C., Cirillo, D., Teti, A., Zambonin-Zallone, A. and Tarone, G. (1987). Rous sarcoma virus-transformed fibroblasts and cells of monocyte origin display a peculiar dot-like organization of cytoskeletal proteins involved in microfilament-membrane interactions. *Experimental Cell Research,* 169: 202-214.

Marcus, R. (1996). The Nature of Osteoporosis. *Journal of Clinical Endocrinology and Metabolism,* 81: 1-5.

Mariotti, A. (1993). The extracelular matrix of the periodontium: dynamic and interactive tissues. *Periodontology 2000,* 3: 39-63.

Marks, S.C. (1983). The origin of osteoclasts: evidence, clinical implications and investigative challenges of an extraskeletal source. *Journal of Oral Pathology,* 12: 226-256.

Marks, S.C. and Walker, D.G. (1981). The hematogenous origin of osteoclasts: experimental evidence from osteoppetrotic (microphthalmic) mice treated with spleen cells from beige mouse donors. *American Journal of Anatomy,* 161: 1-10.

Marks, S.C. and Popoff, S.N. (1988). Bone Cell Biology: The Regulation of Development, Structure, and Function in the Skeleton. *The American Journal of Anatomy,* 183: 1-44.

Marks, S.C. and Chambers, T.J. (1991). The Giant Cells Recruited by Subcutaneous Implants of Mineralized Bone Particles and Slices in Rabbits Are Not Osteoclasts. *Journal of Bone and Mineral Research,* 6: 395-400.

Maroon, J.C. and Bailes, J.E. (1995). Cryoprobe-assisted removal of spinal cord tumors: technical note. *Surgical Neurology,* 43: 265-266.

Martin, T.J., Findlay, D.M., Heath, J.K. and Ng, K.W. (1993). Osteoblasts: Differentiation and Function. *In: Physiology and Pharmacology of Bone.* Mundy, G.R. and Martin, T.J., editors. Springer-Verlag, p. 149-183.

Martin, T.J., Romas, E. and Gillespie, M.T. (1998). Interleukins in the control of osteoclast differentiation. *Critical Reviews in Eukaryotic Gene Expression,* 8: 107-123.

Martland, M., Hansman, F.S. and Robinson, R. (1924). Phosphoric esterase of blood. *Biochemical Journal,* 18: 1152-1158.

Mason, J.T. and O'Leary, T.J. (1991). Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *The Journal of Histochemistry and Cytochemistry,* 39: 225-229.

Matsuda, E. (1992). Ultrastructural and Cytochemical Study of the Odontoclasts in Physiologic Root Resorption of Human Deciduous Teeth. *Journal of Electron Microscopy,* 41: 131-140.

Matthews, J.B. (1981). Influence of clearing agent on immunohistochemical staining on paraffin-embedded tissue. *Journal of Clinical Pathology,* 34: 103-105.

Matthews, J.B. (1982). Influence of decalcification on immunohistochemical staining of formalin-fixed paraffin-embedded tissue. *Journal of Clinical Pathology,* 35: 1392-1394.

Matthews, J.B. and Mason, G.I. (1984). Influence of decalcifying agents on immunoreactivity of formalin-fixed, paraffin-embedded tissue. *Histochemical Journal,* 16: 771-787.

Mazur, P. (1963). Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *Journal of General Physiology,* 47: 347-369.

Mazur, P. (1984). Freezing of living cells: mechanisms and implications. *American Journal of Physiology,* 247: C125-C142.

McCulloch, C.A.G. (1995). Origins and functions of cells essential for periodontal repair: the role of fibroblasts in tissue homeostasis. *Oral Diseases,* 1: 271-278.

McCullough, C.A.G., Nemeth, E. and Lowenberg, B. (1987). Paravascular cells in endosteal spaces of alveolar bone contribute to periodontal ligament cell populations. *The Anatomical Record,* 219: 2233-2242.

McNicol, A.M. and Richmond, J.A. (1998). Optimizing immunocytochemistry: antigen retrieval and signal amplification. *Histopathology,* 32: 97-103.

McSheehy, P.M. and Chambers, T.J. (1986). Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. *Endocrinology,* 118: 824-828.

Meikle, M.C. (1997). Control mechanisms in bone resorption: 240 years after John Hunter. *Annals of the Royal College of Surgeons of England,* 79: 20-27.

Meikle, M.C., McGarrity, A.M., Thomson, B.M. and Reynolds, J.J. (1991). Bone-derived growth factors modulate collagenase and TIMP (tissue inhibitor of metalloproteinases) activity and type 1 collagen degradation by mouse calvarial osteoblasts. *Bone and Mineral,* 12: 41-55.

Meikle, M.C., Bord, S., Hembry, R.M., Compston, J., Croucher, P.I. and Reynolds, J.J. (1992). Human osteoblasts in culture synthesize collagenase and other matrix metalloproteinases in response to osteotropic hormones and cytokines. *Journal of Cell Science,* 103: 1093-1099.

Melcher, A.H. and Eastoe, J.E. (1969). The connective tissues of the periodontium. *In: Biology of the Periodontium.* Melcher, A.H. and Bowen, W.H., editors. Academic Press, London, p. 167-343.

Meryman, H.T. (1970). The exceeding of a minimal tolerable cell volume in hypertonic suspension as a cause of freezing injury. *In: The Frozen Cell.* Wolstenholme, G.E.W. and O'Connor, M., editors. Churchill, London, p. 51-69.

Metzger, Z. (2000). Macrophages in periapical lesions. *Endodontics and Dental Traumatology,* 16: 1-8.

Meunier, P.J., Coindre, J.M., Edouard, C.M. and Arlot, M.E. (1980). Bone histomorphometry in Paget's disease. Quantitative and dynamic analysis of pagetic and non-pagetic bone. *Arthritis and Rheumatology,* 23: 1095-1103.

Micklem, K.J., Rigney, E., Cordell, J., Simmons, D., Stross, P., Turley, H., Seed, B. and Mason, D. (1989). A human macrophage-associated antigen (CD68) detected by six different monoclonal antibodies. *British Journal of Haematology,* 73: 6-11.

Mighell, A.J., Hume, W.J. and Robinson, P.A. (1998). An overview of the complexities and subtleties of immunohistochemistry. *Oral Diseases,* 4: 217-233.

Minkin, C. (1982). Bone Acid Phosphatase: Tartrate-Resistant Acid Phosphatase as a Marker of Osteoclast Function. *Calcified Tissue International,* 34: 285-290.

Minkin, C. and Jennings, J.M. (1972). Carbonic anhydrase and bone remodeling: sulphonamide inhibition of bone resorption in organ culture. *Science,* 176: 1031

Miyamoto, T., Ohneda, O., Arai, F., Iwamoto, K., Okada, S., Tagaki, K., Anderson, D.M. and Suda, T. (2001). Bifurcation of osteoclasts and dendritic cells from common progenitors. *Blood,* 98: 2544-2554.

Mizuno, A., Amizuka, N., Irie, K., Murakami, A., Fujise, N., Kanno, T., Sato, Y., Nakagawa, N., Yasuda, H., Mochizuki, S., Gomibuchi, T., Yano, K., et al. (1998). Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor / osteoprotegerin. *Biochemical and Biophysical Research Communications,* 237: 610-615.

Mjor, I.A. (1984). The morphology of dentin and dentinogenesis. *In: Dentin and Dentinogenesis. Volume I.* Linde, A., editor. CRC Press, Boca Raton, Florida, p. 1-18.

Mochizuki, H., Hakeda, Y., Wakasuki, N., Usui, N., Akashi, S., Sato, T., Tanaka, K. and Kumegawa, M. (1992). Insulin-like growth factor-1 supports formatiom and activation of osteoclasts. *Endocrinology,* 131: 1075-1080.

Modderman, W.E., Tuinenburg-Bol Raap, A.C. and Nijweide, P.J. (1991). Tartrate-resistant Acid Phosphatase is not an Exclusive Marker for Mouse Osteoclasts in Cell Culture. *Bone,* 12: 81-87.

Morony, S., Capparelli, C., Lee, R., Shimamoto, G., Boone, T., Lacey, D.L. and Dunstan, C.R. (1999). A chimeric form of osteoprotegerin inhibits hypercalcaemia and bone resorption induced by IL-beta, TNF-alpha, PTH, PTH rP, and 1,25(OH)2D3. *Journal of Bone and Mineral Research,* 14: 1478-1485.

Morony, S., Capparelli, C., Sarosi, I., Lacey, D.L., Dunstan, C.R. and Kosteniuk, P.J. (2001). Osteoprotegerin inhibits osteolysis and decreases skeletal tumor burden in syngenic and nude mouse models of experimental bone metastasis. *Cancer Research,* 61: 4432-4436.

Morris, M.L., Harper, E. and McKenzie, M. (1993). The presence of an inhibitor of human skin collagenase in the roots of healthy and periodontally diseased teeth: changes that will occur with age. *Journal of Periodontology,* 64: 363-365.

Moseley, J.M. and Gillespie, M.T. (1995). Parathyroid related protein. *Critical Reviews in Clinical and Laboratory Science,* 32: 299-343.

Moss, D.W. (1986). Multiple forms of acid and alkaline phosphatase: genetics, expression and tissue specific modification. *Clinica Chimica Acta,* 161: 123-135.

Mostov, K. and Werb, Z. (1997). Journey Across the Osteoclast. *Science,* 276: 219-220.

Mukai, K., Yoshimura, S. and Anzai, M. (1986). Effects of decalcification on immunoperoxidase staining. *American Journal of Surgical Pathology,* 10: 2413-2419.

Mullink, H., Henzen-Logmans, S.C., Tadema, T.M., Mol, J.J. and Meijer, C.J. (1985). Influence of fixation and decalcification on the immunohistochemical staining of cell-specific markers in paraffin-embedded human bone biopsies. *The Journal of Histochemistry and Cytochemistry,* 33: 1103-1109.

Mundy, G.R. (1983). Monocyte-Macrophage System and Bone Resorption. *Laboratory Investigation,* 49: 119-120.

Mundy, G.R. (1989). Local Factors in Bone Remodeling. *Recent Progress in Hormone Research,* 45: 507-531.

Mundy, G.R. (1993a). Hormonal Factors Which Regulate Bone Resorption. *In: The Physiology and Pharmacology of Bone.* Mundy, G.R. and Martin, T.J., editors. Springer-Verlag, Berlin, p. 215-247.

Mundy, G.R. (1993b). Cytokines of Bone. *In: The Physiology and Pharmacology of Bone.* Mundy, G.R. and Martin, T.J., editors. Springer-Verlag, Berlin, p. 186-213.

Mundy, G.R. (1996). Regulation of Bone Formation by Bone Morphogenetic Proteins and Other Growth Factors. *Clinical Orthopaedics and Related Research,* 323: 24-28.

Mundy, G.R. and Roodman, G.D. (1987). Osteoclast ontogeny and function. *In: Bone and Mineral Research 5.* Peck, W.A., editor. Elsevier Science Publishers B.V. Amsterdam, p. 209-279.

Mundy, G.R., Altman, A.J., Gonder, M.D. and Bandelin, J.G. (1977). Direct Resorption of Bone By Human Monocytes. *Science,* 196: 1109-1111.

Mundy, G.R., Shapiro, J.L., Bandelin, J.G., Canalis, E.M. and Raisz, L.G. (1979). Direct stimulation of bone resorption by thyroid hormones. *Journal of Clinical Investigation,* 58: 529-534.

Murata, M., Miyashita, S., Yokoo, C., Tamai, M., Hanada, K., Hatayama, K., Towatari, T., Nikawa, T. and Katunuma, N. (1991). Novel epoxysuccinyl peptides: Seletive inhibitors of Cathepsin B. *FEBS Letters,* 280: 307-310.

Murphy, G. and Reynolds, J.J. (1993). Extracellular matrix degradation. *In: Connective Tissue and its Heritable Disorders.* Royce, P.M. and Steinmann, B., editors. Wiley-Liss Inc. New York, p. 287-316.

Nakane, P.K. (1968). Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: a study on pituitary glands of the rat. *Journal of Histochemistry and Cytochemistry,* 16: 557-560.

Nakane, P.K. and Pierce, G.B. (1967). Enzyme-labeled antibodies for the light and elctron microscopic localization of tissue antigens. *Journal of Cell Biology,* 33: 307-318.

Nakane, S. and Kameyama, Y. (1987). Root resorption caused by mechanical injury of the periodontal soft tissues in rats. *Journal of Periodontal Research,* 22: 390-395.

Nakayama, F., Nishihara, J., Iwasaki, H., Kudo, T., Okubo, R., Kaneko, M., Nakamura, M., Karube, M., Sasaki, K. and Narimatsu, H. (2001). CD15 expression in mature granulocytes is determined by alpha 1,3-fucosyltransferase IX, but in promyelocytes and monocytes by alpha 1,3-fucosyltransferase IV. *Journal of Biological Chemistry,* 276: 16100-16106.

Narayanan, A.S. and Page, R.C. (1983). The effect of chronic inflammation on the composition of collagen types in human connective tissue. *Collagen Related Research,* 3: 323-334.

Nelson, P.N., Pringle, J.A.S. and Chambers, T.J. (1991). Production and Characterization of New Monoclonal Antibodies to Human Osteoclasts. *Calcified Tissue International,* 49: 317-320.

Nesbitt, S.A. and Horton, M.A. (1997). Trafficking of Matrix Collagens Through Bone-Resorbing Osteoclasts. *Science,* 276: 266-269.

Nesbitt, S.A., Nesbit, A., Helfrich, M. and Horton, M. (1993). Biochemical characterization of human osteoclast integrins: Osteoclasts express $\alpha_v\beta_3$, $\alpha_2\beta_1$ and $\alpha_v\beta_1$ integrins. *Journal of Biological Chemistry,* 268: 16737-16745.

Nicholson, G.C., Moseley, J.M., Sexton, P.M., Mendelsohn, F.A.O. and Martin, T.J. (1986). Abundant calcitonin receptors in isolated rat osteoclasts. Biochemical and autoradiographic characterization. *Journal of Clinical Investigation,* 78: 355-360.

Nijweide, P.J., Vrijheid-Lammers, T., Mulder, R.J.P. and Blok, J. (1985). Cell surface antigens on osteoclasts and related cells in the quail studied with monoclonal antibodies. *Histochemistry,* 83: 315-324.

Nilsen, R. (1977). Electron microscopy of induced heterotropic bone formation in guinea pigs. *Archives of Oral Biology,* 22: 485-493.

Ockerse, T. (1959). The anatomy of the teeth of the vervet monkey. *Journal of the Dental Association of South Africa,* 14: 209-226.

Oddie, G.W., Schenk, G., Angel, N.Z., Walsh, N., Guddat, L.W., de Jersey, J., Cassady, A.I., Hamilton, S.E. and Hume, D.A. (2000). Structure, function, and regulation of tartrate-resistant acid phosphatase. *Bone,* 27: 575-584.

Okada, Y., Naka, K., Kawamura, K. and Matsumoto, T. (1995). Localization of Matrix Metalloproteinase 9 (92-Kilodalton Gelatinase/Type IV Collagenase = Gelatinase B) in Osteoclasts: Implications for Bone Resorption. *Laboratory Investigation,* 72: 311-322.

Okamura, K., Maeda, M., Takeda, R., Wato, M. and Tsutsui, M. (1980). The Localization of Plasma Proteins in Odontoclasts. *Journal of Dental Research,* 59: 2075

Okamura, T., Shimokawa, H., Takagi, Y., Ono, H. and Sasaki, S. (1993). Detection of Collagenase mRNA in Odontoclasts of Bovine Root- Resorbing Tissue by *In Situ* Hybridization. *Calcified Tissue International,* 52: 325-330.

Ordonez, N.G., Manning, J.T. and Brooks, T.E. (1988). The effect of trypsinisation on the immunostaining of formalin-fixed paraffin-embedded tissues. *American Journal of Surgical Pathology,* 12: 121-129.

Orlandini, S.Z., Formigli, L., Benvenuti, S., Lasagni, L., Franchi, A., Masi, L., Bernabei, P.A., Santini, V. and Brandi, M.L. (1995). Functional and structural interactions between osteoblastic and preosteoclastic cells in vitro. *Cell and Tissue Research,* 281: 33-42.

Osdoby, P., Collin-Osdoby, P. and Oursler, M.J. (1992a). The immunobiochemistry of the osteoclast. *In: The Biological Mechanisms of Tooth Movement and Craniofacial Adaptation.* Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, p. 55-65.

Osdoby, P., Oursler, M.J., Li, L., Pyfferoen, J., Riggs, B.L. and Spelsberg, T. (1992b). The regulation of osteoclast activity. *In: Chemistry and biology of mineralized tissues.* Slavkin, H. and Price, P., editors. Elsevier Science Publishers, p. 339-346.

Oursler, M.J. and Osdoby, P. (1988). Osteoclast Development in Marrow Cultured in Calvaria-Conditioned Media. *Developmental Biology,* 127: 170-178.

Oursler, M.J., Bell, L.V., Clevenger, B. and Osdoby, P. (1985). Identification of osteoclast-specific monoclonal antibodies. *Journal of Cell Biology,* 100: 1592-1600.

Oursler, M.J., Landers, J.P., Riggs, B.L. and Spelsberg, T.C. (1993). Oestrogen effects on osteoblasts and osteoclasts. *Annals of Medicine,* 25: 361-371.

Page, A.E., Warburton, M.J., Chambers, T.J., Pringle, J.A.S. and Hayman, A.R. (1992). Human osteoclastomas contain multiple forms of Cathepsin B. *Biochimica Biophysica Acta,* 1116: 57-66.

Park, Y., Chung, K.R., Lee, T., Kim, C. and Davidovitch, Z. (2000). Constitutive synthesis of progelatinase-A by mouse calvarial osteoblasts and effects of interleukin 1, indomethacin and dexamethasone on suppression of collagenolysis and bone resorption. *In: Biological Mechanisms of Tooth Movement and Craniofacial Adaptation.* Davidovitch, Z. and Mah, J., editors. EBSCO Media, Birmingham, Alabama, p. 131-136.

Pavlin, D. and Gluhak-Heinrich, J. (2001). Effect of Mechanical Loading on Periodontal Cells. *Critical Reviews in Oral Biology and Medicine,* 12: 414-424.

Pazzaglia, U.E. and Pringle, J.A.S. (1989). Bone resorption *in vitro:* macrophages and giant cells from failed total hip replacements *versus* osteoclasts. *Biomaterials,* 10: 286-288.

Pearse, A.G.E. (1972). Oxidoreductases II. *In: Histochemistry.* Churchill Livingstone, Edinburgh and London, p. 921-961.

Peters, M., Meyer zum Buschenfelde, K.H. and Rose-John, S. (1996). The function of the soluble IL-6 receptor *in vivo. Immunology Letters,* 54: 177-184.

Picton, D.C.A. (1976). Tooth movement as mesial and lateral drift. *In: The Eruption and Occlusion of Teeth.* Poole, D.F.G. and Stack, M.V., editors. Butterworths, London, p. 108-119.

Pierce, A.M. (1989a). Experimental basis for the management of dental resorption. *Endodontics and Dental Traumatology,* 5: 255-265.

Pierce, A.M. (1989b). Attachment to and phagocytosis of mineral by alveolar bone osteoclasts. *Journal of Submicroscopic Cytology and Pathology,* 21: 63-71.

Pierce, A.M. and Lindskog, S. (1987). The effect of an antibiotic/corticosteroid paste on inflammatory root resorption *in vivo*. *Oral Surgery Oral Medicine Oral Pathology,* 64: 216-220.

Pierce, A.M. and Lindskog, S. (1989). Early responses by osteoclasts *in vivo* and dentinoclasts *in vitro* to corticosteroids. *Journal of Submicroscopic Cytology and Pathology,* 21: 501-508.

Pierce, A.M., Heithersay, G.S. and Lindskog, S. (1988). Evidence for direct inhibition of dentinoclasts by a corticosteroid/antibiotic paste. *Endodontics and Dental Traumatology,* 4: 44-45.

Pierce, A.M., Lindskog, S. and Hammarström, L. (1991). Osteoclasts: Structure and Function. *Electron Microscopy Review,* 4: 1-45.

Piper, K., Boyde, A. and Jones, S.J. (1992). The relationship between the number of nuclei of an osteoclast and its resorptive capability in vitro. *Anatomy and Embryology,* 186: 291-299.

Pitaru, S., McCulloch, C.A. and Narayanan, S.A. (1994). Cellular origins and differentiation control mechanisms during periodontal development and wound healing. *Journal of Periodontal Research,* 29: 81-94.

Porcu, E. (2001). Oocyte freezing. *Seminars in Reproductive Medicine,* 19: 221-230.

Postlethwaite, A.E. and Kang, A.H. (1976). Collagen and collagen peptide-induced chemotaxis of human blood monocytes. *Journal of Experimental Medicine,* 143: 147-152.

Postlethwaite, A.E., Seyer, J.M. and Kang, A.H. (1978). Chemotactic attraction of human fibroblasts to type I, II, and III collagens and collagen-derived peptides. *Proceedings of the National Academy of Science USA,* 75: 871-875.

Puchtler, H. and Meloan, S.N. (1985). On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions. *Histochemistry,* 82: 201-204.

Pulford, K.A., Rigney, E.M., Micklem, K.J., Jones, M., Stross, W.P., Gatter, K.C. and Mason, D.Y. (1989). KP1: a new monoclonal antibody that detects a monocyte/macrophage associated antigen in routinely processed tissue sections. *Journal of Clinical Pathology,* 42: 414-421.

Quinn, J., Athanasou, N.A. and McGee, J.O. (1991). Extracellular matrix receptor and platelet antigens on osteoclasts and foreign body giant cells. *Histochemistry,* 96: 169-176.

Quinn, J.M.W., Sabokbar, A. and Athanasou, N.A. (1996). Cells of the mononuclear phagocyte series differentiate into osteoclastic lacunar bone resorbing cells. *Journal of Pathology,* 179: 106-111.

Quinn, J.M.W., Sabokbar, A., Denne, M., de Vernejoul, M.C., McGee, J.O. and Athanasou, N.A. (1997). Inhibitory and stimulatory effects of prostaglandins on osteoclast differentiation. *Calcified Tissue International,* 60: 63-70.

Quinn, J.M.W., McGee, J.O. and Athanasou, N.A. (1998a). Human tumour-associated macrophages differentiate into osteoclastic bone-resorbing cells. *Journal of Pathology,* 184: 31-36.

Quinn, J.M.W., Neale, S., Fujikawa, Y., McGee, J.O. and Athanasou, N.A. (1998b). Human osteoclast formation from blood monocytes, peritoneal macrophages, and bone marrow cells. *Calcified Tissue International,* 62: 527-531.

Quintero, J.C., Piesco, N.P., Langkamp, H.H., Bowen, L. and Aggarwal, B.B. (1995). LPS responsiveness in periodontal ligament cells is regulated by tumor necrosis factor-alpha. *Journal of Dental Research,* 74: 1802-1811.

Rabin, Y. and Shitzer, A. (1996). A new cryosurgery device for controlled freezing. *Cryobiology,* 33: 82-92.

Rainbow, R.D. (1994). Immunohistochemistry. *In: Laboratory Histopathology. A Complete Reference.* Woods, A.E. and Ellis, R.C., editors. Churchill Livingstone, Edinburgh, p. 8.2-1-8.2-72.

Ralph, S.J., Thomas, M.L., Morton, C.C. and Trowbridge, I.A. (1987). Structural variants of human T220 glycoprotein (leukocyte common antigen). *EMBO Journal,* 6: 1251-1257.

Ramponi, G., Manao, G., Camici, G., Cappuci, G., Ruggiero, M. and Bottaro, D.P. (1989). The 18kDa cytosolic acid phosphatase from bovine liver has phosphotyrosine phosphatase activity on the autophosphorylated epidermal growth factor receptor. *FEBS Letters,* 250: 469-473.

Rani, C.S. and MacDougall, M. (2000). Dental cells express factors that regulate bone resorption. *Molecular and Cellular Biology Research Communications,* 3: 145-152.

Ravesloot, J.H., Eisen, T., Baron, R. and Boron, W.F. (1995). Role of Na^+-H^+ exchangers and vacuolar H^+ pumps in entracellular pH regulation in neonatal rat osteoclasts. *Journal of General Physiology,* 105: 177-208.

Reddy, S.V., Scarcez, T., Windle, J.J., Leach, R.J., Hundley, J.E., Chirgwin, J.M., Chou, J.Y. and Roodman, G.D. (1993). Cloning and characterization of the 5'-flanking region of the mouse tartrate-resistant acid phosphatase gene. *Journal of Bone and Mineral Research,* 8: 1263-1270.

Redlich, K., Hayer, S., Maier, A., Dunstan, C.R., Tohidast-Akrad, M., Lang, S., Turk, B., Pietschmann, P., Woloszczuk, W., Haralambous, S., Kollias, G., Steiner, G., et al. (2002). Tumor necrosis factor alpha-mediated joint destruction in inhibited by targeting osteoclasts with osteoprotegerin. *Arthritis and Rheumatology,* 46: 785-792.

Reeve, C.M. and Wentz, F.M. (1962). The prevalence, morphology and distribution of epithelial rests in the human periodontal ligament. *Journal of Oral Medicine, Oral Surgery and Oral Pathology,* 15: 785-793.

Reichel, H., Koeffler, H.P. and Norman, A.W. (1989). The role of the vitamin D endocrine system in health and disease. *New Engand Journal of Medicine,* 320: 980-991.

Reinholt, F.P., Widholm, S.M., Ek-Rylander, B. and Andersson, G. (1990). Ultrstructural Localization of a Tartrate-Resistant Acid ATPase in Bone. *Journal of Bone and Mineral Research,* 5: 1055-1061.

Reitan, K. (1974). Initial tissue behaviour during apical root resorption. *Angle Orthodontist,* 44: 68-82.

Reitan, K. and Kvam, E. (1971). Comparative Behaviour of Human and Animal Tissue During Experimental Tooth Movement. *Angle Orthodontist,* 41: 1-8.

Reponen, P., Sahlberg, C., Munaut, C., Thesleff, I. and Tryggvason, K. (1994). High expression of 92-kD type IV collagenase (gelatinase B) in the osteoclast lineage during mouse development. *Journal of Cell Biology,* 124: 1091-1102.

Reynolds, J.J. and Meikle, M.C. (1997). Mechanisms of connective tissue matrix destruction in periodontitis. *Periodontology 2000,* 14: 144-157.

Rifkin, B.R. and Heijl, L. (1979). The occurrence of mononuclear cells at sites of osteoclastic bone resorption in experimental periodontitis. *Journal of Periodontology,* 50: 636-640.

Rifkin, B.R. and Gay, C.V. (1992). *In: Biology and Physiology of the Osteoclast.* Rifkin, B.R. and Gay, C.V. editors. CRC Press, Boca Raton,

Rifkin, B.R., Baker, R.L. and Coleman, S.J. (1979). An Ultrastructural Study of Macrophage-Mediated Resorption of Calcified Tissue. *Cell and Tissue Research,* 202: 125-132.

Roberts, W.E. (2000). Bone physiology, metabolism, and biomechanics in orthodontic practice. *In: Orthodontics: Current Principles and Techniques.* Graber, T.M. and Vanarsdall, R.L., editors. Mosby, St Louis, Missouri, p. 231-234.

Roberts, W.E. and Chamberlain, J.G. (1978). Scanning electron microscopy of the cellular elements of rat periodontal ligament. *Archives of Oral Biology,* 23: 587-589.

Roberts, W.E., Wood, H.B., Chambers, D.W. and Durk, D.T. (1987). Vascularly orientated differentiation gradient of osteoblast precusor cells in rat periodontal ligament: Implications for osteoblast histogenesis and periodontal bone loss. *Journal of Periodontology,* 22: 461-467.

Robertson, J.D., Bodenheimer, T.S. and Stage, D.E. (1963). The ultrastructure of Maunther synapses and nodes in goldfish brain. *Journal of Cell Biology,* 19: 159-165.

Robey, P.G. (1996). Bone Matrix Proteoglycans and Glycoproteins. *In: Principles of Bone Biology.* Bilezikian, J.P., Raisz, L.G., Rodan, G.A., editors. Academic Press, San Diego, p. 155-165.

Robinson, D.B. and Glew, R.H. (1981). Substrate specificity of Gaucher spleen phosphoprotein phosphatase. *Archives of Biochemistry and Biophysics,* 210: 186-199.

Rodan, G.A. and Martin, T.J. (1981). Role of osteoblasts in hormonal control of bone resorption. A hypothesis. *Calcified Tissue International,* 33: 348-351.

Rody, W.J.J., King, G.J. and Gu, G. (2001). Osteoclast recruitment to sites of compression in orthodontic tooth movement. *Americal Journal of Orthodontics and Dentofacial Orthopedics,* 120: 477-489.

Romas, E., Gillespie, M.T. and Martin, T.J. (2002). Involvement of receptor activator of NFkappaβ ligand and tumor necrosis factor-α in bone destruction in rheumatoid arthritis. *Bone,* 30: 340-346.

Roodman, G.D. (1996). Advances in bone biology: the osteoclast. *Endocrine Reviews,* 13: 66-80.

Roodman, G.D. (1999). Cell biology of the osteoclast. *Experimental Hematology,* 27: 1229-1241.

Rosenbloom, J. (1993). Elastin. *In: Connective Tissue and its Heritable Disorders.* Royce, P.M. and Steinmann, B., editors. Wiley-Liss, New York, p. 167-188.

Ross, R. (1968). The connective tissue forming cell. *In: Treatise on Collagen, volume 2: Biology of Collagen (Part A).* Gould, B.S., editor. Academic Press, London, p. 2-82.

Ross, R. (1973). The elastic fibre, a review. *Journal of Histochemistry and Cytochemistry,* 21: 199-208.

Ross, F.P., Cao, X., Chiba, M., Mimura, M. and Teitelbaum, S.L. (1994). Steroid hormone regulation of the integrin $\alpha_v\beta_3$ on avian osteoclast precursors. *In: The Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants.* Davidovitch, Z. and Mah, J., editors. EBSCO Media, Birmingham, Alabama, p. 227-237.

Ruoslahti, E. and Pierschbacher, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. *Science,* 238: 491-497.

Rustin, P., Bourgeron, T., Parfait, B., Chretin, D., Munnich, A. and Rotig, A. (1997). Inborn errors of the Krebs cycle: a group of unusual mitochondrial diseases in human. *Biochimica Biophysica Acta,* 1361: 185-197.

Rygh, P. (1973). Ultrastructural changes of the periodontal fibers and their attachment in rat molar periodontium incident to orthodontic tooth movement. *Scandinavian Journal of Dental Research,* 81: 467-480.

Rygh, P. (1976). Ultrastructural changes in tension zones of rat molar periodontal ligament incident to orthodontic tooth movement. *American Journal of Orthodontics,* 70: 269-281.

Rygh, P. (1977). Orthodontic Root Resorption Studied by Electron Microscopy. *Angle Orthodontist,* 47: 1-16.

Rygh, P. (1984). Periodontal responses to orthodontic forces. *In: Malocclusion and the periodontium.* McNamara, J.A. and Ribbens, K.A., editors. Center for Human Growth and Development, Ann Arbor, Michigan, p. 17-42.

Rygh, P. (1992). The response of the periodontal ligament to orthodontic forces. *In: Monograph No. 27. Craniofacial Growth Series.* Carlson, D.S. and Goldstein, S.A., editors. Center for Human Growth and Development, Ann Arbor, Michigan, p. 257-279.

Sabatini, M., Boyce, B., Aufdemorte, T., Bonewald, L. and Mundy, G. (1988). Infusions of recombinant human interleukins 1 alpha and 1 beta cause hypercalcemia in normal mice. *Proceedings of the National Academy of Science USA,* 85: 5235-5259.

Sabokbar, A., Fijikawa, Y., Neale, S., Murray, D.W. and Athanasou, N.A. (1997). Human arthroplasty derived macrophages differentiate into osteoclastic bone resorbing cells. *Annals of the Rheumatic Diseases,* 56: 414-420.

Saffar, J.L., Lasfarques, J.J. and Cherruau, M. (1997). Alveolar bone and the alveolar process: the socket that is never stable. *Periodontology 2000,* 13: 76-90.

Sahara, N. (1998). Resorption and repair of human deciduous teeth prior to shedding. *In: Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants.* Davidovitch, Z. and Mah, J., editors. EBSCO Media, Birmingham, Alabama, p. 383-396.

Sahara, N., Okafuji, N., Toyoki, A., Suzuki, I., Deguchi, T. and Suzuki, K. (1992). Odontoclastic Resorption at the Pulpal Surface of Coronal Dentin Prior to the Shedding of Human Deciduous Teeth. *Archives of Histology and Cytology,* 55: 273-285.

Sahara, N., Okafuji, N., Toyoki, A., Ashizawa, Y., Yagasaki, H., Deguchi, T. and Suzuki, K. (1993). A histological study of the exfoliation of human deciduous teeth. *Journal of Dental Research,* 72: 634-640.

Sahara, N., Okafuji, N., Toyoki, A., Ashizawa, Y., Deguchi, T. and Suzuki, K. (1994). Odontoclastic resorption of the superficial nonmineralized layer of predentine in the shedding of human deciduous teeth. *Cell and Tissue Research,* 277: 19-26.

Sahara, N., Toyoki, A., Ashizawa, Y., Deguchi, T. and Suzuki, K. (1996). Cytodifferentiation of the Odontoclast Prior to the Shedding of Human Deciduous Teeth: An Ultrastructural and Cytochemical Study. *The Anatomical Record,* 244: 33-49.

Sakata, M., Shiba, H., Komatsuzawa, H., Fujita, T., Ohta, K., Sugai, M., Suginaka, H. and Kurihara, H. (1999). Expression of osteoprotegerin (osteoclastogenesis inhibitory factor) in cultures of human dental mesenchymal cells and epithelial cells. *Journal of Bone and Mineral Research,* 14: 1486-1492.

Salo, J., Metsikko, K., Palokangas, H., Lehenkari, P. and Väänänen, H.K. (1996). Bone-resorbing osteoclasts reveal a dynamic division of basal plasma membrane into two different domains. *Journal of Cell Science,* 109: 301-307.

Salo, J., Lehenkari, P., Mulari, M., Metsikko, K. and Väänänen, H.K. (1997). Removal of Osteoclast Bone Resorption Products by Transcytosis. *Science,* 276: 270-273.

Sandy, J.R., Farnedale, R.W. and Meikle, M.C. (1993). Recent advances in understanding mechanically induced bone remodelling and their relevance to orthoodntic theory and practice. *Americal Journal of Orthodontics and Dentofacial Orthopedics,* 103: 212-222.

Sasaki, T. (1996). Recent Advances in the Ultrastructural Assessment of Osteoclastic Resorptive Functions. *Microscopy Research and Technique,* 33: 182-191.

Sasaki, T. and Garant, P.R. (1993). Multinucleated fibroblastic cells in the periodontal ligament of aged rats. *Journal of Periodontal Research,* 28: 65-71.

Sasaki, T. and Ueno-Matsuda, E. (1992). Immunocytochemical Localization of Cathepsins B and G in Odontoclasts of Human Deciduous Teeth. *Journal of Dental Research,* 71: 1881-1884.

Sasaki, T. and Ueno-Matsuda, E. (1993). Cystein-proteinase localization in osteoclasts: An immunocytochemical study. *Cell and Tissue Research,* 271: 177-179.

Sasaki, T., Takahashi, N., Watanabe, C., Suzuki, H., Higashi, S. and Suda, T. (1988a). Cytodifferentiation of odontoclasts in physiologic root resorption of human deciduous teeth. *In: The Biologic Mechanisms of Tooth Eruption and Root Resorption.* Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, p. 321-328.

Sasaki, T., Motegi, N., Suzuki, H., Watanabe, C., Tadokoro, K., Yanagisawa, T. and Higashi, S. (1988b). Dentin Resorption Mediated by Odontoclasts in Physiological Root Resorption of Human Deciduous Teeth. *The American Journal of Anatomy,* 183: 303-315.

Sasaki, T., Shimizu, T., Suzuki, H. and Watanabe, C. (1989). Cytodifferentiation and Degeneration of Odontoclasts in Physiologic Root Resorption of Kitten Deciduous Teeth. *Acta Anatomica,* 135: 330-340.

Sasaki, T., Watanabe, C., Shimizu, T., Debari, K. and Segawa, K. (1990). Possible role of cementoblasts in the resorbant organ of human deciduous teeth during root resorption. *Journal of Periodontal Research,* 25: 143-151.

Sasaki, T., Hong, M., Udagawa, N. and Moriyama, Y. (1994). Expression of vacuolar H^+-ATPase in osteoclasts and its role in resorption. *Cell and Tissue Research,* 278: 265-271.

Sato, Y., Shibasaki, Y. and Sasaki, T. (2000). Effects of bisphosphonate administration on root and bone resorption during experimental movement of rat molars. *In: Biological Mechanisms of Tooth Movement and Craniofacial Adaptation.* Davidovitch, Z. and Mah, J., editors. EBSCO Media, Birmingham, Alabama, p. 243-252.

Savill, J. (1997). Apoptosis in resolution of inflammation. *Journal of Leucocyte Biology,* 61: 375-380.

Saygin, N.E., Giannobile, W.V. and Somerman, M.J. (2000). Molecular and cellular biology of cementum. *Periodontology 2000,* 24: 73-98.

Schenk, R.K., Felix, R. and Hofstetter, W. (1993). Morphology of connective tissue: Bone. *In: Connective Tissue and Its Heritable Disorders.* Royce, P.M. and Steinmann, B., editors. Wiley-Liss, New York, p. 85-101.

Schmidt, B.L. and Pogrel, M.A. (2001). The use of enucleation and liquid nitrogen cryotherapy in the management of odontogenic keratocysts. *Journal of Oral and Maxillofacial Surgery,* 59: 720-725.

Schour, I. and Massler, M. (1971). The Teeth. *In: The Rat in Laboratory Investigation.* Farris, E.J. and Griffith, J.J.Q., editors. Hafner Publishing Co. New York, p. 104-165.

Scott, B.L. and Pease, D.C. (1956). Electron microscopy of the epiphyseal apparatus. *The Anatomical Record,* 125: 465-495.

Seaman, W.E., Talal, N., Herzenberg, L.A. and Ledbetter, J.A. (1981). Surface antigens on mouse natural killer cells: use of monoclonal antibodies to inhibit or to enrich cytotoxic activity. *Journal of Immunology,* 127: 982-986.

Seligman, A.M. and Rutenburg, A.M. (1951). The Histochemical Demonstration of Succinic Dehydrogenase. *Science,* 113: 317-320.

Selvig, K.A. and Selvig, S. (1962). Mineral content of human and seal cementum. *Journal of Dental Research,* 41: 624-632.

Serotec (1999). Serotec Product Guide. Oxford, United Kingdom.

Shackleford, J.M. (1971a). The indifferent fibre plexus and its relationship to principal fibres of the periodontium. *American Journal of Anatomy,* 131: 427-442.

Shackleford, J.M. (1971b). Scanning electron microscopy of the dog periodontium. *Journal of Periodontal Research,* 45-54.

Shalaby, M.R., Aggarwal, B.B., Rinderknecht, E., Svedersky, L.P., Finkle, B.S. and Palladino, M.A. (1985). Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. *Journal of Immunology,* 135: 2069-2073.

Shalhoub, V., Faust, J., Boyle, W.J., Dunstan, C.R., Kelley, M., Kaufman, S., Scully, S., Van, G. and Lacey, D.L. (1999). Osteoprotegerin and osteoprotegerin ligand effects on osteoclast formation from human peripheral blood mononuclear cell precursors. *Journal of Cellular Biochemistry,* 72: 251-261.

Sheibani, K. and Tubbs, R.R. (1984). Enzyme immunohistochemistry: technical aspects. *Seminars in Diagnostic Pathology,* 4: 235-250.

Shen, V., Kohler, G., Huang, J., Huang, S.S. and Peck, W.A. (1989). An acidic fibroblast growth factor stimulates DNA synthesis, inhibits collagen and alkaline phosphatase synthesis and induces resorption in bone. *Bone and Mineral,* 7: 205-219.

Shi, S.R., Key, M.E. and Kalra, K.L. (1991). Antigen retrieval in formalin-fixed paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *The Journal of Histochemistry and Cytochemistry,* 39: 741-748.

Shiba, H., Nakanishi, K., Sakata, M., Fujita, T., Uchida, Y. and Kurihara, H. (2000). Effects of ageing on proliferative ability, and the expressions of secreted protein, acidic and rich in cysteine (SPARC) and osteoprotegerin (osteoclastogenesis inhibitory factor) in cultures of human periodontal ligament cells. *Mechanisms of Ageing and Development,* 117: 69-77.

Shigeyama, Y., Grove, T.K., Strayhorn, C. and Somerman, M.J. (1996). Expression of adhesion molecules during tooth resorption in feline teeth: a model for aggressive osteoclast activity. *Journal of Dental Research,* 75: 1650-1657.

Shioni, A., Teitelbaum, S.L., Ross, F.P., Welgus, H.G., Suzuki, H., Ohara, J. and Lacey, D.L. (1991). Interleukin 4 inhibits murine osteoclast formation *in vitro*. *Journal of Cellular Biochemistry,* 47: 272-277.

Shiozawa, S. and Kuroki, Y. (1994). Osteoporosis in rheumatoid arthritis: a molecular biological aspect of connective tissue gene activation. *Tohoku Journal of Experimental Medicine,* 173: 189-198.

Shiraishi, C., Hara, Y., Abe, Y., Ukai, T. and Kato, I. (2001). A histopathological study of the role of periodontal ligament tissue in root resorption in the rat. *Archives of Oral Biology,* 46: 99-107.

Shore, R.C. and Berkovitz, B.K.B. (1979). An ultrastructural study of periodontal ligament fibroblasts in relation to their possible role in tooth eruption and intra-cellular collagen degradation in the rat. *Archives of Oral Biology,* 24: 155-164.

Simasaki, M. and Yagi, T. (1960). Histochemistry of carbonic anhydrase with special reference to the osteoclast. *Dental Bulletin of Osaka University,* 1: 89-93.

Simonet, W.S., Lacey, D.L., Dunstan, C.R., Kelley, M., Chang, M., Luthy, R., Nguyen, H.Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., et al. (1997). Osteoprotegerin: A Novel Secreted Protein Involved in the Regulation of Bone Density. *Cell,* 89: 309-319.

Sims, M.R. (1975). Oxytalan-vascular relationships observed in histological examination of the periodontal ligaments of man and mouse. *Archives of Oral Biology,* 20: 713-717.

Sims, M.R. (1976). Reconstitution of the human oxytalan system during orthodontic tooth movement. *American Journal of Orthodontics,* 70: 38-58.

Sims, M.R. (1980). Angular changes in collagen cemental attachment during tooth movement. *Journal of Periodontal Research,* 15: 638-645.

Sims, M.R. (1983). Electron-microscopic affiliations of oxytalan fibres, nerves and the microvascular bed in the mouse periodontal ligament. *Archives of Oral Biology,* 28: 1017-1024.

Sismanidou, C., Hilliges, M. and Lindskog, S. (1996). Healing of the root surface-associated periodontium: an immunohistochemical study of orthodontic root resorption in man. *European Journal of Orthodontics,* 18: 435-444.

Slavkin, H.C. (1988). Molecular biology of dental development: a review. *In: The Biological Mechanisms of Tooth Eruption and Root Resorption.* Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, p. 107-116.

Sloan, P., Shellis, R.P. and Berkovitz, B.K.B. (1976). Effect of specimen preparation on the rat periodontal ligament in the scanning electron microscope. *Archives of Oral Biology,* 21: 633-635.

Sloan, P., Carter, D.H., Kielty, C. and Shuttleworth, C.M. (1993). An immunohistochemical study examining the role of collagen type IV in the rat periodontal ligament. *Histochemical Journal,* 25: 523-530.

Sminia, T. and Dijkstra, C. (1986). The Origin of Osteoclasts: An Immunohistochemical Study on Macrophages and Osteoclasts in Embryonic Rat Bone. *Calcified Tissue International,* 39: 263-266.

Sminia, T. and Jeurissen, S.H. (1986). The macrophage population of the gastro-intestinal tract of the rat. *Immunology,* 172: 72-80.

Sobue, T., Hakeda, Y., Kobayashi, Y., Hayakawa, H., Yamashita, K., Aoki, T., Kumegawa, M., Noguchi, T. and Hayakawa, T. (2001). Tissue inhibitor of metalloproteinases 1 and 2 directly stimulate the bone-resorbing activity of isolated mature osteoclasts. *Journal of Bone and Mineral Research,* 16: 2205-2214.

Sodek, J. and McKee, M.D. (2000). Molecular and cellular biology of alveolar bone. *Periodontology 2000,* 24: 99-126.

Solari, F., Domenget, C., Gire, V., Woods, C., Lazarides, E., Rousset, B. and Jurdic, P. (1995). Multinucleated cells can continuously generate mononucleated cells in the absence of mitosis: a study of cells of the avian osteoclast lineage. *Journal of Cell Science,* 108: 3233-3241.

Somerman, M.J., Shroff, B., Argraves, W.S., Morrison, G., Craig, A.M., Denhardt, D.T., Foster, R.A. and Sauk, J.J. (1990). Expression of attachment proteins during cementogenesis. *Journale de Biologie Buccale,* 18: 207-214.

Somerman, M.J., Sauk, J.J., Foster, R.A., Norris, K., Dickerson, K. and Argraves, W.S. (1991). Cell attachment activity of cementum: bone sialoprotein II identified in cementum. *Journal of Periodontal Research,* 26: 10-16.

Spouge, J.D. (1980). A new look at the rests of Malassez. *Journal of Periodontology,* 51: 437-444.

Storey, E. (1973). The nature of tooth movement. *American Journal of Orthodontics,* 63: 292-314.

Strauss, W. (1979). Peroxidase procedures: technical problems encountered during their application. *Journal of Histochemistry and Cytochemistry,* 27: 1349-1351.

Stutzmann, J., Petrovic, A. and Shaye, R. (1980). Extrinsic origin of bone-resorbing cells in orthodontic tooth movement. *Journal of Dental Research,* 59: 440

Suda, T., Tanaka, S., Udagawa, N., Tamura, T. and Takahashi, N. (1992a). The role of osteoblastic cells in osteoclast differentiation. *In: Chemistry and biology of mineralized tissues.* Slavkin, H. and Price, P., editors. Elsevier Science Publishers, p. 329-338.

Suda, T., Takahashi, N. and Martin, T. (1992b). Modulation of Osteoclast Differentiation. *Endocrine Reviews,* 13: 66-80.

Suda, T., Udagawa, N., Nakamura, I., Miyaura, C. and Takahashi, N. (1995). Modulation of osteoclast differentiation by local factors. *Bone,* 17: 87S-91S.

Suda, T., Udagawa, N. and Takahashi, N. (1996). Cells of Bone: Osteoclast Generation. *In: The Biology of Bone.* Bilezikian, J.P., Raisz, L.G., Rodan, G.A., editors. Academic Press, San Diego, p. 87-102.

Suda, T., Nakamura, I., Jimi, E. and Takahashi, N. (1997). Regulation of osteoclast function. *Journal of Bone and Mineral Research,* 869-879.

Suda, T., Takahashi, N., Udagawa, N., Jimi, E., Gillespie, M.T. and Martin, T.J. (1999). Modulation of Osteoclast Differentiation and Function by the New Members of the Tumor Necrosis Factor Receptor and Ligand Families. *Endocrine Reviews,* 20: 345-357.

Suda, T., Kobayashi, K., Jimi, E., Udagawa, N. and Takahashi, N. (2001). The molecular basis of osteoclast differentiation and activation. *Novartis Foundation Symposium,* 232: 235-250.

Sundquist, K., Lakkakorpi, P., Wallmark, B. and Väänänen, H.K. (1990). Inhibition of osteoclast proton transport by bafilomycin A1 abolishes bone resorption. *Biochemical and Biophysical Research Communications,* 168: 309-313.

Takahashi, N., Akatsu, T., Sasaki, T., Nicholson, G.C., Moseley, J.M., Martin, T.J. and Suda, T. (1988). Induction of calcitonin receptors by 1α,25-dihydroxyvitamin D_3 in osteoclast-like multinucleated cells formed from mouse bone marrow cells. *Endocrinology,* 123: 1504-1510.

Takahashi, N., Udagawa, N. and Suda, T. (1999). A new member of the tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. *Biochemical and Biophysical Research Communications,* 256: 449-455.

Takeshita, A., Hanazawa, S., Amano, S., Matumoto, T. and Kitano, S. (1993). IL-1 induces expression of monocyte chemoattractant JE in clonal mouse osteoblastic cell line MC3T3-E1. *Journal of Immunology,* 150: 1554-1562.

Takeshita, S., Kaji, K. and Kudo, A. (2000). Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. *Journal of Bone and Mineral Research,* 15: 1477-1488.

Takimoto, K., Deguchi, T. and Mori, M. (1966). Histochemical detection of succinic dehydrogenase in osteoclasts following experimental tooth movement. *Journal of Dental Research,* 45: 1473-1476.

Tal, H. and Stahl, S. (1986). Healing Following Devitalization of Sites within the Periodontal Ligament by Ultralow Temperatures. *Journal of Periodontology,* 57: 735-741.

Tal, H., Kozlovsky, A. and Pitaru, S. (1991). Healing of sites within the dog periodontal ligament after application of cold to the periodontal apparatus. *Journal of Clinical Periodontology,* 18: 543-547.

Talmage, R.V. (1967). A study of the effect of parathyroid hormone on bone remodeling and on clacium homeostasis. *Clinical Orthopaedics,* 54: 163-173.

Tan, K.B., Harrop, J., Reddy, M., Young, P., Terrett, J., Emery, J., Moore, G. and Truneh, A. (1997). Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and indicible expression in hematopoietic and non-hematopoietic cells. *Gene,* 204: 35-46.

Tani-Ishii, N., Wang, C.Y. and Stashenko, P. (1995). Immunolocalization of bone-resorptive cytokines in rat pulp and periapical lesions following surgical pulp exposure. *Oral Microbiology and Immunology,* 10: 213-219.

Tarquini, R., Perfetto, F. and Tarquini, B. (1998). Endothelin-1 and Paget's bone disease: is there a link? *Calcified Tissue International,* 63: 118-120.

Tashjian, A.H., Voelkel, E.F., Lloyd, W., Derynck, L., Winkler, M.E. and Levine, L. (1986). Actions of growth factors on plasma calcium. Epidermal growth factor and transforming growth factor-α cause elevation of plasma calcium in mice. *Journal of Clinical Investigation,* 78: 1405-1409.

Taylor, C.R., Shi, S.R., Chen, C., Young, L., Yang, C. and Cote, R.J. (1996). Comparative study of antigen retrieval heating methods - microwave, microwave and pressure cooker, autoclave and steamer. *Biotechnic and Histochemistry,* 71: 263-270.

Teitelbaum, S.L., Tondravi, M.M. and Ross, F.P. (1997). Osteoclasts, macrophages, and the molecular mechanisms of bone resorption. *Journal of Leucocyte Biology,* 61: 381-388.

Ten Cate, A.R. (1969). The development of the periodontium. *In: Biology of the Periodontium.* Melcher, A.H. and Bowen, W.H., editors. Academic Press, London, p. 53-89.

Ten Cate, A.R. (1996). The role of epithelium in the development, structure and function of the tissues of tooth support. *Oral Diseases,* 2: 55-62.

Ten Cate, A.R. (1997). The development of the periodontium - a largely ectomesenchymally derived unit. *Periodontology 2000,* 13: 9-19.

Tenorio, D., Cruchley, A. and Hughes, F.J. (1993). Immunocytochemical investigation of the rat cementoblast phenotype. *Journal of Periodontal Research,* 28: 411-419.

Teti, A., Blair, H.C., Schlesinger, P.H., Grano, M., Zambonin-Zallone, A., Kahn, A.J., Teitelbaum, S.L. and Hruska, K.A. (1989a). Extracellular Protons Acidify Osteoclasts, Reduce Cytosolic Calcium, and Promote Expression of Cell-Matrix Attachment Structures. *Journal of Clinical Investigation,* 84: 773-780.

Teti, A., Blair, H.C., Teitelbaum, S.L., Kahn, A.J., Koziol, C., Konsek, J., Zambonin-Zallone, A. and Schlesinger, P.H. (1989b). Cytoplasmic pH Regulation and Chloride/Bicarbonate Exchange in Avian Osteoclasts. *Journal of Clinical Investigation,* 83: 227-233.

Teti, A., Marchisio, P.C. and Zambonin-Zallone, A. (1991). Clear zone in osteoclast function: role of podosomes in regulation of bone-resorbing activity. *American Journal of Physiology,* 261: C1-C7.

The American Society for Bone and Mineral Research President's Committee on Nomenclature (2000). Proposed standard nomenclature for new tumor necrosis factor members involved in the regulation of bone resorption. *Bone,* 27: 761-764.

Thesleff, I. (2000). Genetic basis of tooth development and dental defects. *Acta Odontologica Scandinavica,* 58: 191-194.

Thomas, M.L. (1989). The leucocyte common antigen family. *Annual Review of Immunology,* 7: 339-369.

Tong, H.S., Sakai, D.D., Sims, S.M., Dixon, S.J., Yamin, M., Goldring, S.R., Snead, M.L. and Minkin, C. (1994). Murine osteoclasts and spleen cell polykaryons are distinguished by mRNA phenotyping. *Journal of Bone and Mineral Research,* 9: 577-584.

Tonnaer, E.L., Kuijpers, W., Peters, T.A. and Ramaekers, F.C. (1990). Effect of EDTA on cytokeratin detection in the inner ear. *Journal of Histochemistry and Cytochemistry,* 38: 1223-1227.

Tos, A.P., Doglioni, C., Laurino, L. and Fletcher, C.D. (1993). KP1 (CD68) expression in benign tumours. Further evidence of its low specificity as a histiocytic/myeloid marker. *Histopathology,* 23: 185-187.

Tronstad, L. (1988). Root resorption - etiology, terminology and clinical manifestations. *Endodontics and Dental Traumatology,* 4: 241-252.

Trowbridge, I.A. and Mazaukas, C. (1976). Immunological properties of murine thymus-dependent lymphocyte surface glycoproteins. *European Journal of Immunology,* 6: 557-562.

Trowbridge, I.A., Ralph, P. and Bevan, M.J. (1975). Differences in the surface proteins of mouse B and T cells. *Proceedings of the National Academy of Science USA,* 72: 157-161.

Trowbridge, I.S., Hyman, R. and Mazauskas, C. (1976). Surface molecules of cultured human lymphoid cells. *European Journal of Immunology,* 6: 777-782.

Tsang, W.Y. and Chan, J.K. (1992). KP1 (CD68) staining of granular cell neoplasms: is KP1 a marker for lysosomes rather than the histiocyte lineage? *Histopathology,* 21: 84-86.

Tseng, S.C.G., Jarvinen, M.J., Nelson, W.G., Huang, J., Woodcock-Mitchell, J. and Sun, T. (1982). Correlation of specific keratins with different types of epithelial differentiation: Monoclonal antibody studies. *Cell,* 30: 361-372.

Tsuda, E., Goto, M., Mochizuki, S., Yano, K., Kobayashi, F., Morinaga, T. and Higashio, K. (1997). Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochemical and Biophysical Research Communications,* 234: 137-142.

Tsurukai, T., Udagawa, N., Matsuzaki, K., Takahashi, N. and Suda, T. (2000). Roles of macrophage-colony stimulating factor and osteoclast differentiation factor in osteoclastogenesis. *Journal of Bone and Mineral Metabolism,* 18: 177-184.

Udagawa, N. (2002). Mechanisms involved in bone resorption. *Biogerontology,* 3: 79-83.

Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T.J. and Suda, T. (1990). Origin of osteoclasts: Mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proceedings of the National Academy of Science USA,* 87: 7260-7264.

Udagawa, N., Horwood, N.J., Elliott, J., Mackay, A., Owens, J., Okamura, H., Kurimoto, M., Chambers, T.J., Martin, T.J. and Gillespie, M.T. (1997). Interleukin (IL)-18 (interferon gamma inducing factor) is produced by osteoblasts and acts via granulocyte macrophage-colony stimulating factor and not via interferon-gamma to inhibit osteoclast formation. *Journal of Experimental Medicine,* 185: 1005-1012.

Udagawa, N., Takahashi, N., Yasuda, H., Mizuno, A., Itoh, K., Ueno, Y., Shinki, T., Gillespie, M.T., Martin, T.J., Higashio, K. and Suda, T. (2000). Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. *Endocrinology,* 141: 3478-3484.

Uematsu, S., Mogi, M. and Deguchi, T. (1996). Interleukin (Il-1b), Il-6, Tumor Necrosis Factor-a, Epidermal Growth Factor, and b_2-Microglobulin levels are elevated in gingival crevicular fluid during orthodontic tooth movement. *Journal of Dental Research,* 75: 562-567.

Urra, J.M., Arteta, M., Gomez-Caturla, A. and Garcia-Duran, F. (2001). A chimeric anti-TNFalpha monoclonal antibody (cA2) *in vivo* removes TNFalpha-producing cells in Crohn's disease. *Human Antibodies,* 10: 91-94.

Uy, H.L., Guise, T.A., De La Mata, H., Taylor, S.D., Story, B.M., Dallas, M.R., Boyce, B.F., Mundy, G.R. and Roodman, G.D. (1995). Effects of Parathyroid Hormone (PTH)-Related Protein and PTH on Osteoclasts and Osteoclast Precursors *in Vivo. Endocrinology,* 136: 3207-3212.

Väänänen, H.K. (1996). Osteoclast Function: Biology and Mechanisms. *In: Principles of Bone Biology.* Bilezikian, J.P., Raisz, L.G., Rodan, G.A., editors. Academic Press, San Diego, p. 103-113.

Väänänen, H.K. and Horton, M. (1995). The osteoclast clear zone is a specialized cell-matrix adhesion structure. *Journal of Cell Science,* 108: 2729-2732.

Väänänen, H.K., Karhukorpi, E.K., Sundquist, K., Wallmark, B., Roininen, I., Hentunen, T., Tuukkanen, J. and Lakkakorpi, P.T. (1990). Evidence for the presence of a proton pump of the vacuolar H(+)-ATPase type in the ruffled borders of osteoclasts. *Journal of Cell Biology,* 111: 1305-1311.

Väänänen, H.K., Zhao, H., Mulari, M. and Halleen, J.M. (2000). The cell biology of osteoclast function. *Journal of Cell Science,* 113: 377-381.

Vaes, G. (1968). On the mechanisms of bone resorption. The action of parathyroid hormone on the excretion and synthesis of lysosomal enzymes and on the extracellular release of acid by bone cells. *Journal of Cell Biology,* 39: 676-697.

Vaes, G. (1988). Cellular Biology and Biochemical Mechanism of Bone Resorption. A Review of Recent Developments on the Formation, Activation, and Mode of Action of Osteoclasts. *Clinical Orthopaedics and Related Research,* 231: 239-271.

Vaes, G. and Jacques, P. (1965). Studies on bone enzymes. The assay of acid hydrolases and other enzymes in bone tissue. *Biochemical Journal,* 97: 380-388.

Valderhaug, J.P. (1974). Epithelial cells in the periodontal membrane of teeth with and without periapical inflammation. *International Journal of Oral Surgery,* 3: 7-16.

van de Wijngaert, F.P. and Burger, E.H. (1986). Demonstration of Tartrate-resistant Acid Phosphatase in Undecalcified, Glycomethacrylate-embedded Mouse Bone: A Possible Marker for (Pre)Osteoclast Identification. *The Journal of Histochemistry and Cytochemistry,* 34: 1317-1323.

Van Noorden, S. (1993). Problems and solutions. *In: Immunocytochemistry. A Practical Approach.* Beesley, J.E., editor. Oxford University Press, New York, p. 207-239.

Vandevska-Radunovic, V., Hals Kvinnsland, I., Kvinnsland, S. and Jonsson, R. (1997). Immunocompetent cells in rat periodontal ligament and their recruitment incident to orthodontic tooth movement. *European Journal of Oral Sciences,* 105: 36-44.

Vignery, A. and Baron, R. (1978). Effects of parathyroid hormone on the osteoclastic pool, bone resorption and formation in rat alveolar bone. *Calcified Tissue Research,* 26: 23-28.

Vincent, J.B. and Averill, B.A. (1990). An enzyme with a double identity: purple acid phosphatase and tartrate-resistant acid phosphatase. *FASEB Journal,* 4: 3009-3014.

Vitouladitis, I., Okamoto, Y., Gogen, H., Shanfeld, J.L., Dobeck, J., Guajardo, G., Benning, L. and Davidovitch, Z. (2000). Inflammatory mediators in tooth movement: II. Tumor necrosis factor -α. *In: Biological Mechanisms of Tooth Movement and Craniofacial Adaptation.* Davidovitch, Z. and Mah, J., editors. EBSCO Media, Birmingham, Alabama, p. 161-174.

Vlaskalic, V., Boyd, R.L. and Baumrind, S. (1998). Etiology and Sequelae of Root Resorption. *Seminars in Orthodontics,* 4: 124-131.

Wada, N., Maeda, H., Tanabe, K., Tsuda, E., Yano, K., Nakamuta, H. and Akami, A. (2001). Periodontal ligament cells secrete the factor that inhibits osteoclastic differentiation and function: the factor is osteoprotegerin/osteoclastogenesis inhibitory factor. *Journal of Periodontal Research,* 36: 56-63.

Wakisaka, S., Ichikawa, H., Nishimoto, T., Matsuo, S., Yamamoto, K., Nakata, T. and Akai, M. (1986). A technique for decalcification and demonstration of substance P-like reactivity. *Stain Technology,* 61: 39-42.

Waldo, C.M. and Rothblatt, J.M. (1954). Histologic response to tooth movement in the laboratory rat. *Journal of Dental Research,* 33: 481-486.

Walker, D.G. (1961). Citric acid cycle in osteoblasts and osteoclasts. *Bulletin of the Johns Hopkins Hospital,* 108: 80-98.

Walker, D.G. (1972). Congenital osteopetrosis in mice cured by parabiotic union with normal siblings. *Endocrinology,* 91: 916-920.

Walker, D.G. (1973). Osteopetrosis cured by temporary parabiosis. *Science,* 180: 875

Walker, D.G. (1975). Control of bone resorption by hemapoietic tissue. The induction and reversal of congenital osteopetrosis in mice through use of bone marrow and splenic transplants. *Journal of Experimental Medicine,* 142: 651-663.

Wang, Z. and Gluck, S. (1990). Isolation and properties of bovine kidney brush border vacuolar H^+-ATPase. A proton pump with enzymatic and structural differences from kidney microsomal H^+-ATPase. *Journal of Biological Chemistry,* 265: 21957-21965.

Warnke, R.A., Pulford, K.A., Pallesen, G., Ralfkiaer, E., Brown, D.C., Gatter, K.C. and Mason, D.Y. (1989). Diagnosis of myelomonocytic and macrophage neoplasms in routinely processed tissue biopsies with monoclonal antibody KP1. *American Journal of Pathology,* 135: 1089-1095.

Watanabe, K., Tajino, T., Kusakabe, T., Saitoh, A. and Suzuki, T. (1997). Giant cell tumor of bone: frequent actin immunoreactivity in stromal tumor cells. *Pathology International,* 47: 680-684.

Watanabe, J., Amizuka, N., Noda, M. and Ozawa, H. (2000). Cytochemical and ultrastructural examination of apoptotic odontoclasts induced by bisphosphonate administration. *Cell and Tissue Research,* 301: 375-387.

Wedenberg, C. and Lindskog, S. (1985). Experimental internal resorption in monkey teeth. *Endodontics and Dental Traumatology,* 1: 221-227.

Wedenberg, C. and Lindskog, S. (1987). Evidence for a resorption inhibitor in dentin. *Scandinavian Journal of Dental Research,* 95: 205-211.

Wedenberg, C., Pierce, A.M. and Lindskog, S. (1987). Resorbing potential of different connective tissue types. *Scandinavian Journal of Dental Research,* 95: 270-271.

Weekes, W.T. and Sims, M.R. (1986). The vasculature of the rat periodontal ligament. *Journal of Periodontal Research,* 21: 186-194.

Weir, A.P. (1990). The Marmoset Periodontal Ligament: A T.E.M. Stereological Analysis Following Incisor Crown Fracture, Endodontic Therapy, Orthodontic Extrusion and Long Term Retention. MDS Thesis, The University of Adelaide.

Wergedal, J.E. (1970). Characterization of bone acid phosphatase activity. *Proceedings of the Society for Experimental Biology and Medicine,* 134: 244-247.

Wergedal, J.E. and Baylink, D.J. (1969). Distribution of acid and alkaline phosphatase activity in undemineralized sections of the rat tibial diaphysis. *Journal of Histochemistry and Cytochemistry,* 17: 799-806.

Werner, M., Chott, A., Fabiano, A. and Battifora, H. (2000). Effect of formalin tissue fixation and processing on immunohistochemistry. *American Journal of Surgical Pathology,* 24: 1016-1019.

Wesselink, P.R. and Beertsen, W. (1988). Initiating factors in dental root resorption. *In: The Biological Mechanisms of Tooth Eruption and Root Resorption,* Davidovitch, Z., editor. EBSCO Media, Birmingham, Alabama, 329-334.

Wesselink, P.R. and Beertsen, W. (1993). The prevalence and distribution of rests of Malassez in the mouse molar and their possible role in repair and maintenance of the periodontal ligament. *Archives of Oral Biology,* 38: 399-403.

Wesselink, P.R. and Beertsen, W. (1994). Repair processes in the periodontium following dentoalveolar ankylosis: the effect of masticatory system. *Journal of Clinical Periodontology,* 21: 472-478.

Wesselink, P.R., Beertsen, W. and Everts, V. (1986). Resorption of the Mouse Incisor After the Application of Cold to the Periodontal Attachment Apparatus. *Calcified Tissue International,* 36: 11-21.

West, C.D. and Green, C.J. (1987). The sedative effects of climazolam and climazolam with fentanyl-fluanisone in rats (Rattus novegicus). *Laboratory Animals,* 21: 143-148.

Whitaker, K.B., Cox, T.M. and Moss, D.W. (1989). An immunoassay of human band 5 ("tartrate-resistant") acid phosphatase that involves the use of anti-porcine uteroferrin antibodies. *Clinical Chemistry,* 35: 86-89.

Wilchek, M. and Bayer, E.A. (1989). Avidin-biotin technology ten years on: has it lived up to its expectations? *Trends in Biochemical Science,* 14: 408-412.

Williams, S. (1984). A histomorphometric study of orthodontically induced root resorption. *European Journal of Orthodontics,* 6: 35-47.

Wise, G.E. (1998). Cell and molecular biology of tooth eruption. *In: Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants.* Davidovitch, Z. and Mah, J., editors. EBSCO Media, Birmingham, Alabama, p. 1-8.

Wise, G.E., Lumpkin, S.J., Huang, H. and Zhang, Q. (2000). Osteoprotegerin and osteoclast differentiation factor in tooth eruption. *Journal of Dental Research,* 79: 1937-1942.

Wise, G.E., Frazier-Bowers, S. and D'Souza, R.N. (2002). Cellular, Molecular, and Genetic Determinants of Tooth Eruption. *Critical Reviews in Oral Biology and Medicine,* 13: 323-335.

Wong, B.R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F.S., Frankel, W.N., Lee, S.Y. and Choi, Y. (1997). TRANCE Is a Novel Ligand of the Tumor Necrosis Factor Receptor Family That Activates c-Jun N-terminal Kinase in T Cells. *The Journal of Biological Chemistry,* 272: 25190-25194.

Wong, B.R., Josien, R., Lee, S.Y., Vologodskaia, M., Steinman, R.M. and Choi, Y. (1998). The TRAF Family of Signal Transducers Mediates NF-KB Activation by the TRANCE Receptor. *The Journal of Biological Chemistry,* 273: 28355-28359.

Wu, C.Y., Chen, S.T., Chiou, S.H. and Wang, K.T. (1992). Specific peptide bond cleavage by microwave irradiation in weak acid solution. *Journal of Protein Chemistry,* 11: 45-50.

Wucherpfenning, A.L., Li, Y., Stetler-Stevenson, W.G., Rosenberg, A.E. and Stashenko, P. (1994). Expression of 92 kD type IV collagenase/gelatinase B in human osteoclasts. *Journal of Bone and Mineral Research,* 9: 549-556.

Yam, L.T. (1974). Clinical Significance of the Human Acid Phosphatases. *The American Journal Of Medicine,* 56: 604-616.

Yamamoto, T., Domon, T., Takahashi, S. and Wakita, M. (1996). Cellular cementogenesis in rat molars: the role of cementoblasts in the deposition of intrinsic matrix fibers of cementum proper. *Anatomy and Embryology,* 193: 495-500.

Yamasaki, A., Rose, G.G., Pinero, G.J. and Mahan, C.J. (1987). Ultrastructure and morphometric analyses of human cementoblasts and periodontal fibroblasts. *Journal of Periodontology,* 58: 192-201.

Yao, Y.M., Sheng, Z.Y., Yu, Y., Tian, H.M., Wang, Y.P. and Xu, W. (1995). The potential etiologic role of tumor necrosis factor in mediating multiple organ dysfunction in rats following intestinal ischemia-reperfusion injury. *Resuscitation,* 29: 157-168.

Yasuda, H., Shima, N., Nakagawa, N., Mochizuki, S., Yano, K., Fujise, N., Sato, Y., Goto, M., Yamaguchi, K., Kuriyama, M., Kanno, T., Murakami, S., et al. (1998a). Identification of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG inhibits osteoclastogenesis *in vitro*. *Endocrinology,* 139: 1329-1337.

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., et al. (1998b). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proceedings of the National Academy of Science USA,* 95: 3597-3602.

Yen, E.H.K., Farrell, L.E., Anderson, A.N. and Suga, D.M. (1992). Paradental cell response to orthodontic stress and intermittant stress *in vitro*. *In: The Biologic Mechanisms of Tooth Eruption and Craniofacial Adaptation,* Davidovitch, Z. Editor. EBSCO Media, Alabama, p 367-373.

Yokota, S., Nishimura, Y. and Kato, K. (1988). Localization of cathepsin L in rat kidney revealed by immunoenzyme and immunogold techniques. *Histochemistry,* 90: 277-283.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and Horvitz, H.R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell,* 75: 641-652.

Zaidi, M., Moonga, B., Moss, D.W. and MacIntyre, I. (1989). Inhibition of osteoclastic acid phosphatase abolishes bone resorption. *Biochemical and Biophysical Research Communications,* 159: 68-71.

Zaidi, M., Moonga, B., Bevis, P.J.R., Towhidul Alam, A.S.M., Legon, S., Wimalawansa, S., MacIntyre, I. and Breimer, L.H. (1991). Expression and function of calcitionin gene products. *Vitamins and Hormones,* 46: 87-164.

Zaidi, M., Towhidul Alam, A.S.M., Shankar, V.S., Bax, B.E., Bax, C.M.R., Moonga, B.S., Bevis, P.J.R., Stevens, C., Blake, D.R., Pazianas, M. and Huang, C.L.H. (1993). Cellular Biology of Bone Resorption. *Biology Reviews,* 68: 197-264.

Zamboni, L. and De Martino, C. (1967). Buffered picric acid - formaldehyde: a new, rapid fixative for electron microscopy. *Journal of Cell Biology,* 35: 148A

Zambonin-Zallone, A., Teti, A. and Primavera, M.V. (1984). Monocytes from circulating blood fuse *in vitro* with purified osteoclasts in primary culture. *Journal of Cell Science,* 66: 335-342.

Zernecke, A., Weber, K.S. and Weber, C. (2001). Combined modulation of the mesangial machinery for monocyte recruitment by inhibition of NF-kappaB. *American Journal of Physiology. Cell Physiology,* 281: C1881-C1888.

Zwarych, P.D. and Quigley, M.B. (1965). The intermediate plexus of the periodontal ligament: history and further observations. *Journal of Dental Research,* 44: 383-391.