AN IMMUNOHISTOCHEMICAL STUDY OF EPITHELIAL CELL RESTS OF MALASSEZ, INCIDENT TO ROOT RESORPTION AND REPAIR

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TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

FIGURES

BV	BLOOD VESSEL
C	CELLULAR CEMENTUM
D	DENTINE
E	EPITHELIAL CELL REST OF MALASSEZ
EC	EPITHELIAL CELL
R	RESORPTION
RL	REVERSAL LINE

TEXT

CK	CYTOKERATIN
DAB	DIAMINOBENZIDINE
ECC	EPITHELIAL CELL CLUSTER
EDTA	ETHYLENEDIAMINOTETRA-ACETIC ACID
ERM	EPITHELIAL REST OF MALASSEZ
HERS	HERTWIG'S EPITHELIAL ROOT SHEATH
IHC	IMMUNOHISTOCHEMISTRY
Kd	KILODALTONS
RME	RAPID MAXILLARY EXPANSION
TEM	TRANSMISSION ELECTRON MICROSCOPE
SEM	SCANNING ELECTRON MICROSCOPE

SUMMARY

The role of the epithelial cell rests of Malassez in the periodontal ligament has not yet been clarified. The epithelial cell rests have been suggested to have a role, amongst other possible suggestions, in the prevention of ankylosis and the maintenance of the periodontal space. (LÖE and WAERHAUG 1961; LINDSKOG *et al.* 1988B). The presence of the epithelial cell rests in association with resorption as a result of orthodontic treatment was first demonstrated by BRICE (1988), and BRICE *et al.* (1991). The presence of rests in association with the repair of experimental root resorption in *Macaca fascicularis* has also been demonstrated (LEEDHAM 1990). These recent studies required the use of the transmission electron microscope to positively identify the tonofilaments and desmosomes that are characteristic of epithelial cells.

The present study has sought to develop and apply light microscope methods in association with immunohistochemical techniques to label epithelial cells of the human periodontal ligament. An initial study used premolar teeth from patients undergoing orthodontic treatment that were collected and fixed according to several fixation schedules and decalcified in EDTA. After embedding in paraffin with celloidin, sections were stained using polyclonal antibodies to cytokeratin. The primary antibodies were visualized using anti-rabbit secondary antibodies, and the strept-avidin-biotin method and diaminobenzidine. The sections were dehydrated, counter-stained with haematoxylin, coverslipped, and viewed in the light microscope.

This preliminary study demonstrated that fixation in 4% formalin for 6 hours and decalcification by EDTA, in a cacodylate buffer, was sufficient to enable positive identification of epithelial cells. The method was able to show epithelial cell rests of Malassez along both resorbed and non-resorbed surfaces of the teeth studied. Some examples of epithelial cells within the body of larger resorptive defects were also seen.

The second part of the study used the above methods, except for the substitution of a monoclonal antibody (AE1/3) in place of the polyclonal antibody used in the preliminary study, in an histomorphometric study using adolescent human premolar teeth. The study aimed to quantify the extent of root resorption, and the relationship to epithelial cells as a result of rapid maxillary expansion. Using the previously developed methods, extracted premolar teeth that had been used as anchor teeth in RME patients were collected. These teeth were fixed and decalcified as described. Each tooth root was completely divided into 5μm sections, and sections were selected at 10 equidistant levels (level 0 to level 10) from the cemento-enamel junction to apex. Each of these levels was then stained according to the immunohistochemical protocols, and counter-stained with haemotoxylin. Using histomorphometric methods adapted from ANDERSSON *et al.* (1987) and ANDREASEN (1987), a section from each level was selected, viewed using an octant system, data collected about resorption, epithelial cell rests, blood vessels, and amount of tissue remaining, and the information entered into a spreadsheet, and analysed by the University of Adelaide Vax computer.

The results in this part of the study confirmed the reliability of the immunohistochemical staining methods. The cell rests were clearly visible, although there was considerable variation in their shape and morphology, even in the control teeth. Both control and experimental teeth suffered from the loss of considerable amounts of tissue, limiting the analysis. The analysis did quantify the large amounts of buccal root resorption as a result of the treatment, and the continued presence of epithelial cell rests on all root surfaces after treatment. The cell rests were more numerous in the cervical region, decreasing to the middle levels, and increasing again as the apex was approached. Other parameters could not be statistically examined due to the lack of tissue. Only a few examples of epithelial tissue in the areas of repairing resorption were seen. This may have been as a result of the timing of the extractions which took place well after the RME therapy and repair was well advanced. The small

sample size also restricts the interpretation. The loss of soft tissue from areas of resorption means that many areas of potential interest were lost from examination.

In conclusion, alveolar bone needs to be collected as part of the experimental procedure in order to more completely describe the events occuring as a result of orthodontic resorption. The timing of the repair events appears to be crucial with epithelial cells perhaps an early indicator of repair. Further studies using an animal model may offer more scope for invesigating this aspect as well as the potential to build up three dimensional pictures of the periodontal ligament. The immunohistochemical techniques developed can also be adapted to identify and locate other components of the periodontal ligament.

SIGNED STATEMENT

This report contains no material which has been accepted for the award of any other degree or diploma in any other university or other tertiary institution and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Mark D. Leedham

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CHAPTER ONE

AIMS OF THE INVESTIGATION

1. To evaluate various methods of fixation and decalcification and their effect on the immunohistochemical demonstration of epithelial cell rests in the periodontal ligament.

2. To demonstrate epithelial cell rests using immunohistochemical methods, and with appropriate fixation and decalcification, in collected specimens of teeth from human sources.

3. To examine the effect of orthodontic tooth movement on the presence of epithelial cell rests in the periodontal ligament.

4. To examine the presence of epithelial cell rests in relation to root resorption and repair subsequent to orthodontic tooth movement.

CHAPTER TWO

REVIEW OF THE LITERATURE

I. EPITHELIAL CELL RESTS OF MALASSEZ

2.1 HISTORY

The epithelial cell rests in the periodontal ligament were first described in 1885 by Malassez (MALASSEZ 1885). Many investigators have since looked at the origin, morphology, and function of epithelial cell rests. BLACK (1887, 1899) considered the possibility of a glandular function being one of the roles of epithelial cell remnants. ROBINSOHN (1926) suggested that epithelial cells in the periodontal ligament produced a hormone that prevented the fusion of cementum and alveolar bone. HILL (1930) showed that epithelium is present in periapical granulomas and considered that it was derived from the epithelial cell rests of Malassez in the periodontal ligament. FISCHER (1932) looked at the structure of rests within the periodontal ligament and saw a net-like arrangement, which appeared to be more numerous in the apical and gingival regions.

ORBAN (1924) suggested that epithelial cells may influence cementogenesis by acting as organisers, but DIAB and STALLARD (1965) doubted that the cells had any influence on the deposition of cementum.

CUTRESS and CRIGGER (1974) looked at the network of epithelial cell rests in the sheep periodontium concluded that it was difficult to accept that such an extensive network could be a "mere remnant of a dentally historic event, namely a degenerated outer or inner epithelium."

It has also been suggested that the epithelial cell rests can play a role in the maintenance of the periodontal space (LÖE and WAERHAUG, 1961; SPOUGE, 1980, LINDSKOG *et al.*, 1988B). While REITAN (1961) has suggested that the

absence of epithelial cell rests of Malassez is characteristic of reorganised, formerly hyalinized tissue as a result of orthodontic treatment, other workers have suggested that the epithelial cell rests of Malassez are necessary for not only the maintenance of the periodontal space, but have a role in the prevention of ankylosis, as well as a role in the repair of resorption in teeth (LÖE and WAERHAUG 1961; REEVE and WENTZ 1962; SPOUGE 1980; LINDSKOG *et al.* 1988B).

BRICE (1988) has suggested an association between the presence of epithelial cells and the repair of orthodontically induced root resorption. He has suggested that these epithelial cells act by initiating cytodifferentiation of the cementoblasts required to repair the damaged root surfaces.

More recently, HAMMARSTRÖM *et al.* (1989) have shown that in teeth subject to replantation procedures, less extensive ankylosis was apparent in those teeth where conditions favoured survival of the epithelial cell rests of Malassez and cementoblasts (such as short extra-oral periods). These workers also noted that rest cells seemed less affected by the experimental procedure than other cells of the periodontal ligament.

KITTEL (1990) followed up the work of BRICE (1988), and used 3-D reconstruction techniques to examine the presence of epithelial cell rests in relation to orthodontic root resorption and repair. Although not able to follow the cell rests for great distances, as a result of identification difficulties, KITTEL (1990) demonstrated that there was a close relationship between the rests and blood vessels in areas of repair and resorption. LEEDHAM (1990) looked at the ultrastructural aspects of experimental resorption and repair in *Macaca fascicularis*. The results demonstrated that cells with epithelial features (tonofilaments and desmosomes) could be demonstrated at three weeks not only within the area of the cavity, but in close association with the reparative cementum.

2.2 EMBRYOLOGY

Once tooth crown formation is completed, the cervical margin of the enamel organ lies at the level of the cemento-enamel margin. At this position, the enamel organ consists of several layers of epithelial cells. There appears to be a species variation in this aspect of morphology (GURLING and SAMPSON, 1985).

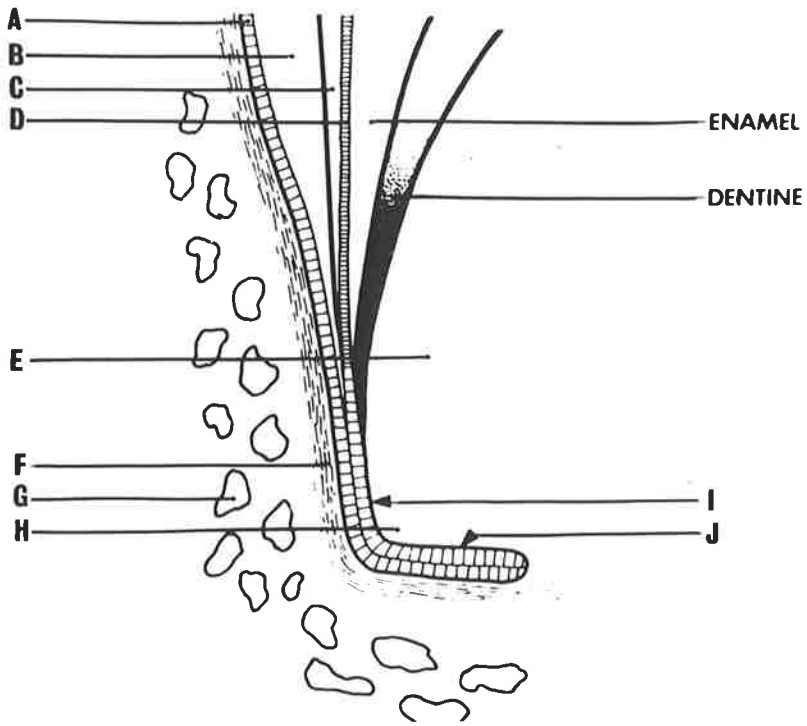


FIGURE 2.1 Diagrammatic representation of the root sheath and early root formation. (Adapted from GURLING, 1982)

A	external enamel epithelium
B	stellate reticulum
C	stratum intermedium
D	internal enamel epithelium
E	dental pulp
F	dental follicle
G	bone
H	lateral epithelial root sheath
I	epithelial diaphragm
J	differentiating odontoblasts

As root formation begins (once crown formation is completed), the layers of epithelial cells, also known as the "cervical loop", are referred to as Hertwig's epithelial root sheath (HERS). The epithelial root sheath was first described in 1874 in amphibia (HERTWIG 1874). The vertical portion of the cervical loop lies along the line of the future dentino-cemental junction (SPOUGE, 1984). There is a parallelled proliferation of the connective tissue cells, both inside the root sheath on its pulpal aspect, and on its outside, the periodontal ligament aspect (Figure 2.1).

As root dentine is formed from odontoblasts on the inner aspect of the root sheath, the sheath itself becomes discontinuous, epithelial cell rests are formed, and move out into the periodontal ligament. According to SPOUGE (1980), cells from the outer aspect of the root sheath move between the epithelial cell rests, and develop into cementoblasts. This view is also held by ARMITAGE (1986), who suggests that contact with the root surface by follicle cells initiates cementoblast formation.

In contrast, JONES and BOYDE (1988) suggest that during root formation, the network of epithelial cells act to hold off blood vessels. THOMAS and KOLLAR (1988, 1989) have identified the cells on the root surface during intermediate cementum formation as being of epithelial origin, and considered that the epithelial root sheath, may play a part in elaboration of the early cementum matrix formation. LEEDHAM (1990) noted a similar close relationship between epithelial cells and reparative cementum in the repair of experimental root resorption. Some workers however, believe that the formation of cementum is dependant on neither the presence or absence of cells of the epithelial root sheath (DIAB and STALLARD 1965). Other workers have demonstrated that as the epithelial root sheath breaks down, some of the sheath cells can also be incorporated into the developing cementum (LESTER, 1969; DIAB and STALLARD, 1965; ARMITAGE, 1986).

LINDSKOG and HAMMARSTRÖM (1982) have demonstrated that the cells of the HERS contribute to the formation of the layer of intermediate cementum. They suggest that it is the exposure of this layer (also known as the hyaline layer of

Hopewell-Smith) as the HERS disintegrates that signals the start of cementogenesis proper.

2.3 DISTRIBUTION, MORPHOLOGY AND OCCURRENCE

1. HUMAN EPITHELIAL CELL RESTS

There have been many descriptions of the organisational structure of the epithelial cell rests of Malassez in the periodontal ligament of both humans and other species.

In the human, SCHOUR (1960) noted that "the meshes are comparatively close in the gingival region ..... the cords becoming scarcer as the apex of the root is approached." However, REEVE and WENTZ (1962) although agreeing that the epithelial cells were more common in the gingival region in the latter part of life, found more epithelial cells in the apical regions in the first two decades of life.

REEVE and WENTZ (1962) found epithelial cell rests of Malassez in all teeth examined in a light microscopic study of over one thousand sections from two hundred and seventy human teeth in subjects with ages from one to seventy-seven years. These workers classified three types of epithelial cell rests (resting, degenerated and proliferated) in the periodontal ligament as a result of their study. The resting type were described as oval groups and strands often forming a net-like arrangement, and were seen in all age groups. The degenerated type were seen most frequently in young people, and usually found in the apical and middle zones of the periodontal ligament "close" to the cementum. The cell rests contained an average of ten cells, and characteristically had small pyknotic nuclei. The proliferated type were usually observed in the later decades of life. The cells generally had large, pale nuclei and the cell groups were often surrounded by a well-differentiated capsule. The total count of epithelial cell rests of Malassez decreased with age, so that by the seventh decade there was only an estimated one quarter of the original number of rests remaining. These workers reported the apparently contradictory increase in the proliferated type in older

subjects, as being due to higher incidences of periapical and periodontal pathology in this age range.

SIMPSON (1965) using an apoxestic technique, observed that in younger specimens, there was a well-developed network, and with increasing age the network became larger meshed with thinner strands. Eventually the network broke up, leaving strands and islands of cells. SIMPSON (1965) indicated however, that such was the rate of degeneration that few periodontal ligaments would be totally free of epithelial cell rests of Malassez, a comment that was confirmed by the findings of VALDERHAUG and NYLEN (1966), who also commented that an extensive network of epithelial rests was seen in almost all of the human periodontal ligaments that they studied. These workers also recognised strands, clusters and small islands which were often found by examination of serial sections to be interconnected.

VALDERHAUG and ZANDER (1967) in a later study, looked at the distances of the epithelial cell rests of Malassez from the cementum in various parts of the periodontal ligament. In fifty human teeth, they found that the average distance of the epithelial cells from the cementum was 27µm in the apical region, increasing to 41µm in the cervical region. Rests were more numerous cervically. No epithelial rests were observed in direct contact with the cementum, the closest being 15µm.

BRICE (1988) has described round, ovoid clusters averaging 6 cells and strands of 5 to 52 cells in a study using adolescent human premolars. He saw equal numbers of strands and clusters, and noted that the epithelial rests were seen mainly in the middle and cervical thirds.

HAMAMOTO *et al.* (1989) looked at the periodontal ligaments of clinically healthy premolars that were extracted for orthodontic reasons. Under the light microscope the epithelial cell rests were located close to the cementum in all specimens. They usually appeared as oval or round clusters of cells in sections cut transversely to the root surface. In longitudinal sections, they were arranged in strands or networks.

YAMASAKI and PINERO (1989) in an *in vitro* study of human periodontal ligament cells from healthy premolars and third molars, described three epithelial cell types (resting, proliferating and migrating). These workers particularly noted that the ultrastructural characteristics of the "proliferating" types were similar to those shown by epithelial cells involved in wound repair.

2. CELL RESTS IN OTHER SPECIES

Epithelial cell rests have been found in many other species. WENTZ *et al.* (1950) looked at the morphology of epithelial cell rests in the rat, and found three types which were considered to represent stages in the life history of the epithelial cell remnants.

1. *Resting type*, which appeared as strands and islands, the frequency of which decreased with age.
2. *Proliferating type*, the most frequent type, with the largest groups of cells. They were seen the nearest to the cervical area, and usually close to the cementum.
3. *Differentiating type*, which showed a distinct difference in cellular morphology, with less, but intensely stained cytoplasm, and a basement membrane.

Other workers have suggested that epithelial cell rests are not normally seen in the periodontal ligament either as a result of degeneration or incorporation into the developing cementum. (DIAB and STALLARD 1965; SHIBATA and STERN 1967; FREEMAN and TEN CATE 1971; TEN CATE 1972). BINNIE and ROWE (1974) have also stated that "...epithelial cell rests of Malassez are known to be absent in the mouse and rat."

Epithelial cell rests have also been noted in the mouse (LISTGARTEN 1975; BEERTSON and EVERTS, 1979). The rests usually appeared as small groups of

epithelial cells, located very close to the surface of the cementum, and more frequent in the coronal half of the periodontal ligament.

FREEZER (1984) in a study of mouse periodontal ligament also found epithelial rests as groups of 3 to 4 cells close to the cementum surface. Some cells appeared to have been incorporated in the developing cementum, while others appeared to have undergone degeneration, in the manner described by LESTER (1969).

In the monkey (species not indicated), REITAN (1961) stated that the epithelial cell rests of Malassez were usually seen to be round and seemed to be larger than in the dog. In general, REITAN (1961) considered that there were fewer rests than in the human periodontal ligament.

LINDSKOG *et al.* (1983) in a study of periodontal repair in replanted teeth of *Macaca fascicularis* saw that epithelial cells were numerous in the connective tissue between the reparative cementum and the alveolar bone. It was observed that strands of cells, two to four cell layers thick, with no distinct lumen between the cells, were close to the reparative cementum surface, a finding confirmed by LEEDHAM (1990).

Epithelial cell rests have also been noted in miniature swine (GRANT and BERNICK 1969), pigs (SPOUGE 1986), dogs (LÖE and WAERHAUG 1961; REITAN 1961; BINNIE and ROWE 1974), and sheep (CUTRESS and CRIGGER 1974).

2.4 ACTIVATION OF EPITHELIAL RESTS

Ultrastructural studies have demonstrated that cells from the epithelial cell rests of Malassez contain the cellular inclusions and organelles that could enable these cells to function in some role if activated. (VALDERHAUG and NYLEN, 1966; NYLEN and GRUPE, 1969; TEN CATE, 1965 and 1972; HAMAMOTO *et al.*, 1989; YAMASAKI and PINERO, 1989). TEN CATE (1965) has suggested that as a result of activation, there is a switch in the metabolism of the cell rests to utilize the hexose mono-phosphate shunt.

Activation of epithelial cell rests has been discussed by several authors, and various factors have been implicated in the activation process. Amongst those factors suggested are pH and local carbon dioxide tension (GRUPE *et al.* 1967), inflammatory processes (BINNIE and ROWE 1974), cholera toxin, dibutyl cyclic-AMP (BRUNETTE 1984A) and epidermal growth factor, EGF (THESLEFF 1987; NÖRDLUND *et al.*, 1991). BINNIE and ROWE (1974) also noted that there appeared to be a higher incidence of epithelial cell rest proliferation and cyst formation in cases of mild inflammation rather than severe or acute inflammation.

THESLEFF (1987) found that the epithelial cell rests of Malassez were able to incorporate ^{125}I-labelled EGF. THESLEFF (1987) was able to demonstrate intense labelling of the epithelial cell rests of Malassez by the labelled EGF. EGF stimulates epithelial cell proliferation in epidermis and oral epithelium, and accelerates wound healing (BROWN *et al.*, 1989). NORDLUND *et al.*, (1991) used monoclonal antibodies against EGF receptor sites in a study of EGF in clinically healthy human gingival epithelium and periodontal ligament. They also described the labelling of epithelial cell rests as "intense", but noted that this intense labelling of cell rests by EGF was not consistent with the suggestion that the number of EGF receptors is related to cell proliferation, since cell rests are normally described as existing in a "passive undifferentiated state".

REITAN (1961) showed that orthodontic forces can also cause changes in the epithelial cell rest network. REITAN (1961) was able to demonstrate changes on both the pressure and tension sides of teeth subject to orthodontic forces. He showed that on the tension side the epithelial cells were compressed between the stretched fibre bundles, and appeared to move slightly away from the root surface. On the compression side, the rests moved closer to the root surface. However, where hyalinisation occurred, the epithelial cells did not reappear, and REITAN (1961) stated that the absence of epithelial cell rests was "characteristic of reorganised, formerly hyalinised periodontal tissue." BRICE (1988) however, has shown the presence of

epithelial cell rests in association with orthodontically induced root resorption, presumably occurring as a result of hyalinisation.

GILHUUS-MOE and KVAM (1972) using rats, noted that their results provided evidence for the idea that mechanical trauma (e.g. orthodontic forces) could influence epithelial cell proliferation. Subsequent work by BRUNETTE (1984B) on mechanical stretching of epithelial cell cultures has provided further evidence for this suggestion. CROWE (1989) using marmosets as a experimental model, saw considerable proliferation of epithelial cell rests in the periodontal ligament when extrusive forces were applied to the teeth. GILHUUS-MOE and KVAM (1972) also observed that the proliferation of epithelial cells appeared to stop with the cessation of the orthodontic forces.

2.5 ULTRASTRUCTURE OF EPITHELIAL RESTS

VALDERHAUG and NYLEN (1966) noted a number of typical features in their study of the ultrastructural morphology of epithelial rests. They included a typical basement lamina, and cells that had rather irregular outlines with many small microvilli. There were often spaces, sometimes large, between individual cells, but no central lumen was present. All cells appeared to have one surface abutting the connective tissue, and this surface appeared quite even, conforming to the smooth contour of the basement lamina. Hemidesmosomes, desmosomes, and tight junctions were observed quite routinely. The nuclei of the epithelial cells varied in shape from round to ovoid and usually showed one or more invaginations. The cytoplasm was usually rather dense with tonofilaments present. Mitochondria were distributed throughout the cell. Profiles of rough endoplasmic reticulum were seen occasionally while the Golgi apparatus was not positively identified.

VALDERHAUG and NYLEN (1966) summarised their findings as indicating that the cellular components and inclusions that they had seen indicated that epithelial cell rests of Malassez did not have a glandular function.

LISTGARTEN (1975) looked at the appearance of epithelial rests in the mouse molar and also noted tonofibrils and desmosomes, characteristic of epithelial cells.

BRICE (1988) examined epithelial cell rests and their relationship to repairing root resorption in orthodontically moved adolescent premolars. He noted the following features where the cell rests were associated with orthodontic root resorption.

1. Increased numbers of ribosomes and polyribosomes.
2. A lack of well developed Golgi apparatus or endoplasmic reticulum.
3. Desmosomes and tonofilaments.
4. Nuclear invagination and nuclear pores.
5. Mitochondria with indistinct christae.
6. A discontinuous basement membrane.
7. Cytoplasmic projections.
8. Many interdigitating cytoplasmic microvilli.
9. The absence of clear cells.

HAMAMOTO *et al.* (1989) have looked at the ultrastructure of epithelial cell rests of Malassez from the human periodontal ligament of extracted human adolescent premolars and they found the following features.

1. High nuclear to cytoplasmic ratio.
2. The nucleus had condensed hetero-chromatin, one or two poorly developed nucleoli, and an irregular nuclear contour.
3. Tonofilaments and abundant mitochondria were seen in most cells.
4. Glycogen granules were present in the cytoplasm.
5. Rough endoplasmic reticulum was poorly developed.
6. A basal lamina was always recognised.
7. Desmosomes and well developed gap junctions
8. A primary cilium was observed in all specimens.

YAMASAKI and PINERO (1989) used an *in vitro* system to study the cells of the epithelial rests and noted resting, proliferating and emigrating cells. They concluded that resting cells had a tendency for differentiation. These workers also described two types of migrating cells - an outer basal-like cell, and an inner, tonofilament-rich, prickle-like cell. This ties in with the original work of NYLEN and GRUPE (1969) who considered that there was ultrastructural evidence that epithelial cell migration was possible. They saw that many of the desmosomes lacked an intercellular layer, and that there was a lack of organisation and a reduction in number of tonofilaments in a study of explants of human epithelial cell rests. The presence of breaks and voids in the basemant membrane, with cell processes extending into the adjacent connective tissue was also seen (NYLEN and GRUPE 1969).

Most recently HAMAMOTO *et al.* (1991) have looked at the role of the basal lamina in the formation of the epithelial rests, and shown in the rat, that the basal lamina plays a role in the organisation and formation of the rests, once the Hertwig's root sheath becomes disorganised after root formation. They considered that the components of the basal lamina are secreted by the epithelial cells and mature as a result of the interaction of the epithelial cells and the mesenchymal cells of the surrounding tissue.

2.6 KERATIN STRUCTURE OF EPITHELIAL RESTS

Keratin filament proteins are the major structural proteins of epithelial cells (LANE *et al.*, 1985). These filaments are the intermediate type with diameters of 7-10nm and are found in the cytoplasm of the epithelial cell. There are other types of intermediate filaments, but keratin is characteristic of epithelial cells (MOLL *et al.*, 1982; DEBUS *et al.*, 1984; COOPER *et al.*, 1985).

Keratin is the most complex of the intermediate filaments. So far, twenty different cytokeratins have been identified (MOLL *et al.*, 1982; MOLL *et al.*, 1990). These cytokeratins have been divided into two classes, coded for by two different gene

families. There is a smaller and more acidic class (keratin type I: cytokeratin numbers nine to twenty) and a relatively large and neutral to basic class (keratin type II: cytokeratins numbers one to eight). The cytokeratins vary in molecular weight from 40,000 to 68,000, usually expressed as kilo-daltons (kD).

A number of "rules" of keratin expression have been devised (COOPER *et al.*, 1985). Keratins are expressed in pairs, consisting of a smaller, acidic cytokeratin in association with a larger, more basic cytokeratin. The difference in molecular weight is usually about 8kD. Each epithelial cell type has a combination of cytokeratins that is characteristic, and can be used to identify its epithelial origin. The keratin patterns of simple epithelia are quite stable, and seem to undergo little if any changes in culture or in malignancy (MOLL *et al.*, 1982).

LANE *et al.* (1985) suggests that the keratin expression of a tissue is closely related to its differentiated state, and is rarely, if ever, organ specific. It has been further established that those cytokeratins associated with simple epithelia are the cytokeratins numbered 7, 8, 18, 19 (MOLL *et al.*, 1982; GAO *et al.*, 1988, GAO *et al.*, 1989).

With the characterization of the cytokeratins of simple epithelia, it is possible to look more closely at the epithelial cell rests of Malassez. GAO *et al.* (1988) have looked at the pattern of keratin expression in epithelial rests of Malassez and periapical lesions. By using immunocytochemistry, it was possible, by examining samples of normal periodontal ligament, as well as samples of periapical granulomas and inflammatory dental cysts, to identify particular cytokeratins. The biopsy material and specimens of normal periodontal tissue were incubated with the specific monoclonal antibodies to the cytokeratins thought to be present in the tissues. The results showed that epithelial cell rests of Malassez stained strongly for cytokeratins number 19, as well as number 5, but only weakly to other cytokeratins. Staining to other cytokeratins indicated that keratins number 7, 8 and 18 did not seem to be expressed by normal rests of Malassez. (Cytokeratins 7, 8, 17, 18, and 19 are thought to be characteristic of simple epithelia.) It was proposed that the most likely basic keratin to be paired with

keratin 19 in normal epithelial cell rests of Malassez was keratin 5, although the specific monoclonal antibodies for this keratin were not available at that time (GAO *et al.*, 1988; MACKENZIE, 1988).

It was further shown that with proliferation of the rests of Malassez, a change in the pattern of keratin expression could be observed. These changes included the expression of keratins more characteristic of a stratified, non-cornifying type of epithelium (keratins 14, 13, 4) (GAO *et al.*, 1988).

2.7 FUNCTION OF EPITHELIAL CELL RESTS OF MALASSEZ

1. EARLY SUGGESTIONS

The earliest suggestions on the possible roles of epithelial cell rests of Malassez were put towards the end of the 19th century. BLACK (1887) and ROBINSOHN (1926) also referred to possible glandular functions. Epithelial cell clusters can often appear to have a glandular appearance. With the use of tangential sections, a net-like appearance became apparent, and prompted suggestions of other roles. NYGAARD OSTBY (1939, 1944) discussed a role in periapical pathology, and LÖE and WAERHAUG (1961) examined the possible role of epithelial rests in protection against root resorption. TEN CATE (1965) however considered that the low metabolic activity of rests, and discounted the possibility of a functional role. VALDERHAUG and NYLEN (1966) felt that the description of "resting" was appropriate for these cells, but did state that the cells contained all the necessary organelles and inclusions to meet any potential functional demands.

2. PROTEIN SYNTHESIS

NYLEN and GRUPE (1969) considered that the structure and organisation of the organelles and inclusions of human epithelial cell rests indicated a role in protein

synthesis. BRUNETTE *et al.* (1979) cultured cells derived from porcine epithelial rests, and showed active protein synthesis.

BIREK *et al.* (1980) have suggested that since cultured porcine epithelial cell rests of Malassez can digest collagen *in vitro*, it may be one of the mechanisms by which extracellular substance can be destroyed in cyst formation involving epithelial cells. LIMEBACK and BRUNETTE (1981) have also demonstrated *in vitro* production of a collagenase by epithelial rest cells.

Conversely, PETTIGREW *et al.* (1980) have shown that epithelial cells from rests of Malassez can, *in vitro*, produce a collagenase inhibitor. These workers suggested that such an inhibitor may protect the periodontal tissues from the pathological destruction that may occur in periodontal disease, and so regulate the balance between collagen synthesis and resorption.

3. BONE RESORPTION

BIREK *et al.* (1983) have shown that cells cultured from porcine epithelial cell rests secrete a bone resorbing factor. These cultured cells were able to cause significant bone resorption and calcium loss when incubated with rat calvaria. LINDSKOG *et al.* (1988B) showed that explants of odontogenic epithelium in experimental cavities in replanted teeth not only prevented ankylosis, but also appeared to encourage resorption of bone around the explants to maintain the width of the periodontal space. They suggested that such a bone resorbing factor produced by dental epithelium may contribute to maintaining the periodontal width.

4. CEMENTOBLAST DIFFERENTIATION

SELVIG (1963) in an electron microscopic study of HERS in young mice considered that it was necessary for disintegration of the root sheath to occur before cementum formation could begin. DIAB and STALLARD (1965) doubted that the epithelial cells had any role in the deposition of cementum, a view shared by SHIBATA and STERN (1967).

However, GURLING (1982) has suggested that if cellular cementum was damaged and the epithelial cell rests of Malassez came into contact with dentine, then it was possible for the epithelial cells to stimulate the formation of cementoblasts from periodontal fibroblasts.

BRICE (1988) has postulated that epithelial cell rests of Malassez could initiate cytodifferentiation of cementoblasts in the repair of root resorption. This conclusion was based on the structural features of epithelial cells found in areas of repairing root resorption in human premolars used as anchor teeth in rapid maxillary expansion (RME). Support for this idea has also come from THOMAS and KOLLAR (1988).

5. MAINTENANCE OF THE PERIODONTAL SPACE

LÖE and WAERHAUG (1961) first offered the hypothesis that not only was an intact periodontal ligament necessary for success of replantation and prevention of ankylosis, but that the epithelial cell rests of Malassez played a role in the maintenance of the periodontal ligament.

SPOUGE (1980) has also suggested that the tendency of bone and cementum to fuse is prevented by the presence of epithelial tissue in the periodontal ligament. He considered that because epithelium and bone do not seem to be directly compatible, the epithelium and its buffer zone of connective tissue may act to limit the encroachment of bone on the network of epithelial rests and the root surface.

More recently, BRICE (1988) and LINDSKOG *et al.* (1988B) have shown that cell rests contribute to maintaining the width of the periodontal space. LINDSKOG *et al.* (1988B) used explants of odontogenic epithelium that were placed in experimental cavities in the roots of monkey incisors. The notable finding was that alveolar bone was resorbed around the explants corresponding to the original width of the periodontal space. BRICE (1988) found epithelial cell rests in some repairing resorption bays in premolar teeth subject to RME, and postulated that the rests were involved in the repair of the root surface and the periodontal ligament.

6. TOOTH ERUPTION

PARTANEN and THESLEFF (1987) have shown that the tissues of the erupting tooth that bind EGF strongly are the dental follicle, blood vessels, and the epithelial cell rests of Malassez. They have suggested that EGF stimulates or maintains the proliferation of undifferentiated cells during embryonic development. The intense staining of the epithelial rests has recently been confirmed by NÖRDLUND *et al.* (1991) who used commercially available monoclonal antibodies against the EGF receptor sites.

2.8 PATHOLOGY OF EPITHELIAL CELL RESTS

The epithelial cell rests of Malassez have been implicated in several pathologic conditions of the periodontal ligament. The mechanisms that might trigger such activation of the rest cells are not known, but may include inflammation, its cells and or its products.

1. PERIODONTAL DISEASE

SPOUGE (1980) has commented that the "anatomical relationship (between the epithelial cell rests of Malassez and the attachment epithelium) would appear to be that of a basketball hoop, which is the attachment epithelium lying around the neck of the tooth, with the network formed by the rests of Malassez "suspended" from and in continuity with its lower border." This means that potentially, they could be continuous with one another (SPOUGE 1980). REEVE and WENTZ (1962) noted that the proliferative nature of epithelium in the presence of inflammation and the existence of epithelial cell rests in the periodontal ligament may be significant in certain phases of periodontal disease.

GRANT and BERNICK (1969) also noted a similar continuity in miniature swine, and saw that this connection could be observed in both clinically healthy and periodontally diseased teeth. CUTRESS and CRIGGER (1974) in looking at the

presence of epithelial cell rests of Malassez in the sheep periodontium, noted a similar association between the network of cell rests and the epithelial attachment.

2. PERIAPICAL PATHOLOGY

TEN CATE (1972) examined the role of epithelial cell rests in the development of the dental cyst, and suggested that intraepithelial cavitation could occur within the activated rests as a mechanism for initial cyst formation, a view shared by SHEAR (1983). SHEAR and PINDBORG (1975) have suggested similar changes for lateral periodontal cysts. MOSKOW and BADEN (1989) considered that odontogenic epithelium was the most common source of gingival and lateral periodontal cysts.

GAO *et al.* (1991) noted however, that despite the role of cell rests in periapical pathology and cyst formation, the epithelial lining of sinus tracts associated with periapical pathology was immunologically similar and continuous with that of the mucosal lining, despite the origins of the cyst epithelium.

3. NEOPLASTIC INVOLVEMENT

All the various types of cells in the periodontal ligament have the potential to undergo neoplastic change. HERROLD (1968) demonstrated that by using a known carcinogen, N.M.U. (N-methyl-N-nitrosourea), the periodontal ligament of syrian hamsters reacted by proliferation of epithelial cells.

McGOWAN (1980) has implicated the cell rests of the periodontal ligament in primary carcinoma of the jaws (primary intra-alveolar epidermoid carcinoma of the jaws).

ADATIA (1982) suggests that there may be neoplastic change in the epithelial cells of the periodontal ligament, giving rise to tumours and that in the right conditions, benign proliferation may also undergo neoplastic alteration.

Epithelial odontogenic hamartomas, although extremely rare, and not strictly neoplastic, are considered by MOSKOW and BADEN (1989) to develop wherever

epithelial odontogenic residues (including epithelial cell rests of Malassez) can be found.

TATEMOTO *et al.* (1989) used immunohistochemical methods to identify the cells involved in the squamous odontogenic tumour, and noted a similarity between the keratin composition of cells of the epithelial cell rests of Malassez and cells of the squamous odontogenic tumour. The role of epithelial cells of the periodontal ligament in relation to the squamous odontogenic tumor has been discussed by several authors since it was first described by PULLON *et al.* in 1975 (KRISTENSEN *et al.*, 1985; YAACOB, 1990).

II ROOT RESORPTION AND REPAIR

Root resorption can be a normal physiological process, such as in the shedding of deciduous teeth, or a process which occurs as a result of pathology. Such pathological causes may include trauma and infection. Human teeth may often show small areas of resorption which could arise as a result of minor trauma, or perhaps even as a result of a tooth coming into occlusion. Larger areas of resorption are less common and are more likely to be associated with severe trauma, infection or orthodontic treatment.

HENRY and WEINMANN (1951) conducted an histologic study of 261 teeth from 15 dentitions, and found areas of resorption on 90.5% of teeth. They found most of the resorptive areas in the apical third (76.8%). Repair was seen in 85% of these areas and these workers also noted that 72% of the areas were anatomically repaired. HENRY and WEINMANN (1951) concluded that it was normal for teeth to incur some degree of resorption during its life.

MASSLER and MALONE (1954) in an X-ray study of untreated and orthodontically treated human teeth from 708 subjects, showed that root resorption was widespread in both the treated and untreated samples. They noted that there was a considerable increase in resorption in the apical areas as a result of orthodontic

treatment. It is interesting that as this was an X-ray study, there would have been many areas of resorption that would not have been detected.

TRONSTAD (1988) contends that transient root resorption is a frequent occurrence as a result of trauma, and periodontal and orthodontic treatment, and that this mechanical or physical irritation will begin a resorptive process that will continue for a short period. However, after this period, the resorptive process cannot be continued without further stimulation. Development of a state of progressive resorption will occur, and may lead, eventually, to destruction of the root and possible loss of the tooth.

2.9 TYPES OF RESORPTION - GENERAL ASPECTS

ANDREASEN (1981) considered that resorption was related to the degree of damage to cells of the periodontal ligament and the distance along the root surface from the site of the original trauma. ANDREASEN (1981) used the results of his studies on resorption to propose that -

1. Minor areas of damage to the periodontal ligament, where a moderate amount of cells survive close to the root surface can be repaired from adjacent surviving parts of the ligament. This may involve resorption of the surface layer of the root, which can be repaired by new cementum if the resorption cavity does not reach dentine. Where the resorption is deeper and reaches dentine, two processes may occur.

    i. if the tooth root contains necrotic material, or infection, inflammatory resorption will occur.

    ii. if the root canal contains normal pulp or an inflamed pulp, repair of the resorption cavity will take place with cementum.

2. Larger areas of damage to the periodontal ligament where few cells survive close to the root surface, will initiate healing processes involving rapid

osteogenesis leading to ankylosis. This ankylosis will be permanent or temporary depending on the degree of damage to the periodontal ligament.

Therefore, minor damage may cause ankylosis that will later be resorbed and repaired, but larger areas of damage will often lead to permanent ankylosis (ANDREASEN and KRISTERSON, 1981; ANDREASEN, 1981).

ANDREASEN (1988) has recently reviewed root resorption systems, using a tooth replantation model with green Vervet monkeys, and has demonstrated that there are three responses as a result of replantation.

1. Surface resorption

This occurs as a result of an injury to the periodontal ligament and possibly also to the root surface. The damaged area is removed, mediated by cell proliferation from the adjacent periodontal ligament, and repaired by the formation of new cementum and periodontal ligament.

2. Inflammatory resorption

Inflammatory resorption occurs as a result of an injury involving the periodontal ligament and the tooth root where bacteria are present in the root canal. Bacterial products can enter the periodontal ligament, causing continuing resorption by clastic-type cells as a result of the inflammatory stimulation.

3. Replacement resorption

This type of resorption, also known as ankylosis, will occur when the damage to the bone, root and the periodontal ligament is more extensive, and the osteogenic healing processes overwhelm normal processes, so that the periodontal ligament begins to be replaced by bone. An ongoing process may see continued replacement of dentine. The fusion of tooth and bone may be temporary or permanent, and is apparently determined by the size of the initial ankylosis (ANDREASEN 1985).

Surface resorption, as previously described by ANDREASEN (1985), is considered by TRONSTAD (1988) to be a type of transient root resorption, and is a frequent sequel to trauma and orthodontic treatment. Such resorption could be thought to be clinically insignificant, unless it was to continue and become progressive (TRONSTAD 1988). This corresponds with earlier work on external root resorption (HENRY and WEINMANN, 1951) who suggested that root resorption associated with orthodontic treatment is usually transitory with little detrimental effect on the prognosis for long term health and function of the periodontal attachment.

2.10 FACTORS PROTECTING AGAINST RESORPTION

In a review of resorption in permanent teeth, BROWN (1982) postulated that if resorption is a universal phenomenon as suggested by early workers (HENRY and WEINMANN 1951; MASSLER and MALONE 1954) then resorption of teeth and repair by cementum could be an essential mechanism for tooth fixation and stability in the dentition. It was further stated that this could be a compensatory mechanism to cope with wear and trauma patterns, so that the relationship of teeth to each other is maintained. BROWN (1982) believed that the periodontal ligament was always in a state of constant responsiveness to any stresses and strains. It was noted that whereas tooth resorption is a common, but usually localized phenomenon, there may be widespread resorption of the tooth socket bone associated with orthodontic tooth movement. It was postulated that bone resorption in preference to tooth resorption could occur for several reasons.

1. The blood supply to the periodontal ligament is richer on the alveolar side of the periodontal space, and therefore the results of any inflammatory response will occur here first.
2. Cementoblasts and Sharpey's fibres may act as a physical barrier to resorption. Because of a reduced density of these fibres at the apex of the

tooth, this may be the reason for the reported increased susceptibility to resorption at the apex.

3. Osteoclasts are more likely to be remodelling the bone at any one time, and therefore are more likely to be able to begin resorption on the alveolar side of the socket more quickly.

1. PERIODONTAL LIGAMENT

In a study of replantation of teeth in six dogs and four monkeys (LÖE and WAERHAUG, 1961) it was shown that under varying conditions, success or failure of the replantation procedure depended upon the presence or absence, respectively, of an intact periodontal ligament. In situations where the periodontal ligament was completely removed, a normal periodontal ligament was not regained, and after less than thirty days a bony attachment had occurred. Where replantation occurred after the periodontal ligament was allowed to dry, a normal periodontal ligament was never found. Bony trabeculae originating from the alveolar side of the periodontal space were seen bridging the periodontal space and areas of ankylosis were encountered along the length of the root. In cases where the air drying was shorter before replantation, small areas of normal periodontal ligament were seen, but in all of these places, epithelial cell rests of Malassez were seen between the fibre bundles. Where not only did the periodontal ligament remain untouched, but replantation was immediate, ankylosis or bony bridging of the periodontal space was not seen. Most of these teeth showed areas of active or arrested resorption, especially in the gingival third, and the resorption lacunae showed the presence of cementum (LÖE and WAERHAUG, 1961).

LINDSKOG and HAMMARSTRÖM (1980) showed that the periodontal ligament contained a potent collagenase inhibitory factor which could play a role in the protection of the root from resorption. It was considered that the inhibitory factor could have arisen from either cells of the periodontal ligament or the cementum.

Further evidence of the importance of the periodontal ligament in the resorption and repair process was provided by LINDSKOG *et al.* (1988B) who used

explants of enamel organ epithelium and placed the explants in experimental cavities of extracted and subsequently replanted incisor teeth of monkeys (*Macaca fascicularis*). The results showed that in the experimental cavities with explants of odontogenic epithelium, there was complete periodontal healing, and a layer of reparative cementum covered the dentine surface. There was a variation in the thickness of the reparative cementum of 10 to 30μm. Furthermore, the alveolar bone around the explants had resorbed to distance equivalent to the original width of the periodontal space next to the experimental cavities.

2. CEMENTUM

It has been noted by several researchers that cementum is more resistant to resorption than either dentine or bone (HAMMARSTRÖM and LINDSKOG, 1985; LINDSKOG *et al.*, 1985). RYGH (1977) also suggested that the surface of cementum is more resistant to resorption than dentine. HAMMARSTRÖM and LINDSKOG (1985) point out that although the composition of bone and cementum is very similar, cementum is less readily resorbed.

LINDSKOG and HAMMARSTRÖM (1980) have suggested that cementum may contain an anti-invasion factor, such as the anti-invasion (protease inhibitor) factor present in cartilage.

LINDSKOG *et al.* (1985) suggested that cementum may lack specific plasma proteins, unlike bone, which have been shown to stimulate attraction of osteoclastic activity and phagocytosis. These workers also felt that cementum could contain inhibitors of phagocytosis yet to be identified. It was also thought that cementum resorbing cells require prior activation. Activating factors could include infection, inflammation, bacterial products, and necrotic debris before resorption can begin. Such factors may also be responsible for the chemo-attraction of macrophages, and other phagocytic cells. These cells may then, themselves, attract further cells, and activate cementum resorbing cells by secretion of other substances. Osteoclast activating factor

and prostaglandins have been suggested, as they are also thought to play a role in bone resorption (HAMMARSTRÖM and LINDSKOG, 1985).

LINDSKOG *et al.* (1987) concluded that the layer of cells covering the root surface protect the root surface integrity by forming a protective barrier.

3. SHARPEY FIBRES

BROWN (1982) considered that the densely packed Sharpey fibres that arise from the cementum may act as a physical barrier to root resorption. In contrast, the less densely packed fibres inserting into the alveolar bone of the socket wall may partly explain why cementum is more resistant to resorption than bone. BROWN (1982) further suggests that the very severe resorption that can sometimes occur in children undergoing orthodontic treatment, may be a result of an unusually low number of Sharpey fibres in the cementum in these particular patients, making these teeth more susceptible to resorption. Other workers have suggested that the progress of root resorption is unaffected by the presence of Sharpey fibres (BARBER and SIMS, 1981) and that Sharpey fibres are no more resistant to resorption than the surrounding mineralised tissue (HARRY and SIMS, 1982).

4. EPITHELIAL CELL RESTS

LÖE and WAERHAUG (1961) considered that not only did the success or otherwise of the replantation procedure depend on a vital periodontal ligament, but that more specifically, the presence of epithelial cell rests of Malassez contributed to the maintenance of the periodontal space.

LINDSKOG *et al.* (1988B) considered that the epithelial cell rests of Malassez contributed to maintaining the width of the periodontal ligament as well as preventing ankylosis. BRICE (1988) provided evidence of this role when epithelial rests were found in areas of repairing root resorption. It was postulated that that the rests were not only involved in the repair of resorbed root surfaces, but also in the restoration of a normal periodontal ligament.

2.11 RESORPTION OF TEETH

The resorption processes in teeth and bone are very similar (HAMMARSTRÖM and LINDSKOG, 1985). In a recent review of osteoclast structure and function, PIERCE *et al.* (1991) note that there is very little difference between the ostoeclast and dentinoclast (odontoclast). Not only are their respective substrates very similar (varying only in their respective proportions of constituents), but they share similar enzymatic properties and function in a similar manner. Other workers have described some differences in these cells. Dentine resorbing cells have been described as being smaller (BOYDE and JONES, 1979), having less nuclei (HAMMARSTRÖM and LINDSKOG, 1985), and smaller clear zones (LINDSKOG *et al.* , 1983). The resorptive cells arrive via the blood stream as mononuclear precursors, from either the spleen or marrow tissue whether they are osteoclasts or dentinoclasts (PIERCE *et al.* , 1991).

1. MORPHOLOGY OF RESORPTION

BOYDE and LESTER (1967) examined the resorption of dentine by odontoclasts, and considered that the demineralization of the surface of the dentine preceded the removal of the organic materials that lined the resorption lacunae. BOYDE and LESTER (1967) also noted the similarities in appearance of resorbing bone and cementum.

LANGFORD and SIMS (1982) in a scanning electron microscope study of root resorption, commented that in resorbed and unrepaired dentine, fibre bundles were often observed orientated predominantly at right angles to the surface of the dentine. They believed that as dentine was resorbed, part of its fibre matrix was retained and could serve to act as an ongoing source of direct attachment.

HAMMARSTRÖM and LINDSKOG (1985) in a review of tooth and bone resorption mechanisms, considered the resorptive processes of teeth and bone to be similar, although there were some differences in the cells resorbing bone and dental

tissue. This difference was attributed to the differences in the composition of the tissue being resorbed.

LINDSKOG *et al.* (1988A) have studied the processes and morphology of dentine resorption using monkey incisors. By extracting, then replanting incisors with infected pulps and artificial resorption cavities prepared in the roots, it was possible to follow the progression of the resorptive process. LINDSKOG *et al.* (1988A) described a number of steps in the process of resorption.

1. After 20 hours the experimental cavities were covered in a thin smear layer of macrophage cells. The cells were 10-15μm in diameter and had a granular, folding surface. The smear layer had completely disappeared by 40 hours.
2. Between 30 and 40 hours, dentinoclasts in three different morphological stages of spreading were observed. After 7 days, all three stages could be seen within the same specimens.

Stage 1. Spherical dentinoclasts, 30μm in diameter, appeared in the central part of the cavities. They had granular dorsal surfaces, numerous long filopodia oriented towards the surface of the dentine, and some of the filopodia projected into open dentinal tubules.

Stage 2. After 40 hours, flattened dentinoclasts, 50μm in diameter were found in the central part of the cavities. These cells had a peripheral fringe of short filopodia, and some cells appeared more flattened than others. Occasional pits and vesicles were seen on a granulated dorsal surface. There were indications of multinuclearity.

Stage 3. After 4 days, completely flattened, tightly attached multinucleated dentinoclasts were seen in crater-like depressions in the dentine surface. They were 50 to 100μm in diameter, and had a peripheral edge of smooth plasma membrane without the earlier peripheral filopod fringe. The plasma membrane was slightly granulated, and some pits and vesicles of less than 1μm were apparent.

3. From one to four weeks after replantation, there was a continuation of the resorption towards the pulp. Numerous new resorption lacunae of 50 to 100μm in diameter appeared, some containing a single dentinoclast, and others empty. The surface of the dentine in the lacunae was smooth, with open dentinal tubules apparent.

SASAKI *et al.* (1989) looked at the process of physiological root resorption in the shedding of the deciduous teeth in kittens. Resorption lacunae were seen at the resorbing surfaces of the root dentine and cementum. These lacunae contained various cells, including multinucleated odontoclasts, pre-odontoclasts, fibroblasts, macrophages, and neutrophils.

2. ULTRASTRUCTURE OF RESORPTION

In a review of the processes that are involved in the physiological resorption of human deciduous teeth, BOYDE and LESTER (1967) saw large numbers of Howships lacunae in the dentine. The resorption bays in cementum had sharp edges, and gave the impression of considerable undermining.

SASAKI *et al.* (1988) in an SEM study of the resorption of human deciduous teeth considered that the resorption lacunae in dentine could be broadly classified into two types: larger, shallower, irregularly shaped lacunae, with intact peritubular dentine, and deeper, round lacunae of 30 to 50μm in diameter, with extensively dissolved peritubular dentine. These workers also suggested that in physiological resorption, odontoclasts are able to demineralize the surface dentine extracellularly, and can also resorb and dissolve some of the released apatite crystals.

The ultrastructure of the cells involved in the shedding of human deciduous teeth has also been examined (SASAKI *et al.* 1988). Odontoclasts were characterised by abundant mitochondria, various lysosomes, stacks of Golgi membranes, many vacuoles, and a well developed ruffled border against the resorption surface. In undecalcified sections, apatite crystals were demonstrated in the extracellular spaces of

the ruffled border, and in adjacent endocytic vacuoles. Lysosomes in the perinuclear region also contained small amounts of crystals.

BRICE (1988) described odontoclasts in areas of active resorption in human premolars (extracted for orthodontic reasons) as having the following features:

1. Large and irregular shape with 1 to 4 prominent nucleoli.
2. A ruffled border close to the tooth surface, but no clear zone was seen.
3. A highly vacuolated cytoplasm, with large numbers of mitochondria and amounts of rough endoplasmic reticulum, and an abundance of microtubules and filaments.

RYGH (1989) observed that in root resorption in rats, the cells which initially penetrate the precementum and cementum are uninuclear cells without any ruffled border, followed later by multinuclear cells.

SASAKI *et al.* (1989) in a study of the cyto-differentiation and degeneration of odontoclasts in physiological resorption, described the features of cells involved in the process, and noted the following features.

1. The mononuclear odontoclast precursor cells contained many mitochondria, cisterns of rough endoplasmic reticulum, numerous free polyribosomes, stacks of Golgi membranes and lysosomes in the perinuclear cytoplasm, but the cytoplasm was not vacuolated.
2. The multinuclear odontoclast precursor cells had similar contents, but did yet have ruffled borders or clear zones. Numerous pale vacuoles (150-500nm), which appeared to have developed from the margins of the Golgi apparatus appeared throughout the cytoplasm.
3. Fully developed odontoclasts located in the resorption lacunae, appeared to arise from joining of preodontoclasts by direct fusion of their cytoplasmic processes. Much of the cytoplasm was occupied by numerous mitochondria, free polyribosomes, rough endoplasmic reticulum, and pale vacuoles. The vacuoles accumulated in the cytoplasm near a clear zone which developed near

the resorbing surface. The odontoclasts also showed a ruffled border, and long broad cellular processes, which had a clear-zone-like cytoplasm.

4. Degenerative odontoclasts were sometimes seen on the surface of the resorbing dentine. As degeneration occurred, the characteristic ruffled border structures disappeared. Eventually the cytoplasm was totally occupied with residual bodies, and numerous pale vacuoles of varying sizes. Degenerated odontoclasts appeared to fuse with granular leukocytes (neutrophils).

2.12 REPAIR OF ROOT RESORPTION

Healing of root resorption, whether its cause is traumatic or infective, takes place by similar mechanisms. The damaged area, which may include areas of periodontal ligament, cementum and dentine, are removed and replaced by new cementum and periodontal ligament components. Repair can be anatomical, or the defects can be overfilled or left as shallow depressions, suggesting some imprecision in the repair processes, at least in the early stages of repair (BARBER and SIMS, 1981). Repair processes can be demonstrated to have begun within a week of the cessation of the resorption. (LINDSKOG *et al.*, 1987)

The repair of root surfaces that are devoid of cementum has been looked at using experimental cavities in monkey teeth (LÖE and WAERHAUG, 1961; LINDSKOG *et al.* 1983, 1987; LEEDHAM, 1990), and by examining human teeth that have been used as anchors in rapid maxillary expansion (BARBER and SIMS, 1981; LANGFORD and SIMS, 1982; LANGFORD, 1982).

The repair processes involved in the reattachment of the fibres of the periodontal ligament have also been examined. STAHL (1979) has suggested several possible modes of re-attachment of connective tissue to tooth root.

1. residual Sharpey fibres could join with fibres in the periodontal ligament.

2. connective tissue fibres could attach to the root surface like a modified periosteum.

3. cementum deposition with fibre insertion on the root surface.

BARBER and SIMS (1981) saw very few Sharpey's fibre depressions in the advancing mineral front of repairing cementum after retention periods of up to 36 weeks following RME They suggested that only limited periodontal fibre reattachment occurs, although conceding that it may occur at a later stage once a normal root surface contour is re-established by the repairing cementum.

LANGFORD and SIMS (1982) stated in a scanning electron microscope study of root resorption, that the repairing tissue was always cellular cementum, regardless of the location of the repairing tissue on the tooth root. This repairing cellular cementum appeared to be histologically and topographically identical with apical cementum. However depressions in the repair surfaces, characteristic of Sharpey fibre attachment, were not a feature. Furthermore, the orientation of the periodontal fibres overlying areas of more advanced repair was seen to be parallel to the root. It was suggested that with time the fibres would become functionally orientated in a more oblique and horizontal direction.

NYMAN *et al.* (1982) looked at periodontal ligament repair where the cementum had been removed, and found two types of repair. Where new cementum had been formed, inserting collagen fibres were observed. However, in some cases where healing had occurred without signs of new cementum and fibrous attachment, the repair was characterized by connective tissue adhesion to the root.

LINDSKOG *et al.* (1983) examined the general processes of periodontal repair in replanted teeth with experimental root resorption. After eight weeks the teeth were extracted and examined histologically. It was seen that the reparative cementum did not fill the cavities, although it was thicker towards the centre of the cavities. The teeth were removed with their associated alveolar bone, and it was observed that the bone filled in the cavities, but maintained a relatively constant periodontal space of about 150µm. It was also noticed that a gap often developed between the reparative cementum and the dentine. Attachment between the reparative cementum and dentine

seemed strongest where there had been associated surface resorption lacunae, and near the edges of the cavities in association with either intermediate cementum or cementum of the root surface. LINDSKOG *et al.* (1983) suggested that good attachment can only occur when the organic matrix of the dentine is exposed.

LINDSKOG *et al.* (1987) noted in an SEM study that in repair of experimental cavities, which are devoid of cementum, there was a superficial resorption of dentine in minor areas prior to repair of the root surfaces. These resorbing cells appeared as early as 24 hours after replantation of the experimental cavities. The cells were described as macrophage-like, and were well attached to the centre of the cavities. After 3 days shallow resorption lacunae were seen in the cavities. Large cells with the characteristics of resorptive cells were seen in about half of the lacunae. It was seen that these resorptive-like cells disappeared as the cavity was gradually filled from the periphery by cells with a fibroblast-like appearance. After six weeks, once the entire cavity was filled with these cells, a mineralized tissue had formed underneath them that had all the characteristics of cementum.

BRICE (1988) considered that repair was characterised by a number of clear features and observed the following.

1. Deposits of cellular fibrillar cementum over the resorbed dentine, with a clear reversal line between them.
2. Epithelial cell clusters were seen in some of the repairing resorption bays.
3. Collagen inserted into the reparative cementum, with the fibres perpendicular to the surface of the dentine.
4. Cementoblasts were present with large round or ovoid nuclei, and with extensive light staining cytoplasm.
5. Pre-cementoblasts were seen located between the repairing surface and the epithelial cell clusters.

BRICE (1988) concluded as a result of his study, that epithelial cell rests of Malassez are present in areas of root resorption, and that they may play a role in the

repair of the cementum surface and the re-establishment of a normal periodontal ligament. He considered that the rests had the structural features suggesting that they might initiate cytodifferentiation of cementoblasts.

2.13 ORTHODONTIC ROOT RESORPTION

The application of orthodontic forces to teeth will create areas of both compression and tension around the teeth within the periodontal ligament. BARBER and SIMS (1981) in a scanning electron microscope study of root resorption as a result of rapid maxillary expansion, demonstrated that there can be widespread root resorption, with unacceptable amounts of iatrogenic tissue damage.

CWYK *et al.* (1984) in a radiological review of post-orthodontic patients has indicated that over 28% of incisors in these patients, 5 to 10 years after completion of treatment, showed some degree of apical resorption. A similar control group indicated that only 3.4% of similar teeth had the same degree of apical resorption. Lateral resorption lacunae were apparent in 9.4% of the orthodontically treated upper central incisors compared with only 0.5% in the control group. In all patients the resorption appeared to be arrested.

The histological response to such forces may involve remodelling of the tooth socket by resorption and deposition of bone, necrosis and re-establishment of the periodontal ligament, and resorption and repair of the teeth involved. It has been suggested that root resorption as a result of orthodontic treatment, seems to be related to local areas of damage to the periodontal ligament, especially where hyalinization occurs (RYGH 1989).

The size and duration of orthodontic forces applied to the teeth are critical in determining the extent of areas of compression and tension within the periodontal ligament. Where compression of the periodontal ligament occurs, two processes will occur according to RYGH (1982). If light forces are used, direct (frontal) resorption will usually occur, and where the pressure is sufficient, "hyalinization" will occur. Lighter forces of the type to cause frontal resorption are more compatible with the

survival of cells within the periodontal ligament according to PROFFIT (1986). He considers that the objective of orthodontic practice should be to move teeth as much as possible by the process of frontal resorption, although he concedes that areas of periodontal necrosis and undermining resorption are probably inevitable.

REITAN (1974) in a study of root resorption as a result of extrusion, intrusion, and tipping of human premolar teeth, noted that resorption was a common occurrence as a result of orthodontic treatment. It was suggested that existing cementum is a barrier to resorption, and that if a thick layer of predentine is present in developing teeth, then apical resorption will not prevent continuing development of the roots. Where moderate forces are used, the resorption areas will be small, and will be filled in by cellular cementum. REITAN (1974) considered that external root resorption was closely related to the type of tooth movement, especially to tipping and intrusion.

RYGH (1977) observed that resorption of the cementum occurs simultaneously with the removal of hyalinized tissue, and is mediated by cells from the adjacent healthy periodontal ligament. It was also observed that the process of tooth resorption can continue even after the removal of hyalinized tissue. RYGH (1977) further demonstrated that resorption of cementum occurs as an undermining process, where odontoclasts attack the cementum from behind in resorption lacunae.

BARBER and SIMS (1981) noted actively continuing resorption after up to 36 weeks of retention in RME patients. They noted that resorption was more severe in these patients in the cervical and middle thirds of the teeth, although apical resorption was also observed. One explanation for this result was possible moulding of the thinner and more flexible alveolar bone of the cervical region exerting a compressive effect leading to resorption.

ENGSTROM *et al.* (1988) examined the effects of inducing orthodontic root resorption in the upper incisors of rats. They saw that resorption lacunae were consistently observed in the compression zones of the periodontal ligament adjacent to the hyaline zones, along with degradative activity, and alveolar bone resorption. In hypocalcaemic rats, the resorption occurred earlier, and was more rapid and more

extensive. It was concluded that orthodontic root resorption was related to the reorganisation occuring in the alveolar bone and periodontal ligament during orthodontic movement, and that the increased severity of root resorption occurring in some patients could be related to a higher than normal rate of bone resorption. ENGSTROM *et al.* (1988) suggested that orthodontists should consider the effects that increased alveolar turnover rate will have, and how to reduce the periods of hyalinization, rather than just the actual amount of force itself.

REMINGTON *et al.* (1989) have stated that apical root resorption did not progress on appliance removal, and that except in severe cases of resorption, function of the teeth was not affected.

RYGH (1989) considers hyalinization to have two important effects. It is the rate limiting factor in orthodontic tooth movement, and it is involved in permanent damage to the teeth.

Repair as a result of hyalinization requires the removal of hyalinized tissue, re-establishment of the periodontal attachment, and repair of root resorption. Providing forces are kept within a reasonable clinical range, necrosis and degeneration could be limited to small areas of the periodontal ligament and should not occur in the adjacent alveolar bone.

LINGE and LINGE (1991) used standardised intra-oral radiographs to review variables associated with apical root resorption as a result of orthodontic treatment in 485 consecutively treated patients. They noted that several factors were significantly associated with apical root resorption. These factors were overjet, history of trauma to maxillary incisors before orthodontic treatment, time of treatment with rectangular wires, time of treatment with Class II elastics, lip/tongue dysfunction, and/or history of finger sucking habits beyond 7 years, and impacted maxillary canines to be corrected orthodontically. However as BARBER and SIMS (1981) have noted, frank apical loss is noted by X-ray examination, but the extensive buccal root resorption seen in their scannining electron microscope study associated with rapid maxillary expansion, was not apparent on long-cone intra-oral periapical radiographs. Clearly the resorption

detectable from radiographs understates the true amount of root resorption associated from orthodontic treatment. The significance of this root resorption lies in the ability of the various repair mechanisms to repair the damage, and its long term effects on tooth prognosis.

VARDIMON *et al.* (1991) looked at external root resorption and repair in Macaque monkeys in an scanning electron microscope study using various palatal expansion devices. They suggested that when the forces applied to teeth reached a critical amount and the periodontal ligament was compressed to a certain level, it would fail to operate as a "physio-immune" system and no longer protect the root from resorption.

III. IMMUNOHISTOCHEMICAL METHODS

There are now a large number of immunohistochemical methods available for both research and diagnostic pathology. All rely on the principle of detection by the use of antigen-specific antibodies. These techniques are available for both light microscope and electron microscope use. The original use by COONS *et al.* (1941) of fluorescent antibodies opened the way for the present day developments. However this technique was not applicable for the electron microscope, and the introduction of ferritin (SINGER, 1959) as an electron dense marker opened a new field of use for immunolabelling.

2.14 METHODS OF IMMUNOLABELLING

1. Immunofluorescence

This technique may be direct (fluorescein labelled primary antibody) or indirect (fluorescein labelled secondary antibody). It is based on the principle that the labelled antibody, once attached to the antigen can be seen on exposure to blue or ultraviolet light, because a green immunofluorescence will be visible at the site of the antibody-antigen interaction. Disadvantages of the method are that some tissues have a natural

immunofluorescence, the fluorescence will fade (requiring a photographic record), and there is limited sensitivity and range of uses (GOSSELIN *et al.*, 1986).

2. Immunoperoxidase

Horseradish peroxidase (MW 40,000) is conjugated to antibodies in place of fluorescein. The original work on the immunoperoxidase labelling technique was done by NAKANE and PIERCE (1966). In the presence of hydrogen peroxide, it forms a complex, which when added to an electron donor substrate (usually DAB - 3'3' diaminobenzidine tetrahydrochloride), forms a second complex that dissociates leaving an insoluble reaction product, that can be seen under the light microscope (GRAHAM and KARNOVSKY, 1966). This method can be used in both light and electron microscope applications. It is most often employed as an indirect technique. Disadvantages most often cited include fixation requirements, toxicity of the DAB substrate, and endogenous tissue peroxidase activity (ROMANO and ROMANO, 1984).

3. Avidin-Biotin

This method was introduced by HSU *et al.* (1981), and uses a biotinylated secondary antibody, which, when combined with an avidin-biotinylated complex utilizes the very high affinity of biotin for avidin, so that the reaction is practically irreversible. Recently streptavidin has been substituted for the avidin to reduce the problems of non-specific binding (due to its near neutral iso-electric point) that were occasionally present and this is reported to further increase acccuracy (DAVIES, 1989). This method is one of the most widely used in routine diagnostic pathology, and is considered accurate and sensitive (MATTHEWS, 1987). Sensitivity can be further increased in this method. Nickel and cobalt in particular can be used to enhance the DAB reaction product (HSU and SOBAN, 1982), or it can be osmicated (DAVIES, 1989).

4. Colloidal Gold

This is the method most applicable to electron microscopy due to its distinctive electron density. It was first introduced by FAULK and TAYLOR (1971). Colloidal gold can be prepared in a variety of sizes from 5nm to 150nm, and can be adsorbed to a number of proteins including immunoglobulins, protein A and lectins in particular. LARSSON (1979) has used gold particles of different sizes to locate two different antigens in the same tissue sections (double labelling).

Colloidal gold can also be used in light microscope applications using the silver staining enhancement technique (HOLGATE *et al.*, 1983; DANSCHER and NÖRGAARD, 1983). This enables the visualization of the gold particles by a silver development technique. It has been suggested that this technique may be applicable to specimens where there has been overfixation or poor processing (HACKER *et al.*, 1985).

CHAPTER THREE

MATERIALS AND METHODS

3.1 INTRODUCTION

This project consisted of two phases. An initial phase was required to test the effects of several fixation schedules, as well as the effect of decalcification on the immunohistochemical protocols that were to be used in the main part of the project. The initial part of the project also allowed testing and development of the immunohistochemical procedures, and preparation of a protocol that could be reliably and routinely used. Previous work has suggested that immunohistochemical methods could be utilized to look at human epithelial cell rests (GAO *et al.* 1988; LEEDHAM 1990).

The second phase of the project used the various protocols developed in the initial phase in an histomorphometric study. This study used the immunohistochemical methods to look at the relationship of epithelial cell rests to resorptive activity in human premolar teeth after rapid maxillary expansion (RME) therapy. The histomorphometric study also assessed the extent of root resorption on premolar teeth as a result of the RME therapy.

3.2 MATERIAL

The material for both phases of this project consisted of human premolar teeth extracted for orthodontic reasons. The teeth for the initial phase were extracted for orthodontic reasons part of the way through treatment as a result of reassessment of the treatment aims, and came from several patients who had had full fixed appliances for varying durations (Table 3.2).

Material for the second part of the project also required the collection of human premolar teeth. Premolar teeth that had been extracted prior to fixed orthodontic

treatment, but after being used as anchor teeth for rapid maxillary expansion were used for this purpose (Table 3.3). It was considered that these teeth would be the most useful for the purposes of the project, as not only would they be subject to extensive resorptive potential as reported by previous authors (BARBER and SIMS, 1981), but also because there should be areas of repair present where epithelial cell rest sections may be demonstrated (BRICE 1991). The lower premolars could act as controls where they had also been extracted as part of the treatment plan. It was also considered that these teeth would be easier to collect and should therefore be more easily available.

In no part of the project was there any deviation from normally accepted treatment planning, or treatment procedures. The procedure was explained, and permission was sought from the patients, and where necessary, the parents, to collect the teeth for participation in the study.

3.3 FIXATION

Several fixation schedules were tested and used in the initial part of the study.

1. Half-strength Karnovsky's fixative (Appendix 1)
2. 10% Formosaline solution (Appendix 2)
3. Microwave fixation

The teeth were fixed immediately after extraction. Where formalin or the half-strength Karnovsky's solution were to be used, fixation was allowed to take place for 6 hours at room temperature, and then the teeth were washed in PBS solution (Appendix 8), and immediately placed in the decalcifying solution. Where microwave fixation was to be utilized, the teeth were immersed in a beaker of 400ml of normal saline, and placed in a temperature controlled microwave oven (National, NE6980, operating at 2480MHz and 600W), brought to $63^{o}C$, and held at that temperature for 2 minutes (LEONG *et al.* 1985), and were then immediately placed in the decalcifying solution.

The second part of the project used 10% formosaline fixation for no more than 6 hours duration for all teeth collected.

3.4 DECALCIFICATION

The teeth were demineralised in 250ml of 4% EDTA solution in cacodylate buffer (Appendix 3) at 4^{o}C (modified from BJURHOLM *et al.*, 1989). The cassettes containing the tissue were suspended in the decalcifying solution, near the top of the container to avoid the heavier consumed EDTA solution. The solution was changed twice a week. The process of demineralization was checked by X-raying the teeth using Kodak DF-49 occlusal film (Kodak, Australia). Decalcification times depended on the number of specimens in the solution, but generally took 6 to 8 weeks.

3.5 DIVISION OF TEETH

The teeth were divided as soon as possible to facilitate the decalcification process. The crown was separated by making a cut coronal to the cemento-enamel junction, and was discarded. After recording which were the mesial and distal surfaces, the remaining tooth root was placed into a labelled cassette, to complete the decalcification process.

3.6 EMBEDDING

A double embedding procedure using celloidin was employed (Appendix 4), in order to give the sections greater strength. Routine paraffin processing was employed, and interrupted at the appropriate times to infiltrate with the celloidin. All tissue blocks were embedded in a similar manner for orientation purposes.

3.7 SECTIONING

Paraffin blocks were mounted in a Leitz 1512 Microtome, and ribbons of 5µm transverse sections cut, and floated on a water bath thermostatically set at 45^{o}C. Four sections at a time were collected and oriented onto chrome-gelatin coated, labelled glass slides (Appendix 5) in a consistent manner. The slides were allowed to air dry, and then were placed into an oven at 35^{o}C for 1 to 2 hours. Preliminary sections from

each block were stained with haematoxylin and eosin (Appendix 6) to ascertain quality of fixation. For the initial study, approximately 100 subsequent 5μm sections from each block were then collected and prepared for antibody labelling (Appendix 7) if there was adequate soft tissue remaining attached to the tooth or areas of interest. These sections were then used to test the labelling and staining procedures.

For the second part of the project, the whole tooth root was transversely sectioned into 5μm sections from the cemento-enamel junction to the apex. Root length varied from 9mm to 12mm, requiring over 14,000 sections to be prepared and collected onto more than 4000 slides.

3.8 HISTOMORPHOMETRIC ANALYSIS

The second part of the project was an histomorphometric study to assess the extent of the effects of orthodontic tooth movement (rapid maxillary expansion) on the epithelial cell rest population. The analysis was based on the principles described by ANDREASEN (1987) and ANDERSSON *et al.* (1987).

Each tooth root was totally divided into 5μm sections and the length of each root calculated using the most coronal section and the most apical section as the first and final sections. The most coronal section was the first section with a complete circumference of cementum, and the most apical section, the last section before the apical foramen was apparent. The sampling procedure used tenths of the root length, so that sections at each ten per cent level were chosen for IHC staining. This produced 11 levels to be examined for each tooth in the study (Figure 3.1).

At each level, the best section on the slide was selected and examined after staining using an octant system (Figure 3.2) adapted from ANDREASEN (1987). A fine cross-hair grid with eight equal octants was placed over the section, always orientated in the same manner, and data collected for each section and recorded on a data sheet (Appendix 11).

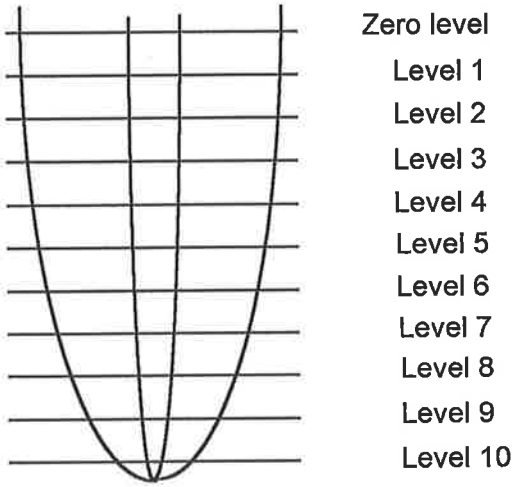


Figure 3.1 A longitudinal diagram of a tooth root showing the levels at which sections were stained using IHC methods, prior to histomorphometric analysis.

The same magnification was used to examine all sections (20X objective lens), although a higher power (40X) was used if required, to positively identify cells or sections of rests. The use of higher power was avoided as much as possible in order not to over estimate values. The octant system was chosen as a result of a review by ANDERSSON *et al.* (1987) examining various histomorphometric methods and which suggested that an eight point registration system was very sensitive in estimating the true value of root resorption.

For analysis purposes, each transverse section was divided into four regions that were related to the octant recording system (Figure 3.2). Region 1 (octants 1 and 8) represented the areas closest to the expected principal effects of the RME therapy (ie the buccal root surface). Region 2 (octants 2 and 7) represented the areas a little further distant from the experimental sites, and was approximately equivalent to the mesiobuccal and distobuccal areas of the tooth root. Region 3 (octants 3 and 6) represented the areas a little further distant again from the experimental sites, and was approximately equivalent to the mesiolingual and distolingual areas of the tooth root. Region 4 (octants 4 and 5) represented the areas most distant from the expected experimental effects and corresponded to the lingual surfaces.

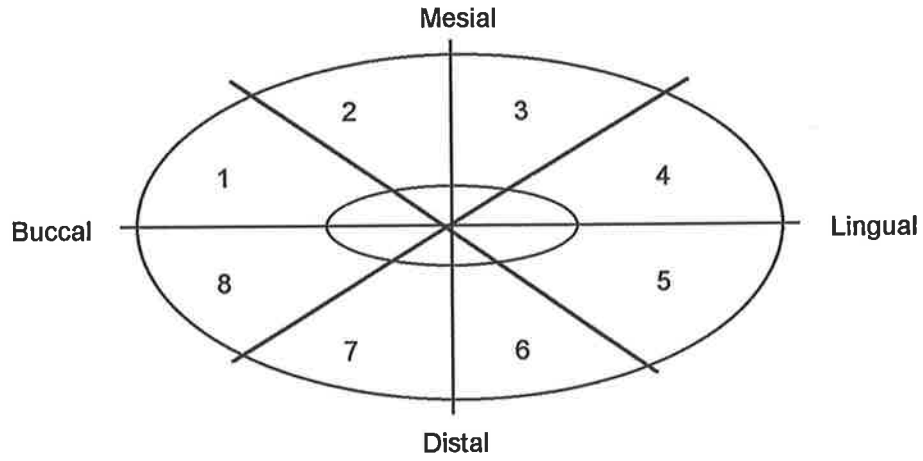


Figure 3.2 Diagram of a transverse root section demonstrating the octant system used to collect data at each level. The numbers identify each octant, and were used consistently for each tooth in the study.

Data were collected for each octant relating, among other things, to the amount of soft tissue present, the extent and type of resorption, the presence of blood vessels and the presence of sections of epithelial cell rests. Table 3.1 lists the variables that were examined for every octant examined. The root surface was examined on the basis of surface integrity, the main divisions being surface intact (ie no signs of resorption or repair), active resorption (ie osteoclastic cells or continuing resorption evident), surface repair (reversal line present and signs of reparative cementum), and an indeterminate category for those areas where it was not possible to classify the root surface into either active or repairing resorption. Field 9 (zone) was placed in the recording system to take account of the fact that most upper first premolars have a buccal and lingual root. Zone 1 represented the tooth root above the bifurcation, zone 2 the buccal root and zone 3 the lingual root. Where both buccal and lingual roots were present, each root surface was completely examined and scored as a separate entity. The amount of soft tissue present, and the extent of resorption present, were recorded on a scale of one to ten, representing 0% and 100% respectively for each octant.

An analysis of the error of the method was also performed. During the early stages of the development of the histomorphometric methods, several teeth were recounted as a measure of the accuracy of identification and counting of the epithelial cell rests sections. All counting of the sections was performed by the one person. Although no statistical analysis of the method was performed, the reliability of identification of the cell rest sections was seen to be very high. This was particularly evident when the monoclonal antibody was used with its low level of background staining and higher specificity.

Information from all the data sheets was entered onto a spreadsheet and then analysed on the University of Adelaide Vax computer, using the program 5v from BMDP Statistical Software (DIXON, 1991). Each variable was analysed in turn by an unbalanced repeated measures analysis of variance, with grouping factors of zone and treatment, and within tooth factors of region and level. This program was chosen for its ability to deal with missing data, which was expected to occur as a result of the extraction process. Furthermore, this program is able to provide tables of "expected values" which are model smoothed versions of sample averages. These model smoothed versions provide the essence or trend of the changes occurring along and around the root surface, despite the missing data, and without the need to provide standard errors or standard deviations.

3.9 IMMUNHISTOCHEMISTRY PROCEDURES

The following methods were used (Appendix 7).

1. ANTIBODIES

The material was stained using monoclonal and polyclonal antibodies diluted appropriately in 3% normal horse serum (Table 3.4). Antibodies were chosen that had been shown in other studies to be active against the proteins of epithelial tissue (ie cytokeratins).

The polyclonal antibody used was human callus antikeratin (Dakopatts; Code A575), at dilutions of 1:500(v/v), and 1:1000(v/v). This antibody was used only during the preliminary stages of protocol development as it was known to give good results with epithelial cell rests from human periodontal ligament (LEEDHAM 1990).

The monoclonal antibody was AE1/3 (Biogenix, USA; Code MU071-UC), at a dilution of 1:450(v/v). This antibody was used for the main study to reduce the extent of background staining and because of its improved specificity compared to the polyclonal antibody. This is actually a mixture of two antibodies (AE1 and AE3), developed by COOPER *et al.* (1985) which these workers suggest, when used together, has a "broader spectrum of activity than any other known antikeratin". Antibody AE1 reacts with cytokeratins of the acidic subfamily, specifically cytokeratins 10, 14, 15, 16, and 18. (Cytokeratins are numbered according to MOLL *et al.,* 1982). Antibody AE3 reacts with all the members of the basic subfamily, ie CK1 to 8 (COOPER *et al.* 1985).

The secondary antibody preparation was chosen to be used with both the polyclonal and monoclonal antibodies employed in this study. The "Multilink" preparation (Dako, Code E 453) is a biotinylated swine antibody active against all classes of mouse, rabbit, and goat immunoglobulins. The recommended dilution for this preparation is 1:50 to 1:150 when used in immunohistochemistry studies. This particular antibody was chosen and tested for use on the recommendation of staff at the Immunocytochemistry Laboratory of the Institute of Medical and Veterinary Science in Adelaide.

2. STAINING PROCEDURE

After deparaffinization, the sections for cytokeratin staining were subject to enzyme digestion with 0.5mg/ml porcine type II trypsin (Sigma, Code T-8128) for 3 to 10 minutes and washed in phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched using 0.6% H_2O_2 in 95% methanol for 30 minutes, and the

sections were washed in PBS to remove reagents. The blocking serum (3% normal horse serum) was applied for 30 minutes.

All primary antibody incubations were then performed overnight at room temperature, followed by the biotinylated secondary antibody ("Multilink", Dako E 453) diluted 1:80 in normal 3% horse serum for thirty minutes.

After washing in PBS, the Streptavidin-biotin complex, diluted 1:200 in normal 3% horse serum, was applied (Amersham Australia, Sydney; Code RPN-1051) for forty five to sixty minutes, and washed in PBS. The DAB substrate was then applied (3'3'-diaminobenzidine tetrahydrochloride; Sigma, Code D9015) as a 0.05% solution, for 5 to 10 minutes. The DAB staining was controlled both macroscopically and where necessary, microscopically, to ensure that the level of background staining was not allowed to rise to unacceptable levels.

After rinsing in running tap water for five minutes, the sections were then counter-stained in haematoxylin (Appendix 6), dehdrated through graded alcohols and xylol, coverslipped with Depex (BDH Chemicals, Melbourne) and viewed.

3. CONTROLS

Positive and negative controls were used in all studies. Negative controls consisted of parallel sections where staining was carried out as described, except for substitution of the primary antibody with the diluent buffer (ie deletion of the primary antibody). Positive controls consisted of sections of either tonsil or preferably gingival tissue, including sections of epithelium. Some of the teeth collected had small amounts of attached gingival tissue, and these proved to be useful controls, as both gingival epithelium, and epithelial cell rests were often present in the same sections. Where possible these sections were used as controls, as the relatively large areas epithelial staining were easily assessed, and gave a good guide to the effectiveness of the staining protocol. This was also preferred as the gingival tissue had also undergone exactly the same processing and fixation protocols.

3.10 PHOTOGRAPHY

Ilford FP4 plate film (4" x 5", ASA 125) was used in a Zeiss Axiomat to photograph selected sections. The best negatives were obtained with the Axiomat set at 100 ASA, and a Zeiss green filter. The film was developed in Microphen developing solution diluted 1:1 with tap water at 20°C, for seven minutes using constant agitation. The film was rinsed in water, and fixed in Hypam rapid fixer for 5 minutes. After washing for 15 minutes, the negatives were briefly rinsed with Kodak Photo-Flo wetting agent, and then dried in a drying cupboard until dry, and stored in labelled cellophane bags.

Sections stained with either haematoxylin and eosin, or labelled with antibodies and counter-stained with haematoxylin were photographed in an Olympus BHT microscope fitted with a PM-10ADS 35mm photographic attachment. Photographs were taken using either Agfapan 25 film for black and white photography, or Fuji HR100 tungsten film for colour photography.

Black and white negatives were printed on Ilfospeed Multigrade glossy photographic paper using a Durst Laborator 54 enlarger and the appropriate filter (usually No 3). Prints were developed in Ilfospeed Multigrade paper developer for 1 minute, fixed in Hypam rapid fixer for 3 minutes, thoroughly washed, and dried in an air dryer (Model R.C.D.-33, F.C. Manufacturing Co. Ltd., Japan). Colour negative film was developed and prints processed using a commercial colour laboratory.

TABLE 3.1 DATA COLLECTED FOR EACH OCTANT

Field	Field ID
1.	Patient ID
2.	Age
3.	Sex
4.	Treatment
5.	Tooth
6.	Duration of Treatment
7.	Level
8.	Octant
9.	Zone
10.	% soft tissue present
11.	*% surface intact*
12.	Number ERM along surface
13.	Blood vessels b/w ERM and tooth
14.	*% active resorption*
15.	Number ERM in bay
16.	Number ERM along surface
17.	Blood vessels b/w ERM and tooth
18.	*% surface repair*
19.	Number ERM in bay
20.	Number ERM along surface
21.	Blood vessels b/w ERM and tooth
22.	*% indeterminate repair*
23.	Number ERM in bay
24.	Number ERM along surface
25.	Blood vessels b/w ERM and tooth

TABLE 3.2 **FIXATION SCHEDULES**

Patient	Age	Tooth	Fixation	Treatment Duration
G.D.	13.5	14	Formaldehyde	10 Months
	"	24	Glutaraldehyde	10 Months
R.S.	17	14	Microwave	14 Months
	"	24	Glutaraldehyde	14 Months
K.T.	24	14	Formaldehyde	16 Months
	"	24	Glutaraldehyde	16 Months
	"	34	Karnovsky's	16 Months
	"	44	Karnovsky's	16 Months

Note:

1. Teeth fixed in glutaraldehyde were not used in this study.

2. All teeth in this part of the study were from patients who had commenced full fixed appliance treatment with a non-extraction treatment plan, but who subsequently had extractions (at treatment duration indicated), as part of a revised treatment plan.

3. Each of the extracted teeth underwent principally alignment procedures. None were severely malpositioned, or partly erupted. It appears from the clinical records that each of the teeth in the study underwent similar "jiggling" tooth movements only.

TABLE 3.3 RAPID MAXILLARY EXPANSION PATIENTS

Patient	Age	Teeth	Duration of Retention
CH	13	14, 24, 34, 44	4 months
DH	14	14, 44	5 months
TM	16	14, 44	12 months

Note:

1. All of these patients were male.

2. Teeth were fixed in 10% formosaline for 6 hours immediately after extraction, and then placed in EDTA solution for transport to the University.

TABLE 3.4 IMMUNOHISTOCHEMICAL REAGENTS

Reagent	Type	Source	Supplier	Code
Human keratin	Polyclonal antibody	Rabbit	Dako	A575
AE1/3	Monoclonal	Mouse	Biogenix	MU071
Second Antibody	"Multilink"	Swine	Dako	E453

CHAPTER FOUR

RESULTS

4.1 MATERIAL

The material used in both parts of this study consisted of human premolar teeth extracted for orthodontic reasons. The material for the initial part of the study was collected from within the Adelaide Dental Hospital, and for the second part from School Dental Service sources outside the dental hospital. The latter material presented some logistical problems in collection and required cooperation from the School Dental personnel in its collection and fixation. To this extent there were some factors outside the investigator's control.

As there was no alveolar bone collected with any of the specimens, there was often little periodontal ligament attached. However, despite the limited tissue available, epithelial cell rests were demonstrable to varying extents in all areas where periodontal tissue remained attached, including some of the areas where sites of resorption were present. The limited tissue however does restrict the interpretation of the material, especially the statistical interpretation and is a handicap in its analysis and discussion.

4.2 FIXATION AND DECALCIFICATION

1. Preliminary study

All tissue appeared to be adequately fixed and there were no signs of tissue degeneration. Each of the fixation schedules was successful in this respect, and morphology was preserved (Figures 4.2 to 4.4).

Decalcification was slightly inadequate in a few blocks, despite the radiographic evidence of X-rays that were taken to check on the progress of decalcification. This problem was discovered when the blocks were sectioned in the microtome. The apical

thirds of some of the teeth were the only areas affected in this way, and the middle and coronal thirds were completely decalcified.

Initially, there was some concern that long periods of decalcification may adversely affect the antibody staining, either by reducing the intensity of staining, damaging the tissue, or in some other way affect the degree of background or antibody staining. This proved not to be so with fixation the most important factor. Of the three methods of fixation used, formalin fixation showed by far the most reliable and consistent results for immunohistochemical (IHC) staining. Glutaraldehyde fixation showed light and variable staining at the lower antibody dilutions only (1/500), and microwave fixation showed little if any staining. However these results must be interpreted with some caution, as there were only a few teeth (one or two) in each sample, and an error in processing could account for these results.

2. Main study

All material in this part of the project was fixed with formalin as a result of the previous findings. However, as stated earlier, the fixation was under control of the School Dental Service personnel collecting the teeth. This required that the teeth were placed in fixative immediately after extraction, and removed after 6 hours and placed in the EDTA solution. Participating School Dental Service personnel were given written instructions on handling of the material, and labelled jars containing the fixative and EDTA were supplied to each clinic prior to the scheduled extractions. The teeth were either collected by the investigator, or collected and delivered by the hospital courier as soon after the extractions as possible. Two of the teeth (DH14 and DH44) showed some signs of poor fixation. Despite this, however, the epithelial cell rests still stained adequately for identification.

4.3 SECTIONING OF BLOCKS

1. Preliminary study

Initial sections were cut on the microtome at 5μm, and from the beginning problems were encountered in the attachment of these tissue sections to the slides. Some sections were lost during the trial IHC procedures. Loss of tissue sections was sometimes seen after the trypsinisation stage. Two slide coatings (APT and chrome-alum) were tried with reasonable success. Other methods were applied to assist in this area. Firstly three or four sections were collected on each slide. The aim of this was to enable viewing of consecutive areas, but also if loss (or more usually, partial loss) of one section occurred, other adjacent sections were available for viewing. Secondly, careful use of heat was employed, by using a hot plate to dry and help adhere the sections to the slides. Caution was required in the application of heat, as high temperatures have the potential to damage the antigens.

It was consistently found that the soft tissue almost always remained attached to the slide (provided an adhesive was employed) and it was the areas of dentine that became detached either completely, or folded over the rest of the section rendering it unfit to view and examine. Often, if little periodontal tissue was present, all that retained the section on the slide was the pulpal tissue.

It was also noted that prolonged heating of the sections either to adhere the sections or to melt the wax, resulted in buckling of the areas of dentine in the sections and separation from the soft tissue of the periodontal ligament as the slides cooled. As a result, during the IHC procedures, the separated areas of dentine often folded over the soft tissue which had remained in place obscuring the results of staining. Separation often seemed to occur in areas of greatest interest, especially those areas associated with epithelial cell rests and areas of resorption. Separation of the periodontal tissue from the hard tissue also prevented any accurate assessment of the relationship between the cell rests and the cementum surface. For these reasons heat was avoided

as much as possible, even to the extent of dissolving the wax on the sections entirely with xylene rather than melting it off with heat.

2. Main study

By the time this part of the study commenced, most of the above problems had been resolved. However, there was still some problem with section adherence to the slides, but greatly reduced. Folding of sections with little periodontal tissue continued to occur. For these reasons, heat was avoided as much as possible, sections were collected and handled carefully, and stored in cool dry conditions.

4.4 IMMUNOHISTOCHEMICAL PROCEDURES

The streptavidin-biotin method worked well, and proved most applicable to sections that had been fixed with formalin, and decalcified in EDTA over a long period. Once the routine was established, then reliable and repeatable results were possible. Initially, the IHC staining results were poor and quite inconsistent. This was a result of two factors. Firstly, the routine was still being established, and the problems associated with organising the routines for the first time were apparent. Primary and secondary antibody dilutions had to be established. The length of antibody incubations were unknown to begin with, as was the required concentration of the biotinylated streptavidin complex. The use of the positive control sections was of considerable help in establishing the routine, and provided a starting point for dilutions of all the reagents. The use of controls consisting of gingival tissue was particularly useful in this respect. As some of the teeth had small amounts of gingival tissue attached above the cemento-enamel junction, this tissue proved to be an excellent source of control tissue (Figure 4.8 and 4.9), and much better than the tonsil epithelium that was also available for use as a control.

The second factor was the unknown effects of the fixation and decalcification on the protocols. Until some indications of positive results were noted, it was not

possible to know if the procedures were practical and indeed if the epithelial cell rests would stain at all.

However once the routine had been run four or five times, and all the procedures checked, staining results began to appear. As it became clear that the formalin fixation routine was superior to the other methods, it was possible to concentrate on these sections and refine the routine, for the best results. The main part of the study used only formalin fixation.

Establishing the correct antibody dilutions, required a determination of the balance between adequate staining of the epithelial rests and the lowest possible level of background staining. Dilutions of 1/500 and 1/1000 with the polyclonal antibody with overnight incubation appeared to give consistent results, although moderate background staining remained (Figure 4.5). It was important to use fresh solutions for each stage of the incubation. One of the early routines was a total failure as a result of "old solutions" (greater than 24 hours old) being used which had on closer inspection afterwards, had started to grow a culture of micro-organisms in the solution. The enzyme digestion required the trypsin solution to be preheated to 37°C for best effect. The secondary antibody step was the cause of some problems. The recommended dilution was 1/80 to 1/150 but it was noted that the best results were obtained with 1/50 dilutions. The addition of the DAB chromagen needed to be carefully monitored both macroscopically and sometimes microscopically so that the reaction did not proceed too far, and background staining did not increase to unacceptable levels.

Each stage of the IHC routine has the potential to affect the results if not properly carried out. Washing steps between each stage were necessary to ensure each stage was stopped before the next commenced. Washing needed to be carefully carried out so that no sections were washed off the slides.

During the main study there were some further observations that improved the staining results. The quality of the chromagen (DAB) proved to be important. Some of the initial results were affected by old stock which did not stain as well. Also filtering of the DAB solution was essential to remove any impurities or undissolved particles of

DAB that could leave unused stain on sections, and which could give the appearance of a false positive result.

The main study used the monoclonal antibody (AE1/3) for all staining for consistency of results. This resulted in further reduction of the background staining to almost nil in most sections (Figures 4.8 and 4.9).

4.5 MORPHOLOGICAL FINDINGS

1. General Observations

There was considerable variation in the width of the periodontal soft tissues remaining attached to the tooth roots. This varied in thickness from more than 150μm in some areas to only a few cell layers or no tissue at all in many other areas. Some of the teeth had extensive areas of soft tissue remaining, whilst others had relatively little. Areas of separation were seen in some sections which often followed along the line of the cell rests (Figure 4.12). Often this line of separation followed the cell rests for a long distance, and left the cell rests right on the torn edge. In these areas, all the cell rests appeared to remain on either one side of the torn ligament or the other. There seemed to be no consistency in whether the rests remained on the tooth side or the bone side, but this aspect was not closely studied.

Some teeth showed large areas of remaining soft tissue adjacent to areas completely devoid of soft tissue (Figures 4.9 and 4.13). There were many examples of extensive tissue remaining adjacent to areas with no tissue at all remaining. This feature seemed to occur on both the control and experimental teeth. The loss of tissue did not appear to be correlated with the presence or absence of resorptive areas, although this was an area that was not closely examined.

2. ERM on Non-Resorbed Surfaces

The epithelial cell rests stained distinctively with the AE1/3 monoclonal antibody and were easily seen. The staining patterns of the cell rests were remarkably

consistent. Staining was principally perinuclear, with more diffuse staining in the cytoplasm. The nuclear areas did not appear to stain (Figure 4.14). Staining was quite intense and there was no mistaking the stained cell rests.

There was considerable variation in the shape and size of the sections of epithelial cell rests. The number of individual cell sections seen in the various cell rests varied from one or two to 10 or 12 in the larger rests (Figures 4.14 and 4.15). The shapes also varied considerably. There were the often described round and ovoid forms as well as the less frequently seen strands and elongated forms. All cell rests were uniformly close to the cementum surface (20 to 30μm) except in the marginal areas (levels 0, and 1), where the cell rests were often at a much greater distance from the cementum surface (Figure 4.11).

The relationship of blood vessels to epithelial cell rests was also examined along non-resorbed surfaces. From the thousands (4,107) of epithelial cell rest sections seen in these slide sections, there were only four instances where the blood vessels appeared to be interposed between the cementum and cell rests.

3. ERM and Resorption

Resorption areas were extensive on the buccal surfaces of the experimental teeth, but also present on other surfaces of these teeth. The control teeth also showed some small areas of resorption, but in comparison the areas were very small and never extensive. Table 4.1 summarises the incidence of the various types of resorption seen in the treated teeth. The overwhelming majority of these resorption sites demonstrated either completed or progressing repair. Active resorption was not seen to any significant degree. There were only six sites of active resorption detected on the experimental teeth, with five of these representing just 10% of an octant, and fourth site representing 50% of the octant.

The relationship of the epithelial cell rests to sites of resorption and repair was also examined. Only a few examples were seen (Figures 4.17 and 4.18). On no occasion was a section of an epithelial cell rest seen in association with active

resorption. There were however a some examples of epithelial cell rests either within (6 examples), or on the surface (13 examples) of repairing resorption bays. Where it could not be decided if there was repair proceeding (indeterminate repair), there were two examples of sections of epithelial cell rests in the resorption bays, and one example of an epithelial rest section along the surface of the bay. This type of "repair" situation was also relatively rare, only being detected on seven occasions.

The presence of blood vessels interposed between the epithelial cell rests and the cementum surface was also examined. Very few examples of blood vessels in such a relationship were encountered. Along non-resorbed surfaces, only four such examples were ever seen. Where resorption had occurred, there were also only four examples seen. Two were in association with repairing resorption, and two in association with indeterminate resorption. None were seen where active resorption was continuing.

There were many situations where all the tissue had been completely torn out of the resorption bays, and from along the nearby cementum surface. As a result, a comprehensive statistical study of these areas was not possible. It is quite likely that many more sites with either epithelial cell rests or blood vessels would have been found that might throw light on these relationships, had it been possible to collect intact teeth and surrounding alveolar bone.

4.6 HISTOMORPHOMETRIC ANALYSIS

The analysis was designed to examine the relationship of the cell rests to the tooth surface in an extreme example (RME) of tooth movement known to cause extensive root resorption (BARBER and SIMS, 1981; BRICE et al., 1991). Numerous variables were to be looked at (Table 3.1; Appendix 11), however there was sufficient variability to assess only four factors. The remaining factors showed insufficient variability to be analysed, and combined with the small sample size, meant that a statistical study of these factors was not possible. The statistics package (program 5v of the BMDP statistical software package) used can deal with small samples and

missing data, but not the large amounts of missing data that became apparent in this study. The factors that were examined were:

1. Soft tissue remaining
2. % surface remaining intact
3. Number of epithelial cell rests per section
4. % surface undergoing repair or repaired.

The results from the analysis of these four variables are recorded in Tables 4.2 to 4.9, and shown graphically in Figures 4.13 to 4.20. These results indicated that there were significant effects (at the 5% level) due to treatment (ie RME), region and level, and that there was a significant interaction between these factors. The tables and graphs represent expected values (generated by the 5v program), which are model smoothed versions of the sample averages.

As described in Chapter 3, the teeth were divided into four regions that were related to the octant recording system (Figure 4.1), and were considered to represent the possible variations in treatment effects as a result of the RME therapy. Region 1 (octants 1 and 8) represented the areas closest to the expected principal effects of the RME therapy (ie the buccal root surface). Region 2 (octants 2 and 7) represented the areas a little further distant from the experimental sites, and was approximately equivalent to the mesiobuccal and distobuccal areas of the tooth root. Region 3 (octants 3 and 6) represented the areas a little further distant again from the experimental sites, and was approximately equivalent to the mesiolingual and distolingual areas of the tooth root. Region 4 (octants 4 and 5) represented the areas most distant from the expected experimental effects and corresponded to the lingual surfaces. In general, region 1 represented the area most likely to be affected by compression as a result of the RME and region 4 the area most likely to be affected by tension. Regions 3 and 4 were the areas most likely to be affected by both compression and tension depending on the tooth morphology.

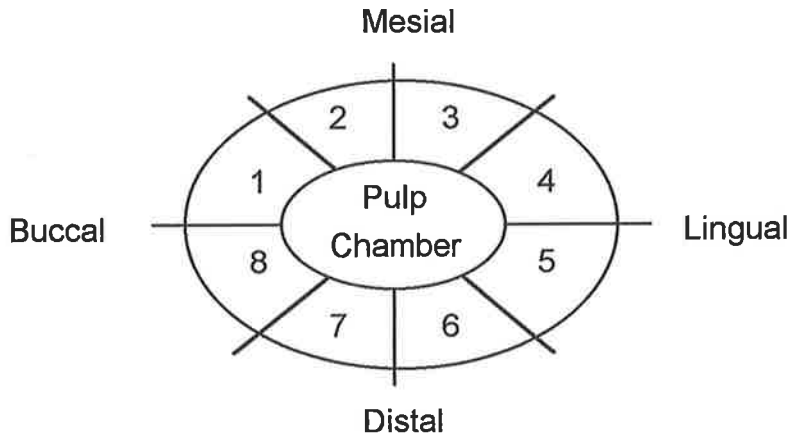


Figure 4.1 Diagram showing the octants used in the morphometric study. Octants 1 and 8 are equivalent to *Region 1*, octants 2 and 7 to *Region 2*, octants 3 and 6 to *Region 3* and octants 4 and 5 to *Region 4*.

1. Soft Tissue Remaining

The results for this section are specifically shown in Tables 4.2 and 4.3, and Figures 4.19 and 4.20. The control teeth showed consistent amounts of soft tissue remaining in each of the four regions, with in general terms less tissue on the buccal and lingual surfaces of these teeth. There was also a trend towards less tissue remaining after extraction in the middle levels (levels 3 to 8), with larger amounts remaining in the apical and coronal thirds.

There was a difference however with the treated teeth. The trend for more tissue apically and coronally held for the regions 1, 2 and 3. However in region 4 (lingual surface) there was more tissue present in the middle levels, with a reverse curvature in the graph. The buccal surfaces showed the least tissue remaining from the zero level to level 6. The treated teeth showed more variation in the amount of tissue present.

2. % Surface Non-Resorbed and Intact

These results are shown specifically in Tables 4.4 and 4.5, and Figures 4.21 and 4.22. The control teeth showed some minor surface changes coronally (levels 0 and 1) in all regions, but more significant changes in the apical half (levels 6 to 10) with up to 25% of the tooth surface being affected at level 10. These changes were consistent for each region.

In contrast there was a significant difference for the experimental teeth. Region 1 (buccal surface) showed considerable surface alterations for all levels. The cervical levels showed almost 20% of the root surface being affected, increasing to 50% in the the apical levels. In comparison, the other regions showed loss of surface integrity from levels 3 to 10, but to a much lesser degree, with maximum root surface changes at about 30% at level 10. The changes in regions 2, 3, and 4, paralleled very closely the changes recorded for the buccal surface. Region 2 did show changes that were slightly greater in extent than region 3.

3. Number of Epithelial Cell Rest Sections per Level

The results for this section are specifically shown in Tables 4.6 and 4.7, and Figures 4.23 and 4.24. The results are recorded as absolute numbers, so that as the levels increase (i.e. towards the apex), the surface area of the octants decreases. The results do not record the surface density of the epithelial cell rests, which would be a more sensitive measure of changes in epithelial cell rests. For these reasons it can be suggested that the epithelial cell rests may not decline very much down the tooth root, but certainly are increased in the apical regions. The control teeth showed epithelial cell rest sections at all levels and and a trend towards a similar pattern in cell sections in each region across the various levels. The highest number of cell rests were seen in the coronal levels, decreasing to a plateau level in the mid-root region, and increasing again slightly in the apical region. There was increasing variation in the apical areas in the actual numbers of sections present. At level 10 for example, there was a range of

3.7 to 14.2 sections seen. The trends for each region were very similar. In the coronal areas, where the numbers of cell sections were highest, there was still a variation in the numbers from 31.2 in region 4 to 39 in region 3 (at level 0).

The treatment teeth showed similar trends in the pattern of cell rests, although the apical areas did not show the higher numbers of cell rest sections demonstrated in the corresponding control sections. The numbers of epithelial cell rest sections begin at a lower level for all regions, but decline in a similar manner to the control teeth. There was a steady decline in the numbers of cell sections in all regions from level 0 down to level 8, with only levels 9 and 10 showing any variation in the underlying trend, probably as a result of the low numbers recorded at these levels. Regions 2 and 3 (proximal surfaces) showed almost identical results from level 0 to level 10. Region 1 (buccal surface) showed lower numbers of epithelial cell rest sections compared to all other regions from level 0 to level 8, and region 4 (lingual surface) showed consistently higher numbers of epithelial cell rest sections than the other regions from level 0 to level 8.

4. % Surface Repairing or Repaired

The results for this section are specifically shown in Tables 4.8 and 4.9, and Figures 4.25 and 4.26. These results complement the results of Tables 4.4 and 4.5, and demonstrate the extent of the repair process in each region. There is generally little repair in most levels and regions of the control teeth, although in the apical regions it reaches 25% at level 10.

The experimental teeth show considerable repair consistent with the extent of surface damage from the RME therapy. Region 1 (buccal surface) shows the greatest repair and this was the region with the most resorption. There is however a lot of repair activity in each of the other regions, although at a lower level.

Figure 4.2 Example from the preliminary study of the use of half strength Karnovsky's solution as a fixative, in association with human polyclonal anti-keratin antibody. There is no keratin staining, although there is a slight tinge of brown staining at the edges of the tissue as a result of the use of DAB chromagen. The section demonstrates an area of resorption which is probably in the early stages of repair, as there is a reversal line (arrowed) just visible at the base of the resorption bay.

Polyclonal anti-human keratin, 1/500. Haematoxylin counterstain.

Magnification: 25 x Enlargement: 4.4 x Bar = 100μm

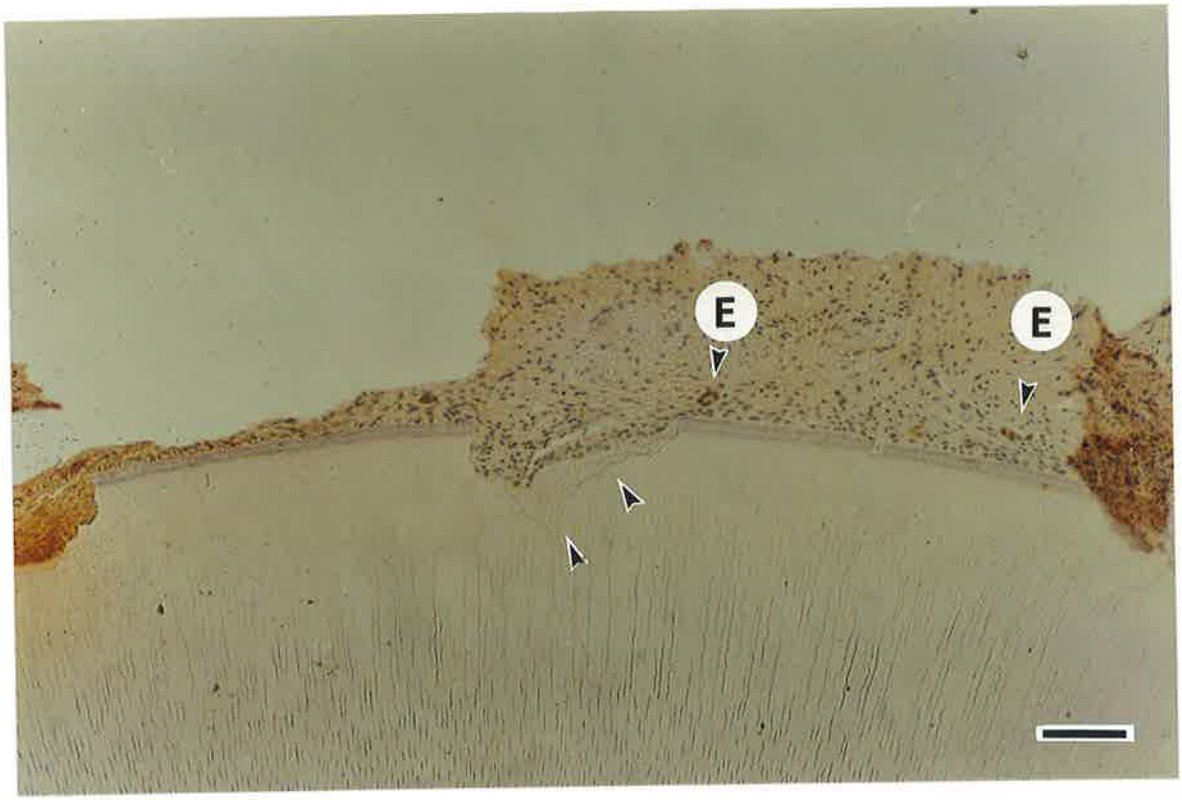


Figure 4.3 Example from the preliminary study of the use of formalin solution as a fixative, in association with human polyclonal anti-keratin antibody. There appear to be two epithelial cell rests present (E), as well as a repairing resorption bay, with a reversal line (arrowed).

Polyclonal anti-human keratin, 1/500. Haematoxylin counterstain.

Magnification: 25 x Enlargement: 4.4 x Bar = 100µm

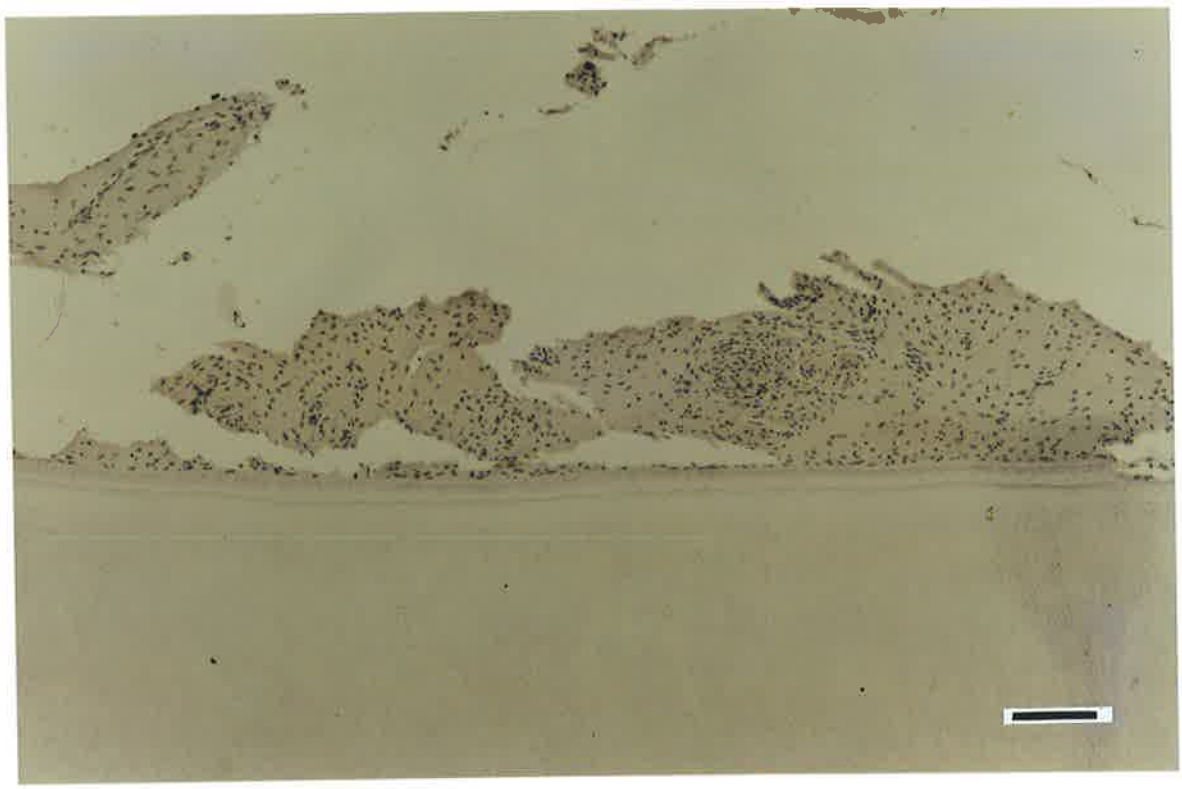


Figure 4.4 Example from the preliminary study of the use of microwave fixation as a fixative, in association with human polyclonal anti-keratin antibody. There is no staining of any cell rests in this section, and in fact no cell rests ever appeared to stain in any of the sections that had been microwave fixed.

Polyclonal anti-human keratin, 1/500. Haematoxylin counterstain.

Magnification: 25 x Enlargement: 4.4 x Bar = 100μm

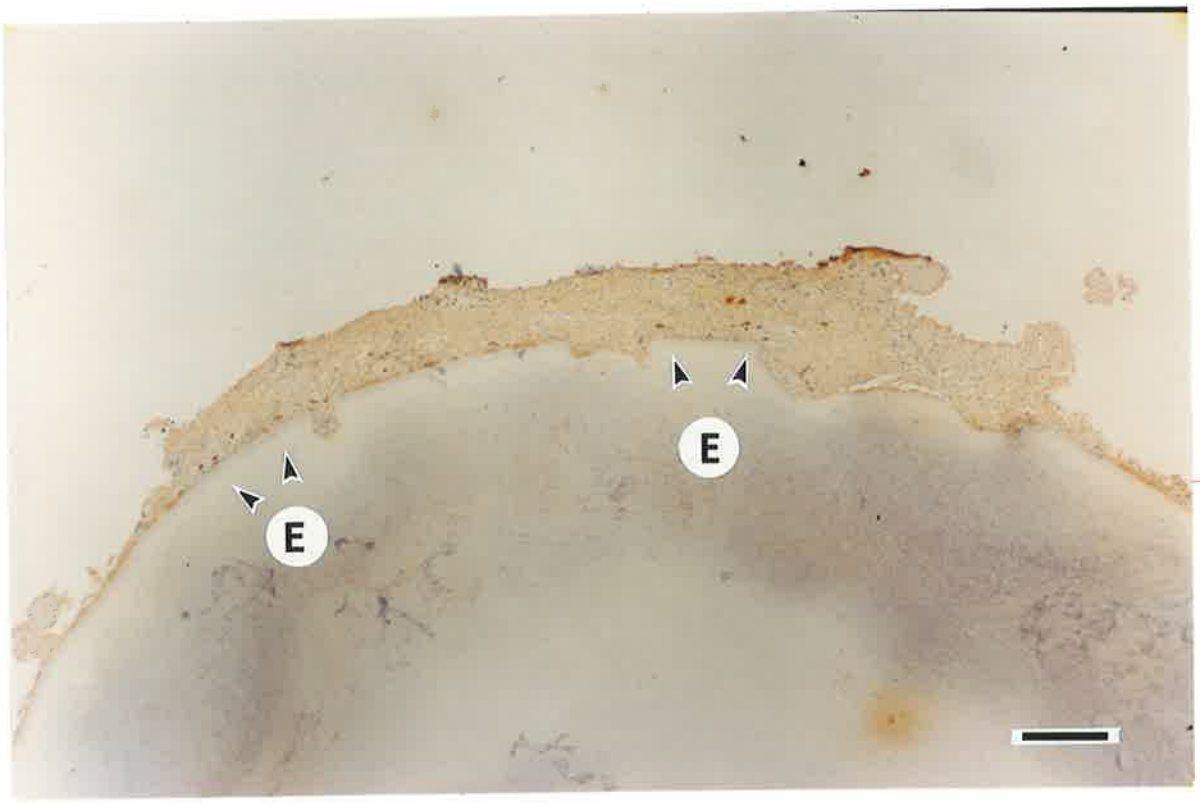


Figure 4.5 A low power view of several areas of resorption. This section is from the buccal surface of the middle third of the root of a human premolar. This section is from the initial trial study and demonstrates the degree of background staining that was evident in this study. Nevertheless, sections of epithelial cell rests (E) are evident. Some separation of tissue from the root surface is also apparent in the larger area of resorption.

Polyclonal anti-human keratin, 1/500. Haematoxylin counterstain.

Magnification: 10 x Enlargement: 4.4 x Bar = 250μm

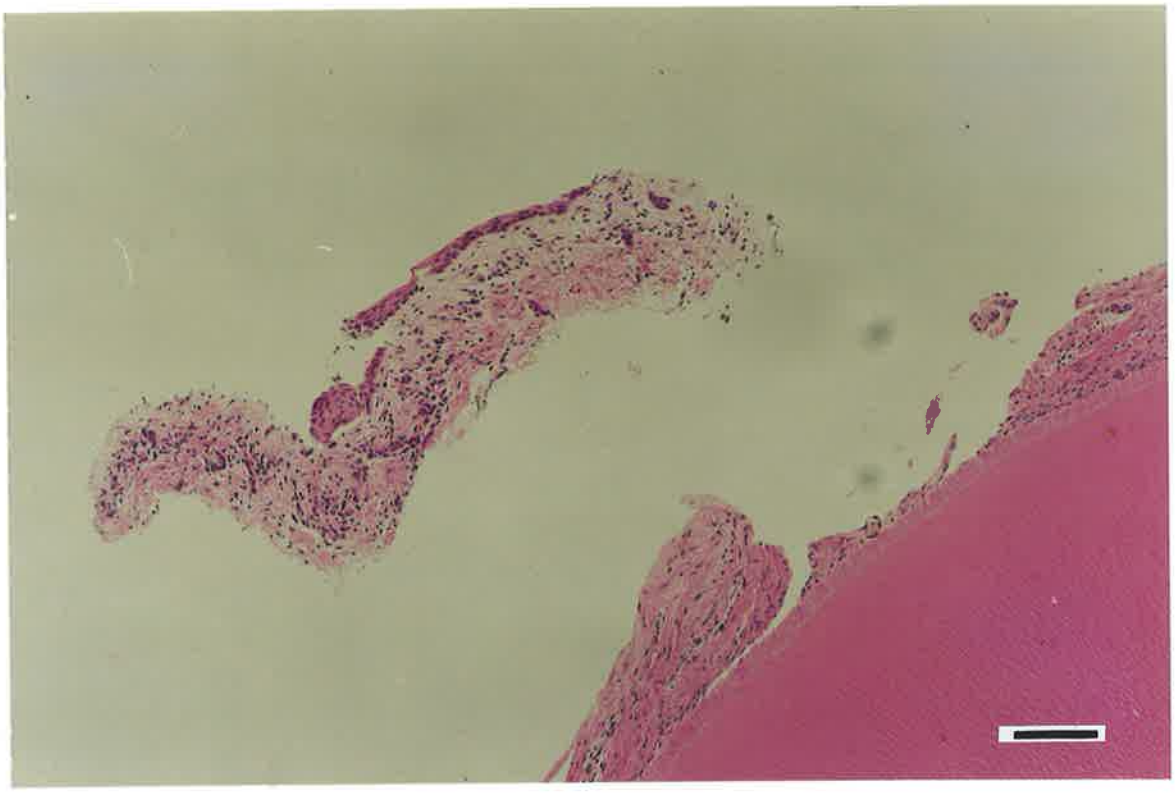


Figure 4.6 Example of haematoxylin and eosin staining from specimen CH24 (slide 58) near the cervical margin, which includes some gingival epithelium and connective tissue as well as some periodontal ligament tissue. The sections in Figure 4.7 (slide 56, negative control) and Figure 4.8 (slide 61, positive control) are similar sections before and after this particular section.

Haematoxylin and Eosin staining.

Magnification: 25 x Enlargement: 4.4 x Bar = 100μm

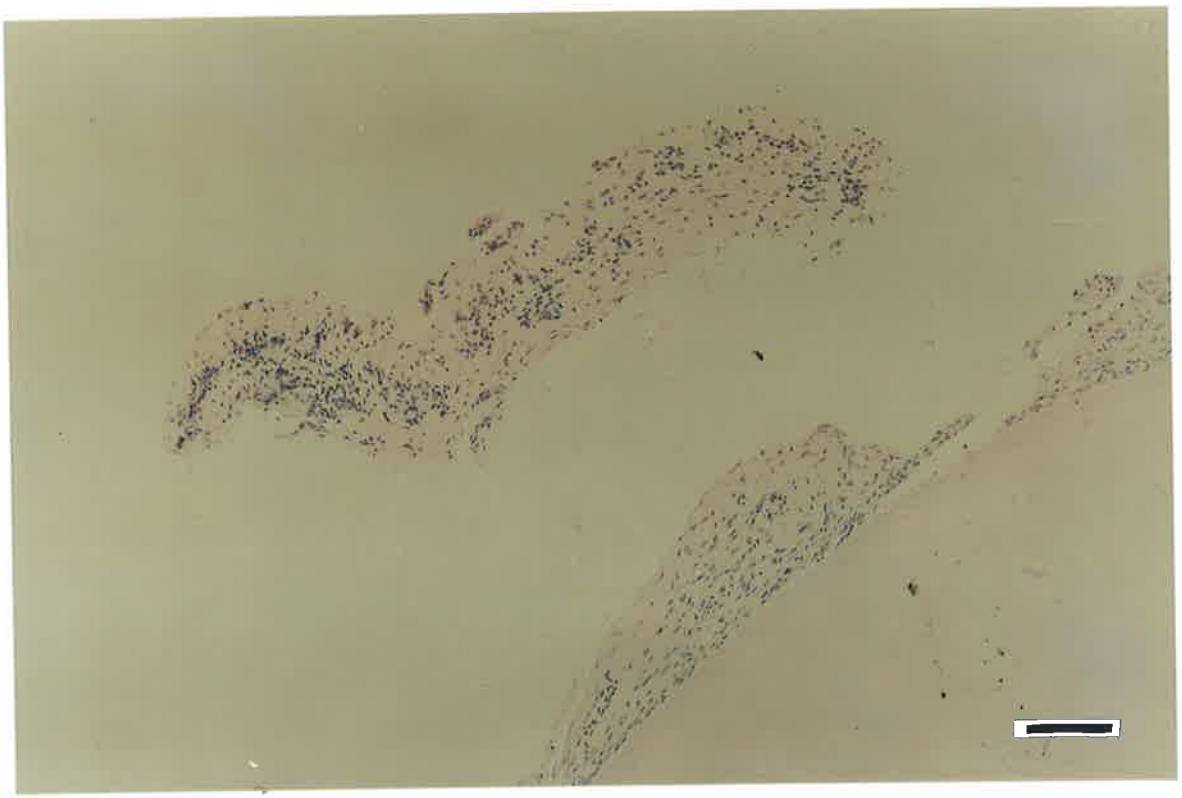


Figure 4.7 Negative control using section CH24/slide 56. Similar section to the previous figure. The primary antibody was left out and the diluent buffer substituted. Otherwise the immunohistochemical procedures were carried out exactly as described for all other staining. There are no signs of any epithelial cell rests, nor has any of the gingival tissue at the top of the figure shown any staining.

Haematoxylin counterstain.

Magnification: 25 x Enlargement: 4.4 x Bar = 100μm

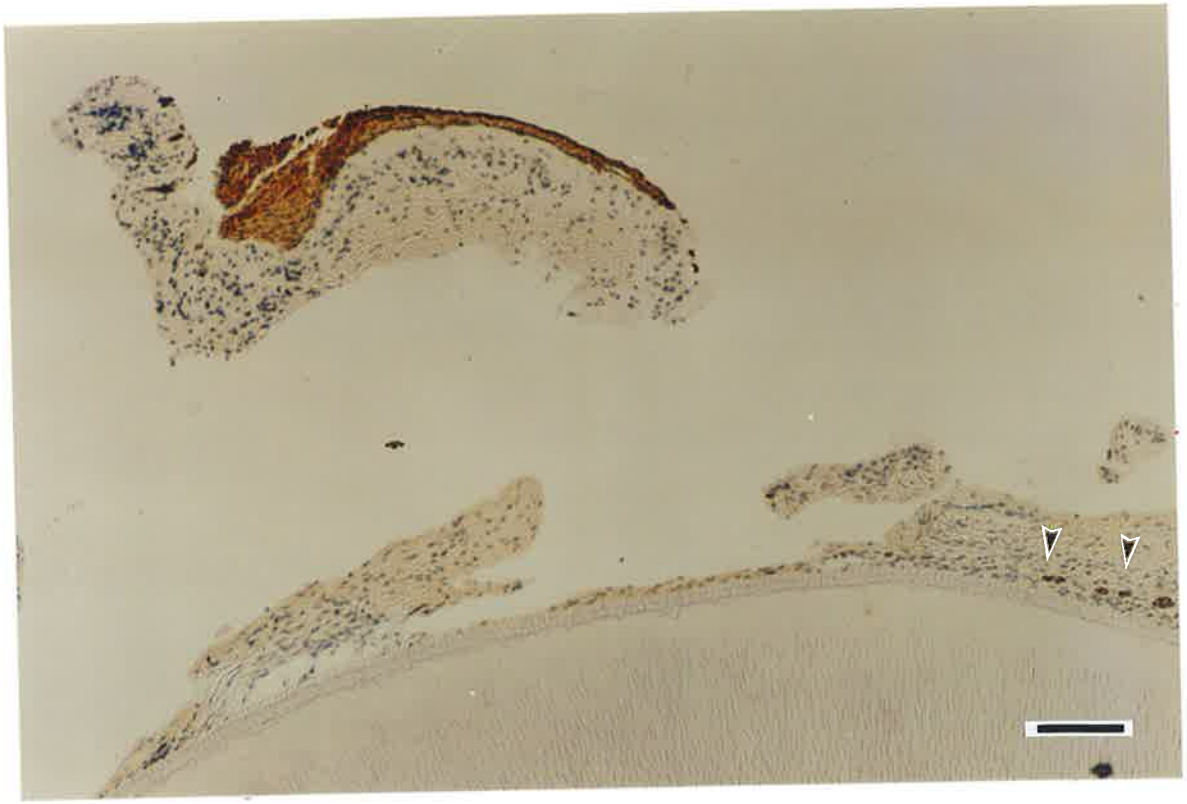


Figure 4.8 Positive Control. Similar section to the previous two figures (CH24/61). There is staining of the epithelial tissue at the top of the figure, as well as some epithelial cell rests (arrowed) along the cementum surface.

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 25 x Enlargement: 4.4 x Bar = 100μm

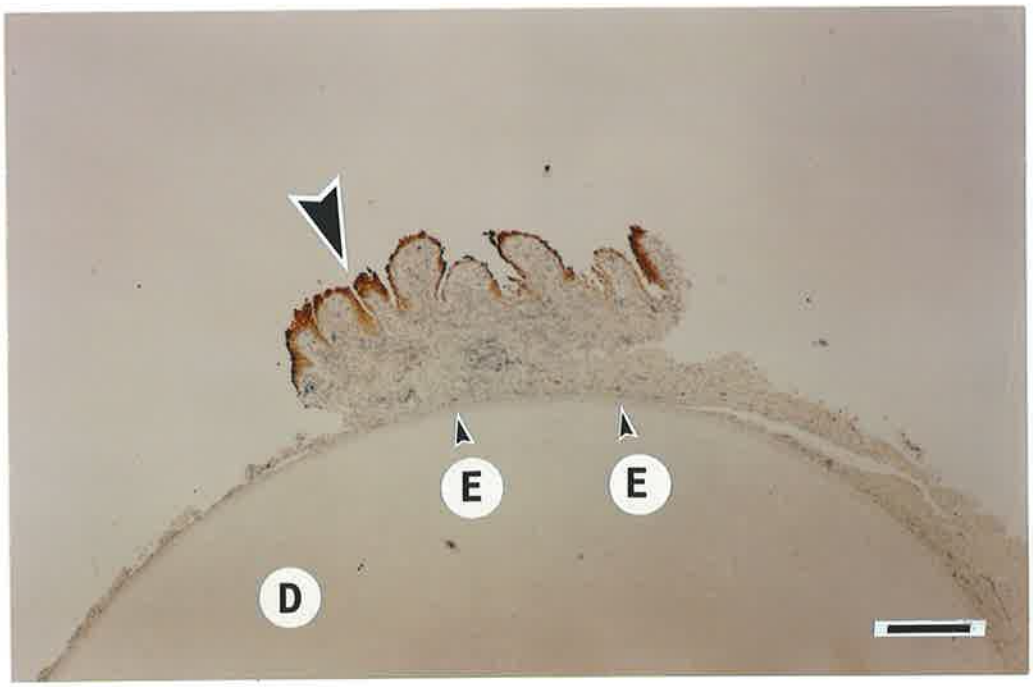


Figure 4.9 Low power view of an experimental tooth near the cervical margin, showing an inclusion of some gingival tissue. Although part of the surface of the gingival epithelium has been lost, during the extraction process, enough remains to demonstrate positive cytokeratin staining (arrowed) with the AE1/3 antibody in the remaining epithelial tissue. The brownish colouration is characteristic of the DAB chromagen. There are also some epithelial cell rests (E) sections present, although not easily seen at this low power. Note also the variation in the tissue present in this section, and dentine (D).

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 10 x Enlargement: 4.4 x Bar = 250µm

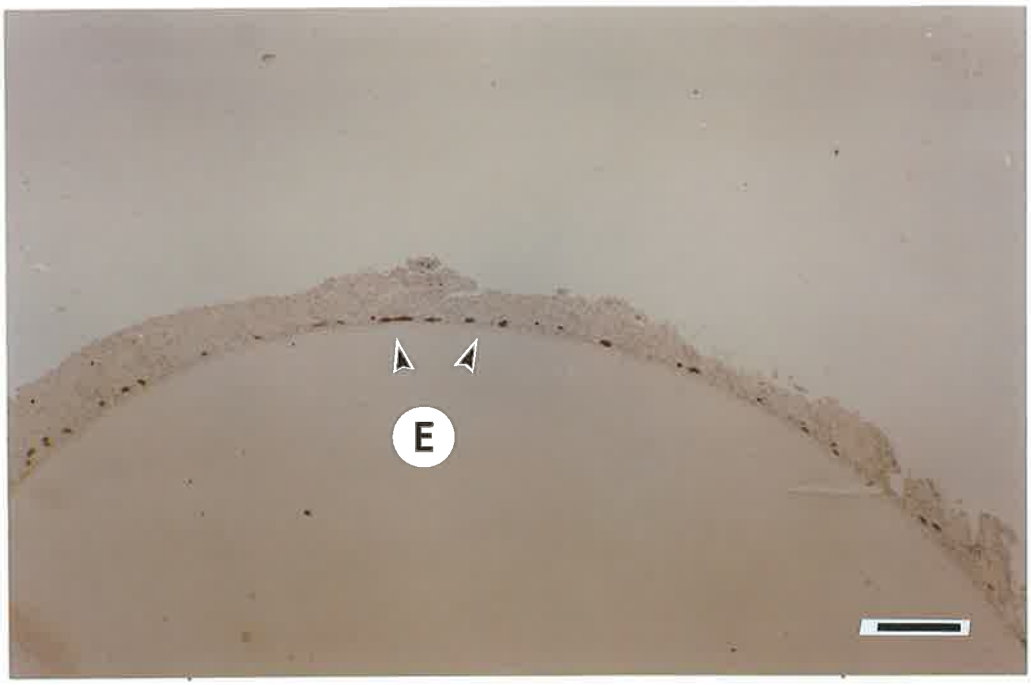


Figure 4.10 Low power view of epithelial staining along the cementum surface of a control tooth. This example is from the cervical region, indicating numerous clearly stained sections of epithelial cell rests (E), close to the cementum surface of the tooth root. There is extensive tissue remaining in this example.

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 10 x Enlargement: 4.4 x Bar = 250µm

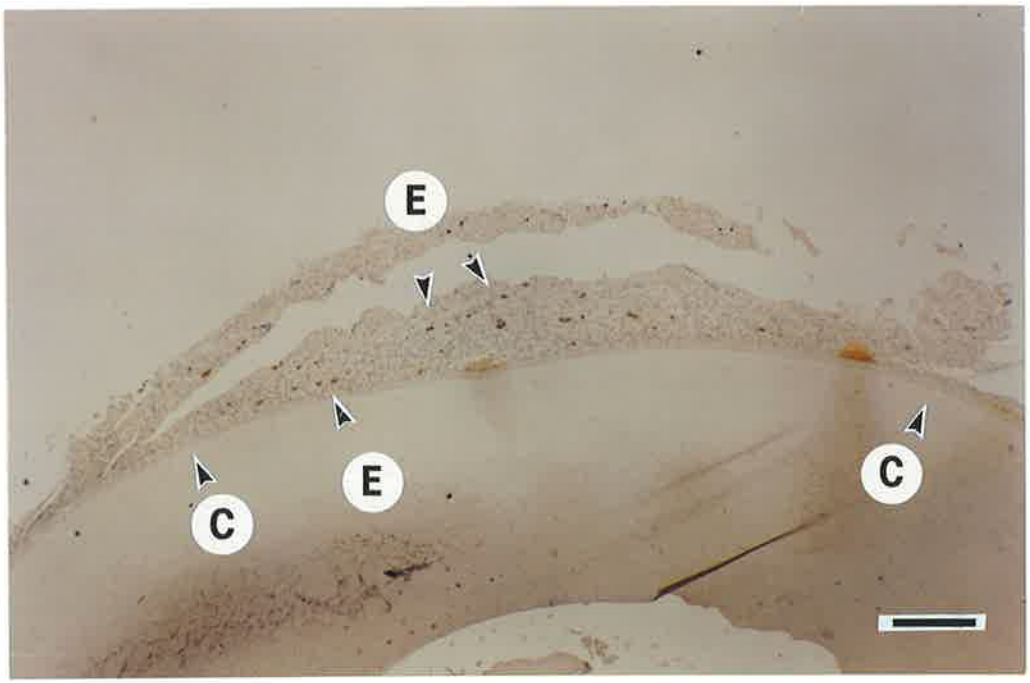


Figure 4.11 An area of cervical epithelial cell rests (E) demonstrating the positional variation that was often evident in the cervical region. The distance of the rests from the cementum surface (C) varies considerably.

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 10 x Enlargement: 4.4 x Bar = 250µm

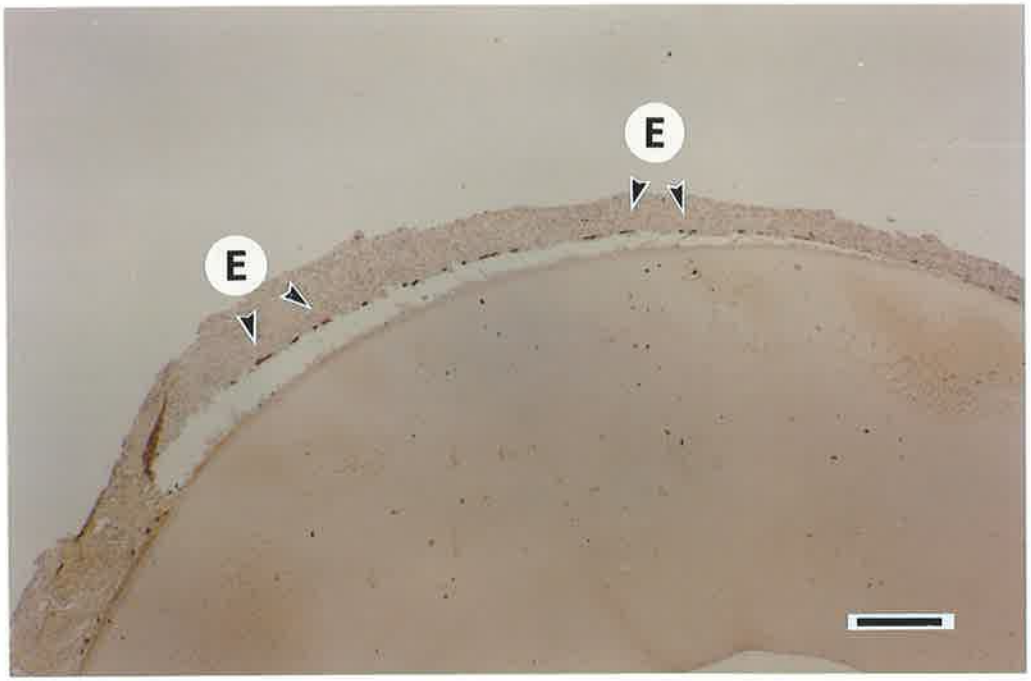


Figure 4.12 Tearing of the periodontal tissue is demonstrated in this experimental tooth at level 2. Note how the epithelial cell rests (E) line the ligament side of the tissue rather than the tooth side.

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 10 x Enlargement: 4.4 x Bar = 250μm

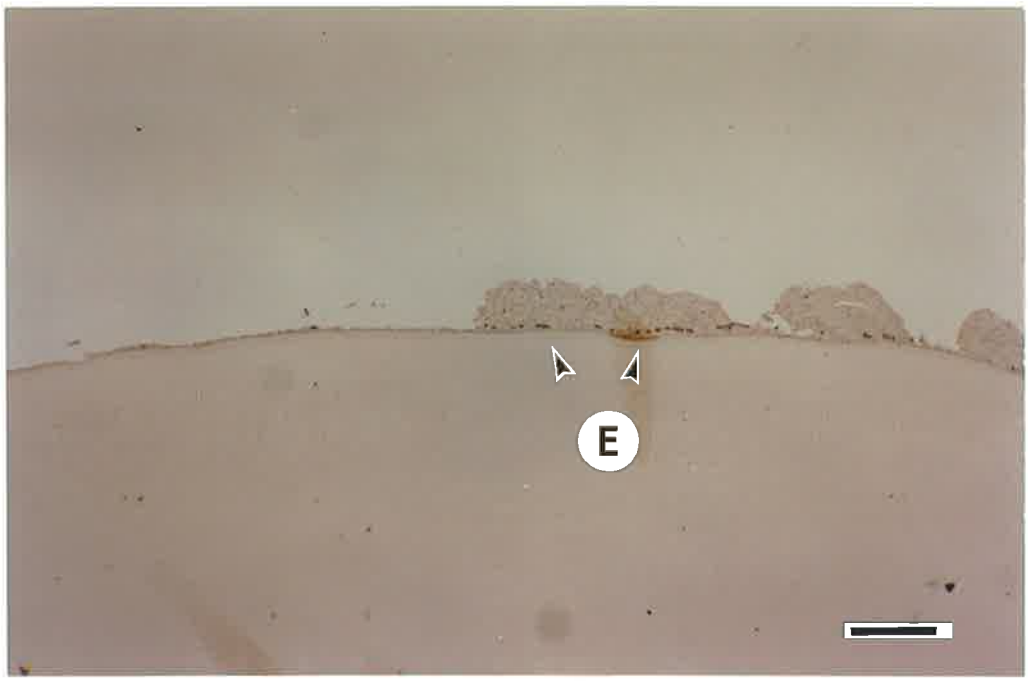


Figure 4.13 Low power view of a section from a control tooth showing the variation in tissue present after extraction. Where tissue remains, epithelial cell rest sections (E) are apparent.

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 10 x Enlargement: 4.4 x Bar = 250µm

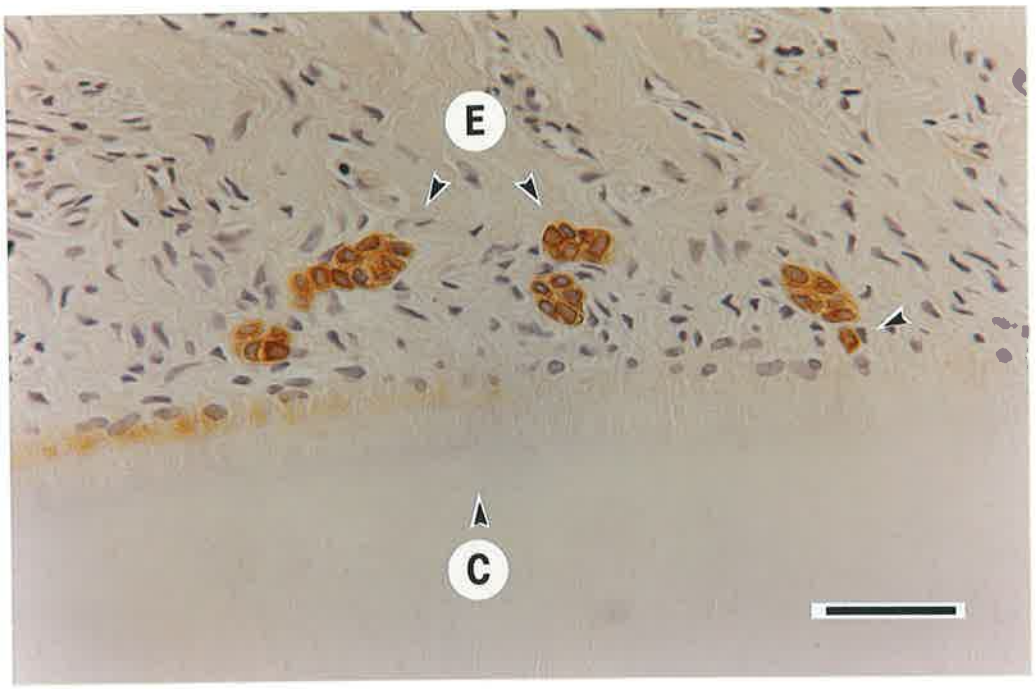


Figure 4.14 View of epithelial cell rest morphology and staining at higher power. These examples of cell rests (E) are from a control tooth. Note how the cell rests are close to the cementum surface (C), and that there is some variation in morphology. Each of the cell rest sections consists of 5 or 6 cells, with the staining limited to the perinuclear and cytoplasmic regions. The nucleus is not stained with the antibody, and shows only the haematoxylin counterstain. Some of the sections could be connected. There appears to be a single cell evident also (arrowed).

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 40 x Enlargement: 4.4 x Bar = 100μm

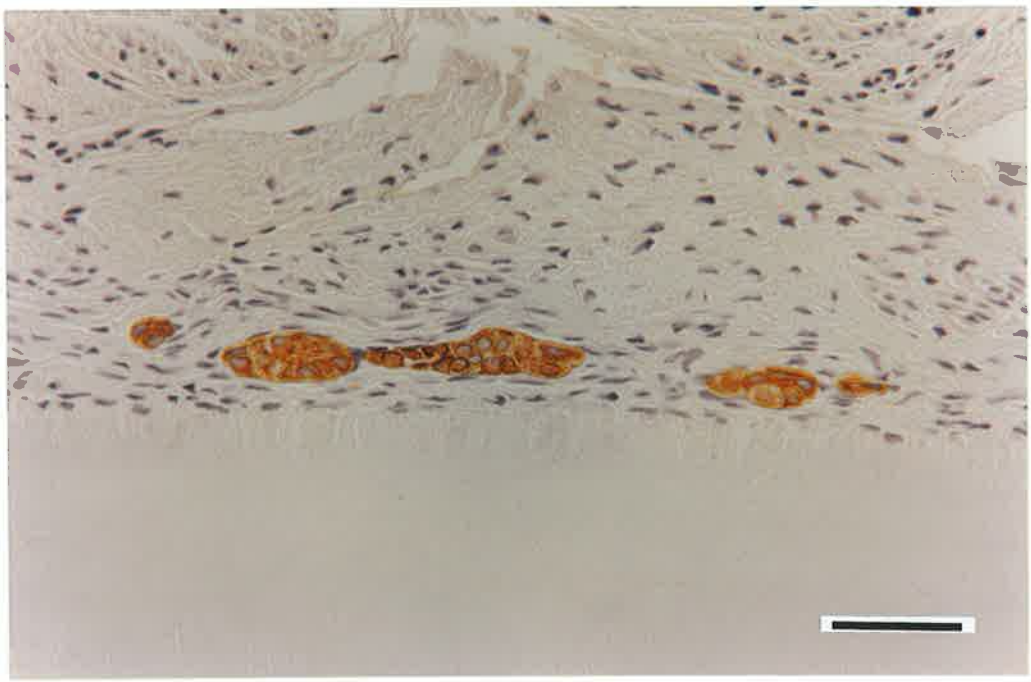


Figure 4.15 A further view of epithelial cell rest sections from a control tooth. Variation in morphology is also apparent, with a more strand like pattern apparent. The staining pattern is consistent with the previous figure. Note the absence of background staining indicating a balance between the incubation period, and antibody dilutions.

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 40 x Enlargement: 4.4 x Bar = 100μm

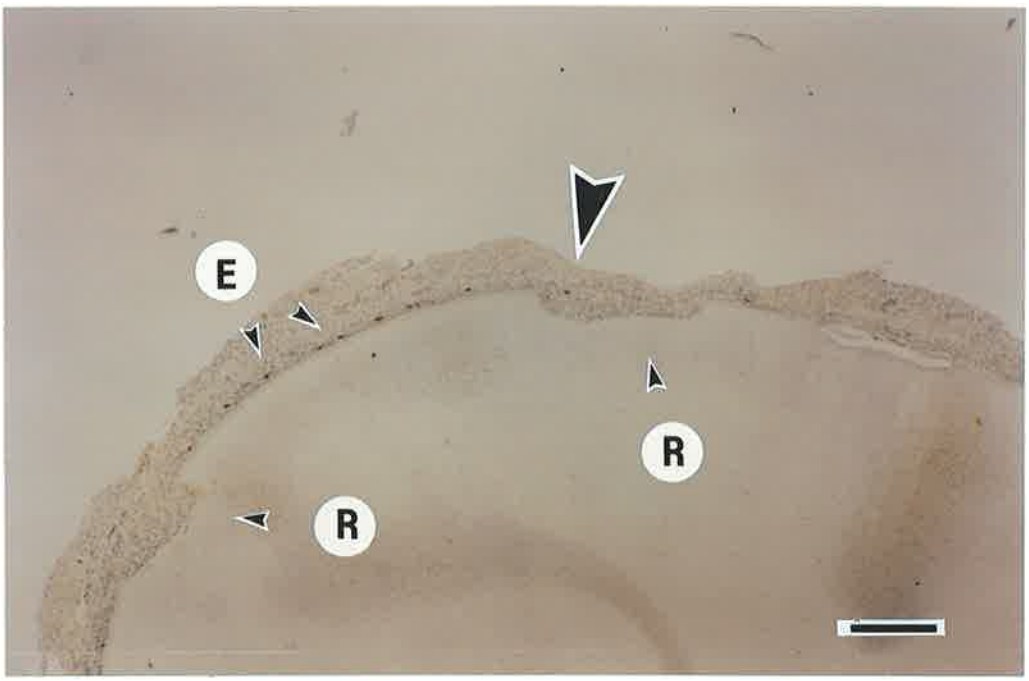


Figure 4.16 Low power view from the mesio-buccal area of the cervical region of an experimental tooth. It shows two areas of resorption (R). Sections of epithelial cell rests (E) are visible along the non-resorbed surfaces, although one epithelial section (arrowed) appears within one of the zones of resorption.

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 10 x Enlargement: 4.4 x Bar = 250μm

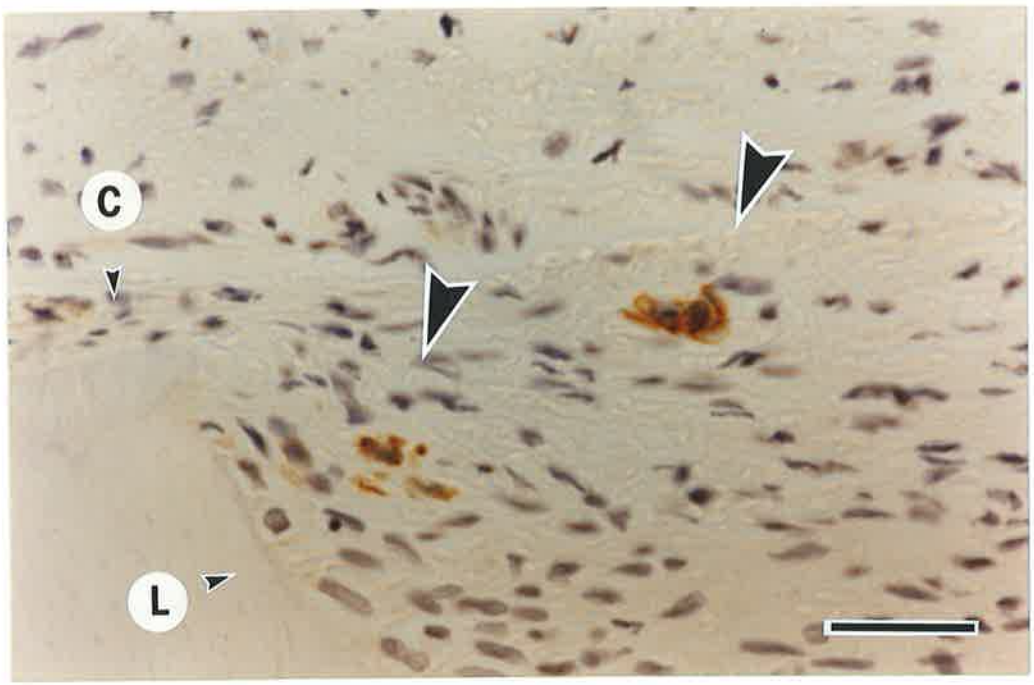


Figure 4.17 High power view of Figure 4.16, showing greater detail from one of the resorption bays in an experimental tooth. Several epithelial cell rest sections are present (arrowed), showing different degrees of staining. Note the cementum surface (C) and the reversal line indicative of repairing resorption (L).

AE1/3 antibody, 1/400. Haematoxylin counterstain.

Magnification: 150 x Enlargement: 4.4 x Bar = 25µm

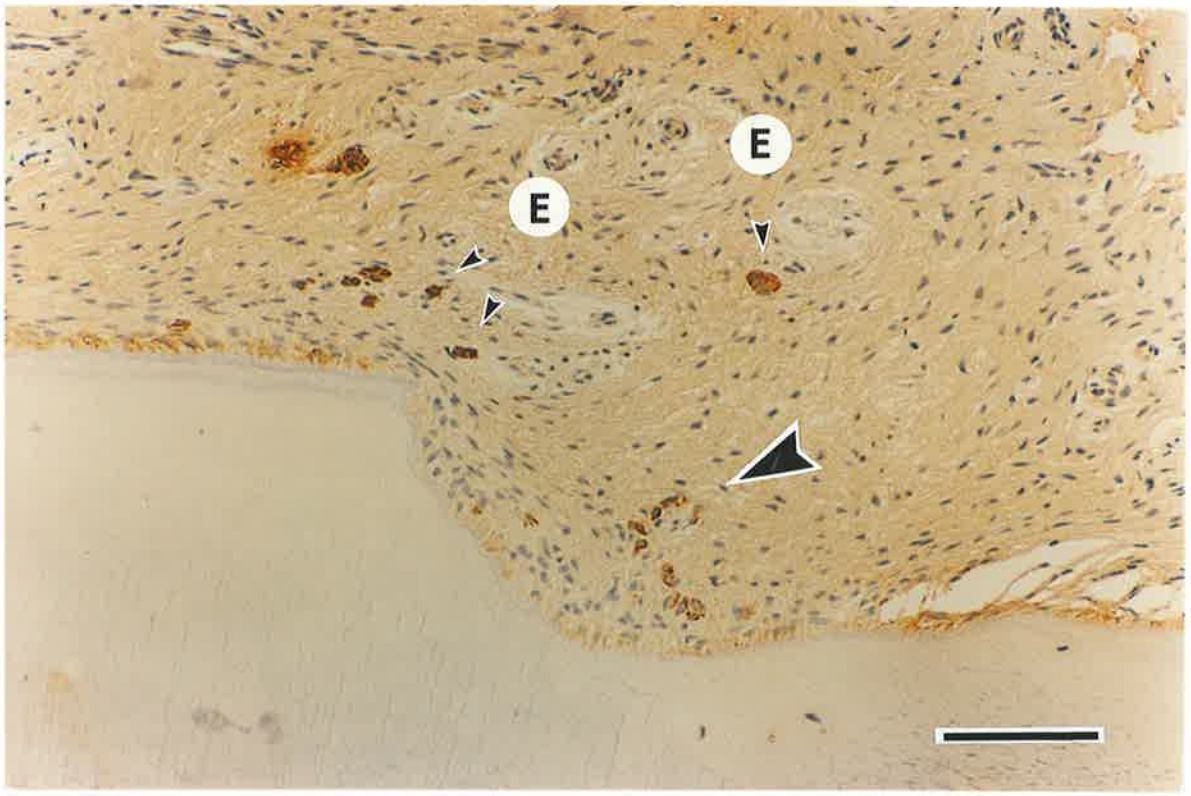


Figure 4.18 Another view of an area of resorption this time from the initial study. This is a higher power view of an area from Figure 4.5. It demonstrates a larger area of resorption with sections of epithelial cell rests at the periphery (E), over the body of the lesion (E) and within the lesion itself (arrowed). Note the relatively high level of background staining compared with the previous figures.

Polyclonal anti-human keratin, 1/500. Haematoxylin counterstain.

Magnification: 50 x Enlargement: 4.4 x Bar = 100μm

Table 4.1 Occurrence of Resorption Types[1]

Resorption Type	*Number*[2]	*ERM within the bay*[3]	*ERM along surface*[4]	*Intervening blood vessels*[5]
Repairing Resorption	174	6	13	2
Active Resorption	6	0	0	0
Indeterminate Resorption	7	2	1	2

Note: 1. The figures in this table relate to the experimental teeth only. There were some sites of resorption seen on the control teeth, but mainly towards the apex, and not as extensive as in the experimental teeth.

2. The values in this column represent the actual number of resorptive sites counted, not the number of affected octants. Some octants demonstrated several sites of resorption within the single octant.

3. Denotes sections of epithelial cell rests seen within the body of the resorptive lesion.

4. Denotes sections of epithelial cell rests seen along the surface of the resorptive lesion but not within.

5. Represents the occurrences of blood vessels between the cementum surface and the epithelial cell rests.

Table 4.2 % Soft Tissue Present: Control

Level	*Region 1*	*Region 2*	*Region 3*	*Region 4*
0	54.5	66.0	66.0	45.7
1	49.5	55.1	54.9	40.4
2	38.6	46.2	45.9	36.2
3	32.6	39.4	39.1	33.0
4	27.8	34.6	34.3	30.8
5	24.4	31.8	31.7	29.7
6	22.3	31.1	31.3	29.5
7	21.4	32.5	32.9	30.4
8	21.9	35.8	36.7	32.3
9	23.7	41.3	42.6	35.3
10	26.7	48.7	50.7	39.3

Figure 4.19 % Soft Tissue Present: Control

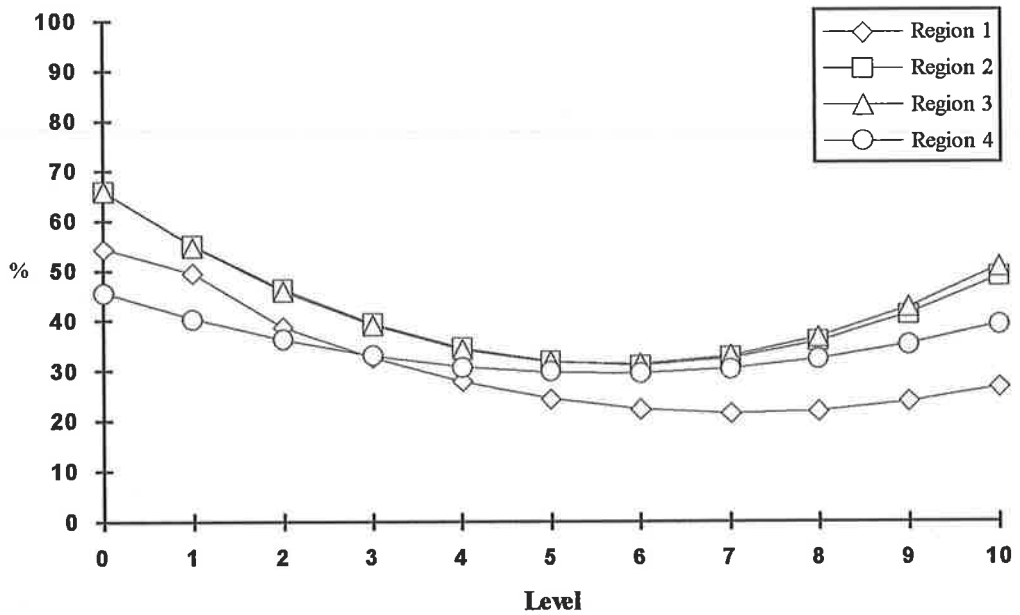


Table 4.3 % Soft Tissue Present: Treatment

Level	*Region 1*	*Region 2*	*Region 3*	*Region 4*
0	37.3	65.2	100.0	59.9
1	32.0	57.9	84.2	64.0
2	28.4	51.8	69.5	66.6
3	26.7	46.8	57.1	67.6
4	26.6	42.9	47.0	66.9
5	28.3	40.1	39.1	64.7
6	31.8	38.4	33.6	60.8
7	37.0	37.8	30.3	55.3
8	43.9	38.4	29.3	48.3
9	52.6	40.0	30.6	39.6
10	63.0	42.7	34.1	29.3

Figure 4.20 % Soft Tissue Present: Treatment

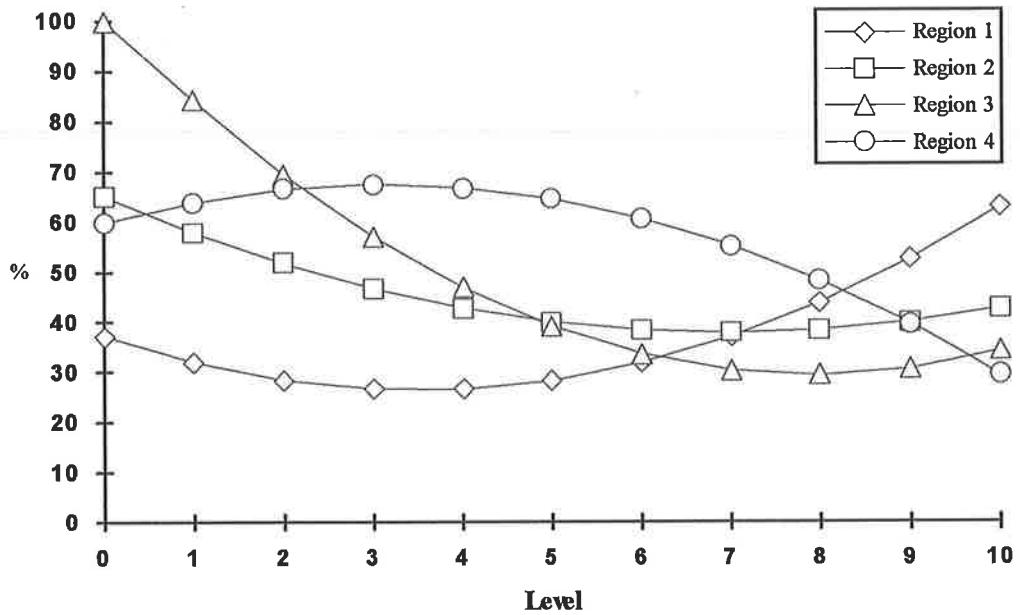


Table 4.4 % Surface Intact: Control

Level	*Region 1*	*Region 2*	*Region 3*	*Region 4*
0	91.8	96.6	96.5	93.7
1	96.4	99.8	100.0	97.0
2	99.7	100.0	100.0	99.1
3	100.0	100.0	100.0	100.0
4	100.0	100.0	100.0	99.8
5	100.0	100.0	100.0	98.5
6	100.0	98.9	97.8	96.0
7	97.3	95.3	93.4	92.3
8	93.0	90.6	87.7	87.5
9	87.4	84.7	80.7	81.6
10	80.6	77.6	72.4	74.4

Figure 4.21 % Surface Intact: Control

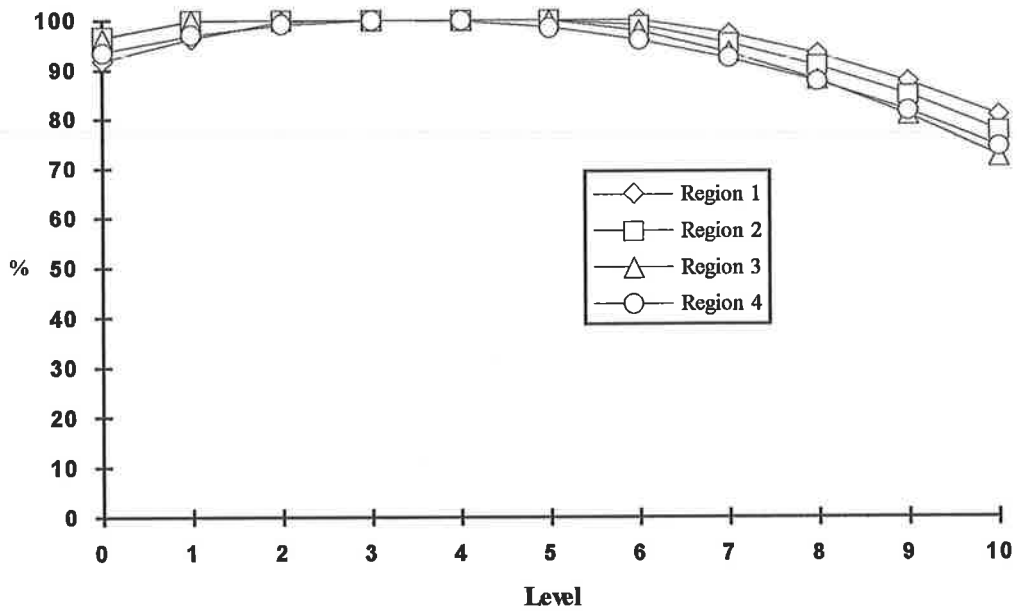


Table 4.5 % Surface Intact: Treatment

Level	*Region 1*	*Region 2*	*Region 3*	*Region 4*
0	83.6	100.0	100.0	100.0
1	77.1	100.0	100.0	100.0
2	71.3	95.9	100.0	100.0
3	66.1	91.2	100.0	98.1
4	61.6	86.7	95.6	94.6
5	57.8	82.5	90.5	91.0
6	54.7	78.6	85.5	87.3
7	52.3	75.0	80.4	83.4
8	50.5	71.6	75.3	79.5
9	49.5	68.5	70.3	75.4
10	49.1	65.7	65.2	71.2

Figure 4.22 % Surface Intact: Treatment

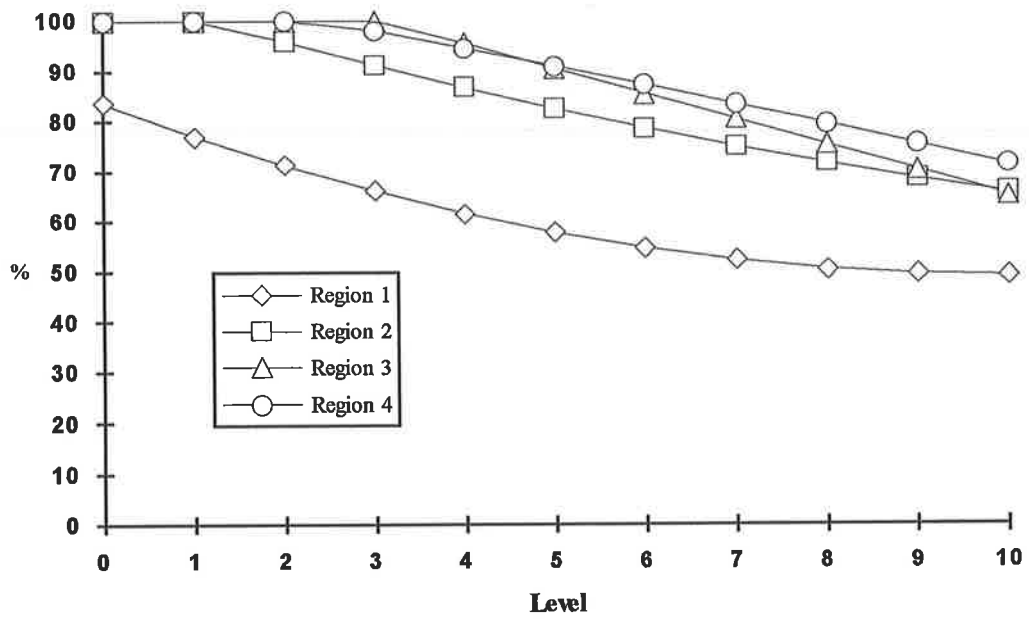


Table 4.6 Number of Epithelial Cell Rest Sections: Control

Level	*Region 1*	*Region 2*	*Region 3*	*Region 4*
0	31.3	35.3	39.0	31.2
1	24.5	26.9	29.7	24.3
2	18.7	19.7	21.8	18.6
3	13.7	13.8	15.3	14.0
4	9.6	9.2	10.3	10.6
5	6.3	5.9	6.7	8.3
6	4.0	3.8	4.6	7.2
7	2.6	3.0	4.0	7.2
8	2.1	3.5	4.7	8.4
9	2.4	5.3	7.0	10.7
10	3.7	8.4	10.6	14.2

Figure 4.23 Number of Epithelial Cell Rest Sections: Control

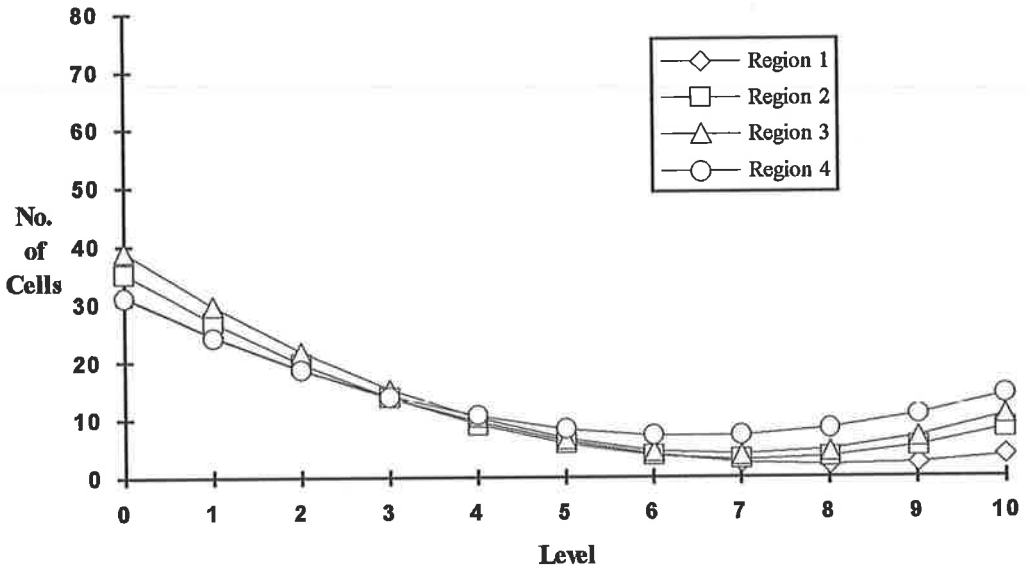


Table 4.7 Number of Epithelial Cell Rest Sections: Treatment

Level	*Region 1*	*Region 2*	*Region 3*	*Region 4*
0	20.8	26.3	26.3	27.7
1	16.3	21.2	21.3	23.7
2	12.4	16.7	16.9	20.0
3	9.2	12.8	13.1	16.6
4	6.7	9.6	9.9	13.4
5	4.7	7.0	7.2	10.5
6	3.4	5.1	5.1	7.9
7	2.7	3.8	3.6	5.6
8	2.7	3.1	2.6	3.5
9	3.3	3.0	2.3	1.7
10	4.5	3.6	2.5	0.2

Figure 4.24 Number of Epithelial Cell Rest Sections: Treatment

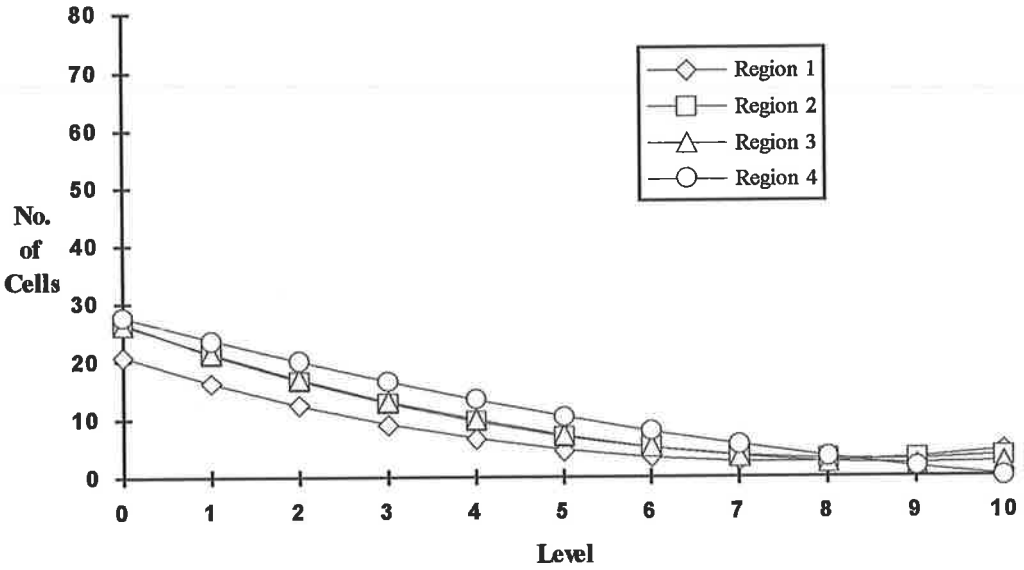


Table 4.8 % Surface Repaired/Repairing: Control

Level	*Region 1*	*Region 2*	*Region 3*	*Region 4*
0	3.5	3.4	3.5	5.2
1	0.3	0.2	0.0	1.5
2	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0
6	0.2	1.1	2.2	2.4
7	3.3	4.7	6.5	6.4
8	7.4	9.4	12.3	11.8
9	12.6	15.3	19.3	18.5
10	18.8	22.4	27.6	26.5

Figure 4.25 % Surface Repaired/Repairing: Control

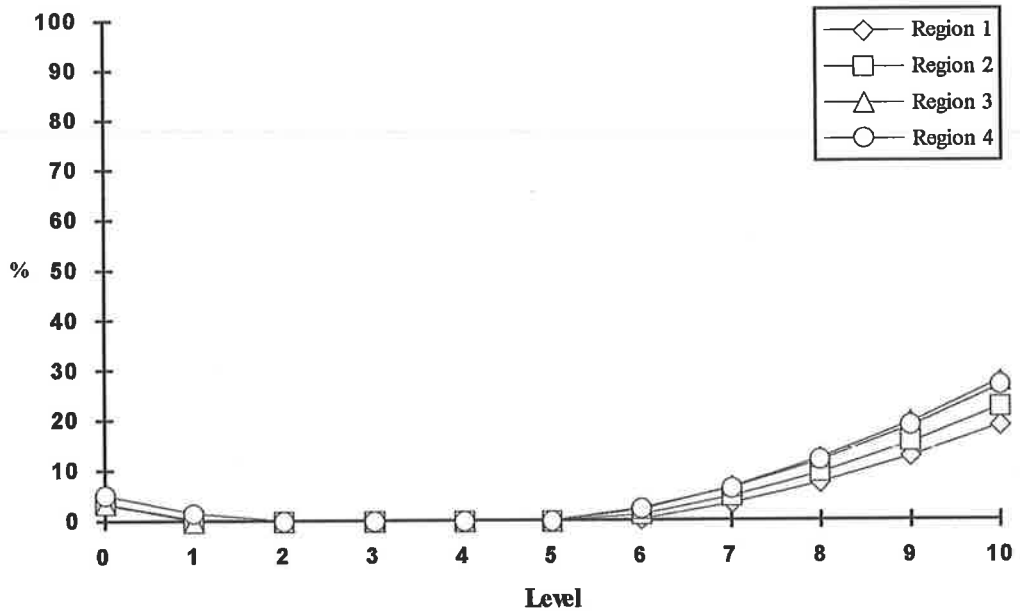
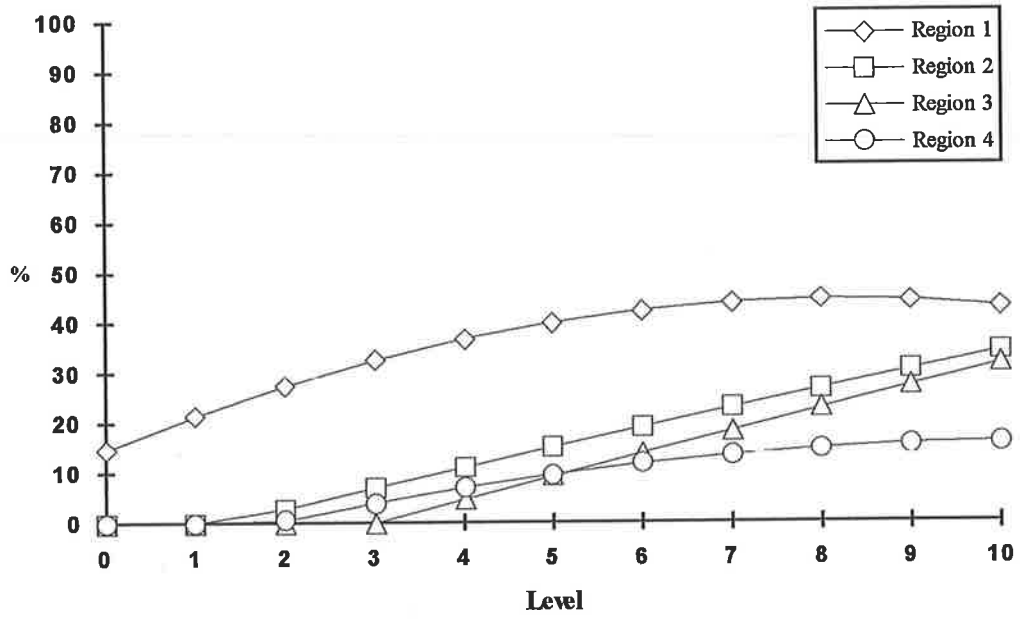


Table 4.9 % Surface Repaired/Repairing: Treatment

Level	*Region 1*	*Region 2*	*Region 3*	*Region 4*
0	14.6	0.0	0.0	0.0
1	21.5	0.0	0.0	0.0
2	27.5	2.8	0.0	0.5
3	32.6	7.0	0.0	4.0
4	36.8	11.1	4.6	7.0
5	40.1	15.1	9.3	9.6
6	42.5	19.1	13.9	11.8
7	44.0	23.0	18.4	13.5
8	44.7	26.8	22.9	14.8
9	44.4	30.6	27.4	15.6
10	43.2	34.3	31.7	16.0

Figure 4.26 % Surface Repaired/Repairing: Treatment



CHAPTER FIVE

DISCUSSION

5.1 MATERIAL

1. Preliminary study

The material collected for both parts of this study consisted of extracted human premolar teeth. As a result the amount of soft tissue available that remained attached to the tooth roots varied considerably. Not only did the tissue vary from site to site, and tooth to tooth, but the remaining tissue had also often been torn and disrupted. Not only therefore has the interpretation of the statistical results been limited by this fact, but the assessment of the morphological relationships must be viewed with caution. This aspect is discussed later in more detail.

The tissue collected for the initial fixation/decalcification study was collected on an opportunistic basis from three patients who had extractions as a result of the review of their progress with full fixed orthodontic appliances (Table 3.1). These teeth would have been subject to alignment forces, and subsequently to "jiggling" forces as the occlusion was modified and archwires were changed. There was no attempt to match these teeth for use as controls, or to examine them in a systematic manner, so the morphological findings need to be interpreted with caution. Nevertheless, some interesting morphological information was collected, although the principal aim of this part of the project was to standardise the fixation, decalcification and immunohistochemical procedures.

In retrospect, it is also possible to conclude that this early tissue was not handled as carefully as it should have been in the laboratory, especially when the teeth were divided, prior to embedding. Initially it was considered that it might be important to complete the decalcification as quickly as possible, so attempts were made to divide

the teeth early. As the later discussion will show, early division of the teeth does not appear to be a critical factor in the preservation of antigenicity, and more harm than good may be done by handling the tissue at too early a stage and too often. Apart from removing the crown, as early as possible to conserve decalcifying fluid, the root can be left to thoroughly decalcify for a long period without any noticeable effect on the immunohistochemical staining of the tissues, a fact also established by BJURHOLM *et al.* (1989). This fact was illustrated when the apical thirds of some of the teeth were found to be incompletely decalcified. It was considered that this probably occurred when the teeth were divided. The apical third, being the narrowest part, was more easily divided, with least pressure using a scalpel blade, despite being incompletely decalcified, yet the other thirds could not be divided at the same time because of their greater diameter. At this stage the apical thirds should have been left in the decalcifying solution with the remainder of the teeth, and not immediately embedded as they were. In this early part of the project it was felt that the least time in the EDTA the better, although in hindsight, this was not such a crucial factor.

2. Main study

The material for this part of the project (Table 4.2) consisted of adolescent human premolar teeth that had been extracted subsequent to rapid maxillary expansion (RME). The material for the second part of the project was collected by School Dental Service (SDS) personnel. As a result of the fixation/decalcification study, fixation was by 10% formosaline for 6 hours maximum. Although this presented some logistic difficulties, and relied on the appropriate handling of the material by the school dental personnel involved, there were few problems with this protocol. The ideal protocol would have all of these factors under the closest possible supervision, with the investigator involved at each step. Patients having these extractions were identified and the extracted teeth collected. Where possible the teeth were collected immediately, otherwise the teeth were placed in fixative by the SDS personnel, and transferred to the EDTA solution after 6 hours. This was not ideal as it was possible if instructions were

not understood for the teeth to be improperly fixed or stored. Ideally these aspects should be under direct control, so that all teeth are collected and processed under completely identical conditions.

The principal problem with this material is clearly the loss of tissue and the lack of alveolar bone. The ideal situation would be to surgically collect all this tissue as a single block, and an animal model would be required. The *Macaca fascicularis* monkey has been used in numerous studies on trauma, and root resorption and would appear to be an ideal model. Such a model would allow a much more accurate assessment of the relationship between epithelial cell rests and root resorption, as well as allow time related studies to be pursued as repair progressed.

5.2 METHODS AND IMMUNOHISTOCHEMICAL TECHNIQUES

A number of factors are crucial in the success of any immunohistochemical (IHC) procedure. In addition, there is a need to discuss the role of the decalcification procedure as it may affect the methods employed.

1. Fixation

There are several aspects of fixation that require careful consideration in the choice of fixative for IHC use, and LARSSON (1988) suggests that each of the following factors are relevant in the choice of fixative.

1. Type of material and species
2. Nature of the fixative itself
3. Rate of penetration and fixation
4. Fixative concentration
5. pH
6. Osmolarity
7. Temperature
8. Procedures to be followed after fixation

In the vast majority of immunohistochemical studies, tissue is fixed prior to immunohistochemical staining. The most commonly used fixatives in IHC are alcohols, formaldehyde, glutaraldehyde, and osmium tetroxide (GOSSELIN *et al.*, 1986) and more recently microwave fixation (LEONG *et al.*, 1985, LEONG *et al.* 1988).

According to LARSSON (1988), adequate fixation for immunohistochemistry results in the need to satisfy several contradictory criteria.

1. Retention of antigens
2. Preservation of antigenicity
3. Preservation of structure
4. Permeability of the tissue for IHC access.

Fixation is essential for structural preservation, and so a balance has to be struck between tissue integrity and antigen availability. The modification of antigens by the various fixatives that may be used is also a serious problem, particularly with the use of strong cross-linking agents such as glutaraldehyde. LARSSON (1988) considers that the fixative may not only extract or displace antigens, but may also act to increase cross-reactivity by promoting or decreasing the reactivity of epitopes on the same antigens.

In this study, although only at the light microscope level, fixation for morphological purposes was quite satisfactory. There were no signs of tissue degradation with any of the fixatives that were used. This was not unexpected as each of the fixatives has a good record for this type of preservation. In addition, several aspects in this study aided fixation. Firstly, the relatively narrow band of tissue requiring fixation enabled rapid penetration and fixation of the periodontal tissues. Secondly, fixation was carried out a $4^{o}C$, slowing any potential for tissue degradation. LEONG and GILHAM (1989) suggest that a period of up to four hours before fixation commences should have no significant effects on antigen preservation. Nevertheless, prompt fixation should be the aim in any study.

In relation to the immunohistochemistry protocols, however, the results were quite different. As LARSSON (1988) states, "... the choice of fixative must be dictated by trial and error experiments."

The following fixatives are discussed because they were used in the preliminary part of the project, to assess their effect on the tissue to be examined.

i. Formaldehyde

Formaldehyde has a number of advantages for IHC use (FOX *et al.*, 1985). It is a standard fixative (LEONG *et al.*, 1985), and its use is well documented in both the light and electron microscope. Formaldehyde is commonly used as a 4% to 10% buffered formalin solution. It is a monoaldehyde (HCHO), and is an efficient cross-linking fixative. Formaldehyde is a mild, and at least partially reversible fixative. BASCHONG *et al.* (1983) reports that formaldehyde fixation can be reversed, reportedly by up to 86% by chemical treatment. LARSSON (1988) also reports that overnight washing of old sections in running water is capable of restoring at least some lost antigenicity. LEONG and GILHAM (1989) reviewed the effects of formaldehyde fixation, and noted that prolonged exposure of tissues to 4% buffered formaldehyde produced progressive loss of tissue antigens. Cytokeratins remained only weakly reactive after one day, and other intermediate filaments were almost completely lost. These workers recommended an exposure to formalin of not more than 6 hours. In this study, formalin fixation proved to be very satisfactory, and for these reasons was used in the main part of this study.

ii. Karnovsky's fixative

This is a combination fixative suggested by KARNOVSKY (1965), and the original formula consisted of a mixture of 4% paraformaldehyde and 5% glutaraldehyde. This fixative is now generally used as a weaker formula for IHC use and LARSSON (1988) suggests that the use of 3% paraformaldehyde and 2%

glutaraldehyde gives the best combination of structural integrity and antigenicity for a period of 15 to 25 minutes. In this study, a half-strength solution was used.

The principal advantage is that whereas the formaldehyde penetrates rapidly for initial fixation, the glutaraldehyde, as a slower penetrating fixative, acts to preserve the structural integrity (GLAUERT, 1975). The glutaraldehyde component is a dialdehyde that is a very effective cross-linking agent, as it reacts with the amino groups of proteins and is said to be capable of cross-linking almost any proteins (BOORSMA, 1983). Glutaraldehyde is generally used at concentrations of 1% to 3%, and LARSSON (1988) suggests that for IHC purposes, it is preferable to use these concentrations for a shorter period (10 to 30 minutes at $4^{o}C$) rather than use very low concentrations (0.1% to 0.3%) for longer periods, as was initially suggested for IHC. In the opinion of LARSSON (1988), this regimen maintains the best possible structural integrity as well as retaining antigenicity. This suggestion is enhanced in the case of fixatives that combine formaldehyde and glutaraldehyde such as Karnovsky's fixative.

In this study, the cytokeratins in the epithelial cell rests were only very weakly reactive under the half-strength Karnovsky's fixative procedure used. This was almost certainly a result of "over-fixation" by the glutaraldehyde component of the fixative. There seems no reason to suspect that Karnovsky's fixative would not work at much shorter fixation periods, such as one hour, or perhaps even less. With such little depth of fixation required, such shorter periods may be possible. The use of this fixative where joint IHC and TEM studies are contemplated, would appear highly desirable.

iii. Microwave fixation

The use of heat generated by microwaves has been suggested as a suitable fixative for light and electron microscopy (LEONG *et al.*, 1985). Recent work by LEONG *et al.* (1988) suggests that microwaves are also suitable for IHC. These workers found that not only were microwaves a rapid method of fixation, but antigen preservation was generally superior to formaldehyde fixation. Of particular interest was the observation that the length of enzyme digestion required for cytokeratin detection

was considerably reduced (or even eliminated), and that staining intensity for cytokeratins was greater in microwave fixed tissue compared to formaldehyde fixed tissue. The results obtained here with microwave fixation are not easily explained. The fact that only one tooth was available for microwaving is also a problem. It may be possible that some fault in the fixation protocol is the most likely explanation. This may have occurred because there was relatively little tissue (ie. what remained of the periodontal ligament), and it was quickly and rapidly fixed. The duration of fixation may have been too long for this tissue. Perhaps there is also the possibility that the dentine and tooth structure somehow acted as a heat-sink and application of heat to the periodontal tissue continued even further past the optimum period of time. Continued use of microwaves for fixation of dental material would demonstrate the optimal temperature and duration of fixation. From a structural point of view, the parameters used demonstrated that at the light microscope level at least, there was adequate fixation. The use of microwaves for fixation offers the advantage of rapid and reliable fixation, and is used routinely in some pathology laboratories for these purposes. Its use in some circumstances may also reduce the needs for some types of fixatives.

2. Selection of antibodies

The selection of the most appropriate antibodies is essential to ensure the most reliable results in any immunohistochemical study. There are numerous polyclonal and monoclonal antibodies available to label cytokeratins (Table 5.1). Most of these antibodies have been derived from various studies and then developed commercially for widespread use in pathology laboratories. The two antibodies used in this study were selected on the basis of a previous study (LEEDHAM, 1990), commercial availability, and demonstrated applicability against the material to be used in the study (human periodontal tissue).

The "Multilink" preparation (Dako, Code E 453) is a biotinylated swine antibody active against all classes of mouse, rabbit, and goat immunoglobulins. It is

TABLE 5.1 Some Commercially Available Antibodies Against Cytokeratins.

Antibody	Specificity	Source
AE1	CK 10,14,15,16,19[1]	Biogenix
AE3	CK 1-8	Biogenix
AEL-KS2	Pan-Keratin	Biogenix
PA071-5P	Pan-Keratin[2]	Biogenix
CAM 5.2	CK 8,18,19	Becton-Dickinson
KL 1	Pan-Keratin	Immunotech
LP 34	Pan-Keratin	Dakopatts
BA 17	CK 19	Dakopatts
MNF 116	Pan-Keratin	Dakopatts
Bovine Keratin	Pan-Keratin[2]	Dakopatts
Human Callus	Pan-Keratin[2]	Dakopatts
PKK 1	CK 8,18,19	Labsystem
35BH11	CK 8	Enzo-Biochem
34BE12	CK 1,5,10,14	Enzo-Biochem
MAK 6	CK 8,14,15,16,18,19	Triton
lu-5	Pan-Keratin	Boehringer
NCL-5D3	CK 8,18,19	Eurodiagnostic
RCK 102	CK 5,8	Eurodiagnostic
RPN.1100	Pan-Keratin[2]	Amersham
RPN.1165	CK 19	Amersham
RPN.1166	CK 8	Amersham

Note: [1] Catalogue numbers according to MOLL (1982)

[2] Polyclonal antisera

reported by the manufacturer to have a very low cross-reactivity with human immunoglobulins because it is solid-phase absorbed with human and ox serum proteins to reduce cross-reactions with human IgG and fetal calf serum proteins. There is also some cross reactivity with pig, rat and sheep immunoglobulins.

3. Background and non-specific staining

Background staining was evident in most sections viewed from the preliminary study. There are a number of factors that can result in non-specific staining (TAYLOR *et al.*, 1978). Amongst these factors are included, poor fixation, electrostatic attachment, serum components, and natural or non-specific antibody. Various steps are available to reduce non-specific staining problems. GOSSELIN *et al.* (1986) suggest that most problems can be eliminated by ensuring that the antibodies to be used are properly purified, that the tissue to be stained is preincubated with neutral serum to remove any non-specific binding sites, and that the highest possible dilutions are used consistent with the retention of specific staining. The latter method also has the added advantage that less of the antibodies are used per section and so costs are kept down. It is also essential that tissues are thoroughly rinsed before staining to remove all traces of EDTA as these may increase background staining (BJURHOLM *et al.*, 1989).

The use in the main study of the AE1/3 monoclonal antibody greatly reduced the level of background staining, as a result of the specificity of the antibody. It was also noted that careful filtration of the DAB chromogen and using fresh DAB also greatly assisted in the reduction of the levels of background staining. This only emphasises the need for careful monitoring of standards at each level, and at each stage of the IHC process.

4. Controls

Adequate controls, both positive and negative are essential in immunohistochemistry, not only to evaluate results, but to exclude staining as a result of the kind discussed above that may be due to non-specific staining or cross-reactivity.

The most commonly used controls consist of a positive control, usually a tissue known to have the antigen present, and a negative control, usually where the primary antibody is replaced by normal serum. GOSSELIN *et al.* (1986) argues that the use of controls should be extended. It is suggested by GOSSELIN *et al.* (1986) that the use of an irrelevant antibody should also be considered in determining whether there are naturally occurring antibodies in the system being used.

In the main study the fortuitous collection of small amounts of gingival tissue, including gingival epithelium, was of great assistance in the assessment of the effectiveness of the antibody staining, by providing antigenic material that had been subject to the same handling, fixation and decalcification routines. Some of this material was used in the staining of each tooth and proved to be the ideal positive control, as it showed very intense and distinctive staining patterns. This material was also used as an effective negative control when the antibody was left out of the staining routine. In the initial phase, tonsil epithelium was used as a positive control and although useful, did not prove as reliable an indicator of the effectiveness of the procedures as the gingival epithelium.

In a discussion of the effective use of controls BOURNE (1984) suggests that a number of different controls should be considered. Where unknown specimens are being stained, the following controls are recommended. Firstly, a specimen processed identically known to contain the antigen in question. This will act as the positive control. Secondly, a specimen processed identically, but which does not contain the antigen in question. Thirdly, a specimen of the unknown specimen that should receive non-immune serum in place of the primary antibody. Where non-immune serum is not available, the diluent buffer can be used (BOURNE 1984). In this study, the diluent buffer was used quite satisfactorily, after consultation with the IMVS Immunocytochemistry Laboratory who advised on a number of the procedures that were used in this study.

5. Decalcification

In studies where decalcification of tissues is required before IHC methods can be applied, it is of interest to determine whether decalcification regimes increase or reduce the intensity of the immunohistochemical staining. This issue is of particular interest where antigens closely associated with teeth and bone require investigation.

The use of a 4% EDTA/cacodylate decalcifying solution has been recommended (BJURHOLM *et al.*, 1989) and a slightly modified version of this solution was used in this project. Although MASSOTH and DALE (1986) considered that extensive decalcification procedures weaken the staining patterns, BJURHOLM *et al.* (1989) believed that decalcification procedures should have little effect on IHC staining results, a comment confirmed by this study. BJURHOLM *et al.* (1989) also noted that where IHC staining was proposed, the intensity of staining only began to decrease after 60 days decalcification, but staining was still evident after 120 days if an EDTA/cacodylate buffer combination was used. MASSOTH and DALE (1986) using Kristensen's solution (0.5N sodium formate in 4N formic acid) and staining for cytokeratins noted that sections subjected to decalcification produced weaker staining than non-decalcified control sections, but did not quantify the differences. Few studies on decalcification protocols for IHC have been reported according to BJURHOLM *et al.* (1989), although HUME and KEAT (1990) have recently reported on the use of a 10% formic acid decalcification regimen. They were able to demonstrate decalcification of adult mouse mandibles in less than half the time (3 days) of a 20% EDTA solution (7 days). Antigenicity was retained in paraffin sections for bromodeoxyuridine, a marker of cell proliferation. The findings of HUME and KEAT (1990) are interesting as they suggest that more aggressive decalcification is possible without compromising antigenicity. As these workers embedded their material in paraffin, an assessment of ultrastructural integrity is not possible, however they made no comment on any problems with morphology or tissue damage as a result of the decalcification procedures that were used.

Most recent studies have used an EDTA solution, with concentrations varying from 24% (BJURHOLM *et al.*, 1989) to 10% (SAWADA *et al.*, 1990), to 5% (MIZOGUCHI *et al.*, 1990), usually in a buffer. A crucial factor, just as important as the concentration of the EDTA, appears to be the pH of the decalcifying solutions, as all the above studies (where stated) used a pH of 7.4 or sometimes 7.3.

The results of this study suggest that more rapid decalcification is likely to be an advantage without damaging the tissue, and that other suitable methods for decalcification should be investigated.

5.3 RESULTS

1. General Comments

There will always be problems in collecting consistent amounts of periodontal tissue from extracted human teeth, unless the alveolar bone is also collected with the teeth. As a result, the quantity of tissue is likely to vary, and the periodontal tissue remaining, must always be viewed with caution. Not only is the extraction procedure itself likely to affect the ligament tissue, but the use of local anaesthetics that contain vasoconstrictors will also have an effect.

It is also important that adequate controls are used to compare the normal distribution of epithelial cell rests.

2. Interpretation of IHC Results

Any use of polyclonal antibodies requires careful attention to the possibility of cross-reactivity. By their nature polyclonal antibodies can react with many epitopes within an antigenic site. Monoclonal antibodies, on the other hand are site specific and react to only one epitope on an antigen. This means the possibility of false positive results must be considered when using polyclonal antibodies. For these reasons only the monoclonal antibody AE1/3 was used in the main study. GOUDIE (1987) suggests that a common cause of cross-reactivity can occur as a result of phagocytosis of

antigenic material by inflammatory cells. No sign of inflammatory cells were seen in the areas of interest in this study.

Positive staining was seen in all teeth and was limited to clusters of cells close along the cementum surface, except in the cervical regions where cell rests were much more variable in the position in relation to the cementum surface. The high level of background staining was reduced in the main study, and the use of monoclonal antibodies allowed most cell rests to be seen even at quite low magnifications, although the smallest rests sometimes required a higher magnification for positive identification.

The staining pattern of individual cell rests was consistent. Nuclear material did not stain, consistent with all reports on cytokeratin staining. The densest staining was often perinuclear, but the remainder of the cytoplasm was also distinctively stained.

3. Morphological Findings

The examination of routine sections stained with haematoxylin and eosin showed clearly that reliable identification of epithelial cell rests at the light microscope level is not possible. The additional use of antibody staining not only allows more reliable identification of epithelial cell rests, but shows up the smaller rests which may not even appear at the light microscope. The possibility of single cell epithelial cell rests has been suggested in a transmission electron microscope study (LEEDHAM 1990) and the use of antibody labelling offers the opportunity to label and identify such cells at the light microscope level, although a lot of effort is required to prepare the material.

The morphology of the cell rests along the "non-resorbed" surfaces varied greatly. The classically described round and ovoid types were seen but there was great variation in the shape and morphology of these rests. This may be a result of several factors. Firstly, all of the patients in the second part of the study were adolescent, and so the epithelial cell network may still be maturing. Secondly, and perhaps more likely since there has been no systematic analysis or three dimensional reconstruction, the morphology seen may be more representative of the real situation. However, as only a

very few sections from each tooth were examined after staining, it is difficult to develop a real appreciation of the epithelial cell structures in the periodontal network. In fact it is yet so be shown conclusively whether or not the epithelial cell network is continuous, nearly continuous or quite discontinuous. SPOUGE (1980) has demonstrated that in the pig at least, there is an extensive network throughout the ligament.

Overfilling of resorptive cavities by reparative cementum was a feature of the repair of some of the lesions seen in teeth from the initial study. Overfilling, or "overcontouring" has previously described in relation to repair resulting from rapid maxillary expansion (BARBER and SIMS, 1981). The reasons for this overfilling are unknown, and may relate to the imprecise or exuberant nature of the repair process, or possibly an attempt to re-establish the periodontal width. It may be a time related matter with excess being removed with the progrees of time. The study by BARBER and SIMS (1981) examined teeth that had been in retention for a while, so it is possible that some excess had been resorbed in the intervening period. This matter could be clarified by correlating areas of overfilling with the type of orthodontic movements applied to teeth, the sites of overfilling, and the time since the completion of orthodontic movement.

The apparent presence of epithelial cell rests in close association with resorption cavities in human teeth confirms the findings of BRICE (1988) who noted epithelial cell rests in a transmission electron microscope study of resorption occurring as a result of RME therapy. However the rests described by BRICE (1988) had a morphology not dissimilar to "resting" types of epithelial cell rests, although the presence of "light" and "dark" cells was noted. The preliminary results of this study suggest that not only may epithelial cells be more prevalent in relation to resorption sites, but also that their morphology may be more variable.

The staining of individual cells within the resorption areas suggests that branching of the rests may be occurring. This may be simply a result of the epithelial network being re-established, or it may occur as a result of the active participation of

cell rests in the repair process. A closer comparison with epithelial cells involved in epithelial wound repair would be interesting.

THOMAS and KOLLAR (1988) have suggested that cells on the forming root suface during the period of intermediate cementum formation are of epithelial origin, and identified them as epithelial by several methods. Ultrastructurally, desmosomes and tonofilaments were noted, and immunohistochemical means were employed that identified the presence of keratin intermediate filaments. THOMAS and KOLLAR (1988) used these results to suggest that during the period of root formation, the cells of the epithelial root sheath do not immediately migrate away from the root surface. They further suggested that these cells close to the cementum surface may undergo an epithelial-mesenchyme transformation, and more closely resemble mesenchymal cells.

The presence of epithelial cells within the resorptive areas, combined with the findings of THOMAS and KOLLAR (1988) allow the possibility that the epithelial cell rests may have more than a passive role in cementum repair. These epithelial cells may either mediate the transformation of reparative cemetoblasts and thus repair of the cementum, or could directly play a role in the initial formation of the first layer of reparative cementum, once the stimulation required to maintain osteoclastic activity is removed. The patchy areas of staining along the cementum, both within the resorptive cavities and along other cementum surfaces may support this contention. This aspect needs further investigation to determine if it is a consistent finding or an artifact. Recent work on cultured epithelial cells from HERS of rats (FARGES *et al.*, 1991), showed that not only was a bilayered structure similar to the root sheath reformed, but that these cells were producing all the major components of basement membranes. This latter aspect was demonstrated by polyclonal antibodies to type IV collagen, mouse laminin, anti-murine basement membrane heparan sulphate proteoglycan, and fibronectin. Monoclonal antibodies to cytokeratins demonstrated the epithelial nature of the cells of the reconstituted "cervical loop" were epithelial in nature.

In conclusion, it seems likely that the epithelial cells have some as yet undefined role in resorption repair and/or protection. This contrasts with the suggestions of

REITAN (1961) who suggested that the absence of epithelial cells was characteristic of hyalinised periodontal tissue, and more recently of ANDREASEN (1988) who considered that that the "significance of the Malassez epithelial cells is questionable in regard to resorption protection". The morphological findings, although limited have clearly identified epithelial cell rests sections in close relationship with some of the areas of repairing resorption. However the role of these cells has not been clarified. It seems most likely that any role they have would be time-sensitive, so that their presence or absence may be crucial either to cessation of resorption, and, or initiation of repair. These events are important events about which little is known. The examination of experimental repair at timed intervals although tedious and time consuming would throw some light on this very interesting area. The use of monoclonal antibodies would enable a very accurate three-dimensional reconstruction of selected areas of resorption that would enable further study of this repair process. KITTEL (1990) showed the difficulties in developing a three dimensional picture without accurate identification of structures.

4. Histomorphometric Results

When this aspect of the study was designed, a large number of variables were to be examined. However, only four of these variables showed sufficient variability to be statistically analysed. The remaining variables occurred so infrequently or perhaps were missing as a result of the missing tissue subsequent to extraction. Consequently, this aspect of the study is limited by the loss of soft tissue, as well as the absence of alveolar surrounding bone. However the statistical method used (program 5v from the BMDP statistical package) is capable of dealing with missing data, and giving the overlying trends in the data via model smoothed averages.

The variation in the soft tissue present between control and experimental teeth was interesting (Figs 4.19 and 4.20). There was a consistency in the results from the control teeth with more tissue remaining in the apical and cervical thirds, a result not inconsistent with the "wine-glass" shape of the periodontal ligament (BERKOVITZ *et*

al., 1982). There was certainly less consistency in the soft tissue present in the four regions in the treatment teeth. With such a small sample, it is difficult to come to any firm cause for this aspect. However region 4 (lingual surface) at least, showed such a different pattern (Fig. 4.20), characterised by a reverse curvature in the graph in the amount of soft tissue present compared to the other regions, with increased soft tissue remaining in the middle third. This region represented the lingual periodontal ligament surfaces of the treatment teeth, the surface subject mostly to tension, rather than compression or a combination of compression and tension. It does seem interesting though that there should be such a difference between the control and treated teeth.

The percentage of the root surface affected by resorption was also examined by region. Not surprisingly, the most extensive resorption was seen on the buccal surface of the treated RME teeth. However the control teeth also showed small amounts of resorption in the cervical region and slightly more in the apical regions. These changes in the control teeth indicate that compensatory tooth movement has occurred here. The occlusal changes as a result of RME therapy are extensive and occur over a short period of time (usually two weeks), with continued settling of the occlusion over a longer period of time. The changes seen in this study in the control teeth, indicate that there are substantial changes in these teeth, and are therefore not the best control teeth. The best controls would obviously be matched upper premolars not subject to any orthodontic therapy. This study has certainly highlighted (and mapped) the fact that there are consistent effects on the opposing dentition as a result of RME treatment on the opposite arch.

The vast majority of these sites of resorption were either repaired or repairing (Figs. 4.25 and 4.26). There were so few sites of active resorption that a statistical analysis was not possible. It would seem logical to expect that the greatest number of resorptive sites will occur initially, with a gradual lessening of these resorptive sites as repair progresses, so that ultimately all are reparative in nature.

The area of this study that it was hoped to look at in some detail was the relationship between RME therapy and the presence of epithelial cell rests. Both the

treated and control teeth showed similar patterns of epithelial cell rest distribution along the tooth root surface. One of the difficulties in interpreting these data, is the length of time these teeth were in retention allowing repair and reconstruction of the periodontal ligament to occur. Nevertheless there were still differences in the number of epithelial cell rests seen, with generally higher levels (approximately 50% higher) of cell rests seen in the treated teeth. This response, while seeming to confirm the idea that epithelial cell rests do respond in some manner to orthodontically induced changes in the periodontal ligament, must be tempered by the fact that so much soft tissue was missing. Nevertheless, the model suggested by the information does point to some interesting possibilities. The results do appear to indicate a correlation between the numbers of epithelial cell rests and repair. However this relationship is not clearly shown because of the nature of the way in which the numbers of cell rests were recorded. As the surface areas of the octants decreased apically, there needed to be a method to compensate so that the surface density of the rests could be calculated. This would give a more accurate representation of the potential role in the repair process. With such a small sample, the effect of variation between the teeth, particularly the experimental teeth can be great.

Similarly, as the octants decrease in size apically, the estimates of resorption (based on the 0 to 10 scale) may tend to over-estimate the extent of resorption in the apical areas. This could suggest that the changes in the apical levels could be over-estimated, and so not as extensive as the results suggest. This may particularly relate to the changes seen in the control teeth (Fig. 4.21).

Another difficulty relates to the timing of the extractions of the teeth in the study. It is apparent that the crucial time in the repair process is the time at which resorption ceases and repair commences. This may be the time at which the epithelial cells (or other cells) may exert an influence if they are involved in the control of repair. Examination of this aspect would involve collecting teeth at various times post-RME therapy. This would probably limit further studies to animal studies for ethical reasons.

In summary, this histomorphometric study points to a number of interesting findings. However, the method does need refining, and with the collection of the associated alveolar bone and therefore the full width of the periodontal ligament, more accurate data collection is possible. Stereological methods offer high accuracy, but are very intensive. The periodontal ligament is a relatively small volume of tissue yet is capable of considerable remodelling activity. The control mechanisms are little understood, and evidence does point to some role for the epithelial cell rests of Malassez in this control. As a result closer study of these cells is essential, using accurate identification methods.

5.4 SUGGESTIONS FOR FUTURE RESEARCH

The present study has so far shown that the use of IHC studies, in association with appropriate decalcification procedures, is capable of identifying epithelial cell rests in areas of orthodontic root resorption and repair. Any systematic association of epithelial cell rests in association with either root resorption and/or repair of resorption has yet to be demonstrated in the literature, although this study has suggested that there may be an increase in epithelial cell rests as a result of orthodontic treatment. This would indicate that one of the potential directions for future research in this project should be to demonstrate more clearly this relationship. As a result of such a study, a more clearly defined role for the epithelial cell rests should become clearer.

There are several possibilities that might enable this approach to be pursued. Firstly, the project could be along the lines of another morphometric study that would not only quantify the presence of root resorption and repair as a result of orthodontic treatment, but also attempt to show an association with epithelial cell rests. An animal study would be required in order to overcome the limitations of this study, whereby alveolar bone could also be collected.

Such a morphometric study however would have several potential limitations. Firstly, the amount of material that might be available would probably be limited by expense, and time constraints. The material available would also need to be divided, as

much as possible, into the types of tooth movement performed, potentially limiting the conclusions of the study. The use of RME type appliances does seem however to be a useful model. The decision on timing of tissue collection would be crucial, although it may be possible to collect material at several intervals. The collection of appropriate controls would be essential. The other potential problem is the method of data collection. Methods to calculate the extent of root resorption are well known as a result of studies on replantation of incisors in monkeys (ANDERSSON *et al.*, 1987; ANDREASEN, 1987). There is a limitation however on the amount of material that can be stained using IHC methods, not only in time and expense, but also in the actual number of slide sections that can be stained. The advantages of adopting one of these morphometric methods are that the techniques are well accepted, and most importantly, a statistically valid study would point to the association of the cell rests with areas of resorption. Most studies however have used extracted monkey incisors for traumatology studies, and this would appear to be an excellent model. A rodent model using rats would also be worth considering, as more material would be available.

The use of IHC methods opens up a wide field of research possibilities as a result of its capability for positively identifying and locating cell types at both the light and electron microscope level. For example, not only can individual cell types, such as epithelial cells be identified, but there are methods of double labelling available that would allow two antigens to be labelled in the one section. Examples of this might include, in reference to the present project, labelling of both epithelial cells and areas of laminin or type IV collagen to show the association of blood vessels to the areas of resorption. Similarly, the labelling of epithelial cells and factors associated with osteoclasts or macrophages might demonstrate a closer association of epithelial cells in relation to either continuing resorption or repair of resorption.

The use of laser scanning confocal microscopes may be a useful aid in this area. Block staining large areas of periodontal ligament for example, may make it possible to build up a three dimensional picture of the association between epithelial cell rests and

areas of resorption. Disadvantages of this method, include the fact that they often require fluorescent probes, and that some tissues have natural fluorescence, and possibily limited penetration of tissues by immunological reagents. However, the advantage is that the depth of tissue to be examined can be increased by the use of the increased resolution of the laser used to focus on the sections. This enables the collection of serial sections from the same tissue section. The depth of tissue in each section depends on the penetration of the laser, as well as the objective lens to be used. The depth of penetration of the immunological reagents into the tissues must also be taken into account. Such systems often require large amounts of computer memory to store each image, although modern image capture systems enable photographic records to be taken. The "BIO-RAD" MRC-500 system for example requires 384KB to store each image, effectively requiring a large hard disk or preferably an optical storage disk if large numbers of images are required to be stored. There is considerable potential in the use of scanning confocal microscopy. It has been demonstrated that such systems can be used to build a picture of tooth resorption (BOYDE, 1986; BOYDE *et al.* 1990), and if its application could be combined with the use of IHC, a three dimensional picture of both the hard tissue and soft tissue relationships could be constructed that should add to the understanding of the processes of resorption and repair.

CHAPTER SIX

CONCLUSIONS

1. Formalin fixation is a suitable fixative that is compatible with the immunohistochemical staining protocol used in this study, and paraffin embedding procedures.
2. Fixation with half-strength Karnovsky's fixative at 6 hours is not satisfactory for IHC purposes, probably as a result of overfixation, although it would probably be quite satisfactory at higher dilutions and for shorter intervals.
3. Microwave fixation as used in this study was unsuccessful for reasons that are unclear, although it was probably a result of the procedures used, rather than the method itself. This method has potential for use that should be further investigated.
4. Decalcification with 4% EDTA does not appear to significantly reduce IHC staining results, and more rapid methods of decalcification should be investigated that might offer the benefit of speeding up the decalcification process without any adverse effects on tissue integrity or IHC staining protocols.
5. Epithelial cell rests are clearly identifiable at the light microscope level when stained with polyclonal and monoclonal antibodies to human cytokeratin. Monoclonal antibodies are preferable because of their greater specificity, and lower background staining.
6. The morphology of epithelial cell rests was quite variable in both the treated and control teeth. Apart from the cervical area, the epithelial cell rests were a uniform distance from the cementum surface.

7. Epithelial cell rests can be found in close relationship to the periphery of some of the resorptive defects that result from orthodontic treatment.
8. Epithelial cell rests are demonstrable within the body of some repairing orthodontic resorptive defects.
9. There was evidence of branching of epithelial cell rests in the vicinity of the areas of resorption.
10. Epithelial cell rests may play a role in the processes involved in the repair of resorptive lesions, particularly the cessation of resorptive activity, and the initiation of repair.
11. There is a need to quantify not only the amount of resorption associated with orthodontic tooth movement, but also to attempt to show the relationship, if any between the epithelial cell rests of Malassez with either continuing resorption, the cessation of resorption, or the repair of resorption as a result of orthodontic tooth movement.
12. Further studies should also collect alveolar bone to ensure the entire width of the periodontal ligament is collected.
13. Three dimensional reconstruction of areas of the periodontal ligament are lacking, and the structure of the human periodontal epithelial cell network is not well characterised either before or after orthodontic treatment.
14. Histomorphometric and stereological studies offer one of the best methods of quantifying the processes and cells involved in the processes of tooth root resorption. Such studies can be time related to follow these processes more closely.
15. Control mechanisms involved in the repair and rebuilding of the periodontal ligament, subsequent to trauma (including orthodontic tooth movement) are little understood, despite our reliance on these processes for successful orthodontic tooth movement.

CHAPTER SEVEN

APPENDICES

1. KARNOVSKY'S SOLUTION

USE: Fixation of tissue

FORMULA: 4% paraformaldehyde
5% glutaraldehyde
0.05% $CaCl_2$

PREPARATION: 2g of paraformaldehyde are dissolved in 25ml of water heated to 60-70°C; add 1 to 2 drops of 1N NaOH, and stir until the solution clears; cool the solution and add 5ml of 50% glutaraldehyde; make up the volume to 50mls with 0.2M cocodylate or phosphate buffer; if using cacodylate, add 25mg $CaCl_2$ anhydrous; final pH is 7.2.

COMMENTS: It is believed that the formaldehyde penetrates more quickly, and temporarily stabilizes structures, which are then stabilized more permanently by the glutaraldehyde. It can be used at half strength.

2. FORMALIN SOLUTION

USE: Fixation of tissue

FORMULA: 4% buffered neutral formosaline

PREPARATION: 900ml distilled water, 6.5g anhydrous sodium phosphate dibasic, 3.5g anhydrous sodium phosphate monobasic, and 100ml of 40% stock formaldehyde solution are mixed together.

COMMENTS: Used for transportation and temporary storage of specimens for immunohistochemistry, to preserve antigenicity.

3. EDTA DECALCIFYING SOLUTION

USE: Decalcification of hard tissues.

FORMULA: Ethylenediaminetetra-acetic acid (EDTA) in sodium cacodylate buffer.

PREPARATION: Dissolve 40g EDTA in 1000ml of distilled water, and add 24.2g sodium cacodylate. Add sodium hydroxide to bring the pH to 7.3 at 4°C.

SHELF LIFE: 7 days at 4°C.

COMMENTS: Solution needs to be changed regularly as the EDTA is consumed.

4. PARAFFIN/CELLOIDIN EMBEDDING

The following 9 day processing procedure was used for double embedding using celloidin/paraffin embedding.

Tuesday — Dehydration and Clearing

1.	30 mins	70% alcohol
2.	1 hour	80% alcohol
3.	1 hour	90% alcohol
4.	1 hour	100% alcohol
5.	1 hour	100% alcohol
6.	1 hour	100% alcohol

Wednesday

7.	1 hour	50:50, Methyl Salicylate: 100% alcohol
8.	2 days	0.5% Celloidin in Methyl Salicylate

Friday

9.	2 days	1% Celloidin in Methyl Salicylate

Monday — Infiltration and Embedding (in 60°C oven)

10.	1 hour	2/3 Methyl Salicylate and 1/3 Paraplast
11.	1 hour	1/2 Methyl Salicylate and 1/2 Paraplast
12.	1 hour	1/3 Methyl Salicylate and 2/3 Paraplast
13.	2 hours	Wax change I
14.	2 hours	Wax change II
15.	12 hours	Wax change III

Tuesday

16. Place specimens in vacuum for 30 to 60 minutes
17. Block specimens in moulds, and place in fridge when cooled.

5. SLIDE COATING PROCEDURES

i) APT

Slides for antibody staining were coated according to the following procedure, using APT (Sigma, code A3648).

1. pre-rinse slides twice in acetone
2. dip for 10 seconds in 2% APT in acetone
3. rinse in acetone
4. rinse in distilled water
5. air dry

ii) CHROME-GELATIN ALUM

1. Add 3g gelatin to 1000ml d.d.water at 60°C
2. Add 0.5g chromium potassium sulphate (chrome alum)
3. Add several crystals of thymol, and stir until all dissolved
4. Filter while hot into a beaker
5. Dip slides into hot solution, and dry vertically in a slide rack in a hot oven
6. Dried slides can stored in boxes until required.

6. STAINS FOR LIGHT MICROSCOPY

i) HAEMATOXYLIN

USE: Staining for light microscopy

FORMULA: Lillie-Mayer Haematoxylin (LYNCH et al., 1969)

PREPARATION: 5g haematoxylin
500ml distilled water
300ml glycerol
50g ammonium alum (mordant)
0.2-0.4g sodium iodate
20ml acetic acid

SHELF LIFE: The glycerol eanables the solution to be kept several years.

COMMENTS: Haematoxylin can be used with eosin, or by itself as a counter stain to antibody staining

ii) EOSIN

USE: Contrast cytoplasmic stain for nuclear stains such as haematoxylin

FORMULA: 1% Eosin Y (LYNCH et al., 1969)

PREPARATION: 1% stock solution Eosin Y
0.25ml of 40% formaldehyde solution per 100ml to prevent bacterial growth
0.2ml acetic acid/100ml

COMMENTS: This solution can be acidified by substituting 80ml of absolute alcohol.

iii) HAEMATOXYLIN-EOSIN STAINING

	xylol	2 mins
	xylol	2 mins
	100% alcohol	2 mins
	100% alcohol	2 mins
	tap water	1 dip
	haematoxylin	10 mins
	running tap water	2 mins
*	acid alcohol	1 dip (<5 secs)
	running tap water	10 mins
**	1% aq $LiCO_3$	10 secs
	running water	10 secs

eosin	30 secs
absolute alcohol	2 mins
absolute alcohol	2 mins
xylol	2 mins
xylol	2 mins

coverslip

* differentiating solution 1% HCl in 70% alcohol
** blueing solution

7. IMMUNOHISTOCHEMICAL STAINING PROCEDURE

1. Deparaffinise Sections
   i) 5 min Xylene
   ii) 5 min Xylene
   iii) 2 min 100% EtOH
   iv) 2 min 100% EtOH
   v) 2 min 95% EtOH
   vi) 2 min 80% EtOH
   vii) 2 min 70% EtOH

2. Trypsinisation
   i) 0.5mg/ml Type II porcine trypsin for 3 mins

3. Quench Endogenous Peroxidase Activity
   30 min 0.6% H_2O_2 in 95% MeOH

4. Wash PBS (pH 7.35)
   3x 3 mins

5. Blocking Serum
   30 mins

6. Primary Antibody
   i) overnight incubation for each dilution
   ii) 3x 3 mins wash PBS

7. Biotinylated Second Antibody
   i) 30 min
   ii) 3x 3mins wash PBS

8. ABC Reagent
   i) 60 mins
   ii) 3x 3 mins wash PBS

9. DAB Substrate (prefiltered)
   i) 5 min
   ii) 5x 3 min rinse tap water

10. Counter-staining and Mounting
    i) stain in haematoxylin
    ii) wash in running tap water for 5 mins
    iii) dehydrate and coverslip

8. BUFFERS

USE: Help maintain pH within specified range

i) CACODYLATE BUFFER

FORMULA: $Na(CH_3)_2AsO_2.3H_2O$

PREPARATION: 25.68g sodium cacodylate in 2,000ml d.d. water. Adjust to pH 7.4 using 1N HCl at 20°C.

SHELF LIFE: 7 days at 4°C

COMMENTS: Stable and does not support micro-organisms. High toxicity to humans (due to arsenic), hence it is suggested that gloves, mask, and a fume cupboard are used.

ii) PHOSPHATE BUFFERED SALINE (PBS)

FORMULA: 10mM sodium phosphate, pH 7.5, 0.9% saline

PREPARATION: Dissolve 27.6g of monobasic sodium phosphate in 1 litre of 0.9% saline solution (solution A). Mix 28.39g of dibasic sodium phosphate in 1 litre of 0.9% saline solution (solution B). Add 16ml of solution A to 84ml of solution B and dilute to 200ml to acheive a final pH of 7.5.

iii) TRIS HCL

FORMULA: 0.1M Tris HCl buffer, pH 7.2

PREPARATION: Dissolve 24.2g tris (hydroxymethyl) aminomethane in 2 litres of distilled water to make a 0.1M solution. Add 50ml of this solution to 44.2ml of 0.2M HCl and make up to a volume of 200ml to acheive a pH of 7.2.

9. LIGHT MICROSCOPY

1. ZEISS AXIOMAT, with a 5"x 4" format film photographic unit, taking Ilford FP-4 film.
2. OLYMPUS BH-2 Microscope, with a PM-10ADS, 35mm format photographic unit.

10. PHOTOGRAPHY

FILM TYPE	DEVELOPER	DILUTION	TIME	FIXER	TIME
Ilford FP4	Microphen	1:1	7 mins	Hypam	3 mins
Ilford Pan F	Microphen	1:1	4 mins	Hypam	3 mins
Kodalith	Kodalith	A:B 1:1	4 mins	Hypam	3 mins

APPENDIX 11. Data Recording Sheet

Patient ID	Age	Sex	Treatment	Tooth	Duration	Level

OCTANT	ZONE	% SOFT TISSUE PRESENT	% SURFACE INTACT	NO. ERM ALONG SURFACE	BV B/W ERM & TOOTH	% ACTIVE RESORPTION	NO. ERM IN BAY	NO. ERM ALONG BAY SURFACE	BV B/W ERM & TOOTH	% SURFACE REPAIR	ERM IN BAY	NO. ERM ALONG BAY SURFACE	BV B/W ERM & TOOTH	% INDETERMINATE REPAIR	ERM IN BAY	NO. ERM ALONG BAY SURFACE	BV B/W ERM & TOOTH
1																	
2																	
3																	
4																	
5																	
6																	
7																	
8																	

CHAPTER EIGHT

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