THE EFFECT OF ORTHODONTIC TOOTH MOVEMENT ON THE MAST CELL POPULATION IN THE RAT PDL

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Master of Dental Surgery

by

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LIST OF ABBREVIATIONS

TEXT

ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Camp	cyclic adenosine monophosphate
cGMP	cyclic guanidosine monophosphate
C3a C4a C5a	components of the complement cascade
CTMC	connective tissue mast cell
DAG	diacylglycerol
FcεRI	high-affinity Fc receptor for IgE.
FITC-avidin	fluorescein isothiocyanate - conjugated avidin
GMCSF	granulocyte-monocyte colony stimulating factor
H_2O_2	hydrogen peroxide
HRP-avidin	horseradish peroxidase-conjugated avidin
IFAA	isotonic formol-acetic acid
IgE	immunoglobulin E
IgG	immunoglobulin G
IL-3	interleukin 3
IL-4	interleukin 4
IL-5	interleukin 5
IL-6	interleukin 6
LTB_4	leukotriene B
LTC_4	leukotriene C
LTD_4	leukotriene D
LTE_4	leukotriene E
MHC	major histocompatibility complex
MMC	mucosal mast cell
PDL	periodontal ligament
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PTH	parathyroid hormone
SRS-A	slow-reacting substance of anaphylaxis
TNF	tumour necrosis factor
TRITC avidin	tetramethylrhodamine isothiocyanate-conjugated avidin
48/80	compound 48/80 - a degranulating agent. A product of condensation of *p*-methoxyphenethylmethylamine

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.

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Melinda E. Barva

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SUMMARY

Orthodontic tooth movement may initially cause an inflammatory process in the periodontal tissues. Mast cells have an important function in the initiation of inflammatory responses either as a reaction to trauma, immediate type of immune reactions, or in delayed-type hypersensitivity. Their role, if any, in orthodontic tooth movement has not been determined.

The principal aim of this study was to test the hypothesis that there would be a reduction in the number of stainable mast cells subsequent to orthodontic tooth movement.

The present study utilised sixty male Sprague-Dawley rats: forty of these were experimental animals and twenty were controls. The experimental animals were 83 or 84 days of age at the time of orthodontic appliance insertion. The rats were anaesthetized by intraperitoneal injection of Ketapex[1] diluted in sodium chloride, and Rompun[2] to enable cementation of the appliances. The appliances consisted of bonding mesh adapted as a band around the two maxillary incisors soldered to a palatally positioned supporting wire. A finger spring was welded to the right side of the supporting wire and was adjusted to provide a force of approximately five grams in a buccal direction to the maxillary right first molar. These appliances were cemented to the maxillary incisors using chemically cured composite resin. The finger spring was maintained in the correct position with the use of a 0.08" stainless steel ligature. During the period of orthodontic tooth movement the animals were fed pulverized standard rat pellets and the appliances were checked every second day.

Following completion of the experimental period (15 minutes, 1 hour, 4 hours, 24 hours, 1 week, 2 weeks, 4 weeks and 8 weeks) the rats were sacrificed and the portion of the maxillae supporting the six molars was removed. These specimens were matched with untreated control animals of the same age.

All of the specimens were immersion fixed in Carnoy's fixative and rehydrated before being demineralized in 4% EDTA in cacodylate buffer. The end point of decalcification was

[1] Ketapex = 100mg/mL ketamine (Apex Laboratories, NSW, Australia)
[2] Rompun = 20 mg/mL xylazine hydrochloride (Bayer, NSW, Australia)

determined radiographically and the specimens were processed for routine paraffin wax embedding.

As this study focussed on early changes in the population and distribution of mast cells (i.e. over the period of one week) all of the animals during this time period were examined (N=33). Only one each of the experimental and control 2 week, 4 week and 8 week animals (N=6) were examined to identify any later trends, and the remaining specimens were processed and set aside for later study. The 39 specimens of the current study were sectioned serially in 5 micron thick sections and mounted on APT[3]-subbed slides. There were on average 600 sections per tooth.

To identify zero levels, every tenth section from the first section to section 300, was stained with haematoxylin and eosin. The zero level was taken as the first connective tissue attachment on the mesial of the first maxillary molar. From zero to the last section per block, every twentieth section (i.e. every 100th micron) was stained for mast cells using 0.5% toluidine blue in HCl at pH 0.5. This resulted in approximately 20 levels studied per tooth, depending on its root length.

The root and surrounding periodontal ligament and bone were divided into four quadrants (buccal, distal, palatal and mesial) with the use of a template in the light microscope eyepiece. In addition, the ligament was further subdivided into horizontal thirds (bone, mid and tooth) utilizing a millimetre scale in the same eyepiece. Therefore, there were twelve possible locations (4 quadrants x 3 thirds) per level per tooth where the mast cells could be located. Also, the level at which the alveolar crest completely surrounded the mesiobuccal root was noted. Furthermore, a note was made if the mast cells were located in close proximity (within 12 microns) to any blood vessels.

These data were provided to the statistician as an Excel spreadsheet and were read into a statistical package, S-plus. The data were reduced into a more manageable form by consolidating the levels studied (averaging 20) into three areas (coronal, middle and apical). The data were subjected to analysis of variance, mean mast cell counts and split-plot analysis.

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[3]APT = aminopropyltriethoxysilane (Sigma Chemical Co. St. Louis, USA)

Analysis of variance indicated there were large treatment differences between the left (control) and right (experimental) teeth within the experimental animals (Mean Sq = 21.83, P = 0.0022).

The present study demonstrated a change in the mast cell numbers when comparing experimental (orthodontically moved) and control teeth. The mean mast cell counts throughout the PDL indicated that the control teeth generally had higher mast cell counts than the experimental teeth, except this trend was reversed in the one hour time group.

This change in mast cell counts was not universal throughout the ligament and was highly affected by position (ie. vertical distribution, quadrant distribution and horizontal distribution). Analysis of variance indicated that the horizontal distribution (ie. bone, mid and tooth thirds) showed the most significant differences, followed by the vertical distribution (i.e. coronal, middle and apical levels), with the least significant differences noted in the quadrant distribution (buccal, mesial palatal and distal).

The distribution of mast cells noted in this study is consistent with the hypothesis that suggests a mast cell role in bone remodelling in orthodontic tooth movement, as the cells were preferentially located next to the bone rather than root surfaces of the PDL. Furthermore, in the experimental teeth, the bone third demonstrated the greatest reduction in stainable mast cells, indicating more of these degranulated following tooth movement.

The mast cells were also more numerous in the coronal and apical regions where tooth movements would be greatest. In the experimental teeth, the coronal numbers dropped dramatically, supporting this interpretation.

In the control teeth there were twice as many mast cells in the buccal and mesial quadrants. In the experimental teeth the numbers became more evenly distributed across the quadrants. This may be due to buccal as well as mesial tooth movement.

The mast cell biochemistry is tantalizing for its potential in bone remodelling in both bone formation (by the actions of prostaglandin and histamine) and bone resorption (by the action of prostaglandin and heparin). There is no contradiction between the stimulatory effect of

prostaglandin on bone formation and resorption since these processes are carried out by different cells.

In addition, the mast cells demonstrated a predilection for blood vessels - more than half of the mast cells in control teeth were within 12.5μm of blood vessels. This proportion was higher in experimental teeth, especially at 4 and 24 hours after starting orthodontic tooth movement. This may indicate either a blood-borne passage of mast cells during tooth movement, or a migration of mast cells within the PDL towards blood vessels where their mediators may have most effect.

In conclusion, this study has shown that the number of mast cells detectable using routine histological staining techniques has decreased following orthodontic tooth movement. Interestingly, this decrease was not uniform throughout the ligament and was highly affected by position (i.e. horizontal distribution, vertical distribution and quadrant distribution).

This investigation also revealed that the rat provided a reliable model for the study of the effect of orthodontic tooth movement on the periodontal ligament.

HORIZONTAL DISTRIBUTION

- There were statistically significant differences between bone, mid and tooth thirds of the ligament ($p \leq 1 \times 10^{-7}$).

- For control teeth, the greatest number of mast cells were found in the bone third, followed by the mid third with the least found adjacent to the tooth.

- For experimental teeth, there was a drop in mast cell numbers in each third, however, the distribution stayed the same i.e. bone > mid > tooth.

VERTICAL DISTRIBUTION

- There were statistically significant differences between coronal, middle and apical levels of the ligament ($p \leq 1 \times 10^{-7}$).

- For control teeth, the greatest number of mast cells occurred in the coronal and apical levels, with considerably less found in the middle level.

- For experimental teeth, this distribution changed such that the coronal mast cell numbers dropped dramatically with essentially no change of the mast cell numbers in the middle and apical levels.

QUADRANT DISTRIBUTION

- Although there were differences between the quadrants of the ligament, these were not statistically significant ($p = 0.1271842$).

- For control teeth, there were nearly twice as many mast cells in the buccal and mesial quadrants as in the palatal and distal quadrants.

- For experimental teeth, this distribution changed such that the buccal and mesial quadrants reduced markedly (by approximately half) whilst the palatal and distal quadrants remained essentially the same.

MAST CELLS NEAR BLOOD VESSELS

- For control teeth (of both experimental and control animals), more than half (54.8%) of the mast cells were located near blood vessels.

- For experimental teeth, more than three-quarters (75.7%) of the mast cells were located near blood vessels.

- There was a significant increase in the number of mast cells located near blood vessels at 4hrs and 24hrs after starting orthodontic tooth movement.

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Changes in mast cells are recognised as one of the earliest stages of inflammation due to secretion of the chemical mediators and enzymes involved in the inflammatory process. These biologically active agents are stored in intracellular granules and include acid mucopolysaccharides (eg. heparin), amines (eg. histamine and serotonin), and many kinds of enzymes.

Previous studies using rats have indicated that following orthodontic mechanical stress there is degranulation of mast cells. This may then be followed by increased vascular permeability, degenerative and reformative changes of the periodontal ligament, prostaglandin synthesis and secretion, followed by bone remodelling.

Current evidence suggests that orthodontic mechanical stress produces:

⇒ damage or perturbation of periodontal tissues
⇒ prostaglandin synthesis
⇒ intracellular cyclic AMP and Ca^{2+} accumulation by monocytic cells
⇒ modulation and activation of osteoclastic activity
⇒ bone resorption
⇒ tooth movement

1.2 HYPOTHESIS

Goodson *et al.* (1974) demonstrated that injections of prostaglandin E_1 stimulated bone resorption. Davidovitch and Shanfeld (1980) reported on a rise in prostaglandin E_2 levels in the alveolar bone of orthodontically treated cats. Yamasaki *et al.* (1982a and 1984) and Yamasaki (1989) describe an experiment in which local prostaglandin E injections increased the rate of orthodontic tooth movement. Conversely, the inhibition of prostaglandin synthesis by indomethacin or flurbiprofen inhibits orthodontic tooth movement (Yamasaki *et al.* 1980; Sandy and Harris, 1984 and Chumbley and Tuncay, 1986). Mast cells are capable of producing prostaglandin (Tolone *et al.*, 1978) and therefore could be directly implicated in orthodontic tooth movement.

Mast cells possess the biological machinery necessary for enhancing bone resorption, and the population density of mast cells is increased in a variety of disorders that are associated with bone resorption, such as chronic otitis media (Berger *et al.*, 1985).

Mast cells may also be indirectly important because of their proximity to blood vessels and the release of mediators which cause increased vascular permeability.

It is hypothesized that the number of stainable mast cells in the rat periodontal ligament will decrease following orthodontic tooth movement due to increased degranulation.

For the present study, the null hypothesis to be tested is that orthodontic tooth movement does not affect the mast cell population in any measurable way following orthodontic tooth movement.

CHAPTER TWO: AIMS OF THE INVESTIGATION

The aim of this study is to provide morphometric data of the mast cell population in the periodontal ligament of the rat. The maxillary right first molar was moved orthodontically whilst the maxillary left first molar acted as a control. In addition, there were control animals matched for species, gender, diet and age which had no orthodontic appliances. Sections were taken at 100μm intervals from the crown to the apex in order to study the periodontal ligament surrounding the mesiobuccal root of the maxillary first molars.

The specific aims of this investigation were:

1. To establish the distribution and morphological characteristics of the normal mast cell population in the periodontal ligament of the rat maxillary first molar with particular reference to the lateral and vertical distributions.

2. To evaluate suitable staining methods for mast cells. In particular, toluidine blue and avidin biotin peroxidase have been used to identify mast cells in the periodontal ligament of the rat.

3. To determine the effect of orthodontic tooth movement on the distribution of the mast cell population in the periodontal ligament.

4. To statistically analyse the data collected to determine whether the mast cell population in the periodontal ligament differed between experimental and control animals, between left and right sides in experimental animals and between different periods of orthodontic activation.

CHAPTER THREE: REVIEW OF THE LITERATURE

3.1 ORTHODONTIC TOOTH MOVEMENT

Tooth movement during orthodontic treatment requires remodelling of alveolar bone and its periodontal tissues. The local changes in bone metabolism associated with orthodontic treatment appears to be a biological response of periodontal tissues to applied mechanical forces (Yamasaki, 1989).

3.1.1 MAST CELLS IN THE PERIODONTAL LIGAMENT

It appears that orthodontic mechanical forces may initially cause an inflammatory process in the periodontal tissues (Cooper and Sims, 1989). Mast cells are considered to have an important function in the initiation of inflammatory responses either as a reaction to trauma, immediate type of immune reactions, or in delayed-type hypersensitivity (Rivero *et al.*, 1995). Changes in mast cells are recognised to be one of the earliest stages of inflammation due to release of chemical mediators and enzymes involved in the inflammatory process. These include acid mucopolysaccharides (for example heparin) some amines (for example histamine and serotonin), neutral proteases, arachidonic acid metabolites and chemotactic factors for neutrophils and eosinophils. The significance of mast cells in inflammatory reactions is further strengthened by the increased number of mast cells in connection with rheumatoid arthritis, atopic dermatitis, chronic bronchitis, oral lichen planus, and gingivitis (Yamasaki *et al.*, 1982b; Matsson, 1993).

In light microscope studies the metachromatic reaction of mast cells with toluidine blue is in response to the presence of acid mucopolysaccharides. Stimulation of the cells causes secretion of these materials which results in the mast cell losing its ability to take up toluidine blue dye. Thus, a decrease in the number of the mast cells in toluidine blue-stained sections might suggest that an inflammatory reaction has begun (Yamasaki, 1989).

This premise has also been used in other studies. Ruokonen (1992) found that in oral erythema multiforme the area which showed the highest degree of inflammation, the superficial lamina propria, showed the lowest mast cell counts. There was a gradual decrease in inflammation intensity and a concomitant increase in stainable mast cells with increasing depth into the lamina propria. Based on studies by Claman 1985 and Claman *et al.*, 1986, this phenomenon may imply mast cell degranulation in areas of high inflammation intensity.

Mast cells in the periodontal membrane were studied by Yamasaki *et al.*, (1982b) in rats after tooth movement using an elastic separator. Orthodontic mechanical stress reduced the number of mast cells that stained with toluidine blue in the periodontal membrane on both the pressure and tension sides of rat molar teeth. This effect was seen immediately after insertion of elastic bands, with the greatest reduction observed after fifteen minutes. The appearance of mast cells returned to normal levels on both pressure and tension sides one hour after the initiation of treatment. On the tension side, mast cells stainable with toluidine blue tended to appear in greater quantities than on the pressure side for most time groups, but there was no significant difference between them for any given time group. The decrease in the number of mast cells suggests an initial process of degranulation. This may then be followed by increased vascular permeability, degenerative and reformative changes of the periodontal membrane, prostaglandin synthesis and secretion followed by bone remodelling (Yamasaki *et al.*, 1982b).

Mast cells were noted by Brudvick and Rygh (1994) in a TEM study of root resorption following orthodontic tooth movement. The mast cells were found near blood vessels in the periodontal membrane and near the root surface. Yamasaki *et al.*, 1982b, in their light microscope study also noted that mast cells can be seen around the blood vessels of the periodontal ligament on both tension and compression sides of the orthodontically moved tooth.

In studies of vascular response, it has been demonstrated that periodontal vascular permeability increased on both the pressure and tension side in rats fifteen to ninety minutes following the application of orthodontic forces. These reports indicate that the initiation of the inflammatory process on both the pressure and tension sides of the periodontal membrane was triggered by chemical mediators such as histamine and bradykinin (Yamasaki, 1989).

The first chemical mediator in areas of inflammation is histamine discharged from mast cells, triggering inflammation within fifteen minutes of an inflammatory stimulus. The second phase of inflammation is mediated by bradykinin and prostaglandins. The vascular permeability of this phase is both stronger and of longer duration than the immediate response. The peak of this phase is at 2 - 4 hours in most cases (Yamasaki, 1989).

Domeij *et al.*, 1991, found when studying the rat larynx that there was an association between the distribution of mast cells and nerves. A moderate to large number of mast cells were found only in regions where there were numerous substance P (SP) and calcitonin gene-related peptide (CGRP) containing nerve fibres. SP and CGRP both cause mast cell degranulation. Nerve fibres observed

at an ultrastructural level were often located within 100nm of mast cells - a distance at which neurons may exert their action on effector cells.

A study by Norevall *et al.* (1995) found after 24 hrs, as well as three days of buccally directed orthodontic tooth movement, a marked increase of both SP and CGRP expressing nerve fibres in the periodontal ligament, pulp and gingival mucosa. An increase of similar magnitude in immunoreactivity of the nerve fibres was also found in the unmoved contralateral side. This contralateral effect may be due to a change in occlusion as a result of the orthodontic tooth movement. These findings contrasted with Davidovitch *et al.* (1988), who noted a clear difference in neuropeptide immunoreactivity between experimental and contralateral teeth. In this study mesially directed forces were applied which may have less effect on the occlusion of the contralateral side than buccal tooth movement would.

Norevall *et al.* (1995) also noted a close association between nerve fibres showing CGRP- and SP-like immunoreactivity, and blood vessels in the PDL and marginal gingiva of both experimental and control animals. Several theories of neurogenic inflammation suggest that activation of neurons with the release of neuropeptides, such as CGRP and SP, is believed to induce mast cell degranulation, which in turn causes the vascular changes seen. Therefore, it is a possibility that mast cells could act as an afferent intermediary of antidromic sensory nerve stimulation (Baraniuk *et al.*, 1990).

3.1.2 MAST CELLS AND BONE METABOLISM

The impact of mast cells on bone metabolism is still unclear. It is conceivable that the proteolytic enzymes could assist in degradation of mineralised and unmineralised bone in sites of bone resorption or that the chemotactic action of a mast cell substance could recruit osteoblasts to sites of bone formation (McKenna and Frame, 1985).

Clinical observations have revealed diverse effects eg. osteoporosis and osteosclerosis (McKenna *et al.*, 1990). Certain mast cell products could cause porosis, such as heparin or prostaglandin, whereas sclerosis could be the result of prostaglandin-stimulated bone formation or histamine-induced peritrabecular fibrosis (McKenna and Frame, 1985; Alho *et al.*, 1991).

The association between mast cells and bone resorption has been suggested in previous studies. The number of mast cells increases in a variety of disorders that are associated with bone resorption: senile osteoporosis, systemic mastocytosis with generalised osteopenia and chronic destructive

periodontal disease (Berger *et al.*, 1985, Bienenstock *et al.*, 1986 and McKenna, 1994). Mast cells may enhance bone resorption by means of several mechanisms that involve demineralisation, as well as removal of the organic matrix.

Heparin, a major constituent of mast cell granules, has been implicated in bone resorption. There is a possibility that heparin acts synergistically with parathyroid hormone to mobilise calcium and also may be involved in removing the collagen matrix of bone (Berger *et al.*, 1985). Long term heparin administration as part of anti-coagulant therapy is known to be associated with osteoporosis (Bienenstock *et al.*, 1986). Parathyroid hormone (PTH) promotes degranulation of the mast cell, and heparin augments PTH-mediated resorption activity in bone culture media. Since the parathyroid gland contains histamine receptors a cycle of biochemical events could be proposed where PTH-stimulates mast cell activity and histamine stimulates parathyroid secretory activity (McKenna and Frame, 1985).

Mast cells are capable of producing prostaglandins (Tolone *et al.*, 1978) which stimulate osteoclastic bone resorption (Yamasaki, 1982a). Yamasaki (1989) described an experiment in which local prostaglandin E injections increased the rate of orthodontic tooth movement. The results indicated that local administrations of PGE_1 or PGE_2 (40μg/site) in the gingiva near the distal of canines caused almost double the rate of tooth movement compared to the vehicle - injected side. Macroscopically, no side effects were observed in the gingiva. These experimental results give support to the hypothesis that local administration of prostaglandins combined with orthodontic tooth movement induces more rapid bone remodelling and accelerates the rate of tooth movement.

There are two possible mechanisms for removal of the decalcified collagen matrix: degradation by a specific collagenase, or digestion by non-specific, possibly lysosomal acid hydrolases, after denaturation of the molecule by the local accumulation of metabolic acids. It is apparent that mast cells are capable of activating both mechanisms in bone resorption (Berger *et al.*, 1985).

3.2 MAST CELLS

The mast cell was first described by Paul Ehrlich in 1877. The name in German means "fattening feed", a reference to the numerous granules, which were erroneously thought to have been engulfed by the cell. He first described the mast cell as a tissue fixed cell containing many granules which stained metachromatically with basic dyes blue (Siraganian, 1992). The advent of the eras of biochemistry and molecular biology has opened the door for the detailed analysis of the mast cell

both as a mediator secreting cell and in its contribution to the pathogenesis of allergic diseases (Holgate *et al.*, 1988).

3.2.1 DISTRIBUTION OF MAST CELLS

Human tissue mast cells are normally distributed widely throughout the body with a predilection for host-environment interfaces (that is, the skin, the respiratory tract and the gastrointestinal tract). In addition, significant mast cell populations have been found in the thymus, uterus, and urinary bladder. Conversely, relatively few mature mast cells are found within normal human liver, spleen or lymph nodes. In most tissues mast cells are generally concentrated around small blood vessels, lymphatic vessels, nerves and glandular tissue (Tharp, 1985 and McKenna and Frame, 1985). Tharp (1985) reports few mast cells are found within the bone marrow, however, McKenna and Frame (1985) report mast cells in the bone marrow of normal individuals, frequently in close proximity to the trabecular surface.

Matsson (1993) studied the oral distribution of mast cells and found differences in distribution between juvenile (one month old) and adult rats (six months old). There were significantly greater numbers of mast cells in the tongue, buccal mucosa, and gingival mucosa of the juvenile rats than in the adult rats. In general, a higher number of cells similar to mucosal mast cells of the intestine were found in the oral sites of the juvenile animals. The differences in the total number of mast cells and the different composition of the mast cell population in the two age groups may indicate an age-related difference in the potential for biological reactions involving mast cells.

Mast cells are found in both inflamed and non-inflamed tissues (Perrini and Fonzi, 1985; and Smith *et al.*, 1989). High relative mast cell numbers mean acute tissue inflammation. Their moderate number and the presence of numerous immature cytoplasmic granules indicate moderate to weak inflammatory activity (Bohne, 1990).

3.2.2 ORIGIN OF MAST CELLS

The origin of mast cells still remains controversial even though the mast cell was formally identified over 100 years ago. Putative mast cell precursors have included primitive mesenchymal cells, fibroblasts, lymphocytes and thymocytes (Fawcett, 1955; Johnstone, 1956; Csaba, 1960).

Ginsburg and Sachs (1963) were one of the first to provide convincing evidence that mast cell precursors were present in mouse thymus and other lymphoid tissues. Ishizaka *et al.* (1976) reported similar findings in the rat. Kitamura *et al.* (1978) confirmed the presence of mast cell

precursors in mouse lymphoid tissue, but found much larger quantities of these precursors in the bone marrow. This group also established that mast cell precursors circulate in the blood of mice, a finding later confirmed by Zucker-Franklin *et al.* (1981) in the rat.

Therefore, it seems that most mast cell progenitors arise in the bone marrow whilst a lesser number originate from progenitors resident within other tissues, such as the thymus gland, thoracic duct lymphocytes, or lymph nodes (Stevens *et al.*, 1986 and Denberg, 1988). Roitt *et al.* (1996) suggest that the precursors of gut mucosal mast cells arise in the mesenteric lymph nodes that drain the gut and then migrate via the thoracic duct into the intestine.

There are two different views on the origin of bone marrow mast cells. One holds that both mast cells and basophils share a common trait ie., the presence of an intermediate cell type seen in chronic myelocytic leukemia (Zucker-Franklin, 1980). The other view holds that the precursor cell is present in the myeloid cell system of neutrophils and monocytes/macrophages because mast cells are stained positively by antibodies against monocytes/macrophages (Horny *et al.*, 1988) and also monocytes/macrophages have been shown to develop into mast cells in culture (Czarnetzki *et al.*, 1984). Saito *et al.* (1991), using antibodies against lactoferrin and lysozyme (both present in the myeloid cell system) supported the proposal that the precursor cell of bone marrow mast cells is nearer to the myeloid cell system than the basophil cell system.

Studies in murine rodents suggest the mitotically active mast cell precursors migrate to the connective tissues as immature cells and undergo differentiation in situ. These precursor cells differ from mature mast cells in that they contain few cytoplasmic granules with little or no heparin. During maturation, the number of granules increases as the proliferative capacity declines (Galli *et al.* 1984; McKenna and Frame, 1985). Their numbers increase slowly in tissues, although irritation may cause local proliferation (Tizard, 1995). Mature, differentiated rat mast cells can increase by cell division but do so in small numbers (Dvorak, 1991). Selye (1965) documented that, at least in animals, mature mast cells appear to undergo mitosis during reactions which elicit their in vivo accumulation.

The origin of mast cells from hematopoietic progenitor cells is now a generally accepted hypothesis. However, little is known about the origin and differentiation pathways of cells that become committed to and differentiate into mature mast cells. It is as yet unknown if the bone marrow remains the constant source of precursor cells throughout life or whether these cells develop from a local pool of precursor cells in extramedullary tissues or circulating stem cells (Födinger *et al.*, 1994).

3.2.3 MAST CELL SUB-POPULATIONS

It has been recognised that the morphology of the mast cell population is not uniform (McKenna and Frame, 1985). Enerbäck (1966b) demonstrated two distinct populations of mast cells in the rat:

- mucosal mast cells (MMCs), (Figure 3.1: right), and
- connective tissue mast cells (CTMCs) (Figure 3.1: left).

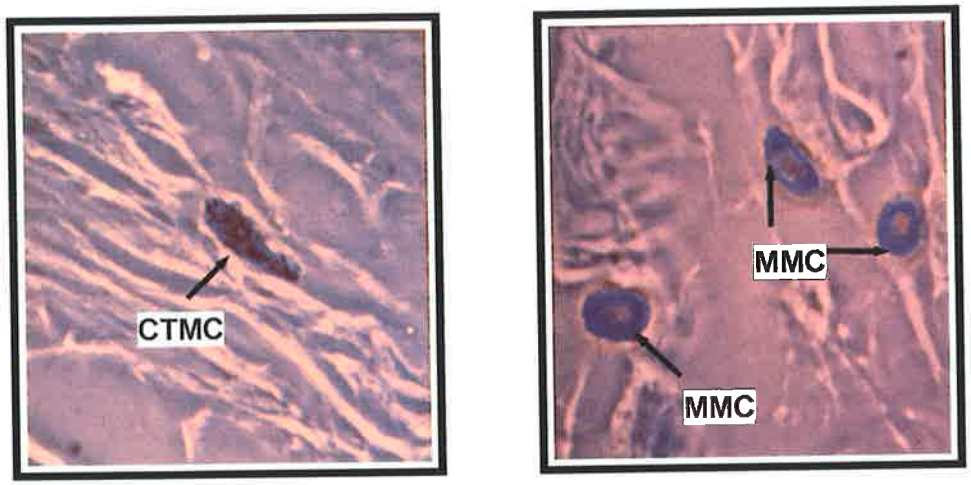

Figure 3.1 Histological appearances of rat ileum mast cells
Left: CTMC showing both blue and brown granules.
Right: Three MMC with only blue granules.
Fixed in formol saline; alcian blue and safranin stain x 600. (From Roitt, *et al.*, 1993).

These sub-types differ in a number of characteristics:

Mucosal mast cells:

- are distributed in the gastrointestinal mucosa
- have few variable-sized granules
- stain blue with alcian blue/safranin O stain due to their granules containing mainly chondroitin sulphate E proteoglycan; anionic sites blocked by aldehyde fixation
- contain serine protease type II (tryptase) (Benfey *et al.*, 1987)
- have a short lifespan, < 40 days
- contain fewer granules than CTMCs
- contain less histamine (1.3 pg/cell)
- contain less 5-hydroxytyptamine, increased in nematode response
- are smaller than CTMCs (9.7μm in diameter) and are of variable shape
- release more LTC_4 and other products of the lipoxygenase pathway
- are resistant to compound 48/80 (Enerbäck, 1966c)
- have a migratory capacity (Enerbäck, 1986)

Connective tissue mast cells:

- are distributed in connective tissues eg. peritoneum, lung, and skin
- have many uniform granules
- stain red with alcian blue/safranin O stain due to their heparin content; anionic sites not blocked by aldehydes
- contain serine protease type I (chymase) and also type II (tryptase)
- have a long lifespan, >6 months
- have prominent granules
- contain large amounts of histamine (15 pg/cell)
- contain more 5-hydroxytryptamine than MMCs
- are larger than MMCs (19.6μm in diameter) and are of uniform shape
- release predominantly PGD_2
- are susceptible to compound 48/80 (Enerbäck, 1966c)

(List compiled from: Sredni *et al.*, 1983; Tharp, 1985; Enerbäck, 1986; Domeij *et al.*, 1991; Siraganian, 1992; Abbas *et al.*, 1994; and Tizard, 1995)

Table 3.1 below summarizes these differences:

CHARACTERISTICS	MUCOSAL MAST CELL	CONNECTIVE TISSUE MAST CELL
GENERAL		
Abbreviation	MMC	CTMC
Distribution	Gut & lung	Most tissues
Differentiation favoured by	IL-3	Fibroblast factor
T-cell dependence	+	-
High affinity Fc receptor	2 x 10^5/cell	3 x 10^4/cell
GRANULES		
Alcian blue and Safranin staining	Blue	Blue & Brown
Ultrastructure	Scrolls	Gratings/lattices
Protease	Tryptase	Tryptase & chymase
Proteoglycan	Chondroitin sulphate	Heparin
DEGRANULATION		
Histamine content	+	++
LTC_4:PGD_2 release	25:1	1:40
Blocked by disodium cromoglycate/theophylline	-	+

Table 3.1: Summary of characteristics distinguishing mucosal mast cells and connective tissue mast cells (Roitt, 1994). **Note:** much of this data is derived from rodent studies and may not apply to man.

Later, these two populations were found in other species, such as mice (Guy-Grand *et al.*, 1984), man (Strobel *et al.*, 1981) and the opossum, a non-eutherian mammal (Chiarini-Garcia and Machado, 1992).

It is unclear if this apparent heterogeneity observed among rodent mast cells reflects differences in cellular origin or represents a variation in the degree of cell differentiation. Some evidence suggests that the two types of mast cell share a common origin (Kitamura *et al.*, 1977, 1981; Galli *et al.*, 1982; Dvorak *et al.*, 1987) and arise from a single type of precursor in the bone marrow. They then spread to tissues via the circulation, and proliferate and/or differentiate at various anatomical sites according to controls imposed by local regulatory forces (Mayrhofer and Pitts, 1986 and Theoharides, 1990). Therefore, the precise nature of the mast cell and the mediators it can produce varies with its anatomical location (Abbas, *et al.*, 1994).

It has also been suggested that the two types of mast cells can interconvert given appropriate stimuli (Kitamura, 1989; Nakano *et al.*, 1985, 1987; Levi-Schaffer *et al.*, 1986; Nakahata *et al.*, 1986). The culture of rodent bone marrow or spleens with IL-3, a growth promoting factor that is elaborated from T-lymphocytes, generates mucosal mast cells (Enerbäck, 1986); the addition of fibroblasts to bone marrow-derived mucosal mast cells causes a phenotypic change to the connective tissue type of mast cell (Theoharides, 1990 and Siraganian, 1992).

According to Befus *et al.* (1986) , Kitamura (1989), Theoharides (1990) and Tainsh and Pearce (1992), this classification into just two subtypes is too simplified. Theoharides (1990) has identified, in addition to MMC and CTMC a third subtype - brain mast cells (dural, perivascular and parenchymal) with their own distinct biochemical, morphological and functional characteristics. Tainsh and Pearce (1992) suggest intermediate phenotypes exist. They studied mast cells from the peritoneal mesentery, lung and skin. It was expected that the peritoneal cells would be MMC whilst the other three would be CTMC, however, this was not the case. The mast cells from these four tissues showed a range of phenotypes following staining with alcian blue/safranin O, suggesting the existence of both chondroitin sulphate and heparin proteoglycans in varying proportions in these cell types. Matsson (1992) found a similar range of phenotypes when studying mast cells from various oral mucosal sites (tongue, buccal mucosa and gingival mucosa). Therefore, no single population can be considered to be representative of another, and that to establish the properties and functions of mast cells in tissues such as the human intestine, lung, genital tract, or the brain, it is essential to study cells derived from these specific sites (Befus *et al.*, 1986). The variation in morphology at different anatomic locations may reflect diverse functional properties (Matsson, 1992 and McKenna and Frame, 1985).

3.2.4 DEVELOPMENT IN VIVO

The sequence of rat mast cell development has been studied using embryonic, newborn, and adult rat tissues examined by light-microscopic histochemistry, autoradiography and electron microscopy (Combs, 1966; Combs *et al.*, 1965).

Histochemical Studies

- *Toluidine blue:* at low pH to identify mast cell granules (Combs *et al.* 1965).
- *Periodic acid-Schiff:* to identify polysaccharides.
- *Alcian blue-safranin:* at low pH to distinguish between weakly sulphated and strongly sulphated mucopolysaccharides by a shift from alcian blue to safranin staining respectively (Enerbäck, 1987).
- *Feulgen-methylene green:* to demonstrate nuclear morphology.
- *O-phthalaldehyde:* to identify histamine (Shore *et al.*, 1959).
- *Phenylproprionyl naphthol-AS:* to demonstrate chymase.

Autoradiographic Studies

- $^{35}SO_4^{2-}$: incorporated into heparin (Lagunoff *et al.*, 1960)
- *[^{3}H] thymidine:* to demonstrate mitotic activity (Yong, 1981).
- *[^{3}H] histidine:* to study histamine release (Anderson and Uvnäs, 1975).

There were four stages of rat mast cell development identified when stained with alcian blue-safranin (Combs *et al.*, 1965):

Stage I mast cells:	lymphocyte-like cells with all cytoplasmic granules staining blue.
Stage II mast cells:	with most cytoplasmic granules staining blue and small numbers of granules staining red.
Stage III mast cells:	with most granules staining red and small numbers of granules staining blue.
Stage IV mast cells:	with all granules staining red.

The histochemical and autoradiographic studies indicated that the incorporation of radiolabelled sulphur into heparin coincided with the appearance of red granules with the alcian blue - safranin stain. The sequence of development of granule contents was shown to be:

i) synthesis and accumulation of nonsulphated heparin precursor in blue granules with the alcian blue-safranin stain;

ii) synthesis and accumulation of highly N-sulphated heparin;

iii) concomitant (or earlier) appearance of chymase; and

iv) presence of histamine only in red granules with the alcian blue-safranin stain and only in fully mature Stage IV mast cells at birth.

Stage IV mast cells did not appear until immediately preceding birth, but all four stages were evident in newborn rats. Only Stage IV mast cells were observed in normal adult rats (Yong, 1981; Dvorak, 1991).

3.2.5 MORPHOLOGY OF MAST CELLS

Mature rat mast cells are approximately 13 μm in diameter, with a single round nucleus (approximate diameter 5.8 μm). The dominant feature of the mast cell, however, is the presence of cytoplasmic granules that are so numerous that they often obscure the nucleus. The cytoplasm also contains the expected normal complement of cellular organelles including single strands of non-dilated, ribosome-bound endoplasmic reticulum, free ribosomes, mitochondria, Golgi's apparatus, centrioles, lipid bodies, microtubules and microfilaments (Figure 3.2) (Lagunoff, 1972; Tharp 1985, and Siraganian, 1992). The mast cell double-layered plasma membrane appears relatively uniform under light microscopy; however, studies using transmission electron microscopy clearly demonstrate the villous or ruffled appearance of this membrane barrier (Tharp, 1985).

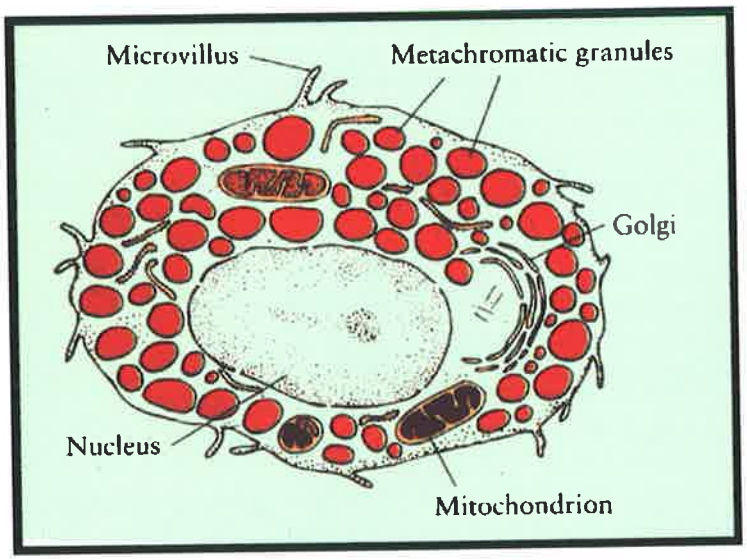

Figure 3.2: The basic features of a connective tissue mast cell (From Tizard, 1995).

The volume of the individual mast cell granule is estimated to be 0.3 μm^3. Stereologically, there are approximately 1020 granules per cell on average which occupy approximately 50% of the cytoplasmic volume (Helander and Bloom, 1974; Martin and Lagunoff, 1984). The majority of granules in the mature rat mast cell are homogeneous and electron dense. In addition there may be a few immature granules which contain irregular aggregates of dense progranules within a membrane-bound chamber with an electron-lucent background. An unusual granule pattern in mature peritoneal rat mast cells has been described by Bloom (1974) and supported by studies by Dvorak (1991). These contain irregular and concentric thick threads superimposed on a lucent background.

In fluid environments (such as the peritoneum) mast cells are round with uniformly distributed narrow surface folds. In tissues, they may be elongated and range in shape from ovoid to spindle-shaped to a "tadpole-like" configuration (Tharp, 1985; McKenna and Frame, 1985 and Dvorak, 1991). Ultrastructurally, mast cells have many long thin projections which appear as villi (Siraganian, 1992) when studied by scanning electron microscopy.

3.2.6 MAST CELL GRANULE STRUCTURE

Mast cell granules are electron-dense organelles measuring between 0.3 and 1.0μm in diameter and are surrounded by a trilaminar membrane (Chi *et al.,* 1975). Transmission electron microscopic studies demonstrate a dense or amorphous material in rodent mast cell granules (Figure 3.3) while similar studies of human tissue mast cells frequently show a scroll or lattice-like granule morphology (Dvorak, 1986). These apparent structural differences between rodent and human mast cells are not understood (Tharp, 1985). Considerable chemical data are available for the rat mast cell granule, but this information can only be applied to humans with major reservations in view of the marked differences in ultrastructural configuration between the granules of the two species (Lagunoff, 1972).

Chemically, in peritoneal mast cells of the rat, the bulk of the granules are composed of glycosaminoglycans (GAG) and several proteins, together accounting for approximately two-thirds of the granular dry mass. These two major granular components form a complex that serves as a matrix for the binding of low molecular-weight components, such as histamine and the monoamines (Enerbäck, 1987). The pH of intracellular granules has been estimated as 5.2 and that of isolated granules as 6.0. It has been postulated that histamine transport to the granule is dependent on a low intragranular pH (Lagunoff and Rickard, 1983).

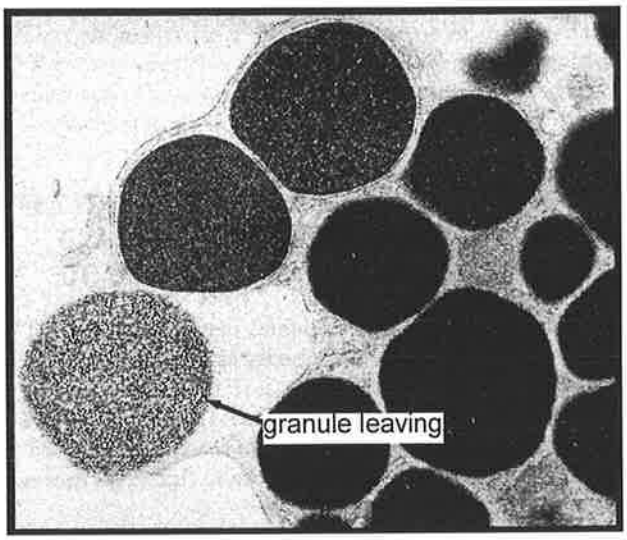

Figure 3.3: TEM of rat peritoneal mast cells indicating a granule leaving the cell X30,000 (From Roitt *et al.*, 1996).

The GAG component of connective tissue mast cell granules is heparin (about 25% of the dry mass of the granules) which is a strongly sulphated polysaccharide; (Bloom and Ringertz, 1960; Parekh and Glick, 1962; and Martin and Lagunoff, 1984). The chondroitin sulphate of mucosal mast cell granules is more soluble than the heparin of connective tissue mast cells. This glycosaminoglycan also demonstrates a lower degree of sulphation (as depicted by berberine fluorescence staining). (Enerbäck, 1966b; Tas and Berndsen, 1977; Wingren and Enerbäck, 1983 and Enerbäck, 1986).

The proteins of the mast cell granules have only been partially characterised. In rat mast cells a chymotrypsin-like enzyme, chymase (MW=25,000) has been isolated (Benditt and Arase, 1959; Kawiak *et al.*, 1971; Yurt and Austen, 1977; Lagunoff and Pritzl, 1976; Lagunoff and Benditt, 1963). Other enzymes that have been identified in mast cells and residing in the granules are *N*-acetyl-ß-glucosaminidase and ß-glucuronidase (Lagunoff *et al.*, 1970), and arylsulphatase A (Martin and Lagunoff, 1984). In addition, several other proteins are present in the mast cells such as a neutrophil chemotactic factor and several small polypeptides, such as the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Wasserman *et al.*, 1974).

3.2.7 ACTIVATION OF MAST CELLS

Degranulation of tissue mast cells results from both immunologic and non-immunologic mediated mechanisms (Table 3.2 and Figure 3.5). The process of degranulation is considered to be an explosive phenomenon during which activation of the mast cell produces expansion of granules with solubilisation of their contents followed by fusion of adjacent granules - a process that is facilitated by the cytoskeleton of microtubules and microfilaments. Finally, there is liberation of the granule

matrix to the external medium by exocytosis (Figure 3.4) (McKenna and Frame, 1985). Degranulated mast cells do not die. Rather, they are difficult to identify because of an absence of characteristic morphological features (Tizard, 1995).

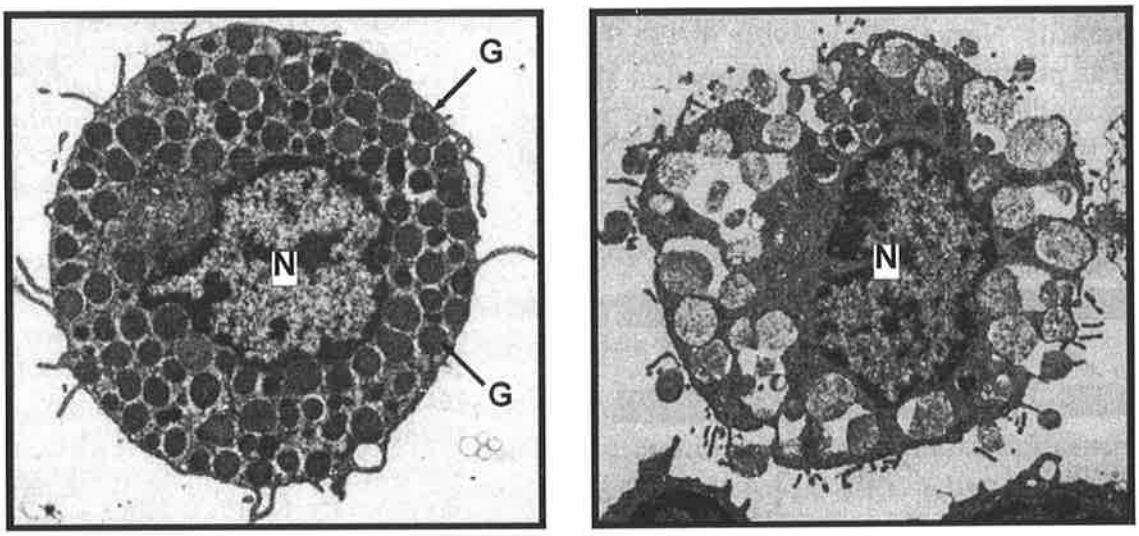

Figure 3.4: Transmission electron micrograph of exocytosis in rat mast cells.
Lead stained sections x 6250. Alberts *et al.*, 1994. (N=nucleus)
Left: A non-degranulated mast cell with its electron-dense granules (G).
Right: A mast cell activated by a soluble extracellular ligand (S anti RIg-FT). Although the cell is almost fully degranulated the rest of the cell organelles (Golgi, mitochondria) appear intact.

Immunologic Mechanisms
IgE-mediated Anaphylatoxins (C3a, C4a and C5a) Lymphokines
Non Immunologic Mechanisms
Medications (Narcotics, Curare, Succinylcholine, Aspirin, Nonsteroidal anti-inflammatory agents, Polymyxin B) Adenine Nucleotides (ATP, ADP) Hormones (Gastrin, ACTH, Oestrogens) Neurotransmitters Peptides Lectins Radiocontrast media Complex carbohydrates Venoms Irradiation ***Trauma?***

Table 3.2: Clinically relevant human mast cell secretagogues

The "typical" mechanism of activating mast cells is by cross linking antigen to IgE molecules bound on the surface of a mast cell (Martin and Lagunoff, 1984; Roitt *et al.*, 1996 and Kuby, 1994). The

number of mast cell surface receptors for IgE ranges from 100,000 to 500,000 with approximately 10% of the receptors being occupied by IgE under normal conditions. In patients with high levels of serum IgE, circulating basophils and probably tissue mast cells, have up to 95% of their receptors occupied by this immunoglobulin (Tharp, 1985). Rodents, unlike humans, also have receptors for IgG molecules, and therefore, circulating IgG can serve as a mast cell-secretory agonist in these animals (Tharp, 1985). In itself, the binding of IgE to the mast cell high-affinity Fc receptors for IgE (FcεRI) apparently has no effect on a target cell. It is only after cross-linkage by allergen of the fixed IgE-receptor complex that degranulation proceeds (Figure 3.5(a)) (Roitt *et al.*, 1996 and Kuby, 1994).

Other experiments have revealed that it is actually the cross-linkage of two or more FcεRI molecules, with or without IgE, that is essential for degranulation. Cross-linkage can be effected by a variety of experimental means that bypass the need for allergen and in some cases for IgE (Kuby, 1994).

For example anti-IgE autoantibodies which are present in sera of allergic patients can stimulate mast cell secretion by cross-linking fixed IgE (Figure 3.5 (b) and (c)). (Martin and Lagunoff, 1984; Kuby, 1994 and Paul, 1993).

Chemical cross-linkage of IgE can also induce degranulation (Figure 3.5 (d)). For example, lectins such as PHA and Concanavalin A (Con A) can cross-link IgE by binding to carbohydrate residues on the Fc region. (Martin and Lagunoff, 1984; Kuby, 1994 and Roitt *et al.*, 1996).

Most significantly, antibodies to the receptor itself, anti-FcεRI antibodies, can induce degranulation in the absence of both allergen and fixed IgE (Figure 3.5 (e)). This demonstrates the crucial role of cross-linkage of FcεRI in initiating the subsequent biochemical events that culminate in degranulation. (Kuby, 1994 and Abbas *et al.*, 1994).

In addition to these stimuli, several non IgE-dependent stimuli activate mast cells. Probably the most important of these *in vivo* are the breakdown products of complement activation, C3a, C4a and C5a (Paul, 1993 and Roitt *et al.*, 1996). These low molecular weight peptides appear to stimulate mast cells through distinct receptors. The complement anaphylatoxins have a rank order of potency in which C5a > C3a C4a with C5a being approximately 100-fold more potent than C3a (Tharp, 1985).

Pharmacological compounds that can directly activate mast cells include calcium ionophores (Figure 3.5 (f)), mellitin, compound 48/80 (a mixture of 4-8 unit lengths of p-methoxy-N-methyl-phenylethylamine units), and drugs such as codeine, morphine and synthetic ACTH. (Martin and Lagunoff, 1984 and Roitt *et al.*, 1996).

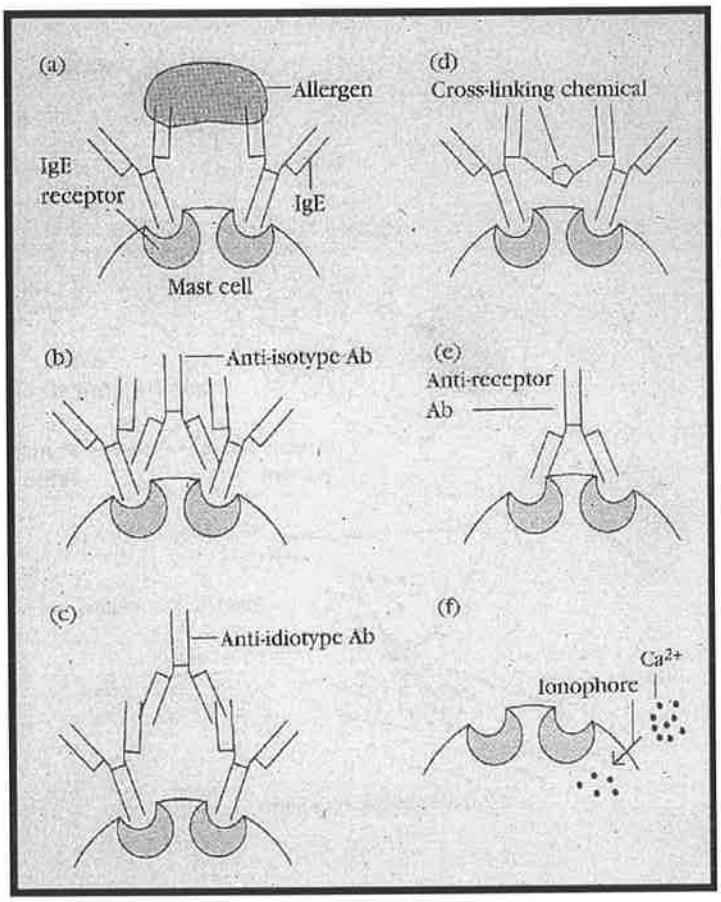

Figure 3.5: Schematic diagrams of mechanisms that can trigger mast-cell degranulation. (a) Allergen cross-linkage of cell-bound IgE molecules. (b,c) Antibody cross-linkage of IgE. (d) Chemical cross-linkage of IgE. (e) Cross-linkage of IgE receptors by antireceptor antibody. (f) Enhanced Ca^{2+} influx stimulated by an ionophore that increases membrane permeability to Ca^{2+} ions. Note that mechanisms (b), (c) and (d) do not require allergen; mechanisms (e) and (f) require neither allergen nor IgE; and mechanism (f) does not even require receptor cross-linkage (from Kuby, 1994).

A number of other naturally occurring exogenous nonimmune agents also appear capable of stimulating mast cell mediator release. The adenine nucleotides, ATP and ADP, have been demonstrated to be potent human mast cell secretagogues both in vitro and in vivo (Tharp, 1985). These molecules have potential clinical relevance since there are several known sources of extracellular ATP and ADP in close proximity to tissue mast cells in the periodontal ligament (cholinergic and adrenergic nerve endings, and endothelial cells) (Tharp, 1985). In addition, cytokines (IL-1, IL-3) and the neuropeptide, substance P, have been cited as nonimmune mast cell stimulants (Paul, 1993).

All of these compounds result in the activation of mast cells by causing an influx of calcium ions. The anaphylactic response induced by these agents is identical to that seen in IgE-mediated reactions although they act by IgE-independent mechanisms (Roitt *et al.*, 1996).

3.2.8 BIOCHEMISTRY OF MAST CELL SECRETION

When mast cells are stimulated a series of reactions are triggered that cause the most peripherally located mast-cell granules to migrate to the cell surface, to fuse with the cell membrane, and to be extruded from the cell, subsequently releasing their contents into the surrounding tissues (Figure 3.6). This process advances towards the cell interior by fusion and opening of the deeper situated granules to the formerly opened granule cavities (Röhlich *et al.,* 1971). Thus, triggering of the receptor initiates several signal transduction pathways (Tizard, 1995).

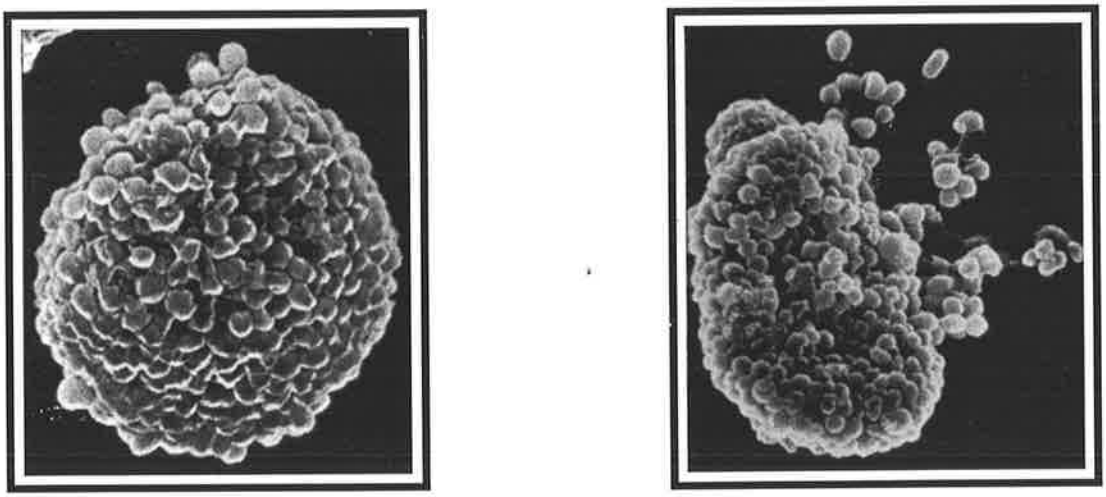

Figure 3.6: Scanning electron micrograph of exocytosis in rat mast cells. SEM x 1500.Roitt *et al.*, 1993. Left: An intact rat peritoneal mast cell with the cell membrane shrunk onto the granules. Right: A rat peritoneal mast cell degranulating following incubation with anti-IgE for 30 seconds.

Three signal transduction pathways lead to mast cell degranulation (Figure 3.7). Allergen cross-linkage of bound IgE activates protein tyrosine kinase, adenylate cyclase and phospholipid methyltransferase I and II (Kuby,1994; Abbas *et al.*, 1994 and Tizard, 1995).

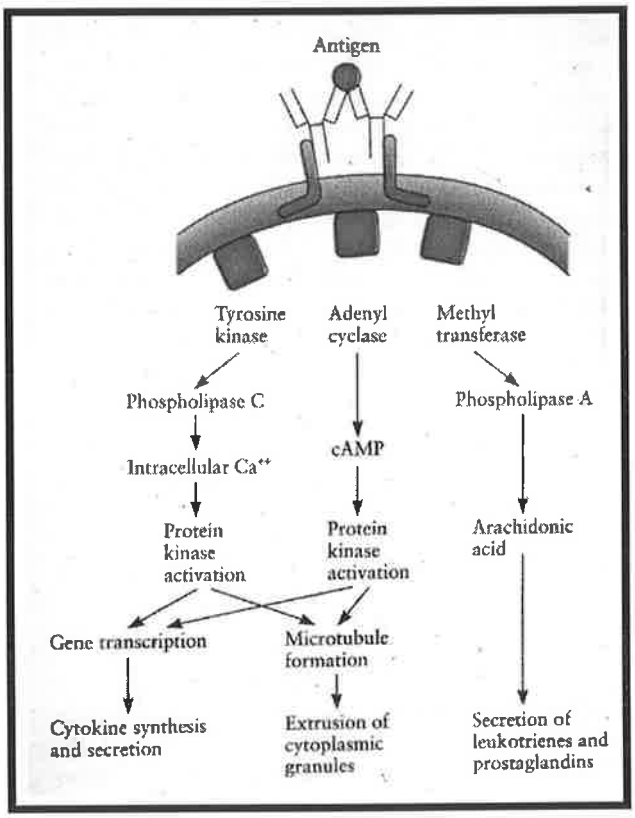

Figure 3.7: Diagrammatic overview of biochemical events in mast-cell activation and degranulation (Tizard, 1995).

3.2.8.1 Tyrosine Kinase

In one pathway, a tyrosine kinase activates phospholipase C that catalyzes phosphatidylinositol biphosphate breakdown to inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). The IP_3 causes elevation of cytoplasmic calcium, and DAG activates protein kinase C (Figure 3.7) (Siraganian, 1992 ;Tizard, 1995, and Abbas, *et al.*, 1994).

3.2.8.2 Adenylate Cyclase

An adenylate cyclase is also activated by cross-linking of the FcεRI receptor. This leads to a transient increase in cAMP (that peaks at 15 seconds post-stimulation) which then activates protein kinase A. The protein kinases together phosphorylate myosin in intracellular filaments and on granules, thereby changing the granules' permeability to water and Ca^{2+}. The consequent swelling of the granules appears to facilitate their fusion to the plasma membrane in degranulation (Tizard, 1995; Abbas *et al.*, 1994 and Kuby, 1994).

3.2.8.3 Methyltransferase

Biochemical studies examining mast cell membrane phospholipid methylation indicate a marked increase in activity within fifteen seconds of mast cell stimulation (Ishizaka *et al.*, 1980 and 1983). In cells that methylate phospholipids, two methyltransferases are usually present with one facing the cytoplasmic side of the cell membrane and methylating phosphatidylethanolamine to phosphatidyl-N-monomethyl-ethanolamine and the second facing the outer cellular membrane surface which actively converts phosphatidyl-N-monomethyl-ethanolamine to phosphatidylcholine (PC) (Hirata and Axelrod, 1980). The resultant accumulation of PC on the exterior surface of the plasma membrane causes an increase in membrane fluidity and facilitates the formation of Ca^{2+} channels (Kuby, 1994).

The influx of Ca^{2+} activates the enzyme phospholipase A_2 which promotes the hydrolysis of PC to form lysophosphatidylcholine and arachidonic acid (Poole *et al.*, 1970). Other enzymes then metabolize the arachidonic acid to leukotrienes and prostaglandins (Figure 3.9). Lysophosphatidylcholine further increases membrane fluidity, facilitating Ca^{2+} influx. The influx of Ca^{2+}also promotes the assembly of microtubules and the contraction of microfilaments, both of which are necessary for the movement of granules to the plasma membrane (Kuby, 1994 and Tizard, 1995).

It is highly conceivable that some mast cell secretagogues provoke mediator release through different biochemical pathways. This is suggested by the observations in human basophils in which an IgE-mediated signal results in the release of histamine and leukotrienes while anaphylatoxin-induced activation produces only histamine release (Findlay *et al.*, 1980).

When mast cells are incubated in fluid containing a soluble stimulant, exocytosis occurs all over the cell surface (Figure 3.4). This is not necessarily a generalised response of the whole cell. This has been demonstrated by attaching a stimulating ligand to a solid bead and placing this in contact with a mast cell. Exocytosis is restricted to the region where the cell contacts the bead (Figure 3.8). The mast cell, unlike a nerve cell, does not respond as a whole when it is triggered; the activation of receptors, the resulting intracellular signals, and the subsequent exocytosis are all localised in the particular region of the cell that has been excited (Alberts *et al.*, 1994).

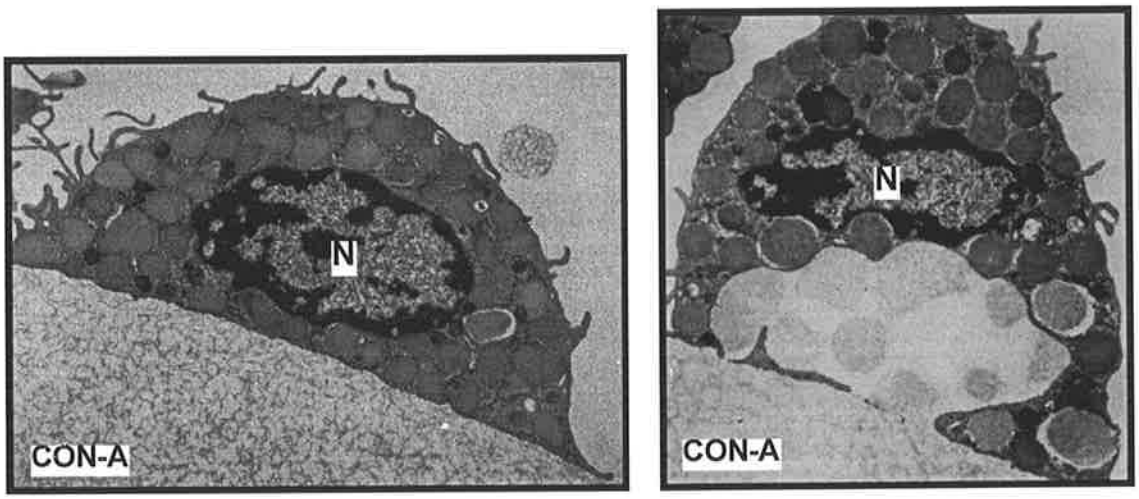

Figure 3.8: Transmission electron micrograph of exocytosis as a localised response (from Lawson *et al.*, 1978). N=nucleus.
Left: Mast cell adhered to a Con A-Sepharose bead (Con-A) in the absence of Ca^{2+}. No degranulation has taken place. x 6900
Right: Mast cell adhered to a Con A-Sepharose bead (Con-A) in the presence of 1.8 $mMCa^{2+}$ for 10 min at 37°C. Degranulation is confined to the region of contact with the bead. x 8550.

When a secretory vesicle fuses with the plasma membrane, its contents are discharged by exocytosis and its membrane becomes part of the plasma membrane. This increase in surface area of the plasma membrane is transient because membrane components are removed from the surface by endocytosis which returns the proteins of the secretory vessel membrane to the trans Golgi network where they can be used again (Alberts *et al.*, 1994).

Since the process of degranulation is a noncytolytic event, restoration of granule content proceeds immediately, and the resultant protein synthesis may be stimulated by histamine. While the mast cell has a limited capacity for replication, it is also possible that the process of degranulation stimulates multiplication, again through the action of a mast cell mediator (McKenna and Frame, 1985).

3.2.9 MAST CELL MEDIATORS

Mast cells are endowed with more mediators destined for secretion than any other secretory cell (Theoharides, 1990). Two categories of regulatory substances have been identified and consist of (a) preformed, granule-associated mediators and (b) newly-formed, unstored mediators that are generated at the time of mast cell activation (Figure 3.9 and Table 3.3). In rat mast cells some preformed mediators such as histamine, serotonin and neutral proteases are rapidly released at the time of mast cell stimulation. However, other preformed mediators (heparin, enzymes), which constitute a significant portion of the mast cell granule, appear to remain granule-bound after

discharge from the cell. The newly-formed mediators (prostaglandins and leukotrienes), which are generated at the time of mast cell stimulation, are released from the mast cell within several minutes after an agonist signal (Tharp, 1985 and Tizard, 1995). The cytokines (interleukins 3-6, GMCSF and TNF) are released hours after mast cell activation (Tizard, 1995).

In general, mast cell mediators, both preformed and newly synthesised, act in concert to promote an inflammatory reaction by increasing vascular permeability, increasing concentration of leukocytes, and producing proteolytic tissue damage.

The mast cell may serve an immunopathologic as well as a protective function (McKenna and Frame, 1985).

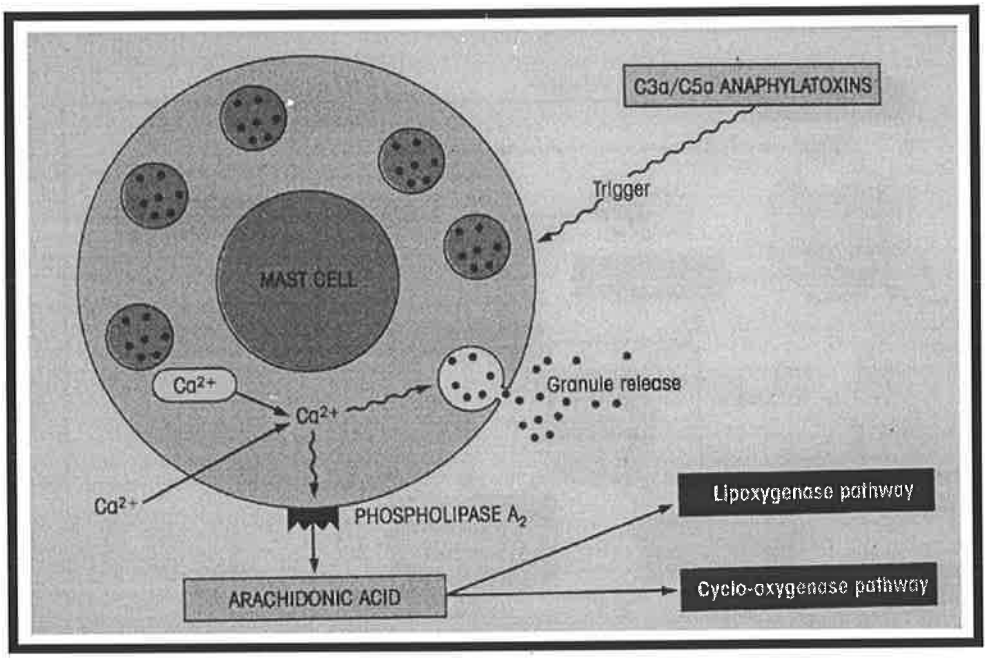

Figure 3.9: Mast cell stimulation leading to release of mediators by two major pathways:
(i) release of pre-formed mediators present in the granules, and
(ii) the metabolism of arachidonic acid produced through activation of a phospholipase. Intracellular Ca^{2+} and cAMP are central to the initiation of these events but details are still unclear.
(Roitt, 1994).

Some of these mediators, however, have opposing effects (eg. heparin and platelet activating factor). The ability of mast cells to release mediators differentially or selectively could have great physiological significance. There is evidence that mast cells may be able to release some preformed mediators differently and without degranualation. Also, different secretagogues appear to favour the release of pre-formed or newly synthesized mediators (Theoharides, 1990).

	PRE-FORMED	EFFECT
Granule release	HISTAMINE H_1	Increases vascular permeability Vascular smooth muscle relaxation? Non-vascular smooth muscle contraction Increases cGMP Promotes prostaglandin synthesis Promotes eosinophil migration
	H_2	Increases vascular permeability Augments gastric acid secretion Increases mucous secretion Increases cAMP Stimulates suppressor T-lymphocytes Inhibits basophil histamine release Inhibits lymphokine release Inhibits eosinophil migration
	PROTEOGLYCAN Heparin	Anticoagulant Binds antithrombin III and platelet factor IV Inhibits complement activation Binds other preformed mediators Promotes plasminogen activator release Promotes phospholipase A release Promotes triglyceride lipase release
	SEROTONIN (5-hydroxytryptamine)	Increases vascular permeability Smooth muscle contraction
	NEUTRAL PROTEASES OTHER ENZYMES Tryptase Arylsulphatase A Hexosaminidase ß -glucosaminidase ß -glucuronidase Superoxide dismutase Peroxidase	Activates C3 Degradation of blood-vessel basement membrane Activates Hageman factor Inactivates SRS-A Cleaves hexosamines Cleaves glucosamine Cleaves glucuronide residues Cleaves oxygen radicals Cleaves H_2O_2
	ECF NCF	Attracts eosinophils Increases eosinophil IgE receptors Neutrophil chemotaxis
	PLATELET ACTIVATING FACTOR (Tizard thinks they are newly synthesized)	Platelet aggregation and degranulation Increases vascular permeability Eosinophil and neutrophil chemotaxis and activation
	INTERLEUKINS 3,4,5 & 6 GMCSF, TNF	Multiple effects including: Stimulation of endothelial cell expression of VCAM-1 and ICAM-1 Macrophage activation Trigger acute phase proteins IL3 also promotes growth of mast cells

Table 3.3a: Pre-formed mediators released on mast cell activation. Adapted from Tharp, 1985; Kuby, 1994; Paul, 1993; Tizard, 1995 and Roitt *et al.*, 1996.

	NEWLY SYNTHESIZED	EFFECT
Lipoxygenase pathway	LEUKOTRIENES C_4, D_4, E_4 (SRS-A) LEUKOTRIENE B_4	Smooth muscle contraction Increased vascular permeability Neutrophil and eonsinophil chemotaxis Increased vascular permeability
Cyclo-oxygenase pathway	PROSTAGLANDINS	Vasodilation Potentiates increased vascular permeability produced by histamine and bradykinin Bronchial smooth muscle contraction Inhibit: Leukocyte chemotaxis and degranulation Mast cell/basophil degranulation Platelet aggregation
	THROMBOXANES	Smooth muscle contraction Platelet aggregation
	BRADYKININ	Smooth muscle contraction Increased vascular permeability
	SEROTONIN (5-hydroxytryptamine)	Smooth muscle contraction and vasospasm

Table 3.3b: Newly-synthesized mediators released on mast cell activation. Adapted from Tharp, 1985; Kuby, 1994; Paul, 1993; Tizard, 1995 and Roitt et al., 1996.

For more detailed information on each of the mast cell mediators refer to Appendix 8.1.

Evidence indicates that many of the mast cell secretory products could modulate mast cell secretion. For example PGE1 and PGE2, histamine, heparin, LTD4 and lipoxin B inhibit secretion, while kinins, LTC4 and PGD2 stimulate secretion. In addition, many of these mediators could be secreted by other cell types, such as endothelial cells, leukocytes and neurons, and could regulate mast cell secretion (Theoharides, 1990).

The physiologic and pathologic consequences of mediator release are dependent upon the tissues in which they are secreted. For example, histamine release in the skin and in the nose result in endothelial cell contraction and increased vasopermeability. Histamine release in the lung results in smooth muscle contraction which causes bronchoconstriction (Tizard, 1995).

3.2.10 PDL SIGNIFICANCE

Of the mast cell mediators released upon activation a few may have significance if released in the periodontal ligament.

Of the preformed mediators:

Histamine:	-	increases vascular permeability
	-	promotes prostaglandin synthesis
	-	increases cAMP
Heparin:	-	anticoagulant

Of the newly synthesised mediators:

PGE_1 and PGE_2:	-	increases cAMP
	-	increases bone resorption by: increasing the number and size of osteoclasts stimulating the action of existing osteoclasts (Lee, 1990)
Leukotrienes	-	increases bone resorption (inhibition of leukotrienes inhibits tooth movement, Mohammed *et al.*, 1989)

3.2.11 RECOVERY FROM DEGRANULATION

There have been a number of studies on recovery from degranulation.

Fawcett (1955) documented by 18 - 20 hours following intraperitoneal injection of compound 48/80 the mast cell numbers in mesenteries returned to nearly normal. He did note that the mast cells were appreciably reduced in size. Also, Weill and Renoux (1982) studied rat peritoneal mast cells stimulated with compound 48/80. They found that 60% of the previously degranulated mast cells had recovered their granules by one day and that 90% had done so by two days. They also showed that restimulation of mast cells after their recovery of granules resulted in a second cycle of degranulation. A study by Dvorak *et al.* (1987) of human lung mast cells stimulated with anti-IgE found that the mast cells had recovered at 24 hours, however, the number of granules was markedly reduced compared with controls. Contrasting these studies Krüger and Lagunoff (1981) found that restoration of peritoneal mast cells after depletion with polymyxin B occurred by 34 days post-stimulation. These differences in recovery times may reflect the different stimulants used to promote mast cell degranulation.

3.3 IDENTIFICATION OF MAST CELLS

3.3.1 FIXATION OF MAST CELL GRANULES

The choice of fixative for mast cells must depend upon what component of the cell one intends to visualise such as proteoglycan, protein, or amines. The general methods for the demonstration of mast cells are based on dye binding to the GAG of the granules. Therefore, the fixative of choice should preserve the structure of the granular proteoglycan, leaving the anionic groups of the GAG (sulphates and carboxyls) available for cationic dye-binding. Procedures aimed at demonstrating granular protein require preservation of enzyme activity of the protein or of its charged groups. The visualisation of the mast cell amines with histochemical methods also requires special considerations with respect to fixation (Lagunoff *et al.*, 1961; Mathiesen, 1973a; Enerbäck *et al.*, 1986 and Enerbäck, 1987).

Due to the ionic linkages between GAG and protein within the mast cell granules, the ideal mast cell fixative should result in an adequate protein fixation and promote such ionic linkages. Failure to preserve the dye-binding may be due to the extraction of soluble GAG by the fixative or due to the blocking of dye-binding groups of the GAG by aldehydes, which are very common components of fixatives. A suitable fixative should therefore have no aldehyde (Carnoy's), a very low aldehyde concentration such as isotonic formol-acetic acid (IFAA) or the fixation times with aldehydes should be kept short (paraformaldehyde) (Enerbäck, 1987).

Aldehyde blocking of dye-binding as a result of routine fixation in formaldehyde or glutaraldehyde may be overcome by trypsinization or by extreme staining times in the order of several days (Lagunoff, 1972; Wingren and Enerbäck, 1983 and Enerbäck, 1986). Addition of salts of heavy metals such as lead to fixatives was often recommended in the past as a means of preventing solubilization of GAG. It should be avoided since heavy metals may block the binding of cationic dyes (Enerbäck *et al.*, 1986).

Strobel *et al.* (1981) compared staining of human mucosal mast cells following fixation in Carnoy's, basic lead acetate (BLA), Baker's, Bouin's, isotonic formol-acetic acid (IFAA), 10% neutral buffered formalin, formol sublimate, and formol saline. They found that substantially more MMC were detected in tissues which had been fixed with Carnoy's or with BLA than in the tissues fixed in formalin-based fixative. Xaubet *et al.*, 1991, also do not recommend formaldehyde fixation and prefer methanol fixation or Carnoy's fixative for visualising CTMC mast cells.

Many human mucosal mast cells are susceptible to fixation by aldehyde to a higher degree than mast cells of other tissue sites and other species, such as rats and mice. For instance, in human gastrointestinal mucous membranes, mast cell counts of tissue fixed in 4% formaldehyde and stained by the conventional method (toluidine blue or alcian blue) are 70 - 80% lower than in IFAA fixed tissue, or in tissue fixed in 4% formaldehyde and stained for five days. Under similar conditions mast cells in normal human skin and mastocytosis lesions are 20 - 30% lower, therefore mucosal mast cells are more sensitive to aldehyde than connective tissue mast cells. The degree of aldehyde blocking of dye-binding may thus be used as a means to define mast cells of a particular site or species (Enerbäck *et al.*, 1986).

Login *et al.*, (1987 and 1992) reported on a rapid microwave fixation technique using peritoneal lavage fluid to study peritoneal mast cells as did Jamur *et al.* (1995) on bone marrow cell suspensions. The ultrastructural morphology of the mast cells fixed by the microwave method was found to be indistinguishable from that of mast cells fixed routinely by aldehyde fixation. Login *et al.*, (1992) found no evidence of cell damage nor induced mast cell activation. This technique, however, would have limited use in specimens of the size used in this study.

3.3.2 STAINING METHODS FOR GRANULAR GLYCOSAMINOGLYCANS

The standard methods for the demonstration of mast cells in tissue sections are based on dye binding to the GAG of the mast cell granules. A large number of cationic dyes may be used for this, most of them exhibiting a metachromatic shift. A few dyes show a nonmetachromatic binding, and two dyes may be used for the demonstration of different staining effects of mast cells or of their granules in different stages of development. Fluorescent dyes have also been used (Enerbäck, 1987).

3.3.2.1 Metachromasia: The Thiazine Dyes

The mechanism underlying metachromatic staining involves the stacking of cationic dye molecules onto the dense, negatively charged heparin polymer, resulting in a shift in light absorbance (Kramer and Windrum, 1955). This shift in the absorption spectrum of the dye toward shorter wavelengths causes a hypochrome colour change of the dye from blue towards violet, red or orange. Metachromatic dyes are the thiazine dyes toludine blue and azure A. Metachromasia is best observed in water solutions of low ionic strength (Enerbäck, 1987).

Tarpley *et al.* (1984) found their toluidine blue procedure inaccurate for mast cell counting due to large amounts of background stain and much preferred the acidified toluidine blue procedure of

Churukian and Schenk (1981) which eliminated this problem. Therefore, the specificity of the metachromatic staining of mast cell granules may be enhanced by staining at controlled acid pH levels. Toluidine blue buffered to pH 4 stains both the GAG of mast cell granules as well as certain other sulphated mucosubstances metachromatically (violet or purple). Weakly acidic polyphosphates, such as nuclear DNA, are stained orthochromatically (blue). At pH 0.5, most of this tissue background staining is lost and only the mast cell granules stain metachromatically; there is no nuclear or other staining (Enerbäck, 1987).

Mucosal mast cells in rodents and humans do not stain with these techniques after fixation in 4% neutral buffered formaldehyde, however, they stain well after fixation in IFAA or in Carnoy's solution (Enerbäck, 1966a, Enerbäck, 1987). Aldehyde fixation results in a protein blocking of the granular GAG, which may be unmasked with very long staining times (for 5-6 days)(Wingren and Enerbäck, 1983; Enerbäck *et al.*, 1989 and Biernacka *et al.*, 1993). Enerbäck (1987) has reported that the long toluidine blue staining is useful for the critical demonstration of mast cells in tissues other than the gut mucosa, especially after aldehyde fixation. Also, it is effective in tissues of high cellular density, such as lymph nodes, where mast cells with few granules may be overlooked.

3.3.2.2 Copper Phthalocyanins

Two copper phthalocyanin dyes are of special interest for the staining of mast cells: alcian blue 8 GX and astrablau 6 GLL. These dyes are of similar or identical structure. Alcian blue, however, formulated by Scott *et al.* (1964), is more easily available and better specified. Alcian blue interacts with polyanions, such as heparin, in aqueous solutions to give insoluble precipitates in which the two components are bound by ionic linkages.

Alcian blue dissolved in 3% acetic acid (pH 2.5) stains mast cell granules and other sites containing strongly acidic sulphated GAG weakly or not at all (Spicer *et al.*, 1967). Mast cell granules therefore have a comparatively low affinity for alcian blue, however, this may be increased by lowering the pH of the stain solution below pH 1.0 or by the addition of salt to the stain solution (Scott and Dorling, 1965).

The staining of mast cell granules with alcian blue is influenced by fixation. Carnoy's fixation was found to strongly enhance the staining of mast cells with alcian blue (Spicer, 1963).

Mast cells of different species and different sites have also been shown to differ in affinity for alcian blue. CTMCs of rats and mice have a low affinity for alcian blue: mast cells in cervical lymph

nodes and in the uterus stain strongly with alcian blue at pH 2.5 (Spicer, 1963). Also, MMCs of the rat have a higher affinity for alcian blue than connective tissue mast cells of skin or tongue (Enerbäck, 1966b).

Differences in affinity for alcian blue of mast cells can be visualised with a sequential staining of alcian blue followed by safranin O (Spicer, 1960). This technique has been used (by Combs *et al.*, 1965) to study the differentiation and proliferation of embryonic rat mast cells. The maturation of mast cells was accompanied by a change in the alcian blue - safranin sequence in the staining properties from blue to red. This was interpreted as a sign of an increased degree of sulphation of the GAG. This staining pattern may also be attributable to other properties of the GAG-protein complex unrelated to the structure of the GAG *per se*, such as the spatial relations between the GAG and protein, resulting in variable degrees of protein blocking of anionic sites (Enerbäck, 1987).

3.3.2.3 Berberine Fluorescence

Berberine is one of the few naturally cationic dyes. In solutions of very low ionic strength, this weakly fluorescent dye forms a strongly fluorescent complex with heparin in mast cell granules, the fluorescence intensity being proportional to heparin content (Enerbäck, 1974). The potential histochemical value of the fluorescent berberine binding is suggested by a strongly fluorescent staining of CTMCs of the rat known to contain heparin: the gut MMCs do not stain at all (Wingren and Enerbäck, 1983).

This dye stains mast cells selectively at pH 4 or below, resulting in a strong yellow fluorescence, contrasting with the green fluorescence of nuclei. This green fluorescence is greatly suppressed by fixation in IFAA and to a somewhat lesser extent by Carnoy's fixation when compared to fixation in 4% buffered formaldehyde. Therefore, mast cell granules in tissues fixed in IFAA appear strongly fluorescent against a background of very low fluorescence intensity. For critical studies of berberine fluorescence in mast cells IFAA fixation is therefore recommended (Enerbäck, 1987).

3.3.3 DEMONSTRATION OF GRANULE PROTEIN

The protein of the mast cell granules can be demonstrated with two different types of histochemical methods. One of these utilises the cationic property of the protein for its visualisation with anionic dye binding; the other type of methodology rests on the demonstration of esterase activity of the major class of the granular protein (Enerbäck, 1987).

3.3.3.1 Anionic Dye Binding: Biebrich Scarlet

The anionic bis-azo dye Biebrich scarlet has a strong affinity for basic proteins and can be used to stain them over a wide pH range, thereby defining them histochemically according to their relative basic strength (Spicer and Lillie, 1961). A large number of sites containing GAG can be stained with this dye within a narrow alkaline pH range after fixation in formaldehyde-free fixatives. Thus, mast cells stain intensely at pH 8.0 - 10.5, but not at lower pH levels. The interaction of the anionic dye Biebrich scarlet with a number of cationic polymers has been suggested to represent a metachromatic reaction (Enerbäck, 1987).

It is not known to what extent mast cells of various sites or in different pathologic processes differ with respect to the availability of the basic protein to the staining with Biebrich scarlet or other anionic dyes. The staining properties of the basic protein of mast cell granules, which seem to be largely neglected in clinicopathologic studies, may provide information about the functional differentiation of mast cells in pathologic processes (Enerbäck, 1987).

3.3.3.2 Esterase Methods

The chloroacetate esterase stain selectively demonstrates mast cells and neutrophils in tissue sections, and depends upon cleavage of the ester linkage by mast cell granule - associated proteolytic enzymes (Benditt and Lagunoff, 1964). Mast cells and cells of the myeloid series hydrolyze ∝-chloroacyl esters of ∝-naphthol and of naphthol AS at a faster rate than the corresponding regular acyl esters. With naphthol-AS-chloroacetate as a substrate and subsequent diazo coupling, mast cells and neutrophils (after fixation in acetone or neutral formalin), stain strongly within a few minutes while no other tissue elements show any appreciable reaction. IFAA fixation is especially suited for the demonstration of esterase activity, as indicated by good structural details and shorter incubation times compared with normal formaldehyde fixation (Enerbäck, 1987). This histochemical method, however, has the disadvantage of staining both mast cells and neutrophils.

3.3.4 MAST CELL AMINES

Biogenic amines are important constituents of mast cells. Histamine occurs in mammalian mast cells and blood basophils. The monoamines dopamine and serotonin only occur in a limited number of species and are normally absent from human mast cells.

3.3.4.1 Histamine

Definite proof for the localisation of histamine in mast cells has been obtained by chemical analysis of mast cells isolated from the peritoneal fluid of rats and mice (Benditt *et al.*, 1955). Histamine can be demonstrated histochemically with a fluorescent reaction using 0-phthalaldehyde (OPT). The OPT reaction in itself has limited specificity. Fluorescent products are formed with amino acids, polypeptides, proteins, and amines, such as histamine and serotonin. By careful standardisation of the reaction conditions, a reasonably specific and sensitive biochemical assay procedure has been devised for histamine (Shore *et al.*, 1959). However, purification of histamine from interfering compounds, using chromatography or solvent extraction procedures, is usually required.

Fixation in non-polar fixatives followed by vacuum embedding in paraffin and flattening of sections on warmed slides while avoiding contact with water adequately preserves the histamine of CTMCs for visualisation with OPT (Enerbäck, 1969). After a trial of several non-polar and dehydrating fixatives, Carnoy's fixation was found to give the best results, with sharply localised yellow fluorescence in connective tissue of mast cells of the rat. Histamine in very low concentrations may escape detection by this method. Gut MMCs of the rat have only 10% of the histamine content of connective tissue mast cells (Enerbäck, 1981; Befus *et al.*, 1982). The MMCs cannot be demonstrated in Carnoy's fixed and paraffin embedded tissues, but can be in freeze-dried cryostat sections which have been reacted with an OPT vapour phase. This results in a weak blue fluorescence (Enerbäck and Wingren, 1980).

3.3.4.2 Monoamines

Serotonin, also known as 5-hydroxytryptamine (5-HT) is normally found in mast cells of rats and mice (Benditt *et al.*, 1955). The quantity of serotonin in rat mast cells is very low, amounting to only 1-2% of that of histamine. Serotonin and histamine are bound to identical storage sites in the mast cell granules and appear to be freely exchangeable. The rat mast cells have a very avid uptake mechanism for 5-HT. Injections of 5-HT in very low doses results in a rapid uptake of 5-HT, which is retained in the mast cells and eliminated very slowly, with a half-life of approximately three weeks. Histochemical reactions, such as fluorescence, diazo reactivity, and reducing properties can indicate the presence of 5-HT.

Dopamine is only known to occur in mast cells in ruminants (Falck *et al.*, 1964). An uptake of dopamine may be induced in rat mast cells after an injection of L-dopa or dopamine, but it is apparently stored at a different site from that of histamine and 5-HT (Rundquist *et al.*, 1982).

The monoamines can be demonstrated histochemically with the formaldehyde condensation reaction of Falck and Hillarp (Falck *et al.*, 1962). The formaldehyde condensation method is based on freeze drying of the tissues followed by a reaction with gaseous formaldehyde in a closed vessel and with controlled humidity. Formaldehyde reacts with the monoamines by condensation and cyclization followed by a dehydrogenation step yielding strongly fluorescent derivates. The fluorescent product of 5-HT exhibits a yellow fluorescence whilst catecholamines, including dopamine, exhibit a green fluorescence.

A major advance in monoamine histochemistry is the introduction of 5-HT antibodies for immunocytochemical demonstration of the amine. These immunocytochemical methods have a considerably increased sensitivity compared with the formaldehyde condensation methods and may reveal mast cell stores of 5-HT (Enerbäck, 1987).

3.3.5 CONJUGATED-AVIDIN PREPARATIONS

Avidin binds to biotin with very high affinity and great specificity and this interaction remains unimpaired when enzymes are covalently linked to avidin. Biotin can be conjugated to a variety of molecular probes such as antibodies, lectins and nucleic acids without affecting the antigen binding capacity. These probes recognise specific tissue substrates, which in turn are detected by avidin conjugated to enzymes or fluorescent dyes (Bergstresser *et al.*, 1984; Tharp *et al.*, 1985, and Jones *et al.*, 1987).

First reports of avidin-biotin-peroxidase complexes (ABC) staining mast cells came about accidentally when Bussolati and Gugliotta (1983) stained human carcinomas to detect tumor-associated antigens. These cells were devoid of specific antigenic activity and endogenous peroxidase, but bound the ABC "nonspecifically" even without any previous treatment of the sections. These cells were later identified as mast cells with toluidine blue. They suggested to avoid this "non-specific staining" of mast cells to use an ABC solution at high pH, which does not affect specific binding.

Jones *et al.* (1987) reported on another procedure which eliminated "non-specific staining" with avidin peroxidase conjugates. Avidin peroxidase conjugates (5μg/mL), diluted in standard 0.05M tris-buffered saline, pH 7.6, containing 0.139M NaCl produced considerable background colouration and intense mast cell staining. This background staining diminished as the ionic strength of the buffer was raised. At 0.125M tris-buffered saline (containing 0.347M NaCl) the background was completely unstained, with elimination of all binding to mast cells.

Conjugated-avidin preparations are highly sensitive for identifying mast cells in tissues (Bergstresser *et al.* 1984; Tharp *et al.* 1985). Avidin conjugated either to the enzyme horseradish peroxidase (HRP), or to the fluorochrome dyes (fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) binds specifically to mast cells in tissues. The specificity of avidin for mast cells was confirmed by the absence of conjugated avidin-positive cells in the skin of mice deficient in mature dermal mast cells. (Fritz *et al.*, 1984; Bergstresser *et al.*, 1984 and Tharp *et al.*, 1985).

Avidin-biotin-peroxidase complex (ABC) has advantages over FITC-avidin and TRITC-avidin. It dispenses with the special optical equipment essential for fluorescence techniques and retains the conventional morphologic characteristics of tissue in paraffin embedded sections. Sensitivity of the ABC technique is high as avidin can be bound to 3 molecules of biotin that have been previously bound with horseradish peroxidase. The remaining free binding site on the avidin molecule can then be bound to a biotinylated anti-immunoglobulin specific for the primary antibody (Milios and Leong, 1988).

Conjugated avidin can serve as a sensitive method for identifying both rodent and human mast cells in tissue specimens and in isolated cell suspensions. The evidence that avidin, and not its conjugate, is the important molecule in this staining process is supported by the observation that FITC-avidin, TRITC-avidin, and HRP-avidin identify mast cells equally well while FITC alone and FITC or HRP conjugated to irrelevant immunogobulins fail to stain mast cells. In addition, pretreatment of mast cells with excess unlabelled avidin results in almost complete blocking of subsequent mast cell staining with FITC-avidin (Bergstresser *et al.*, 1984 and Fritz *et al.*, 1984).

It appears that avidin binds specifically to individual mast cell granules rather than to other cellular structures. Individual granules of fixed mast cells appeared fluorescent; granules liberated from unfixed cells stained readily with avidin, and tissues from the mast cell-deficient mice failed to exhibit avidin binding. This has been confirmed with electron microscope studies resulting in two observations. First, varying amounts of HRP reaction product, ranging from only surface deposition to staining throughout the matrix, were observed in granules that had been released from mast cells. Secondly, HRP-avidin stained granules within mast cells, although the reaction product was frequently limited to those granules lying adjacent to the intact cell membrane. These studies do not rule out the possibility that avidin also binds to the cytoplasmic membrane of mast cells, but this seems unlikely since FITC-avidin failed to stain the surface of unfixed cells while it readily stains granules that had been released from the cells (Tharp *et al.*, 1985).

The binding of avidin to mast cell granules may be explained in two different ways: firstly, the binding of avidin to a mast cell product structurally related to biotin, and secondly, the polar binding of basic avidin to the acidic contents of mast cells where heparin is though to be the major constituent (Lagunoff and Chi, 1980).

Bergstresser *et al.* (1984) tested the hypothesis that because of avidin's great affinity for biotin the avidin binding to mast cells might be biotin related. They preincubated FITC-avidin with biotin and then used this to stain mast cells. Even at a 400-fold excess, biotin failed to block avidin staining of mast cells suggesting the mast cell recognition site for avidin is not biotin or a biotin-like molecule.

The results of the studies by Fritz *et al.* (1984) suggest that avidin binds to heparin or heparin-like substances within mast cell granules, a theory that is supported by the observation that exogenous heparin inhibits staining of mast cells by avidin peroxidase.

Arizono *et al.* (1987) found proliferating lung mast cells in early stages of infection stained with toluidine blue and alcian blue but were negative for ABC and berberine sulphate. Both of the latter methods have been reported to stain CTMC but not MMC (therefore both are believed to be bound to heparin within mast cell granules). Histochemical studies of the tissue at a later stage of infection showed positive mast cells with berberine sulphate and ABC. They proposed a phenotypic change in mast cells from the mucosal to the connective tissue type may have occurred.

Tharp *et al.*, 1985, note several advantages in using the ABC technique over conventional metachromatic stains. Avidin staining of tissue mast cells is not limited by methods of fixation, dehydration, small changes in pH or by specific embedding and cutting procedures that are frequently employed to preserve mast cell morphology when using metachromatic stains. Although tissue fixation is required for avidin staining the type of fixative appears to be relatively unimportant. No appreciable differences in staining were observed among tissue mast cells fixed in acetone, formalin or Helly's fixative (Bergstresser *et al.*, 1984). This technique already has proven to be a simple and reliable method for identifying and enumerating mast cells in tissues. In addition, fluorochrome-labeled avidin eliminates the problem of differentiating true metachromasia from non-specific staining (Kasper *et al.*, 1987).

3.3.6 SUMMARY OF METHODS FOR THE IDENTIFICATION OF MAST CELLS

Identification of mast cells are directed towards their main components such as proteoglycan, proteinase or IgE. Proteoglycan is the single, most distinctive constituent of the mast cell granules,

where its glycosaminoglycan (GAG) component is responsible for the specific metachromatic dye-binding.

3.3.6.1 Fixation Of Mast Cell Proteoglycan:

Recommended procedures:

- IFAA: is recommended as a general fixative for connective tissue mast cells. Dye-binding of GAG is excellently preserved.

- Carnoy's: was found to be one of very few of the common fixatives which preserved the dye-binding of mucosal mast cells. It may be used as a general mast cell fixative. It does not block dye-binding, however, structural details are not as well preserved as they are after fixation in IFAA.

3.3.6.2 Fixation Of IgE And Mast Cell Antigens:

Currently, there is no universal fixative that will preserve mast cell granules reliably and also be suitable for the detection of all antigens. Carnoy's has been of considerable use and has allowed simultaneous demonstrations of mast cells and sheep immunoglobulin, rat IgE, rat mast cell protease and a mast cell-specific surface antigen (Enerbäck *et al.*, 1986).

3.3.6.3 Staining Of Mast Cell Proteoglycan:

- Toludine Blue: Staining at pH 0.5 results in stained mast cell granules against a virtually unstained background whilst staining at pH 4 helps to identify related cells or tissue components. It has been recommended to identify both CTMC and MMC (Enerbäck, 1987 and Strobel *et al.*, 1981).

- Alcian Blue: Staining at low pH (dye dissolved in 0.7N HCl) as a general mast cell stain, alone or in a sequential staining with Safranin to differentiate between MMC and CTMC.

According to Xaubet *et al.*, (1991) the alcian blue - safranin O method has not been demonstrated as superior to toluidine blue in the identification of mast cells.

Since Carnoy's fixative allowed for staining of GAG and also IgE and mast cell antigens it was chosen as the preferred fixative for this study.

The 0.5% toluidine stain at pH 0.5 was chosen as it minimised background staining so that mast cells could be easily identified (Enerbäck *et al.*, 1986).

The avidin-biotin peroxidase complex stain was chosen as a more modern stain to act as a comparison with the acidified toluidine blue stain.

CHAPTER FOUR: MATERIALS AND METHODS

4.1 THE EXPERIMENTAL ANIMALS

The Sprague-Dawley rats used in this study were kept in an animal house where temperature, humidity and lighting were controlled. These sixty animals were all males and 83 or 84 days of age at the start of the experiment.

4.1.1 THE PILOT STUDY

The pilot study was conducted using six of the Sprague-Dawley rats to satisfy the Animal Ethics Committee regarding the level of tolerance to the orthodontic appliance and the suitability of Nembutal[1] as the proposed anaesthetic for the experiment proper.

During the pilot study one of the rats died two hours after the administration of Nembutal[1] and also a colleague, Dr. Manosudprasit, had found this a difficult anaesthetic to work with (refer to Results Section 5.1.1). Therefore, it was decided to change to Ketapex[2] for the experiment proper. An additional rat was obtained and substituted for the lost pilot study rat in the experiment proper.

Tolerance of the appliances was assessed by observation of the rats after appliance insertion and also by reference to weight loss over the period of 24 hours (N=2) and 7 days (N=3). None of the remaining five pilot study rats showed signs of distress or weight loss over the review period. Based on this result the Animal Ethics Committee approved the provision of the additional rats required for the experiment proper.

4.1.2 THE EXPERIMENT PROPER

The experiment proper was conducted using the five remaining pilot study rats which had the appliances already inserted, and 55 additional rats obtained from the animal house. From the 55 rats, 20 were used as controls ("the control rats"). The 35 remaining rats as well as the five pilot study rats (referred to as the "experimental rats") had orthodontic appliances placed. Refer to Table 4.2 for the allotment of the 60 animals into experimental (N=40) and control (N=20) groups.

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[1] Nembutal = 60mg/L pentobarbitone sodium. Boehringer Ingelheim, NSW, Australia
[2] Ketapex = 100mg/ml ketamine. Apex Laboratories, NSW, Australia.

4.1.3 ANAESTHESIA

To cement the appliances the rats from the experiment proper were anaesthetised by intraperitoneal injection of Ketapex[2] diluted in 0.9% sodium chloride, and Rompun[3], using a 1.0mL disposable syringe with a 26.5 gauge needle.

Approximately 0.4mL Ketapex was used for a 500g rat. This varied proportionately with the rat's weight and was diluted in a 1:1 ratio with sodium chloride. Then 0.15mL of Rompun[3] was injected, irrespective of the weight of the rat.

4.1.4 THE ORTHODONTIC APPLIANCES

The orthodontic appliances were constructed on a model of one of the pilot study rats. Two impregum[4] impressions were made in special trays, one following a gingivectomy of the two incisors. The impressions had molten inlay wax slowly laid into them to minimise shrinkage. A wax base was made for each and the wax-ups were removed from the impressions. These were sprued through their bases, invested and the wax burnt out in the conventional manner for cast dental work. These were cast in cobalt chrome to create a working model (Figure 4.1 Left) that enabled close adaptation of bonding mesh around the two incisors without damaging the model. The gingivectomy model proved to be more suitable for close approximation of the band.

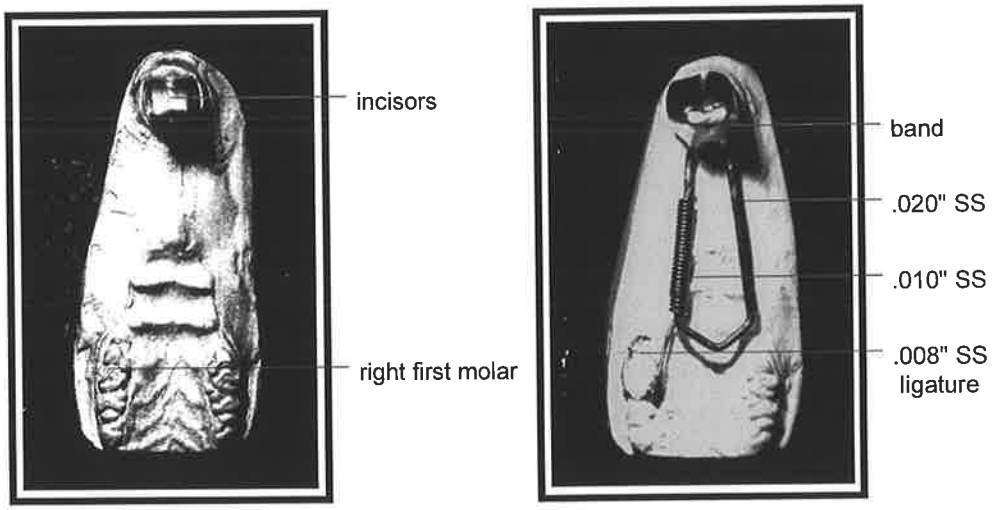

Figure 4.1. Model of a rat maxilla showing two maxillary incisors with three molars on each side.
Left: Cobalt chrome working model for appliance construction.
Right: Orthodontic appliance used to move right first maxillary molar buccally.

[2] Ketapex = 100mg/ml ketamine. Apex Laboratories, NSW, Australia.
[3] Rompun = 20mg/L xylazine hydrochloride. Bayer, NSW, Australia.
[4] ImpregumF = polyether impression material. Espe, Dental-Medizin GmbH & Co. KG, Seefeld, Germany.

The appliances (Figure 4.1 Right) consisted of bonding mesh material closely adapted around the two maxillary incisors soldered to a supporting wire of 0.020" in diameter (Wilcock "Special Plus" stainless steel). This supporting wire diverged backwards lateral to the mid-palatal suture and converged again in the midline distal to the first molars. This design allowed anteroposterior adjustment. A finger spring of 0.010" diameter (Wilcock "Premium Plus" stainless steel) was attached on the right side level with the first maxillary molar in the form of a coil wrapped around the supporting wire. The anterior part of the coil was welded to the supporting wire to prevent rotation. The finger spring was adjusted to apply a known force to the molar.

The maxillary incisors were etched with 37% phosphoric acid for 60 seconds, rinsed with sterile saline, dried with a puffer (no compressed air was available) and painted with bonding resin before the appliances were cemented with chemically-cured composite resin (Concise, 3M Unitek). A 0.008" stainless steel ligature was threaded through a circle bent into the end of the spring and tied around the first molar to prevent dislodgement of the spring occlusally during mastication.

4.1.5 FORCE VALUES

The finger spring of the orthodontic appliance produced a buccally-directed force to the palatal surface of the right first maxillary molar. During anaesthesia, the appliances were adjusted to apply five grams of force as measured by a GEC tension gauge[5].

4.1.6 CHECKING RATS

Every second day over the duration of appliance wear the condition of the appliances was examined. The rats were lightly anaesthetised in a suitable chamber with a mixture of halothane, nitrogen oxide and oxygen as follows:

Gas	Volume (litres/minute)
Halothane	2.0 - 2.5
N_2O_2	0.2
O_2	1.0

Table 4.1: Anaesthetic mixture for checking appliances.

The animals were removed and quickly checked for appliance integrity and were also weighed to see if appliance wear caused weight loss from inadequate food intake. During the period of orthodontic tooth movement the animals were fed pulverised standard rat pellets and water.

[5] GEC tension gauge = General Electric Company of England. British Patent No. 323282 441164, range 4-24g.

4.2 TISSUE PREPARATION

4.2.1 SPECIMEN RETRIEVAL

Following the completion of the experimental periods (15 minutes, 1 hour, 4 hours, 24 hours, 1 week, 2 weeks, 4 weeks and 8 weeks) each rat was sacrificed by carbon dioxide inhalation. Immediately after death, the portion of the maxilla holding the six molars was removed together with a block of supporting bone. These specimens were matched with controls of the same age, namely 83 day, 90 day, 97 day, 111 day and 139 day old rats.

EXPERIMENTAL RATS		
Experimental Period	**Age at time of sacrifice**	**No. of Rats**
15 minutes	83 days	5
1 hour	83 days	5
4 hours	83 days	5
24 hours	83 days	5
1 week	90 days	5
2 weeks	97 days	5
4 weeks	111 days	5
8 weeks	139 days	5
		TOTAL 40
CONTROL RATS		
Age at Time of Sacrifice	**Equivalent Expt. Period**	**No. of Rats**
83 days	15 min, 1 hr, 4 hrs, 24 hrs	4
90 days	1 week	4
97 days	2 weeks	4
111 days	4 weeks	4
139 days	8 weeks	4
		TOTAL 20

Table 4.2: Allotment of the 60 animals into experimental and control groups.

4.2.2 FIXATION

Following collection the specimens were immersion fixed in Carnoy's solution (refer to Appendix 8.2) at 0°C for 18 hours.

Carnoy's fluid penetrates very rapidly and gives excellent nuclear fixation. It has been recommended by many researchers for the fixation of carbohydrates. It causes considerable shrinkage and destroys or dissolves most cytoplasmic elements. The degree of shrinkage can be reduced by fixation at 0°C for 18 hours. Following fixation, blocks should be transferred to 95% or absolute alcohol.

4.2.3 REHYDRATION

Since the specimens were fixed in a predominantly alcoholic fixative it was necessary to rehydrate the tissue prior to placement in the EDTA decalcifying solution. This fixative is normally a bonus for soft tissue sections as it is unnecessary to dehydrate the tissue prior to processing.

The specimens were rehydrated by placing them in decreasing concentrations of alcohol: 90%, 80%, 70% and 50% alcohol and double distilled water for four hours each.

4.2.4 DECALCIFICATION

Once rehydrated, the maxillae were washed in cacodylate buffer and placed into labelled histokinette baskets (Tissue-Tek III megacassette, Lab-Tek division, Miles Laboratories Inc, Naperville, Illinois) which were suspended in a 4% ethylenediaminetetra-acetic acid (EDTA) in sodium cacodylate buffer solution at pH6 (refer to Appendix 8.3). This solution was maintained at 4°C and was continuously agitated using a magnetic stirrer.

The exact concentration of EDTA used is relatively unimportant as long as uncombined reagent is available to the tissue at all times. In practice, a volume of EDTA 20-30 times that of the tissue has been found satisfactory, which is renewed every 5-7 days until completion of decalcification. Decalcification takes from 4 to 40 days depending on the composition of the specimen but prolonged exposure to EDTA is not detrimental to subsequent staining of tissues.

4.2.5 RADIOGRAPHIC EXAMINATION

Radiographic examination was used to identify the end point of decalcification. The specimens were radiographed at various exposures to check the ideal exposure time. This was found to be 0.2 seconds with the x-ray tube positioned 10cm away from the specimens.

The radiographs were developed by hand following conventional processing methods used in developing dental radiographs. Once the end point of decalcification was reached the tissues were washed in 70% alcohol and processed.

4.2.6 PARAFFIN WAX EMBEDDING

Routine paraffin wax embedding involves four stages: dehydration, clearing, infiltration and embedding. These stages have been discussed in detail in Appendix 8.4.

The following paraffin wax embedding regime was adhered to in this study:

Dehydration: under vacuum (25 inches of mercury) at room temperature.

1. 70% Ethanol overnight to several days
2. 80% Ethanol ½ day
3. 90% Ethanol ½ day
4. 100% Ethanol ½ day
5. 100% Ethanol ½ day
6. 100% Ethanol overnight
7. Chloroform 1 hour

Clearing: under vacuum (25 inches of mercury) at room temperature.

1. Clearene*[1] 1 day
2. Clearene*[1] overnight

*[1] Clearene is manufactured by Surgipath - Grayslake, Illinois

Infiltration: under vacuum (25 inches of mercury) 60°C

1. Wax*[2] ½ day - 1 day depending on the size of the tissue block.
2. Wax*[2] ½ day - 1 day
3. Wax*[2] ½ day - 1 day By Nose: if the specimen has an orange smell (Clearene) it will need longer and another change of wax.

*[2] Wax is blue ribbon infiltrating medium manufactured by Surgipath - Richmond, Illinois

Embedding: In tissue embedding media*[3] in a metal mould to fit the specimen.

*[3] Tissue embedding medium is manufactured by Surgipath - Richmond, Illinois

4.3 SECTION PREPARATION

4.3.1 SECTIONING

A single-sided razor blade was used to trim the block face to a divergent sided trapezoidal mesa. The block was orientated such that the occlusal surfaces of the molars were perpendicular to the block face. This time-consuming horizontal sectioning plane was chosen in order to evaluate the entire periodontal ligament. If the teeth were sectioned along their long axes, two surfaces of the teeth would be lost to evaluation (Andreasen, 1987).

The specimens were sectioned serially in 5 micron thick sections using a Leitz 1512 microtome and a Leitz knife. Serial sectioning consists of the preparation of consecutive sections at a uniform thickness throughout the entire mass of the specimen. A ribboning medium is desirable for serial sectioning and paraffin wax is mainly used for serial work. The resultant ribbons were divided into smaller ribbons of three sections each and floated in a warm water bath to flatten them.

4.3.2 MOUNTING

The sections were mounted on glass slides. Three sections were used per slide in case a section became folded during mounting or staining. It was also more economical to mount multiple sections per slide.

To aid in adhesion of the sections to the glass slides the slides were coated in a subbing solution of APT (see Appendix 8.5). Also the slides were placed in an oven at 60°C immediately after mounting to help adhere the sections to the slides.
Every section was mounted even though only every twentieth section was stained for this study. This allowed the remainder of the tissue to be used in later studies. There were approximately 600 sections per tooth.

4.3.3 STAINING

Three staining methods were utilised in this study. These were:

1) Haematoxylin and eosin (see Appendix 8.6) for orientation purposes and identifying zero point
2) Toluidine blue (pH 0.5) for identification of mast cells (Appendix 8.7.1)
3) Avidin-biotin-peroxidase complex for identification of mast cells (Appendix 8.10)

4.3.4 ZERO LEVELS

To identify zero levels every tenth section from the first section to section 300 was stained with haematoxylin and eosin. The first connective tissue attachment on the mesial of the first maxillary molar was used as zero point for each tooth (see rationale for this in Discussion 6.3.1). The average section number for zero for the blocks used in this study was 95 and ranged from 1 to 260. This varied firstly due to the differing heights of crowns and secondly, due to the number of sections lost whilst aligning the block such that the occlusal surfaces of the molars were parallel to the cutting surface of the block.

In addition, these haematoxylin and eosin sections were used to locate other landmarks such as: first cementum visible (mesial and distal), when the mesiobuccal root split off, first alveolar bone visible and when the alveolar bone surrounded the mesiobuccal root.

4.3.5 MAST CELL STAINING REGIME

From zero level, previously identified with haematoxylin and eosin, every twentieth section (i.e. every 100th μm) was stained for mast cells to the last section per block. If the ideal section was missing because it was stained with haematoxylin and eosin or lost whilst cutting or picking up the sections the closest section to ideal was used instead. The mast cell stain used was 0.5% toluidine blue in HCl at pH 0.5 (see Appendix 8.7.1).

In those blocks where the right and left side zeros did not occur in the same section the right side zero became the determinant for the mast cell staining run. For example, if the right side zero was at section 53 the mast cell staining run was 53, 73, 93, 113, 133 etc. and section 53 was level 1 on the right side.

If the left zero was more apical to the right zero, say at section 68 in the example above, the next available toluidine blue section (73) was taken as level 1 for the left side. The left mast cell staining run was then: 73, 93, 113, 133 etc. If the left side zero was more coronal to the right zero, say at section 10 in the example above, the staining run was backstained every 100μm from the right side zero: 33, 13 so section 13 was taken as level 1 for the left side. The left viewing run was then: 13, 33, 53, 73, 93 etc.

4.4 MICROSCOPY AND DATA MANAGEMENT

4.4.1 LIGHT MICROSCOPY AND PHOTOMICROGRAPHY

An Olympus BH-2 light microscope was used to examine tissue sections. The photographic unit attached to the microscope was an Olympus model PM 10ADS. Micrographs were obtained with Kodachrome 64 film using various combinations of photographic eye tubes (1.67, 2.5 and 3.3) and objective lenses (10X, 20X, 40X, 100X) for different magnifications.

		OBJECTIVE LENSES			
		10X	20X	40X	100X
PHOTOGRAPHIC EYE TUBES (PET)	1.67	16.7	33.4	66.8	167
	2.5	25	50	100	250
	3.3	33	66	132	330

RESULTANT MAGNIFICATIONS POSSIBLE

Table 4.3: Possible magnifications with objective lenses and photographic eye tubes.

The total magnification of the photographic images in the text have been calculated as PET x objective x digitized image enlargement.

4.4.2 COUNTING PROCEDURE

The mesiobuccal (MB) root and surrounding periodontal ligament and bone were divided into quadrants with the use of a template (Figures 4.2 and 4.3) in the eyepiece. The mast cells in each quadrant (buccal, mesial, palatal and distal) were counted.

The template was initially aligned such that the centre of the crosshairs was in the centre of the pulp chamber of the MB root of the first molar. It was initially orientated so that one line of the cross-hairs bisected the two distal roots of the first molar (Figure 4.2). This meant that the crosshairs all traversed the periodontal ligament at the mid palatal, mesial, buccal and distal of the MB root.

The template was then rotated 45°, maintaining the centre of the crosshairs in the centre of the pulp chamber (Figure 4.3). This meant that the crosshairs traversed the periodontal ligament at the boundaries between the palatal, mesial, buccal and distal quadrants. It is in these quadrants that the mast cells were counted.

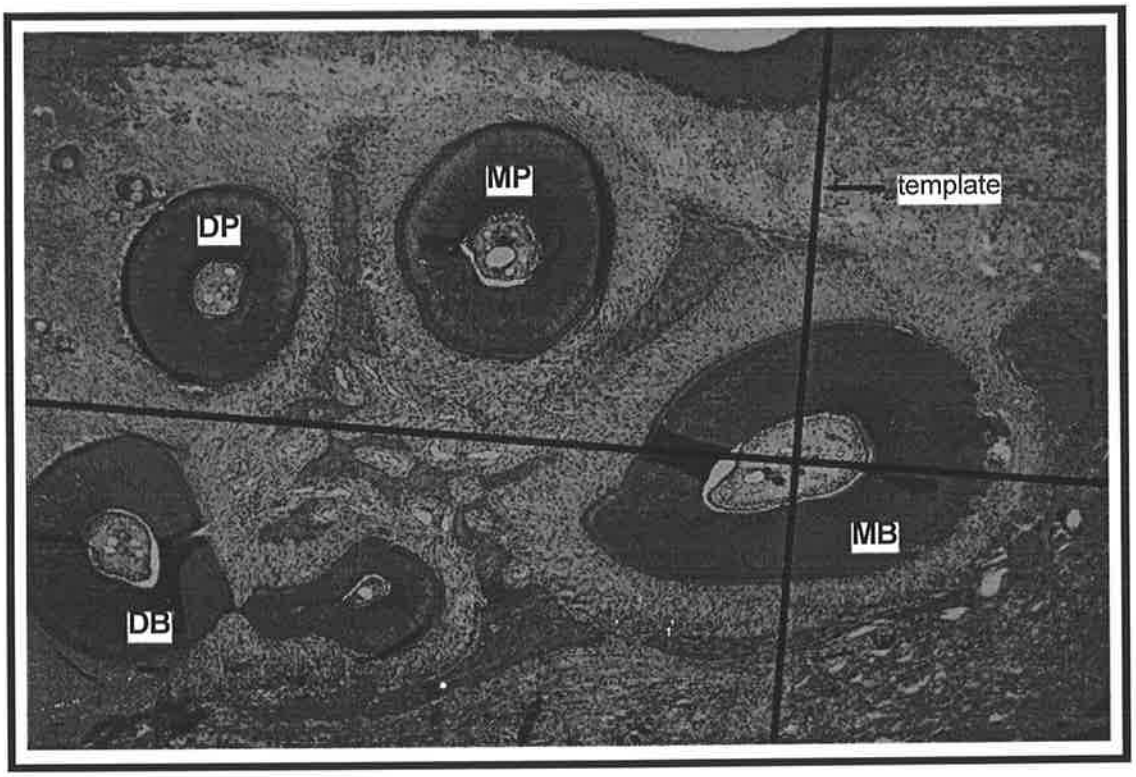

Figure 4.2: Initial orientation of the template. Illustrated on a haemotoxylin and eosin stained section. Total magnification = 41.77
MB = mesiobuccal root, MP = mesiopalatal root, DP = distopalatal root and DB = distobuccal root.

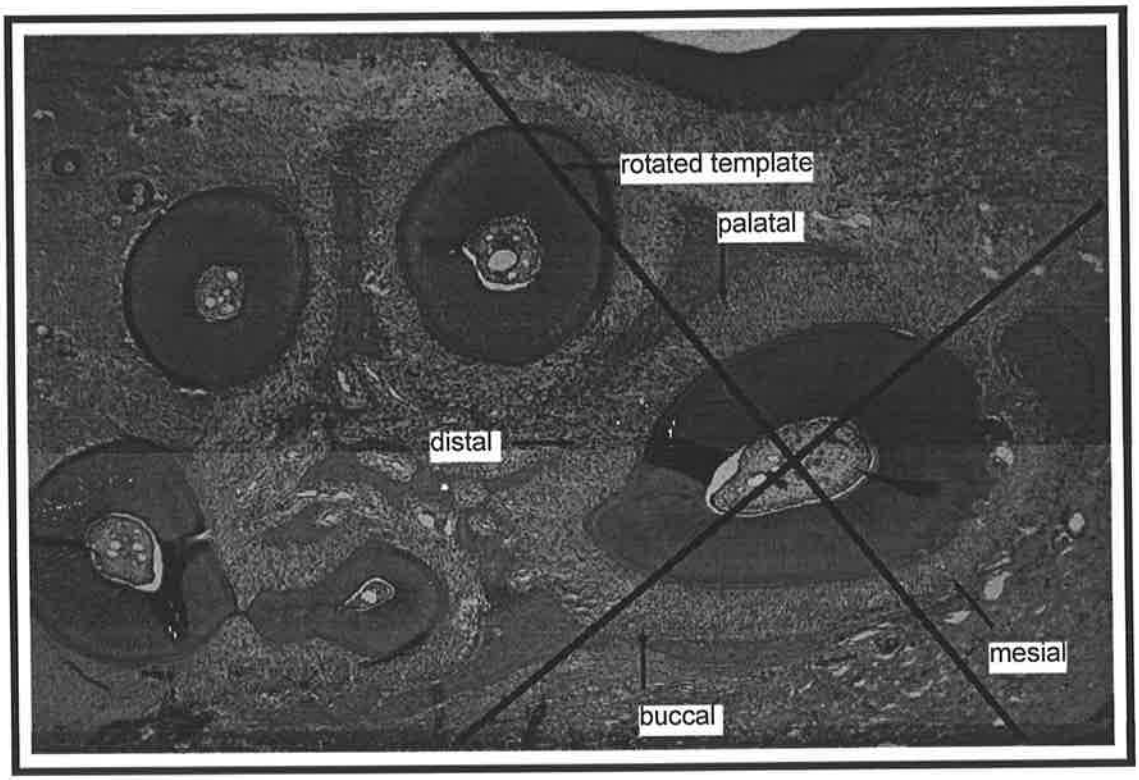

Figure 4.3: Final orientation of the template for counting of mast cells in the PDL. Illustrated on a haemotoxylin and eosin stained section. Total magnification = 41.77

4.4.2.1 Mast Cells And Bone

Originally, it was intended to only subdivide the ligament into quadrants and state the total number of mast cells in each quadrant, However, it was noticed after viewing a quarter of the blocks that the highest number of mast cells in the periodontal ligament seemed to be near the bone side of the ligament. Therefore, it was decided to subdivide the ligament further into horizontal thirds (tooth, middle and bone) within each quadrant of the ligament (Figure 4.4). This was achieved with a millimetre ruler graticule within the eyepiece of the microscope. Wherever mast cells were found the ligament width was measured using the graticule and then subdivided horizontally to determine which third the mast cells were located in. The recording sheet was modified to the one seen in Table 4.4 and the first quarter of the blocks already viewed were recounted to record this additional information.

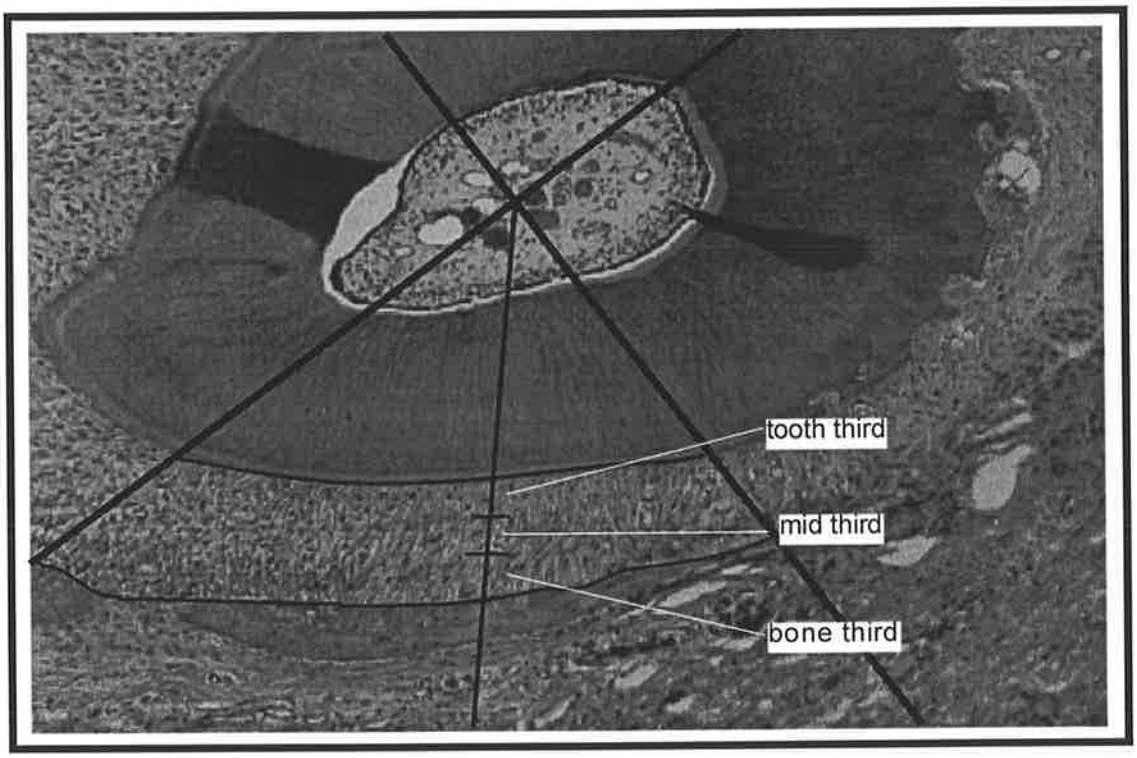

Figure 4.4: Further subdivision of the PDL into thirds. Illustrated is the buccal quadrant with a ruler in the eyepiece that has divided the ligament into thirds (tooth, mid and bone). The boundaries of this quadrant have been highlighted.

4.4.2.2. Mast Cells And Blood Vessels

After viewing a quarter of the blocks it was observed that many of the mast cells were in close proximity to blood vessels in the periodontal ligament. It was then decided to record whether the

mast cells in the periodontal ligament were within a certain distance from any blood vessels or not. This distance was measured using a millimetre ruler graticule within the eyepiece of the microscope.

The mast cells were designated as being "near" a blood vessel if they were within "3mm" of the blood vessel using the 25X objective lens. This distance was later confirmed as being 12 microns by using a 1mm graticule on a glass slide viewed at 25X.

For details of the microscopic procedure for counting mast cells in the ligament refer to Appendix 8.11.

4.4.3 DATA COLLECTION

This study was to focus on early changes in the mast cell population of the PDL. Hence all of the 83 and 90 day old animals (experimental and control) (N=33) and one each of the 97, 111 and 139 day old animals (N=6) were used in this study. The remaining animals (N=21) were dissected, fixed, rehydrated, decalcified and processed into blocks for later study.

The areas studied for each mesiobuccal root of the maxillary first molar were: the buccal, mesial, palatal and distal quadrants (which were further subdivided into tooth, mid and bone thirds) of every twentieth section (i.e. every 100 μm) from the first connective tissue attachment to the apex. As there were approximately 400 sections per tooth from zero to the apex this resulted in 15 - 20 samples per tooth, depending on the length of the tooth. The average length was 2000 μm, i.e. 2mm.

In summary, data for this study were collected for:

- 39 rats (28 experimental, 11 controls),
- 2 teeth for each rat (left side and right side),
- 15 - 20 vertical levels on average per tooth, depending on its length,
- 4 quadrants (buccal, mesial, palatal and distal) for each level,
- 3 horizontal thirds (tooth, mid and bone) for each level and each quadrant.

The data were recorded as follows (Table 4.4):

Animal Number:

Control/Experimental Tooth:

Level	buccal			mesial			palatal			distal			Bone	Near BV
	T	M	B	T	M	B	T	M	B	T	M	B		
1														
2														
3														
4														
5														
6														
Etc														
25														

Table 4.4: Recording sheet for mast cell distribution within the PDL of one mesiobuccal root.

Key:
B = bone third of the PDL
M = mid third of the PDL
T = tooth third of the PDL
Bone = indicates the level at which the alveolar crest completely surrounds the mesiobuccal root.
Near BV = near a blood vessel. "Near" meaning within "3mm" at 25X (ie. 12 microns) as measured with the ruler graticule in the eyepiece.
99 = No viable ligament. Either the area of the section is above the epithelial attachment, or, the ligament is damaged.
77 = No more tooth left i.e. below apex.

4.4.4 ORGANISING THE DATA

The data was provided to the statistician as a Microsoft Excel spreadsheet with 39 arrays (representing 39 rats) each containing a 25x29 (i.e. left side data, right side data and level) matrix. The data were read into S-PLUS[6] as a single matrix of size 975x29. Data analysis was performed in consultation with Prof. R. Jarrett from the Dept. of Statistics and Computing Science, The University of Adelaide.

[6] References for the S-PLUS language are: Becker *et al., (1988),* and Venables and Ripley (1994).

Since there were differing numbers of levels in each tooth there were difficulties analysing the data. The aim was to reduce the data in a consistent and reproducible manner as follows:

- for each tooth and each quadrant, the first 4 levels after 99 finished were summed (i.e. the first four levels below the epithelial attachment);
- for each tooth and each quadrant, the last 4 levels before 77 started were summed (i.e. the last four levels before the apex of the tooth); and
- for each tooth and each quadrant, the middle 4 of the levels that remained were summed.

There were some missing values and there were some cases where there were not 12 levels available to be used. In that case, the sum of the central 4 levels were used even if that meant that one row was used twice, so that each number of mast cells referred to the same number of levels. Where there were missing values among the four rows chosen, the program was allowed to skip one such missing value and take the next appropriate entry.

The data were converted using an S-PLUS code to an 117 x 24 array containing just 3 rows (corresponding to the vertical levels) for each rat (N=39) and 24 columns corresponding to the combinations of tooth, quadrant and horizontal third.

There were some missing values where the program was not able to sort out what to do which were corrected manually. The only missing values remaining were those corresponding to rat 26 (a two week experimental rat) for which all the data on the control tooth was missing. It was decided to delete rat 26 from the data set.

In summary, the data were then represented as:

- 38 rats: 27 experimental animals
  - RHS experimental tooth
  - LHS control tooth
  - 10 control animals
    - RHS control tooth
    - LHS control tooth
- 2 teeth for each rat,
- 3 vertical levels (coronal, middle and apical) for each tooth,
- 4 quadrants (buccal, mesial, palatal and distal) for each level,
- 3 horizontal thirds (bone, mid and tooth) for each level and each quadrant.

The final data file consisted of 38 rats with 72 (2T x 3V x 4Q x 3H) readings on each.

4.4.5 DATA ANALYSIS

Analysis of variance was used to compare the numbers of mast cells observed between control teeth (no orthodontic movement) and experimental teeth (orthodontic movement) in 28 experimental animals firstly, to see if there was a significant overall difference, secondly, to see if position (horizontal, vertical and quadrant) had any effect and thirdly, whether there was any relationship to time. A split plot analysis was also used to determine whether the effect of treatment changed with time. Also where appropriate an analysis of logs was done to see if any changes were proportional.

For the experimental animals data were tabulated to give mean mast cell counts throughout the PDL, in each horizontal third (bone, mid and tooth), each vertical level (coronal, middle and apical), and each quadrant (buccal, mesial, palatal and distal).

Ratios of means with standard errors were given for the vertical levels and for each quadrant and vertical level. Chi-square tests were used to compare significant ratios.

Mean mast cell counts were also tabulated in each of the three positions (horizontal, vertical and quadrant) for the 11 control animals (control/control teeth) with R:L ratios and standard errors so a comparison could be made with the experimental rats.

An analysis of deviance and empirical logits were used to compare whether orthodontic tooth movement had any effect on the closeness of mast cells to blood vessels using the control and experimental rats.

CHAPTER FIVE: RESULTS

5.1 THE EXPERIMENTAL ANIMALS

5.1.1 ANAESTHESIA

When appliances were cemented for the pilot study the anaesthetic used was Nembutal[1] (pentobarbitone sodium) which was administered as an intraperitoneal injection (60 mg/mL) diluted in a 1:1 ratio with 0.9% sodium chloride. The amount of Nembutal administered was approximately 0.09 mL per 100g of weight of the animal.

One of the six pilot study rats died two hours after administration of Nembutal for appliance insertion. Due to the small "window" of action with this anaesthetic, a decision was made to switch to Ketapex[2] (ketamine) and Rompun[3] (xylazine hydrochloride). Approximately 0.4mL Ketapex was used for a 500g rat, this varied proportionately with the rat's weight, diluted in a 1:1 ratio with 0.9% sodium chloride. After this injection 0.15mL of Rompun was injected, irrespective of the weight of the rat.

The Ketapex and Rompun combination proved to be a much better anaesthetic than Nembutal as it had a larger "window" between the animals being awake and dead. Of the remaining 38 animals[4] which were anaesthetised only one died. This animal stopped breathing within ten seconds after the Rompun injection and then started breathing again but stopped again after five minutes.

5.1.2 ORTHODONTIC APPLIANCES

The orthodontic appliances proved to be successful over the time frame studied. There were only two failed appliances out of forty experimental animals. Both of these appliances failed at the soldered area between the orthodontic band on the incisors and the 0.020" supporting wire. Two extra animals were obtained and substituted for those that had failed.

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[1] Nembutal = 60mg/L pentobarbitone sodium. Boehringer Ingelheim, NSW, Australia.
[2] Ketapex = 100mg/L ketamine. Apex laboratories, NSW, Australia.
[3] Rompun = 20mg/L xylazine hydrochloride. Bayer NSW, Australia.
[4] 38 animals = 40 experimental rats - 6 original pilot study rats + replacements for 1 Nembutal death (in the pilot study), 1 Ketapex death (in the experiment proper) and 2 failed appliances.

5.1.3 WEIGHT

During the period of orthodontic tooth movement the animals were fed pulverized standard rat pellets and water ad libitum. When the appliances were checked and also when the animals were sacrificed they were weighed to see if appliance wear caused weight loss from inadequate food intake. The treated animals tolerated the orthodontic appliance and were of similar weight to external controls throughout the study.

5.2 TISSUE PREPARATION

5.2.1 FIXATION

All the tissue appeared to be adequately fixed and there were no signs of tissue degeneration in the sections studied. Therefore, Carnoy's fixative was successful in this respect, and morphology was well preserved.

5.2.2 DECALCIFICATION

Decalcification seemed slightly inadequate in a few blocks, despite radiographic evidence of complete decalcification. This problem was discovered when the blocks were being sectioned.

Decalcification took a longer period of time than was originally expected (one month). The blocks generally took two months to decalcify, according to radiographic evidence. However, due to earlier difficulty with sectioning, the remaining blocks were left for two to four weeks longer in EDTA, resulting in a decalcification period of two and a half to three months.

5.3 SECTION PREPARATION

5.3.1 SECTIONING

The serial sectioning of the tissue at 5μm thickness went well except for the initial problems in decalcification as stated earlier. One of the control teeth of a two week experimental animal (rat 26) was pulled out of the embedding wax due to inadequate decalcification. The remaining unprocessed blocks were decalcified for a longer period of time to prevent this from occurring again.

The sections adhered to the APT-coated slides well with only the occasional section falling off or creasing during staining. The collection of three sections per slide was an advantage as it enabled

one of two consecutive sections to be viewed if the intended section to be viewed was lost, torn or creased.

5.3.2 HAEMATOXYLIN AND EOSIN STAINING

The haematoxylin and eosin stain was predominantly used to locate landmarks on each block.

Earlier on in the histological phase of this experiment every twentieth section (ie. every 100th micron) was stained with haematoxylin and eosin. This was to gain an understanding of the normal anatomy of the first maxillary molar, particularly the mesiobuccal root. These series were used as a guide when viewed alongside the toluidine blue stained sections (in which it was more difficult to identify tissue types clearly).

Once the nuances of staining of these tissue types with toluidine blue were understood, entire series of haemotoxylin and eosin stained teeth were no longer necessary and only the following landmarks were recorded: zero point, when the mesiobuccal root split off, when the first alveolar bone was visible and when the alveolar bone surrounded the mesiobuccal root.

A series of low power views depicting some important landmarks have been included in Figure 5.1 A-H and a further series of low power views depicting the anatomy at the end of the mesiobuccal (MB) root have been included in Figure 5.2 A-D.

Figure 5.1 Lower power views depicting important landmarks (83 day old rat).

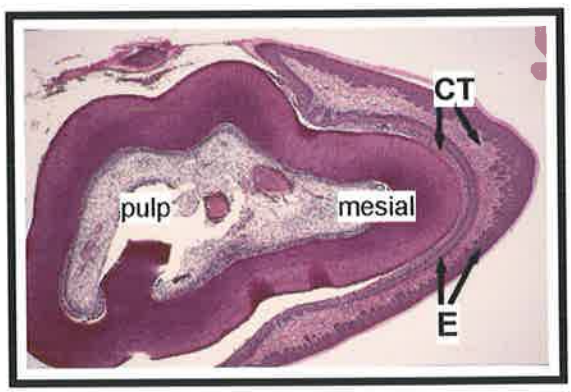


Figure 5.1A: Just below zero. CT = connective tissue, E = epithelium
Slide: MBC13 - Section 115 RHS (mirror image to correspond with pictures below)
Stain: Haematoxylin and eosin
Magnification: PET 2.5, Objective 4X. Total mag. = 20X

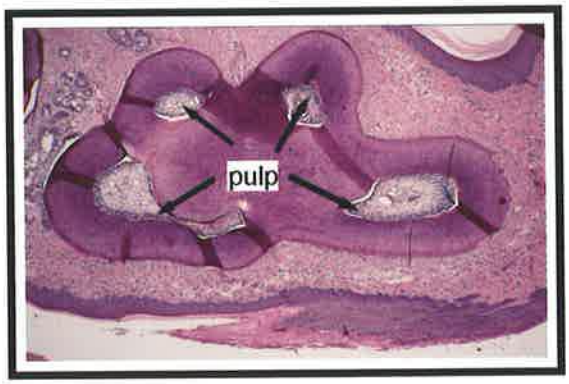


Figure 5.1B: Furcation of the pulp
Slide: MBC3 - Section 160 LHS(Note: LHS zero for MBC3 = 85)
Stain: Haematoxylin and eosin
Magnification: PET 2.5, Objective 4X. Total mag. = 20X

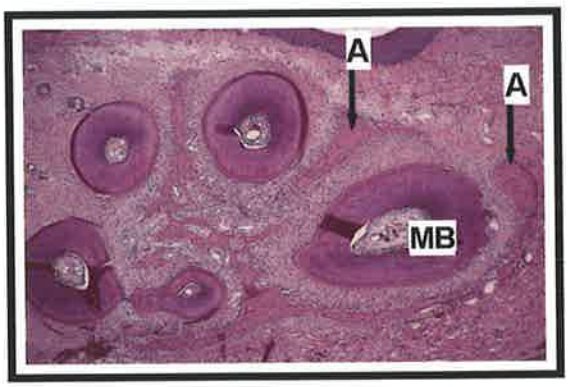


Figure 5.1C: Furcation of the roots (MB = mesiobuccal) and the alveolar crest (A) is visible.
Slide: MBC3 - Section 201 LHS
Stain: Haematoxylin and eosin
Magnification: PET 2.5, Objective 4X. Total mag. = 20X

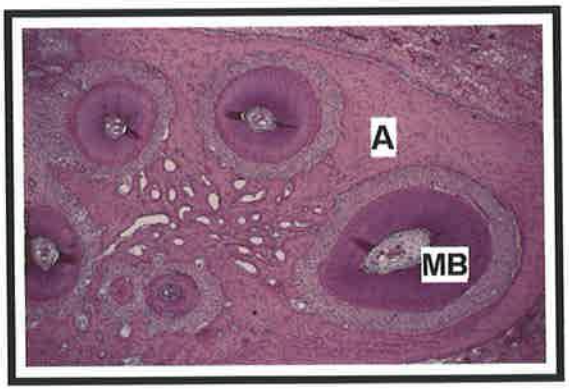


Figure 5.1D: Alveolar bone (A) completely surrounds the MB root.
Slide: MBC3 - Section 239 LHS
Stain: Haematoxylin and eosin
Magnification: PET 2.5, Objective 4X. Total mag. = 20X

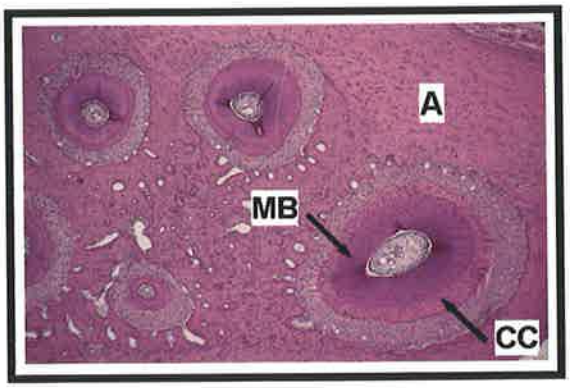


Figure 5.1E: Cellular cementum (CC) is present around the MB root.
Slide: MBC3 - Section 300 LHS
Stain: Haematoxylin and eosin
Magnification: PET 2.5, Objective 4X. Total mag. = 20X

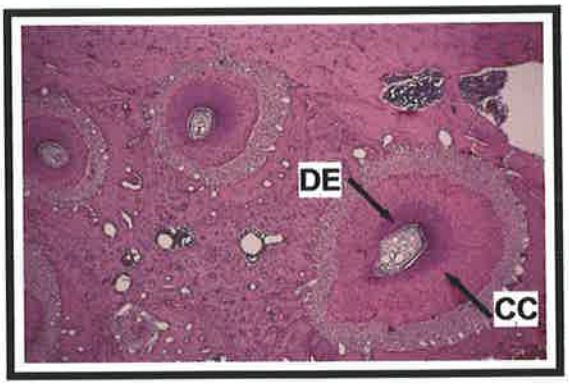


Figure 5.1F: More cellular cementum (CC) is present than dentine (DE) in the MB root.
Slide: MBC3 - Section 382 LHS
Stain: Haematoxylin and eosin
Magnification: PET 2.5, Objective 4X. Total mag. = 20X

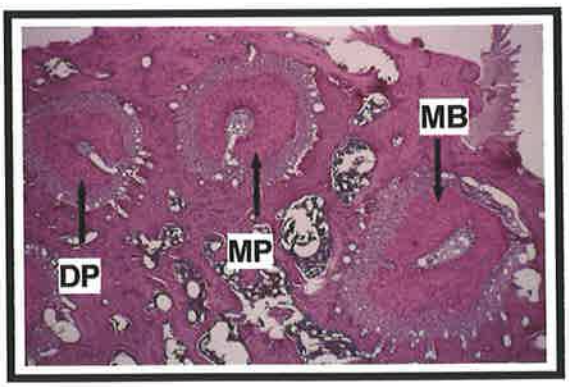


Figure 5.1G: Just above the apical foramen in the MB root. The apical formina of the mesiopalatal (MP) and distopalatal (DP) roots are visible.
Slide: MBC3 - Section 458 LHS
Stain: Haematoxylin and eosin
Magnification: PET 2.5, Objective 4X. Total mag. = 20X

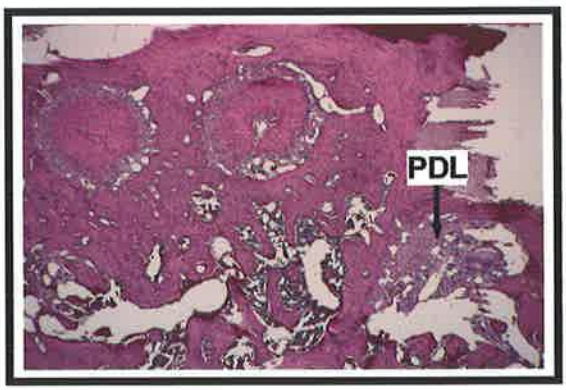


Figure 5.1H: Below tooth in MB root region - only PDL is present.
Slide: MBC3 - Section 495 LHS
Stain: Haematoxylin and eosin
Magnification: PET 2.5, Objective 4X. Total mag. = 20X

Figure 5.2 Higher power views of the end of the MB root (84 day old rat)

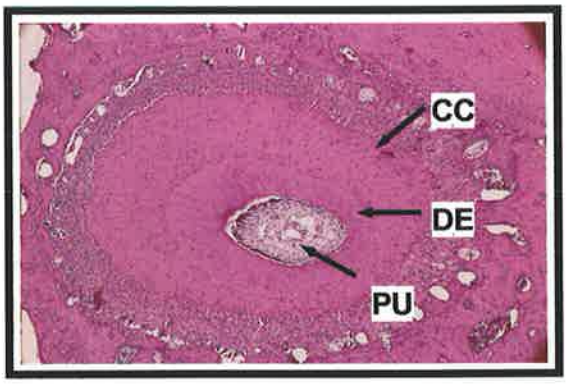


Figure 5.2A: More cellular cementum (CC) than dentine (DE) (PU = pulp).
Slide: MBE6 - Section 355 LHS
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 33.4X

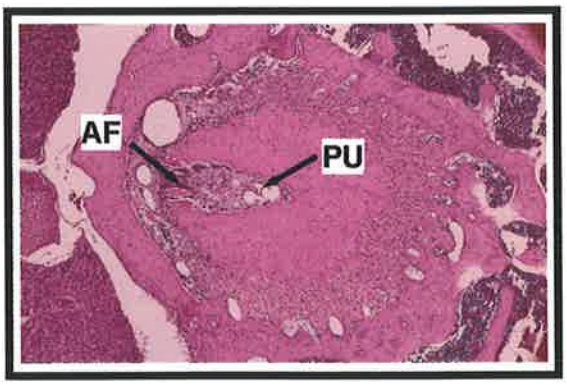


Figure 5.2B: Apical foramen (AF) of the pulp (PU).
Slide: MBE6 - Section 384 LHS
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 33.4X

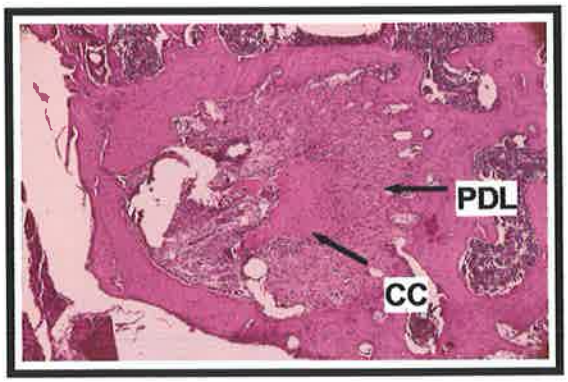


Figure 5.2C: Cellular cementum (CC) and PDL only (no more dentine or pulp).
Slide: MBE6 - Section 405 LHS
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 33.4X

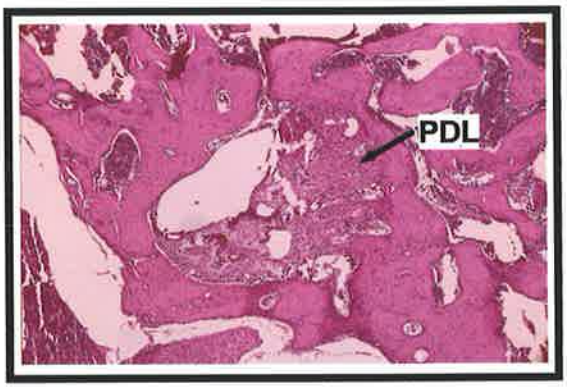


Figure 5.2D: PDL only (no more tooth).
Slide: MBE6 - Section 428 LHS
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 33.4X

The most important role of the haematoxylin and eosin staining was in its use to identify zero point, as this determined the staining run for the mast cell staining. The first connective tissue attachment to the tooth on the mesial of the first maxillary molar was used as zero (Figure 5.3). This proved to be a reliable landmark as it was simple to identify and easily reproducible.

The right hand side zero point determined the mast cell staining run with 0.5% toluidine blue. If the left hand side zero was more apical to the right the staining run was backcounted from the right zero until the left zero was included in the run.

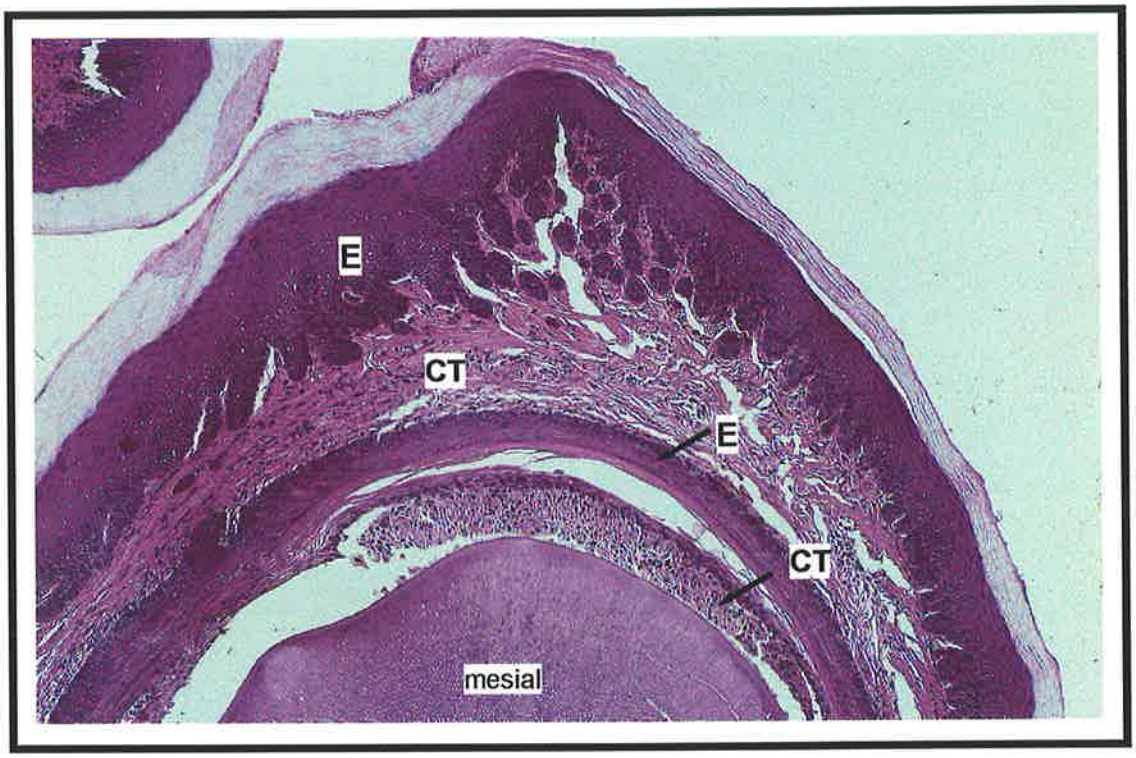


Figure 5.3: Zero (or just below zero) - the first connective tissue attachment (CT-arrowed) on the mesial of the root (84 day old rat). Adjacent is the epithelium (E-arrowed) of a rete peg which is surrounded by a band of connective tissue (CT) and epithelium (E).
Slide: MBE6 - Section 72 LHS
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 68.76X

5.3.3 TOLUIDINE BLUE STAINING

The toluidine blue stain used in this experiment worked well. This was buffered to pH 0.5 to decrease background staining and only stain the mast cell granules.

Initially the acidified toluidine blue sections were difficult to orientate because of this pale background staining. The very slight colour changes between tissue types made it difficult to distinguish between bone, periodontal ligament and tooth, however, the mast cells were easily identified especially due to their metachromatic properties. To confirm that a mast cell was being viewed (and not a speck of dirt) the fine focus was racked up and down slightly to view a colour shift from blue to reddish-purple (Figure 5.4 vs Figure 5.5) where in contrast, a speck of dirt would remain black (Note: these mast cells depicted in Figures 5.4 and 5.5 are located in connective tissue outside the bone). The use of the haematoxylin and eosin stained series in conjunction with the toluidine blue series was of considerable help in orientation early on in the viewing phase until the nuances of the acidified toluidine blue staining were understood.

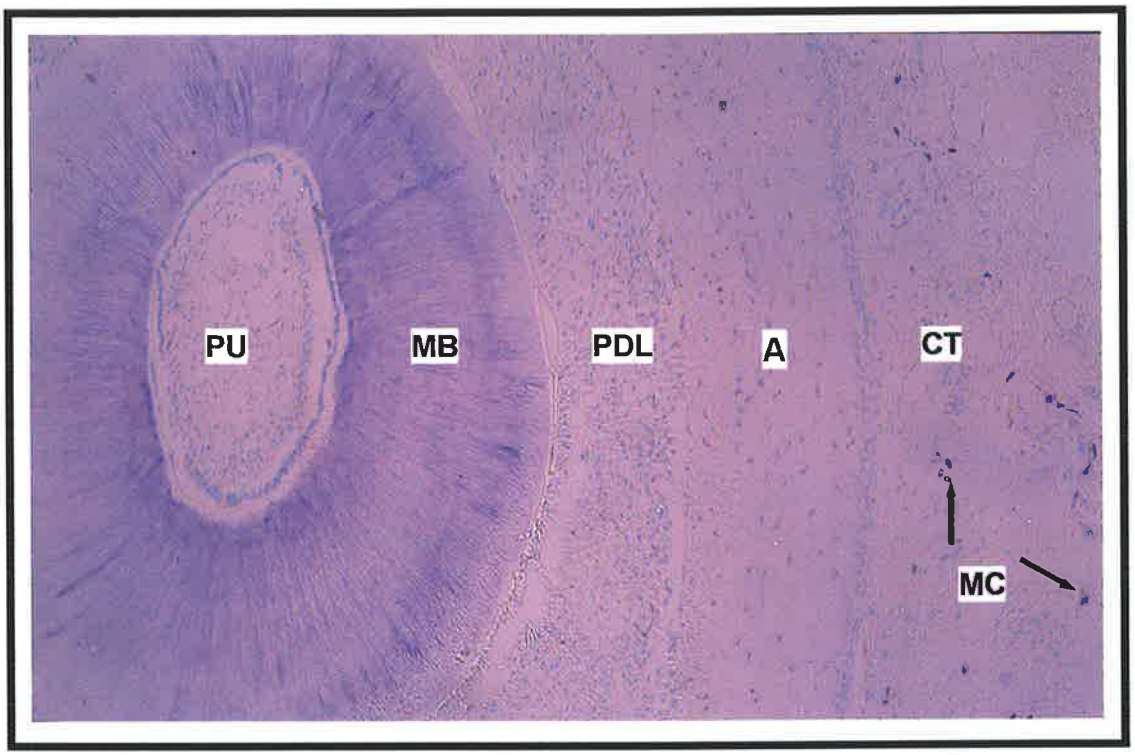


Figure 5.4: Pale background staining of pulp (PU), mesiobuccal root (MB), PDL, alveolar bone (A) and connective tissue (CT) but good mast cell (MC) staining (1 hr activation)
Slide: MBE22 - Section 243 RHS
Stain: 0.5% toluidine blue pH 0.5
Magnification: PET 2.5, Objective 10X. Total mag. = 103.68X

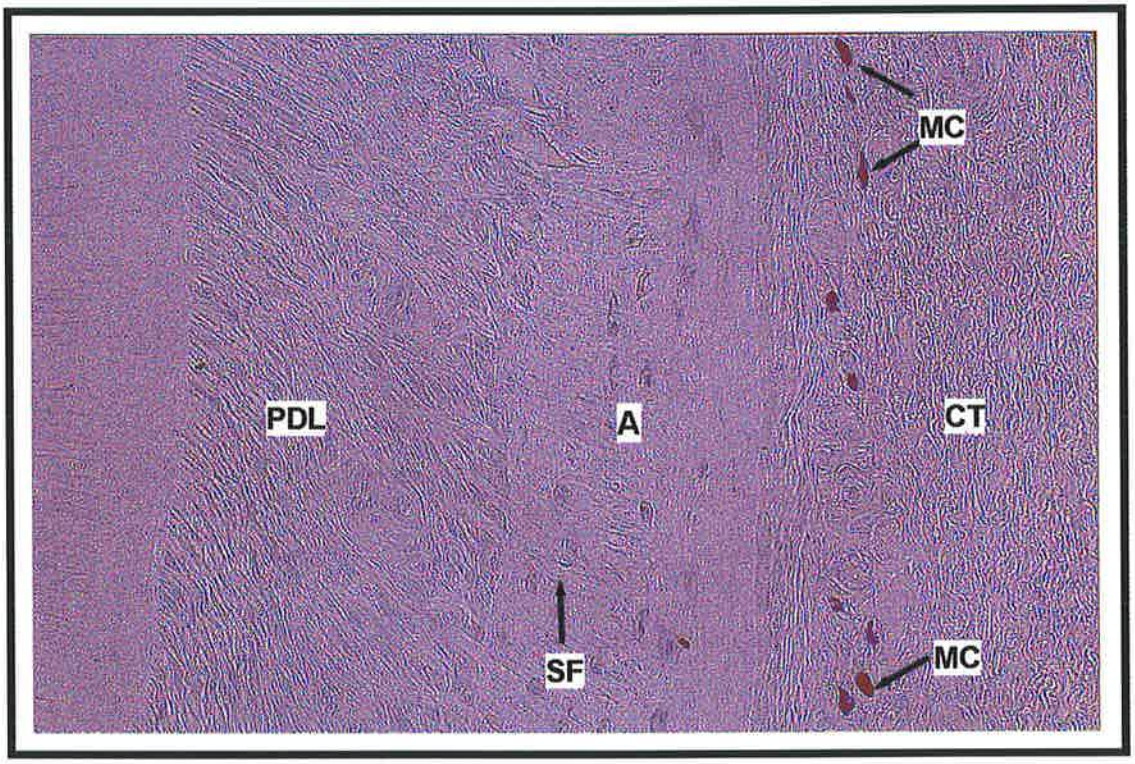


Figure 5.5: Pale background staining of PDL, alveolar bone (A) and connective tissue (CT) but good mast cell (MC) staining. SF = Sharpey's fibres. (90 day old rat).
Slide: MBE41 - Section 278 LHS
Stain: 0.5% toluidine blue pH 0.5
Magnification: PET 3.3, Objective 20X. Total mag. = 272.74X

The mast cell granules stained well with the acidified toluidine blue. This can be seen in Figure 5.6 which shows a mast cell degranulating. Individual granules that have been liberated can be seen near this mast cell.

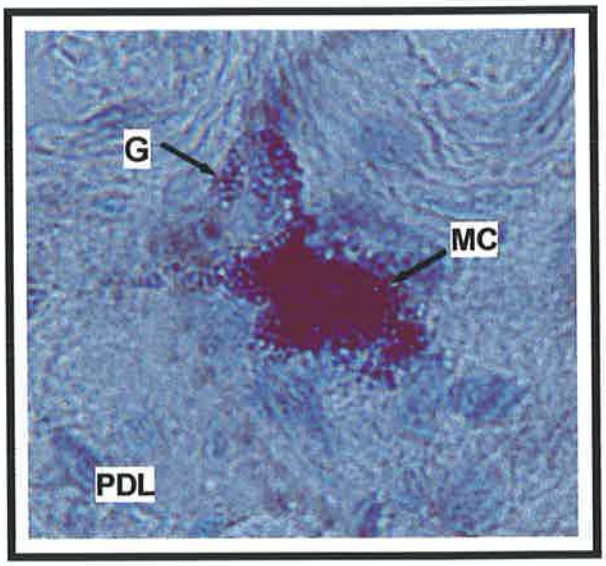


Figure 5.6: A degranulating mast cell (MC) showing liberated granules (G) (8 week experimental rat - experimental tooth) in the periodontal ligament (PDL)
Slide: MBE17 - section 465 RHS
Stain: 0.5% toluidine blue pH 0.5
Magnification: PET 3.3, Objective 100X. Total mag. = 2442X

5.3.4 HRP-AVIDIN STAINING

In addition to toluidine blue, it was intended to use a conjugated avidin preparation to identify mast cells. The conjugated stain is purportedly a very sensitive method for identifying mast cells. The results of the conjugated avidin stain were to be compared to the toluidine blue staining.

The HRP-avidin-DAB histochemical staining trialed on rat palate tissue obtained from the experimental rats that lost orthodontic appliances failed to exhibit any positive reactions for mast cells. The sections demonstrated a large amount of background staining and not one particular cell type demonstrated a stronger staining reaction over other cell types. Different concentrations of avidin-horseradish peroxidase (1:50, 1:100, 1:200, 1:400 and 1:800) proved to be just as disappointing.

The current experiment was completed only using 0.5% toluidine blue at pH 0.5 as the results of this stain were encouraging and consistent.

5.4 HISTOLOGIC OBSERVATIONS

5.4.1 GENERAL OBSERVATIONS

Histological examination of the control teeth demonstrated the periodontal ligament to be of even thickness around the MB root (Figure 5.7A). The experimental teeth, on the other hand, demonstrated the periodontal ligament on the buccal side to be under compression whilst the palatal side to be under tension (Figure 5.7B)

Of all the experimental teeth viewed in the present study (28) only one tooth (one week after orthodontic tooth movement) demonstrated hyalinisation of the periodontal ligament (Figure 5.8A and B).

When examined histologically the coronal portion of the ligament of the experimental teeth demonstrated tissue disruption due to the presence and/or removal of the ligature as compared with control teeth (Figure 5.9A and B). After having seen this tissue damage histologically it may be advisable to change the method of holding the spring in place. Perhaps a preferable way to prevent occlusal dislodgement of the spring would be to bond it to the palatal surface of the first molar. This may cause plaque accumulation in the area but a least it would not cause tissue disruption or possible mesial movement of the tooth.

5.4.2 ROOT RESORPTION

In the present study varying amounts of root resorption were seen. Interestingly, resorption was noted in both control and experimental teeth, usually to a greater degree in the latter (Figures 5.10 A and B and 5.11A and B). Initially when viewing the experimental animals' sections it was thought that perhaps the resorption seen on control teeth could be due to either a "mirroring" effect of tooth movement causing root resorption on the contralateral side or due to an increase in bruxing on control teeth due to orthodontic tooth movement on the experimental side. However, when viewing the control animals' sections it was noted that resorption occurred in these teeth also (Figure 5.12A and B) indicating it is a normal occurrence.

Löe and Waerhaug (1961) and Lindskog *et al.* (1988) suggested a protective role of epithelial cell rests of Malassez within the periodontal ligament. 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 (Leedham, 1992). A possible epithelial rest was identified in one of the experimentally moved teeth of the present study (Figure 5.13).

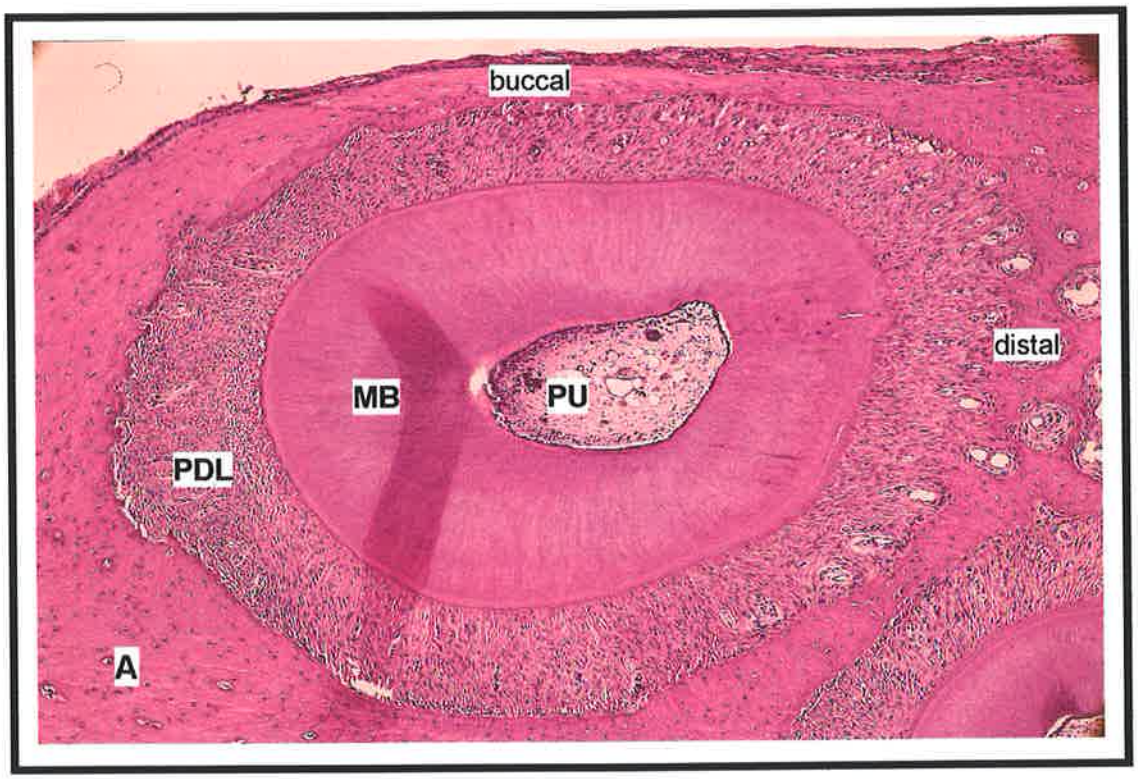


Figure 5.7A: Control Tooth - PDL evenly spaced around the mesiobuccal (MB) root.
Slide: MBE6 - Section 208 LHS
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 68.76X

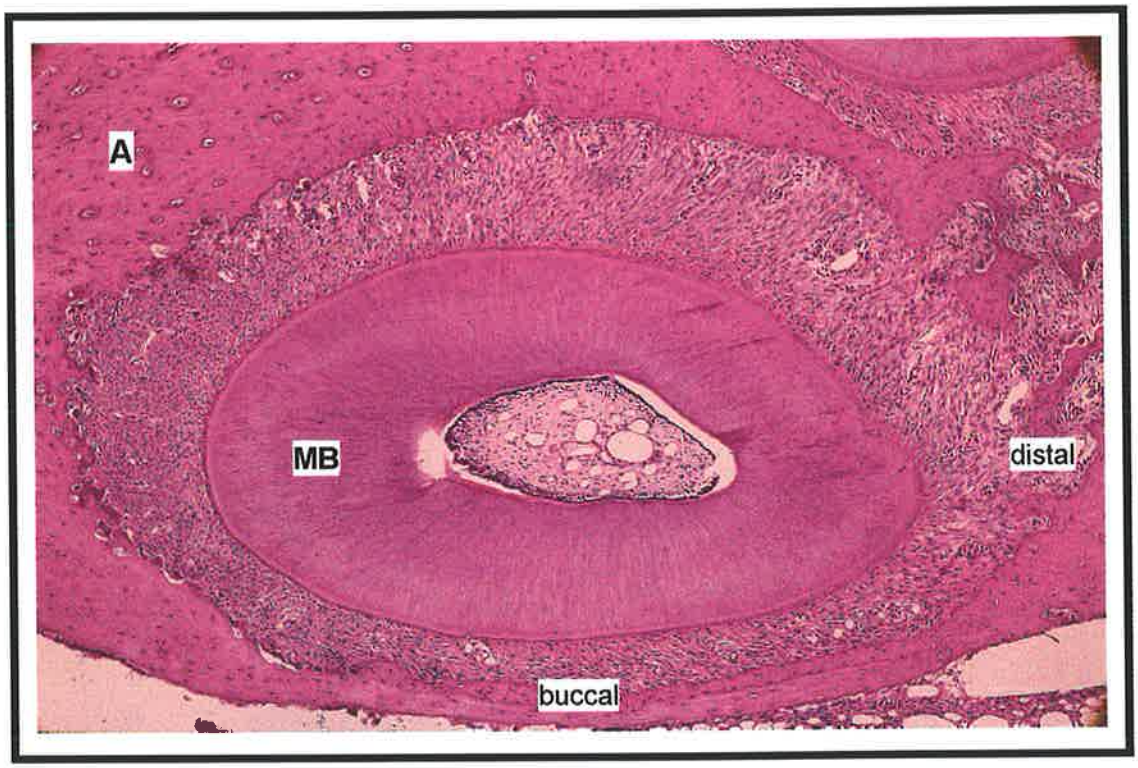


Figure 5.7B: Experimental tooth - PDL around mesiobuccal (MB) root compressed on buccal (24hr activation).
Slide: MBE6 - Section 208 RHS
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total Mag. = 68.76X

Figure 5.8A: Control tooth - NAD - Note the large amount of cellular cementum (CC) around the MB root, particularly on the mesial.
Slide: MBE42 - Section 291 LHS (zero = section 118) ∴ Level = 173
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 69.81

Figure 5.8B: Experimental tooth - an area of hyalinization (H) and undermining resorption filled with osteoclasts (O) on the mesial (1 week activation).
Slide: MBE42 - Section 291 RHS (zero = section 199) ∴ Level = 92
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 69.81

Figure 5.9A: Control Tooth - normal appearance of the PDL above the alveolar crest.
Slide: MBE6 - Section 165 LHS
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 69.81X

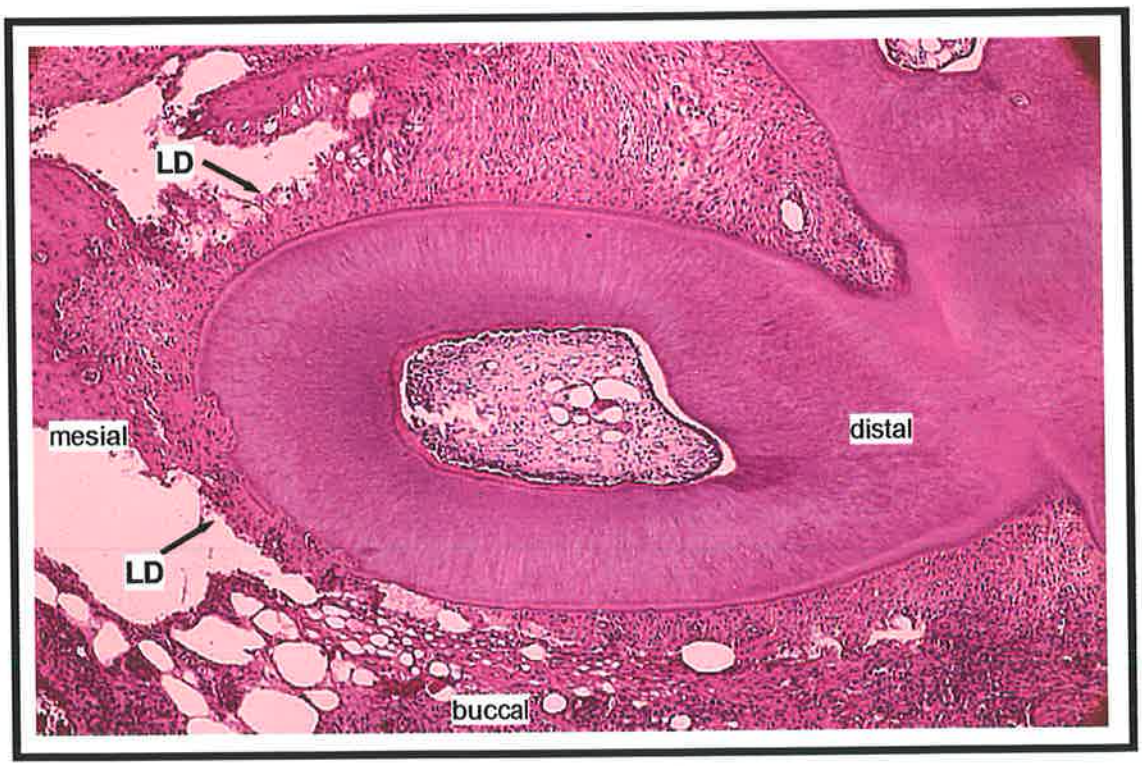


Figure 5.9B: Experimental tooth - root resorption and PDL damage from the ligature (LD) placed around the tooth to support the orthodontic spring (24hr activation).
Slide: MBE6 - Section 165 RHS
Stain: Haematoxylin and eosin.
Magnification: PET 1.67, Objective 10X. Total mag. = 69.81X

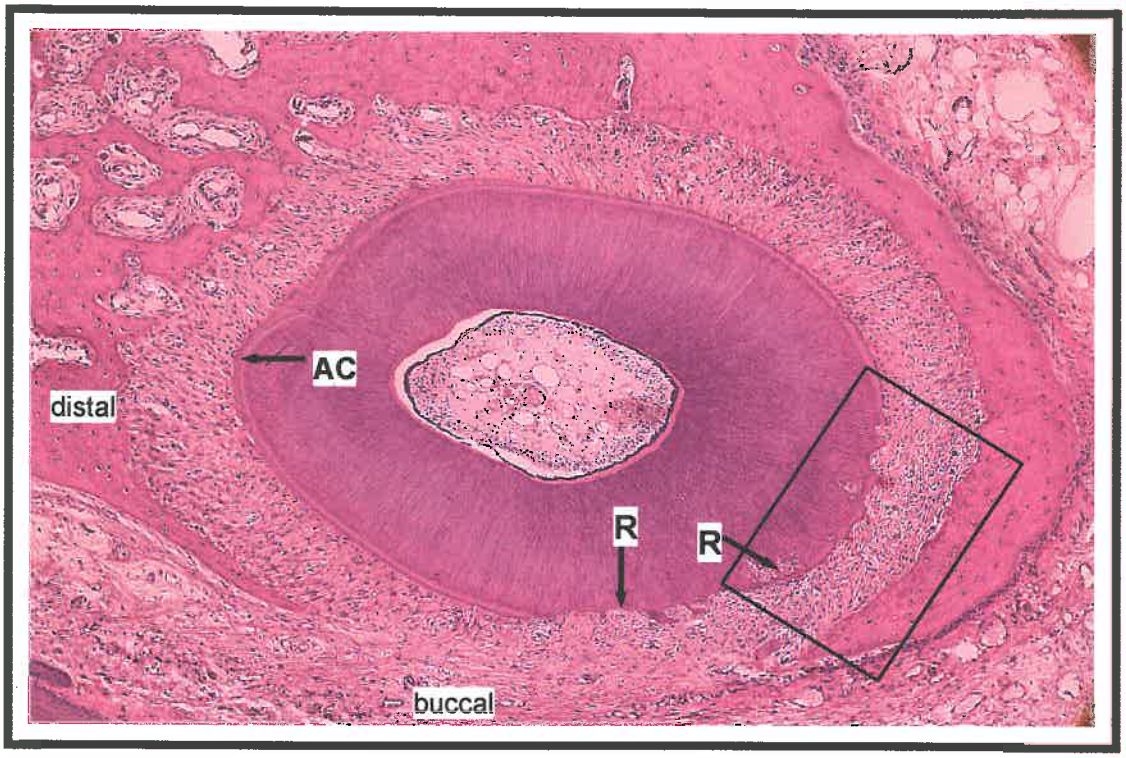


Figure 5.10A: Resorption (R) in a control tooth of an experimental animal (1 week activation). Acellular cementum (AC) is visible in the non-resorbed areas.
Slide: MBE40 - Section 259 LHS
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 69.26X

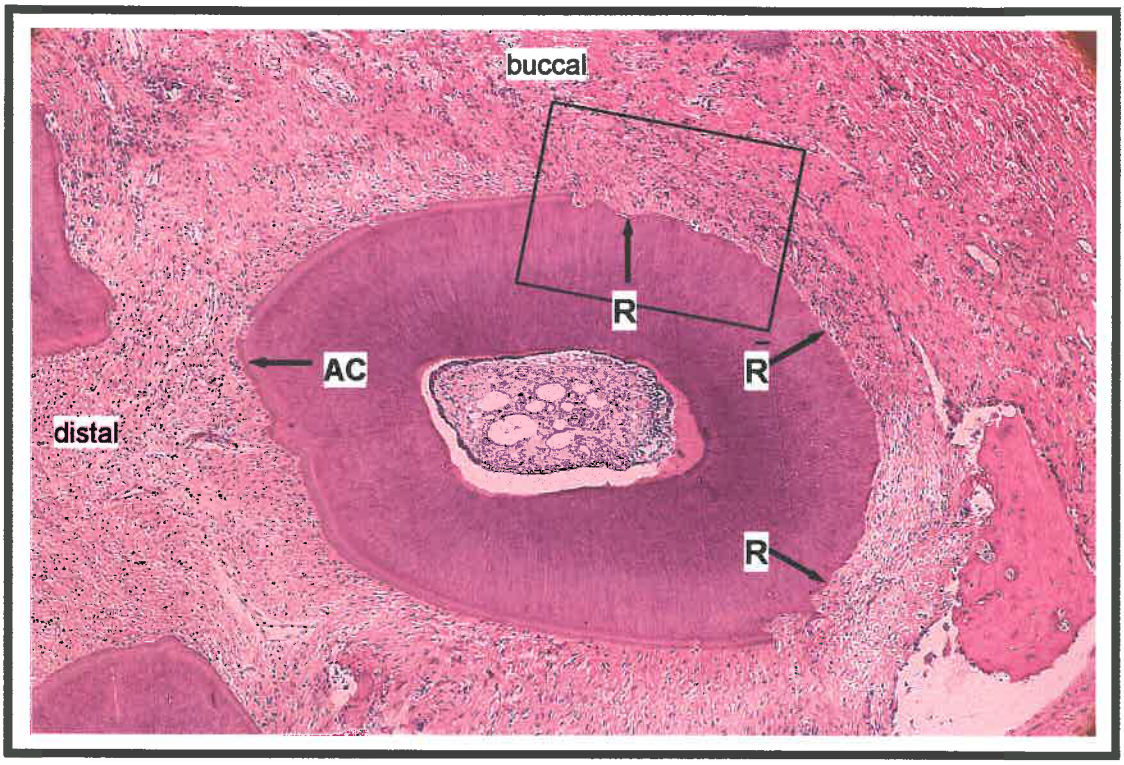


Figure 5.10B: Resorption (R) in an experimental tooth of an experimental animal (1 week activation).
Slide: MBE40 - Section 259 RHS.
Stain: Haematoxylin and eosin
Magnification: PET 1.67, Objective 10X. Total mag. = 69.26X

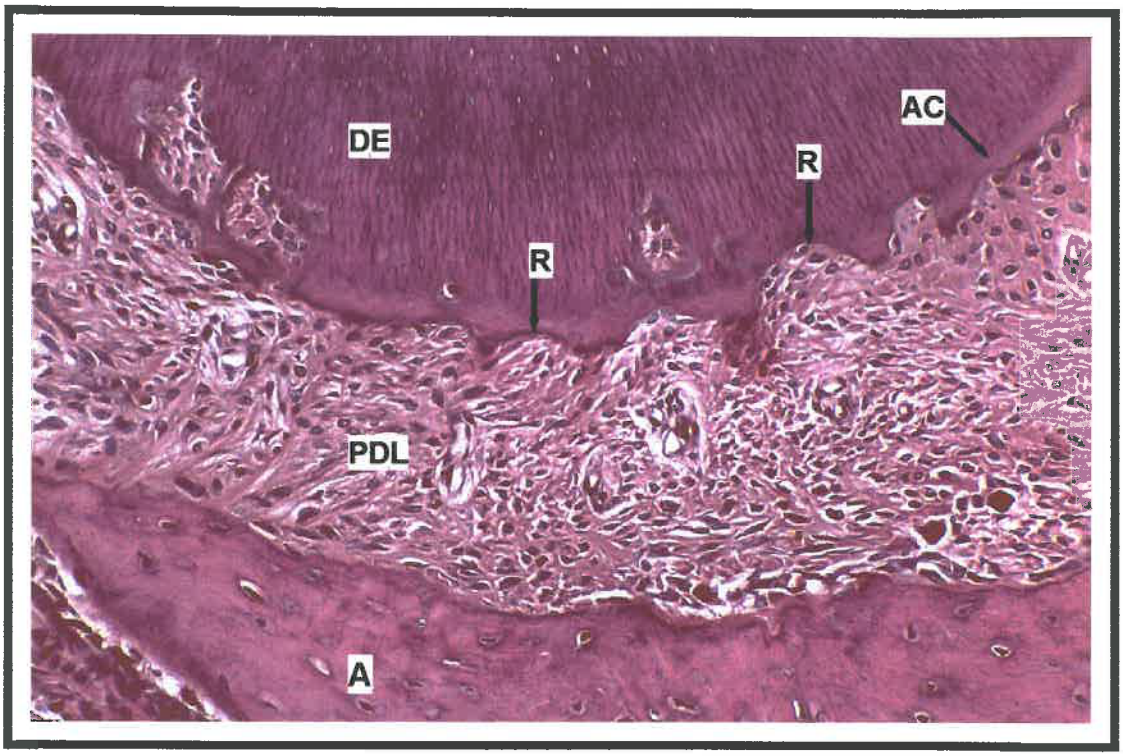


Figure 5.11A: High power view of Figure 5.10A (boxed area) showing resorption lacunae (R). (DE = dentine, AC = acellular cementum, A = alveolar bone).
Slide: MBE40 - Section 259 LHS
Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 20X. Total mag. = 272.74X

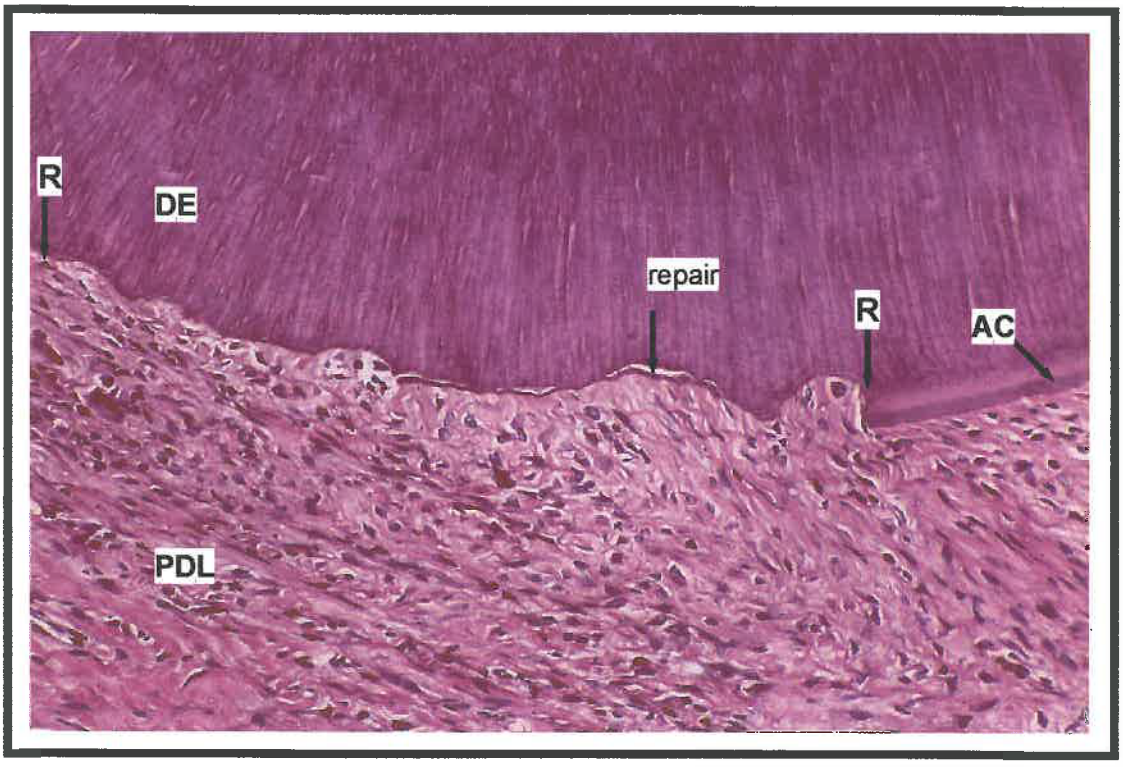


Figure 5.11B: High power view of Figure 5.10B (boxed area) showing a large area of resorption (R-R) with repair evident. (DE = dentine, AC = acellular cementum).
Slide: MBE40 - Section 259 RHS
Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 20X. Total mag. = 272.74X

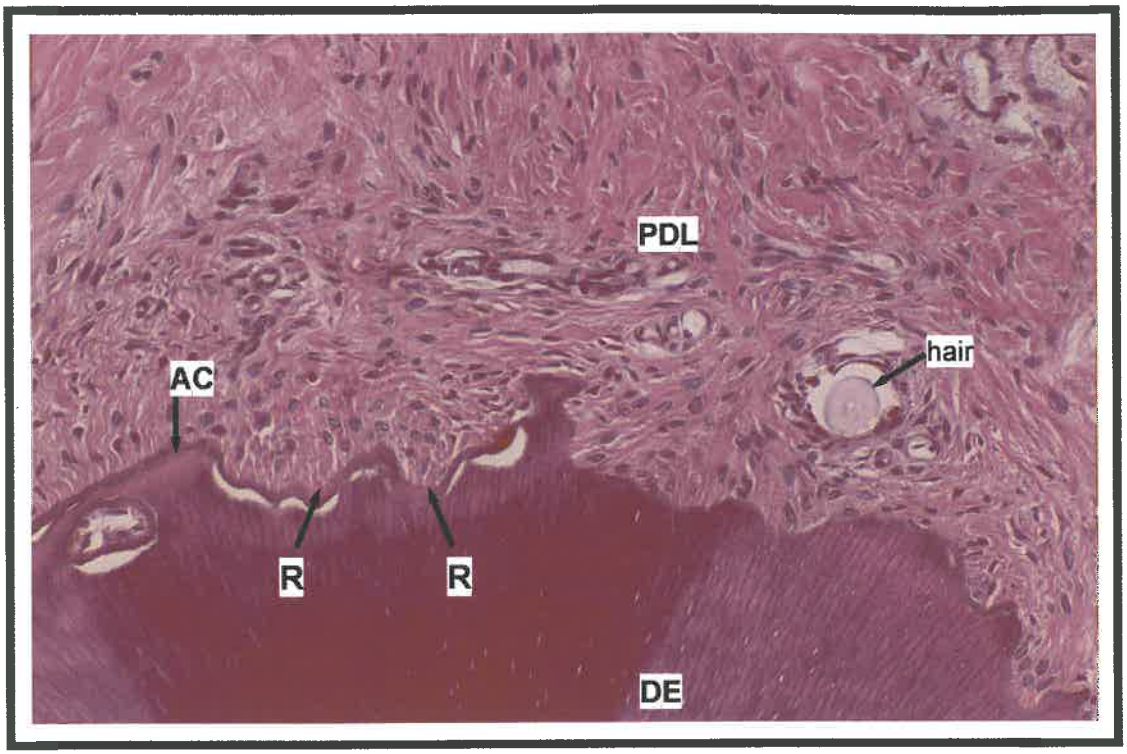


Figure 5.12A: Resorption (R) in a control tooth (83 day old rat). (AC = acellular cementum, DE = dentine). Note the embedded hair.
Slide: MBC3 - Section 181 LHS - mesial aspect
Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 20X. Total mag. = 273.71X

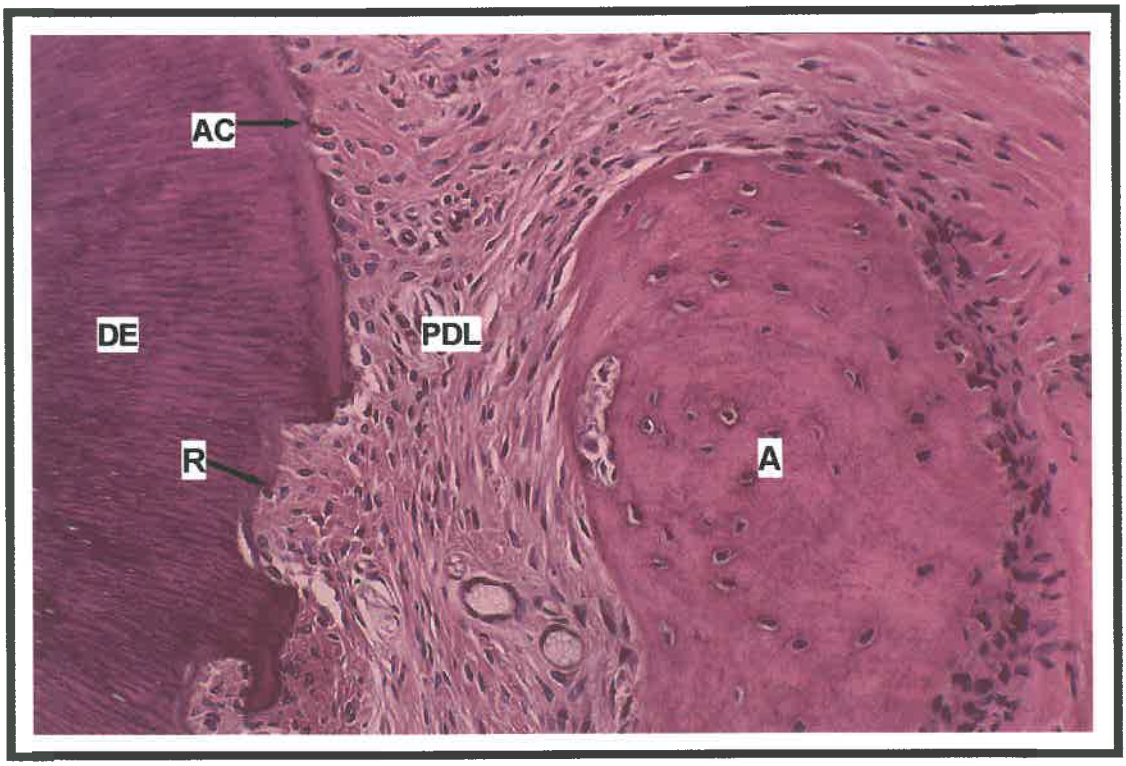


Figure 5.12B: Resorption (R) in a control tooth. (DE = dentine, AC = acellular cementum, A = alveolar bone).
Slide: MBC3 - Section 201 LHS - mesial aspect
Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 20X. Total mag. = 272.74X

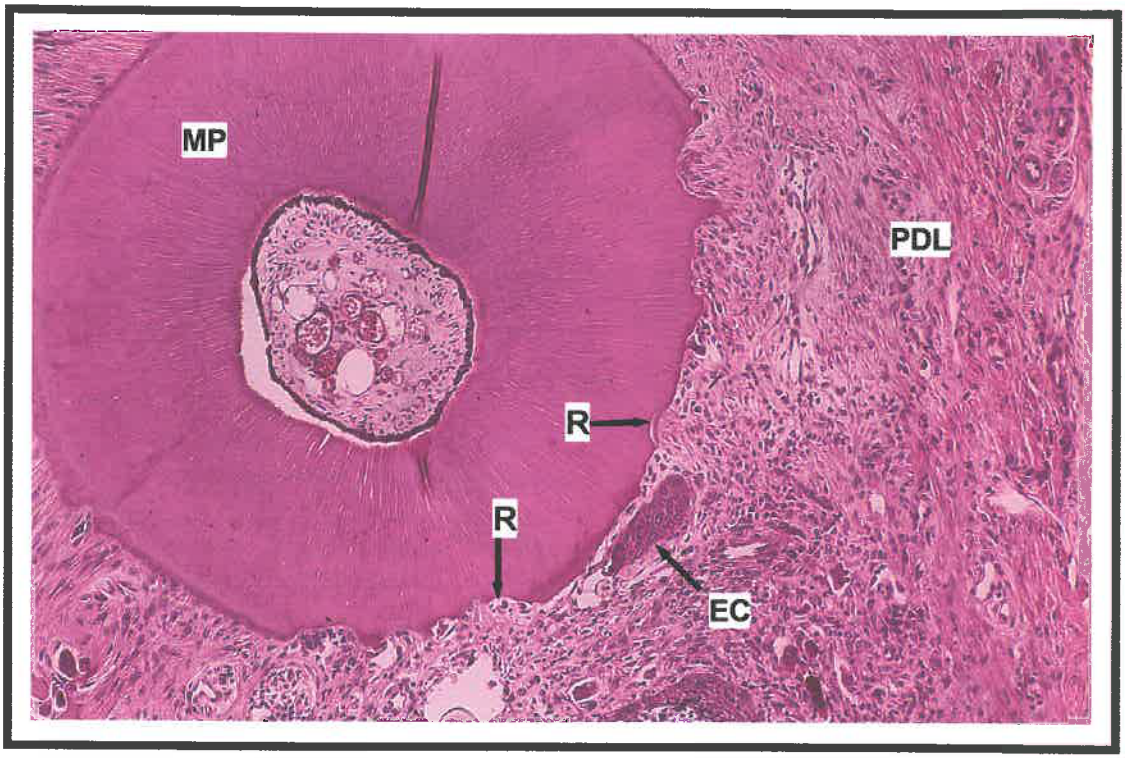


Figure 5.13: A cluster of epithelial cells (EC) adjacent to an area of root resorption (R) of the midpalatal root (1 wk activation)
Slide: MBE42 - Section 285 RHS - mid palatal root
Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 10X. Total mag. = 136.85X

This cluster of epithelial cells may be an epithelial rest, however due to its size and coronal location it is unlikely to be one. It is more likely to be a rete peg or a sulcular epithelial downgrowth as an inflammatory response to the ligament placed around the tooth to hold the orthodontic spring in place.

It is interesting to note, however, that there is a reduced resorption of the tooth in the area directly adjacent to the epithelial cells as opposed to the more advance resorption either side of this cluster of cells.

5.4.3 BONE RESORPTION

In this study only one of the 28 experimental teeth (one week activation) demonstrated hyalinization within the periodontal ligament and concomitant undermining bone resorption (Figure 5.14 A - C and 5.15 A and B).

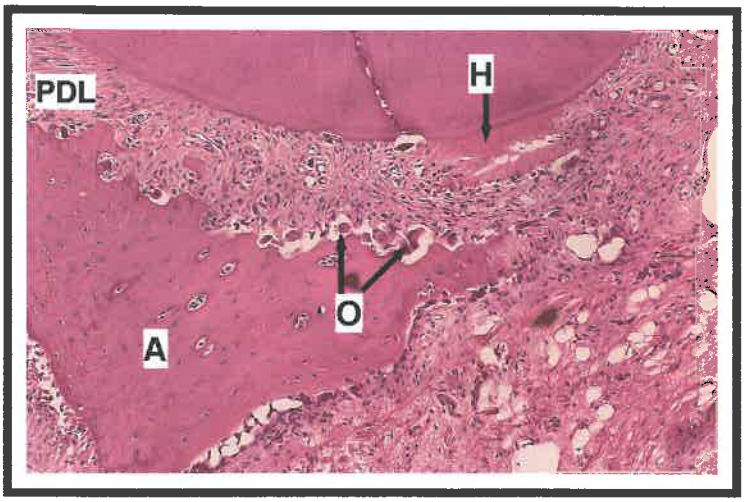


Figure 5.14A: A small area of hyalinization (H) adjacent to the tooth. O = osteoclasts.
Slide: MBE42 - Section 269 RHS Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 10X. Total mag. = 88.32X

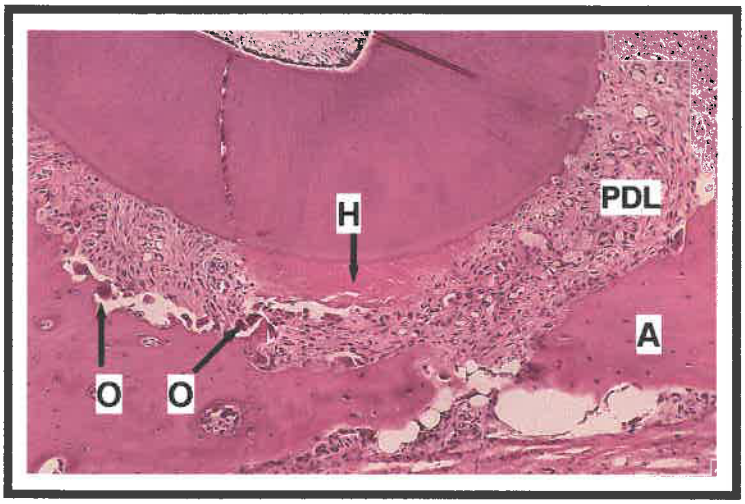


Figure 5.14B: A larger area of hyalinization (H) further down the tooth. A band of osteoclasts (O) can be seen adjacent to the alveolar bone (A).
Slide: MBE42 - Section 276 RHS Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 10X. Total mag. = 88.32X

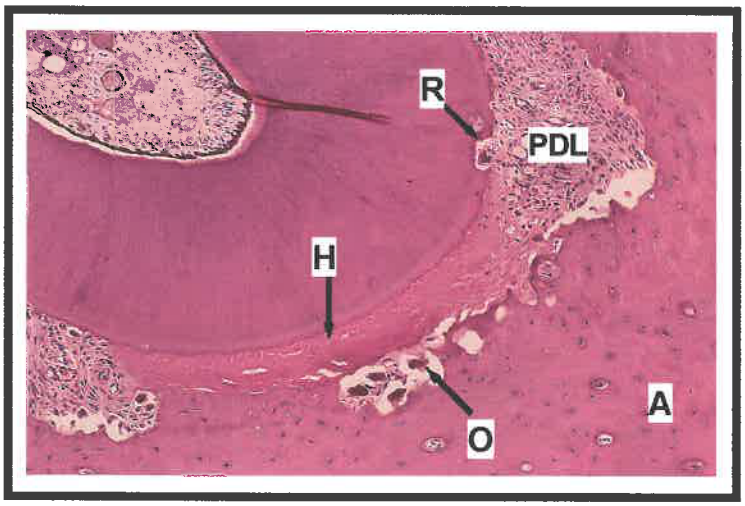


Figure 5.14C: A complete area of hyalinization (H) from tooth to alveolar bone (A) with undermining resorption lacunae filled with osteoclasts (O).
Slide: MBE42 - Section 291 RHS Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 10X. Total mag. = 88.32X

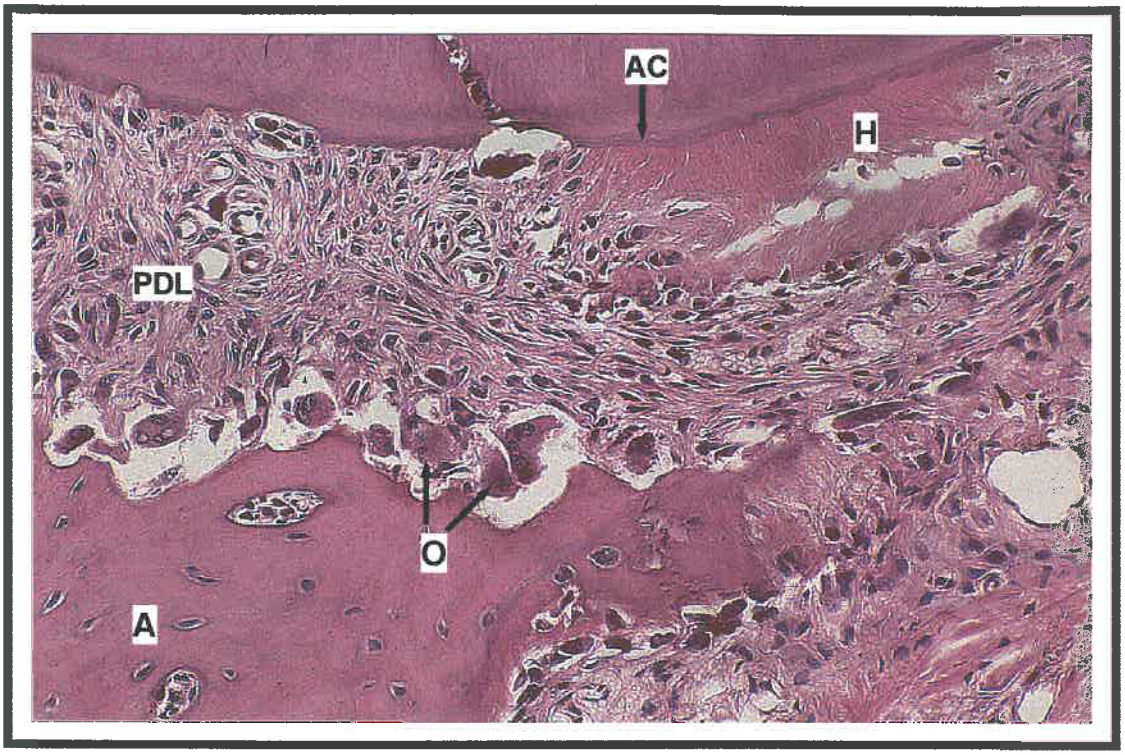


Figure 5.15A: High power view of 5.14A. (H = hyalinization, O = osteoclasts).
Slide: MBE42 - Section 269 RHS
Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 20X. Total mag. = 272.74

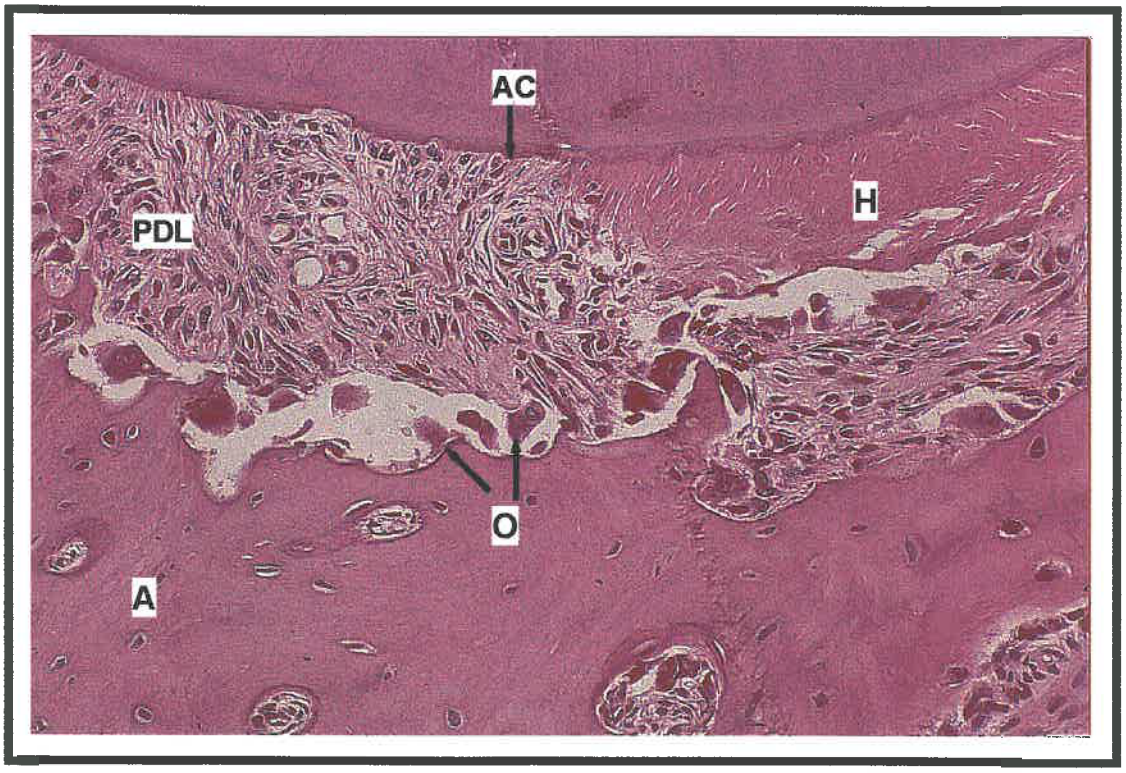


Figure 5.15B: High power view of 5.14B. (H = hyalinization, O = osteoclasts).
Slide: MBE42 - Section 276 RHS
Stain: Haematoxylin and eosin
Magnification: PET 3.3, Objective 20X. Total mag. = 272.74X

5.4.4 MAST CELLS AND BONE

Whilst initially counting only the quadrant distribution of mast cells it became apparent that most of the mast cells seemed to be closer to the bone side rather than the tooth side of the periodontal ligament. It was then decided to further subdivide the ligament into horizontal thirds and recount the material so that this apparent trend could be measured statistically (refer to Results section 5.5.3.1).

5.4.5 MAST CELLS AND BLOOD VESSELS

During the initial counting sessions it also became apparent that most of the mast cells seemed to be in close proximity to blood vessels as shown in Figures 5.16 and 5.17. When the material was recounted it was noted whether the mast cells in the periodontal ligament were within a certain distance (12 microns) of any blood vessels so this apparent trend could be measured statistically (refer to Results section 5.5.5).

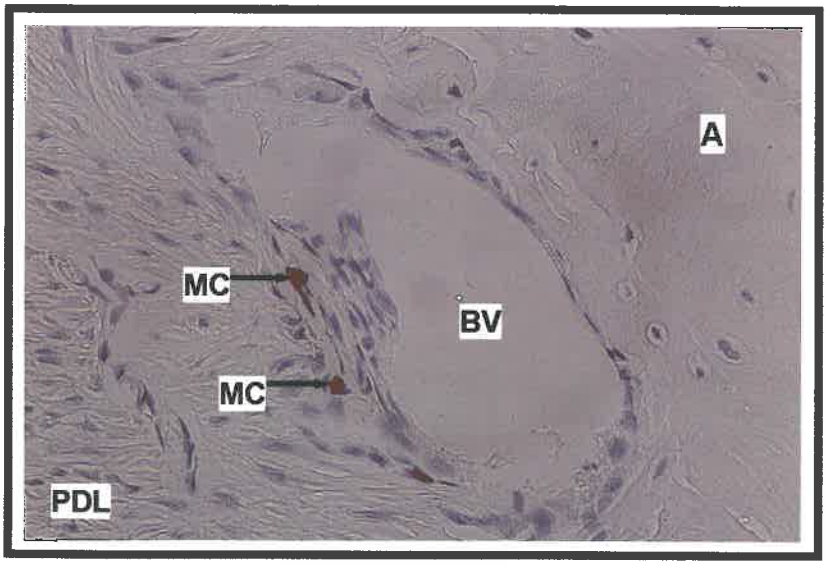


Figure 5.16: Three mast cells (MC) in close proximity to a blood vessel (BV) (83 day old control rat). A = alveolar bone.
Slide: MBC3 - Section 350 RHS
Stain: 0.5% toluidine blue pH 0.5
Magnification: PET 3.3, Objective 40X. Total mag. = 398.37X

In a couple of instances, mast cells were even found in the lumen of blood vessels (Figure 5.17). This was an interesting finding that would not have been discovered had the tissue been perfusion fixed.

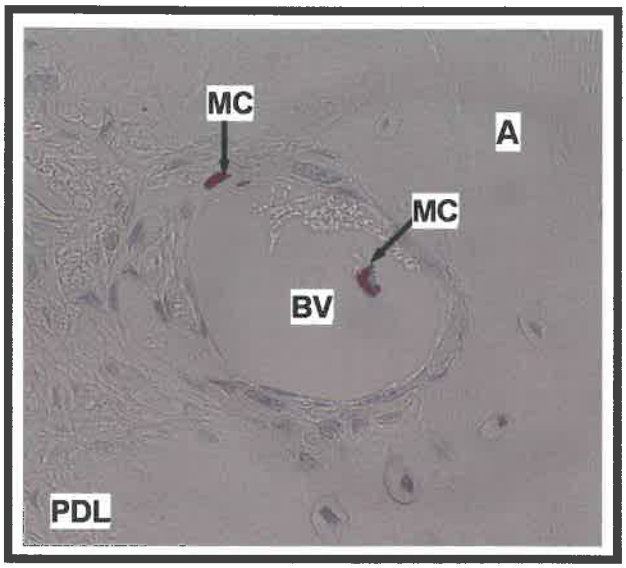


Figure 5.17: One mast cell (MC) in close proximity to and one mast cell (MC) in a blood vessel (BV). (111 day old control rat). A = alveolar bone.
Slide: MBC9 - Section 358 RHS
Stain: 0.5% toluidine blue pH 0.5
Magnification: PET 3.3, Objective 40X. Total mag. = 472.15X

5.4.6 MAST CELL MITOSIS

From the thousands of sections viewed in this study one mitotic figure was observed in the periodontal ligament of a control animal (Figure 5.18). From reports in the literature (Galli *et al.*, 1984 and 1986; Dvorak, 1991) mitosis of mature mast cells is a rare occurrence so finding this solitary figure provided considerable excitement!

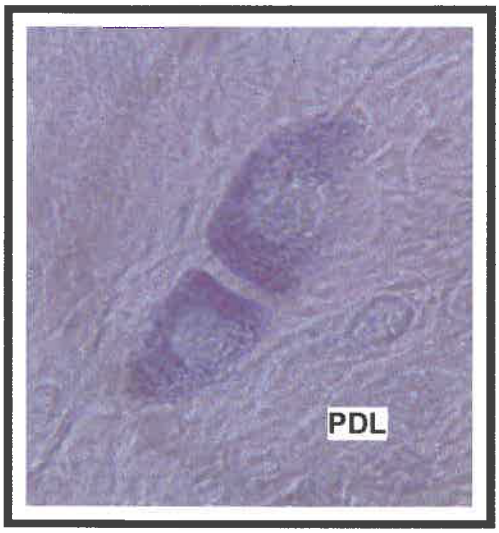


Figure 5.18: A mitotic mast cell in the PDL of a 90 day control
Slide: MBC7 - Section 203 LHS
Stain: 0.5% toluidine blue pH 0.5
Magnification: PET 3.3, Objective 100X. Total mag. = 1500X

5.5 STATISTICAL ANALYSES

5.5.1 TREATMENT EFFECT - THE EXPERIMENTAL RATS

The treatment effect was ascertained by statistical analysis of a subset of data collected for each of the 27 experimental animals (28 animals less rat 26 mentioned earlier).

The statistical analysis sought to determine whether there was a co-relation in the changes observed in the number and distribution of mast cells in the PDL of the tooth activated by the appliance, the right first maxillary molar (the "experimental tooth" or "experimental teeth") using the corresponding left first maxillary molar as a control (the "control tooth" or "control teeth").

The following Table (5.1) highlights the results obtained from an analysis of variance of the overall data, containing only the experimental rats:

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
rat	26	87.787	3.376	1.455	0.065
tooth	1	21.829	21.829	9.404	0.002
rat:tooth	26	41.476	1.595	0.687	0.879
Residuals	1890	4387.111	2.321		

Table 5.1: Analysis of variance of treatment effect.

This analysis indicated that there were quite large treatment differences between the left (control) and right (experimental) tooth (Mean Sq = 21.83, Pr = .002).

Observation One: There were quite large treatment differences between control and experimental teeth.

The mean mast cell count for each of the rats was obtained by counting the mast cells in 3 levels (coronal, mid, apical) x 4 quadrants (buccal, mesial, palatal and distal) x 3 horizontal thirds (bone, mid, tooth) ÷36 (areas studied). The following Table (5.2) summarises the mean mast cell count for each of the experimental rats. The average for each of the time groups is highlighted (in bold).

Table 5.2 indicates that generally, the control teeth had higher mast cell counts than the experimental teeth. However, this trend was reversed in the one hour time group where three of the five rats demonstrated more mast cells in the experimental teeth.

TIME	RAT	CONTROL TOOTH	EXPERIMENTAL TOOTH
15 min	1	0.722	0.750
	2	1.056	0.472
	3	1.139	0.583
	4	0.778	1.056
	5	0.639	0.333
	AVERAGE	**0.867**	**0.639**
1 hour	6	0.389	0.556
	7	0.444	0.750
	8	0.444	0.722
	9	0.778	0.667
	10	0.889	0.528
	AVERAGE	**0.589**	**0.644**
4 hours	11	0.944	0.556
	12	1.222	0.611
	13	0.583	0.556
	14	0.778	0.500
	15	1.389	0.639
	AVERAGE	**0.983***	**0.572***
24 hours	16	0.667	0.250
	17	0.917	0.528
	18	0.861	0.472
	19	0.667	0.139
	20	0.694	0.361
	AVERAGE	**0.761***	**0.350***
1 week	21	0.139	0.167
	22	0.278	0.194
	23	0.722	0.361
	24	0.583	0.222
	25	0.361	0.389
	AVERAGE	**0.417**	**0.267**
2 weeks	26	N/A	N/A
4 weeks	27	0.556	0.722
8 weeks	28	0.306	0.139

Table 5.2: Mean mast cell counts throughout the PDL of 28 experimental animals. Asterisks (*) indicate significant differences between means ($p \leq 0.005$)

Observation Two: Although control mast cell numbers were generally higher than experimental numbers, treatment differences were highly significant at 4 hrs and 24 hrs.

5.5.2 TREATMENT AND TIME EFFECT

Having identified that there is a significant treatment effect evident in the subset of data containing only the experimental rats, further analysis was undertaken to determine whether the effect of treatment changed over time. A split-plot analysis was performed using the 54 means corresponding to the two teeth for each of the 27 rats to see if there was an interaction of treatment and time.

The first part of this analysis examined the average of the left and right teeth, so it includes control and experimental teeth, and identified any significant changes over time, relative to the differences between rats within a time group. The second part of the analysis sought to identify any overall difference between the control tooth and the experimental tooth and whether that difference changed with time.

Error: between rats (i.e. how the rats differ from one another)

	Df	Sum of Sq	Mean of Sq	F Value	Pr(F)
Time	6	1.527	0.254	5.583	0.002
Residuals	20	0.912	0.046		
TOTAL	**26**	**2.439**			

Error: within rats (i.e. how left teeth within rats differ from right teeth.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Tooth (L vs R)	1	0.606	0.606	17.498	0.000*
Time: Tooth[1]	6	0.460	0.077	2.213	0.085
Residuals	20	0.693	0.035		
TOTAL	**27**	**1.759**			

Table 5.3: Split-plot analysis of treatment and time effect. Asterisk (*) indicates a significant treatment effect (P=0.00046).

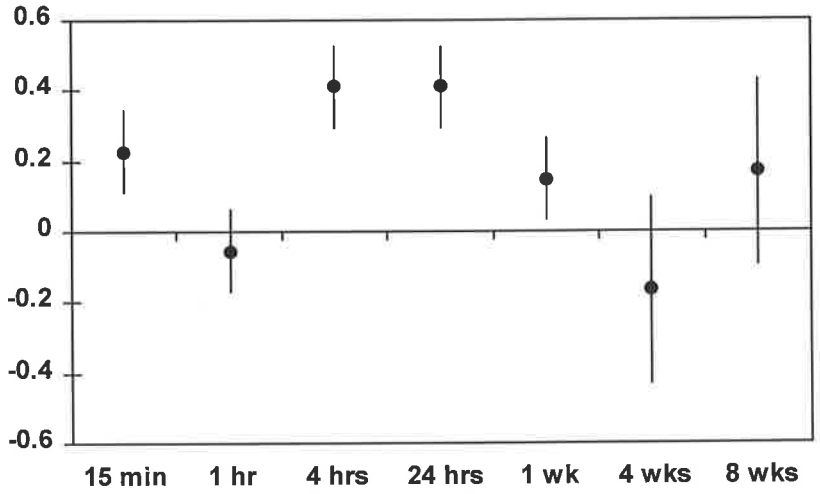
The results obtained from the first part of this analysis (Table 5.3) indicated that there is a highly significant difference between time groups (Time P=0.002). However, this difference was not considered to be important because of the low F value (5.583).

[1] Time:Tooth = left vs right difference over time groups

The results from the second part of this analysis (Table 5.3) indicated a highly significant effect related to treatment, ie. comparing control and experimental sides (Tooth: P=0.000) which is important (F value = 17.498). However, the analysis did not indicate any relationship between time and treatment (Time:Tooth P=0.085).

The treatment effect (control tooth vs experimental tooth) at each time is a difference between two means. In terms of the model developed, each tooth mean can have a variance of about 0.040 which is the average of the two residual mean squares above ((0.046+0.035)÷2). The difference between the tooth means for a particular rat will have a variance of twice the bottom residual mean square ie. 2 x 0.035=0.07. The first five time means are all means of 5 rats, which equates to 0.014. The differences were then plotted in Figure 5.19, along with appropriate error bars. A horizontal line at zero represents no difference between control and experimental teeth. In this graph negative values would imply that the number of mast cells increases under treatment.

Figure 5.19: Plot of differences between control and experimental teeth vs time for experimental animals



(±one standard error of the mean)

A paired *t*-test of experimental periods of 4 and 24 hours was performed:

Difference between controls and treated teeth

Standard error of the difference

$$= \frac{0.411}{0.12}$$

= 3.4 at 2° of freedom

∴ **is significant at P = 0.005**

This indicates the differences between controls and experimental teeth at 4 and 24 hours were highly significant. In Figure 5.19 this can be seen graphically as at 4 hrs and 24 hrs the differences and their error bars are quite removed from zero line (ie. no difference). This is in contrast to the other time slots which may be close enough to zero line to question if there are any differences between control and experimental teeth.

5.5.3 POSITIONAL EFFECTS - THE EXPERIMENTAL RATS

The positional effect relates to the influence of orthodontic treatment on the mast cell distribution at all of the recorded positions within the ligament. This is demonstrated in the following analysis of variance (Table 5.4) which specifies the effect of position (by level, quadrant and horizontal distribution) for the experimental animals.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
time	6	54.965	9.161	5.350	0.000
tooth	1	21.829	21.829	12.748	0.000
level	2	129.262	64.631	37.745	0.000*
quadrant	3	9.772	3.257	1.902	0.127
horizontal	2	888.188	444.094	259.350	0.000*
time:tooth	6	16.565	2.761	1.612	0.140
time:level	12	29.643	2.470	1.443	0.139
time:quadrant	18	22.090	1.227	0.717	0.797
time:horizontal	12	64.417	5.368	3.135	0.000
tooth:level	2	58.427	29.214	17.061	0.000*
tooth:quadrant	3	18.224	6.075	3.548	0.014
tooth:horizontal	2	15.908	7.954	4.645	0.010
Residuals	1874	3208.913	1.712		

Table 5.4: Analysis of variance of positional effect. Asterisks (*) indicate significant positional effects: horizontal $p \leq 1 \times 10^{-7}$, level $p \leq 1 \times 10^{-7}$, tooth: level $p \leq 1 \times 10^{-7}$.

The significance of the positional effect is indicated by the mean square of the mast cell distribution - the higher the number, the higher the effect. This shows that the horizontal position (ie. bone, mid, tooth) of the mast cells within each section is most significant (Mean Sq = 444.09). The next important positional effect is level (Mean Sq = 64.63) with least being quadrant (Mean Sq = 3.26). Also important, is the interaction of level (coronal, mid, apical) and tooth (experimental, control) (Mean Sq = 29.21).

5.5.3.1 Horizontal Distribution

Further analysis of some of the means was undertaken to understand where the changes occurred. Note that all of these means refer only to experimental rats. The following Table (5.5) indicates the effect of the horizontal position which was identified as the most significant in Table 5.4.

CONTROL TEETH			
TIME	MEAN MAST CELL COUNTS IN EACH HORIZONTAL THIRD		
	bone	mid	tooth
15 min	2.050	0.400	0.150
1 hour	1.583	0.150	0.033
4 hours	2.400	0.517	0.033
24 hours	2.017	0.233	0.033
1 week	1.083	0.117	0.050
4 weeks	1.417	0.250	0.000
8 weeks	0.833	0.083	0.000
Left Average:	**1.775**	**0.275**	**0.056**

EXPERIMENTAL TEETH			
TIME	MEAN MAST CELL COUNTS IN EACH HORIZONTAL THIRD		
	bone	mid	tooth
15 min	1.600	0.233	0.083
1 hour	1.750	0.133	0.050
4 hours	1.617	0.083	0.017
24 hours	0.983	0.033	0.033
1 week	0.683	0.100	0.017
4 weeks	2.167	0.000	0.000
8 weeks	0.333	0.083	0.000
Right Average:	**1.321**	**0.111**	**0.037**

Table 5.5: Mean mast cell counts dividing PDL into horizontal thirds.

Table 5.5 contains the means for each time for each side (ie. control and experimental), and the averages over time as well. There appears from the ANOVA (Table 5.4) to be an interaction of

time and horizontal distribution and an interaction of treatment and horizontal distribution. In particular, the analysis in Table 5.5 indicates that:

Observation Three: There were huge differences between bone, mid and tooth thirds of the ligament ($p \leq 1 \times 10^{-7}$).

Observation Four: The greatest number of mast cells were found in the bone third, followed by the mid third, with the least found adjacent to the tooth.

Observation Five: There were more mast cells in each horizontal third on the control side than on the experimental side.

The data collated in Table 5.5 has been expressed graphically in Figure 5.20.

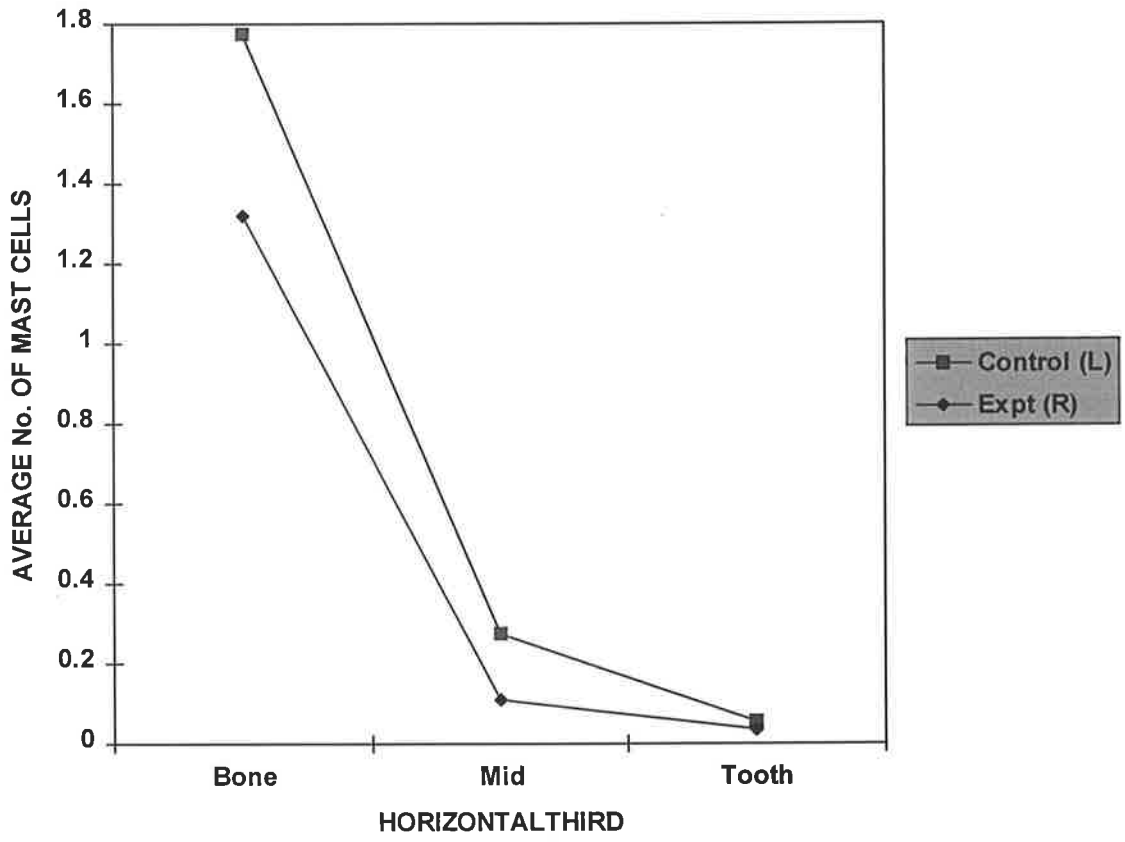


Figure 5.20: Plot of horizontal third means L vs R for experimental animals.

The significance of the changes shown in the horizontal distribution as a response to tooth movement is demonstrated in the following analysis of variance for the experimental animals (Table 5.6).

Error: between rats.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
time (tm)	6	4.580	0.763	5.582	0.002
Residuals	20	2.735	0.137		
TOTAL	**26**	**7.316**			

Error: within rats.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
treatment (tr)	1	1.819	1.819	20.748	0.000
horizontal (hor)	2	74.016	37.008	422.098	0.000
tm:tr	6	1.380	0.230	2.624	0.021*
tm:hor	12	5.368	0.447	5.102	0.000*
tr:hor	2	1.326	0.663	7.560	0.001*
tm:tr:hor	12	1.747	0.146	1.661	0.087
Residuals	100	8.768	0.088		
TOTAL	**135**	**94.424**			

Table 5.6: Analysis of variance of treatment, horizontal distribution and time interactions. * = significant.

This shows that the interactions of time: treatment (P=0.021), time: horizontal (P=1 x 10^{-6}) and treatment: horizontal (P = 0.001) are significant.

Analysing the square roots of the actual counts demonstrates that the estimated residual variance is 0.3008. This shows that the counts are essentially Poisson (close to 0.25) and hence we can base comparisons on Poisson variability (Snedecor and Cochran, 1967).

x

Considering the analysis on the log scale shows if any changes demonstrated in Table 5.6 are proportional; such changes would appear additive on the log scale.

Error: between rats.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
time (tm)	6	12.290	2.048	2.746	0.041
Residuals	20	14.916	0.746		
TOTAL	**26**	**27.206**			

Error: within rats.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
treatment (tr)	1	6.309	6.309	17.848	0.000
horizontal (hor)	2	268.464	134.232	379.764	0.000
tm:tr	6	4.144	0.691	1.954	0.079
tm:hor	12	5.127	0.427	1.209	0.288
tr:hor	2	2.990	1.495	4.229	0.017*
tm:tr:hor	12	4.847	0.404	1.143	0.335
Residuals	100	35.346	0.354		
TOTAL	**135**	**327.227**			

Table 5.7: Analysis of variance (log scale) of treatment, horizontal distribution and time interactions. * = significant.

In Table 5.7 of the three significant interactions shown in Table 5.6 only the treatment:horizontal interaction remains significant (P=0.017) and the others disappear. This implies that all other interactions are essentially "proportional" in nature.

From Table 5.6 the first significant interaction is time:treatment (P = 0.021) for which further data is provided in Table 5.8.

TIME	TOOTH	
	Left	Right
15 mins	0.87	0.64
1 hr	0.59	0.64
4 hrs	0.98	0.57
24 hrs	0.76	0.35
1 week	0.42	0.27
4 weeks	0.56	0.72
8 weeks	0.31	0.14
Mean	**0.702**	**0.490**

Table 5.8: Time: Treatment Interaction indicating the mean mast cell counts throughout the PDL in each time group.

From the analysis of the logs (Table 5.7) there is a proportional change between the two columns in Table 5.8. This is best estimated by the ratio Right: Left (0.490:0.702) where the values for the right teeth are 69.8% of the values for the left teeth, consistently across the times. The standard error of this value is 4.2% so that a 95% confidence interval for the Right:Left ratio is 69.8±8.4%

(61.4, 78.2%). This illustrates an interesting effect where orthodontic tooth movement causes a proportional drop in mast cells throughout the ligament.

From Table 5.6 the second significant interaction is time:horizontal distribution ($P = 1 \times 10^{-6}$) for which further data is provided in Table 5.9.

TIME	HORIZONTAL THIRD		
	Bone	Mid	Tooth
15 mins	1.825	0.317	0.117
1 hr	1.667	0.142	0.042
4 hrs	2.008	0.300	0.025
24 hrs	1.500	0.133	0.033
1 week	0.883	0.108	0.033
4 weeks	1.792	0.125	0.000
8 weeks	0.583	0.083	0.000
Mean	**1.548**	**0.193**	**0.046**
SE (Mean)	**0.049**	**0.017**	**0.008**

Table 5.9: Time: Horizontal Distribution Interaction indicating the mean mast cell counts in each horizontal third for each time group.

Again, the evidence from the logs (Table 5.7) is that the changes in Table 5.9 are proportional, and the same at each time. The ratios of the means of bone:mid:tooth thirds of 1.548:0.193:0.046 are the best estimate of these. There are clearly highly significant differences ($P = 1 \times 10^{-6}$) between the horizontal thirds, although it must be noted that these readings are each the average of left and right teeth and therefore include the treatment effect of the right teeth.

From Table 5.6 the third significant interaction is the treatment:horizontal distribution ($P = 0.001$) for which further data is provided in Table 5.10. The analysis of the logs in Table 5.7 indicates that the only significant interaction is between treatment (left vs right) and horizontal distribution. This represents an effect of the orthodontic tooth movement, which is the same regardless of time (Table 5.10).

TOOTH	HORIZONTAL THIRD		
	Bone	Mid	Tooth
Left	1.775	0.275	0.056
Right	1.321	0.111	0.037
R:L	**0.74**	**0.41**	**0.67**
SE (R:L)	**0.048**	**0.081**	**0.239**

Table 5.10: Treatment:Horizontal Distribution Interaction indicating the mean mast cell counts for each horizontal third for treated (right) and control (left) teeth.

It appears that the right counts are depressed much further for the middle third, where the right is 41% of the left, with a 95% confidence interval of (25%, 57%), than for the bone third where the right is 74% of the left, with a 95% confidence interval of (64%, 84%), with the low counts in the tooth third leaving us unsure of how the mast cell counts are changing, if at all.

5.5.3.2 Vertical Distribution

Table 5.11 examines the effect of the level on the mast cell distribution, identified in Table 5.4 as the next most significant positional effect.

CONTROL TEETH			
TIME	MEAN MAST CELL COUNTS IN EACH VERTICAL THIRD		
	coronal	middle	apical
15 min	1.050	0.333	1.217
1 hour	0.533	0.167	1.067
4 hours	1.750	0.217	0.983
24 hours	1.150	0.200	0.933
1 week	0.600	0.167	0.483
4 weeks	0.833	0.083	0.750
8 weeks	0.250	0.167	0.500
Left Average:	**0.981**	**0.210**	**0.914**

EXPERIMENTAL TEETH			
TIME	MEAN MAST CELL COUNTS IN EACH VERTICAL THIRD		
	coronal	middle	apical
15 min	0.683	0.350	0.883
1 hour	0.400	0.350	1.183
4 hours	0.233	0.333	1.150
24 hours	0.050	0.250	0.750
1 week	0.067	0.233	0.500
4 weeks	0.583	0.667	0.917
8 weeks	0.000	0.250	0.167
Right Average:	**0.287**	**0.315**	**0.867**

Table 5.11: Mean mast cell counts dividing PDL into vertical thirds.

On the control side the greatest number of mast cells occurred in the coronal and apical levels with considerably less found in the middle third. The distribution changes in the experimental side such that the coronal mast cell numbers drop dramatically with essentially no change of the mast cell numbers in the middle and apical levels.

Observation Six: Anova for tooth: vertical level gave a $p \leq 1 \times 10^{-7}$ indicating a difference between these levels for control and experimental teeth, but not specifying which direction.

This can be seen in the analysis of variance of positional effect (Table 5.4) where Mean Sq = 64.631 and $p \leq 1 \times 10^{-7}$ for level distribution.

Observation Seven: A large difference was evident between coronal levels in control and experimental teeth (control > experimental).

Observation Eight: Data from control teeth indicate that both coronal and apical values were much higher than mid-areas.

The data from Table 5.11 has been expressed graphically in Figure 5.21.

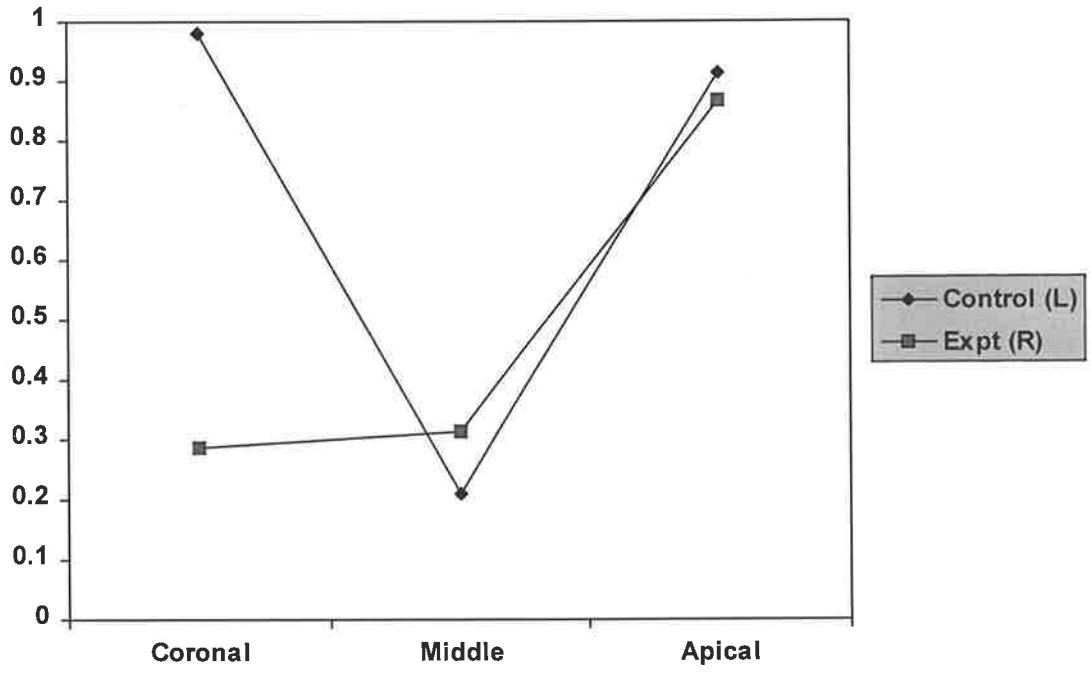


Figure 5.21: Plot of vertical distribution L vs R for experimental animals.

The significance of the changes shown in the vertical distribution as a response to tooth movement is demonstrated in the following analysis of variance for the experimental animals (Table 5.12).

Error: between rats.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
time (tm)	6	4.580	0.763	5.582	0.002
Residuals	20	2.735	0.137		
TOTAL	**26**	**7.316**			

Error: within rats.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
treatment (tr)	1	1.819	1.819	14.436	0.000
level (lv)	2	10.772	5.386	42.742	0.000
tm:tr	6	1.380	0.230	1.826	0.102
tm:lv	12	2.470	0.206	1.634	0.094
tr:lv	2	4.869	2.434	19.320	0.000*
tm:tr:lv	12	2.707	0.226	1.790	0.060
Residuals	100	12.601	0.126		
TOTAL	**135**	**36.618**			

Table 5.12: Analysis of variance of treatment, vertical distribution (level) and time interactions. * = significant.

This shows that the interaction of treatment: level is the only significant interaction ($P = 1 \times 10^{-7}$) and that there are not significant differences between the levels overall (Table 5.14), only differences in how they react to the treatment (Table 5.16).

The analysis in Table 5.12 is considered on the log scale to see if the interactions are essentially proportional (Table 5.13).

Error: between rats.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
time (tm)	6	21.631	3.605	5.230	0.002
Residuals	20	13.786	0.689		
TOTAL	**26**	**35.417**			

Error: within rats.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
treatment (tr)	1	7.188	7.188	11.860	0.001
level (lv)	2	44.097	22.049	36.379	0.000
tm:tr	6	6.232	1.039	1.714	0.125
tm:lv	12	5.275	0.440	0.725	0.724
tr:lv	2	26.856	13.428	22.156	0.000*
tm:tr:lv	12	8.859	0.738	1.218	0.281
Residuals	100	60.608	0.606		
TOTAL	**135**	**159.116**			

Table 5.13: Analysis of variance (log scale) of treatment, vertical distribution (level) and time interactions. * = significant.

Again, all interactions on the log scale are non-significant except for the treatment: level interaction which is highly significant ($p \leq 1 \times 10^{-7}$) and therefore non-proportionate in nature.

TIME	VERTICAL LEVEL		
	Coronal	**Middle**	**Apical**
15 mins	0.867	0.342	1.050
1 hr	0.467	0.258	1.125
4 hrs	0.992	0.275	1.067
24 hrs	0.600	0.225	0.842
1 week	0.333	0.200	0.492
4 weeks	0.708	0.375	0.833
8 weeks	0.125	0.208	0.333
Mean	**0.634**	**0.262**	**0.890**
SD	**0.031**	**0.020**	**0.037**

Table 5.14: Time:Vertical level interaction indicating the mean mast cell counts in each vertical level for each time group.

As before, the lack of interaction on the log scale (Table 5.13) suggests that, for each time, the three levels are proportional, and those proportions are best estimated as 0.634:0.262:0.890 (Table 5.14).

To calculate the standard deviations on these means they need to be converted into counts. Since each is a mean of 648 observations (27 rats x 4 quadrants x 3 levels x 2 teeth = 648), the standard

deviation of each is the square root of its value divided by the square root of 648. For example, the coronal mean 0.634 has a standard deviation of sqrt (0.634) ÷sqrt (648) = 0.031.

Ratios of the means of the vertical levels gives Table 5.15:

	Ratio	SE (Ratio)
Coronal: Middle	2.42	0.22
Coronal: Apical	0.71	0.046
Middle: Apical	0.29	0.026

Table 5.15: Ratios of the means of the vertical levels with their standard errors.

The standard errors for these ratios are given by: SE(x/y) = (x/y) x sqrt ($1/x$ + $1/y$) where x and y are the original counts. So, for example, the coronal:middle ratio 0.634/0.262 = 2.42 has an estimated standard error of: 2.42 x sqrt (1/(0.634 x 648) + (0.262 x 648)) = 0.22.

From Table 5.12 the only significant interaction is the treatment:vertical level distribution (P = 1 x 10^{-7}) for which further data is provided in Table 5.16.

The analysis of the logs in Table 5.13 indicates that the significant interaction is between treatment (left vs right) and vertical level. This represents an effect of the orthodontic tooth movement, which is the same, regardless of time (Table 5.16).

TOOTH	VERTICAL LEVEL		
	Coronal	Middle	Apical
Left	0.981	0.210	0.914
Right	0.287	0.315	0.867
R:L	**0.29**	**1.50**	**0.95**
SE (R:L)	**0.034**	**0.234**	**0.023**

Table 5.16: Treatment: Vertical level interaction indicating the mean mast cell counts for each vertical level for treated (right) and control (left) teeth.

It appears that the right counts are markedly depressed for the coronal level, where the right is 29% of the left. The right counts are elevated for the middle level, where the right is 150% of the left. The apical level shows essentially no change where the right is 95% of the left.

5.5.3.3 Quadrant Distribution

Table 5.17 examines the effect of the tooth quadrants on the mast cell distribution, identified in Table 5.4 as having the third most significant positional effect.

CONTROL TEETH				
TIME	MEAN MAST CELL COUNTS IN EACH QUADRANT			
	buccal	mesial	palatal	distal
15 min	0.822	0.978	0.889	0.778
1 hour	0.689	0.533	0.489	0.644
4 hours	1.244	1.533	0.600	0.556
24 hours	0.933	1.089	0.556	0.467
1 week	0.822	0.289	0.311	0.244
4 weeks	0.778	0.444	0.444	0.556
8 weeks	0.556	0.111	0.111	0.444
Left Average:	**0.885**	**0.840**	**0.547**	**0.535**

EXPERIMENTAL TEETH				
TIME	MEAN MAST CELL COUNTS IN EACH QUADRANT			
	buccal	mesial	palatal	distal
15 min	0.667	0.889	0.578	0.422
1 hour	0.733	0.289	0.778	0.778
4 hours	0.333	0.533	0.622	0.800
24 hours	0.244	0.467	0.311	0.378
1 week	0.156	0.356	0.111	0.444
4 weeks	0.444	1.000	0.556	0.889
8 weeks	0.111	0.333	0.111	0.000
Right Average:	**0.416**	**0.519**	**0.469**	**0.556**

Table 5.17: Mean mast cell counts in each quadrant of the PDL.

The analysis in Table 5.17 indicates that:

Observation Nine: On the control side there are generally more mast cells in the buccal and mesial quadrants than in the palatal and distal quadrants.

The distribution changes in the experimental side such that the mast cell numbers in the buccal and mesial quadrants are markedly reduced but there is essentially no change in the numbers in the palatal and distal quadrants.

Observation Ten: A large difference was evident between the buccal and mesial quadrants in the control and experimental teeth (control > experimental) indicating a large treatment effect).

On the control side if the buccal and mesial quadrant averages are added together they are always greater than the combined palatal and distal quadrant averages for each time interval.

On the experimental side if the buccal and mesial quadrant averages are added and the palatal and distal quadrant averages are added the mast cell distribution is:

15 min	-	same pattern as for the control side ie. B+M>P+D
1 hr & 4 hrs	-	strong reversal ie. P+D>B+M
24 hrs, 1 week & 4 weeks	-	equivalence ie. B+M= P+D
8 weeks	-	return to original pattern ie. B+M>P+D

The data collected in Table 5.17 has been expressed graphically in Figure 5.22.

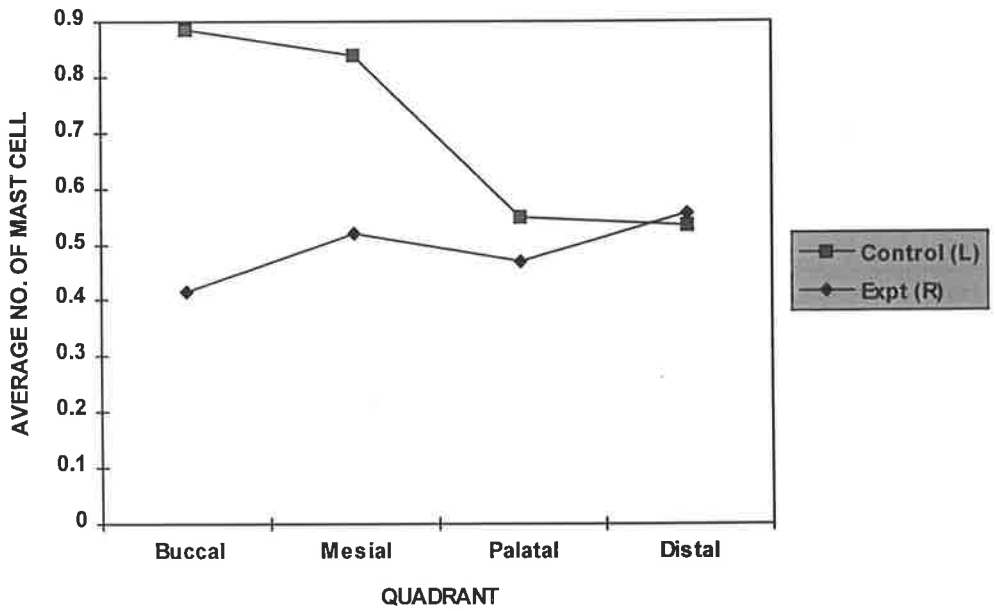


Figure 5.22: Plot of quadrant distribution L vs R for experimental animals.

The significance of the changes shown in the quadrant distribution as a response to tooth movement is demonstrated in the following analysis of variance for the experimental animals (Table 5.18).

Error: between rats.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
time (tm)	6	6.107	1.018	5.582	0.002
Residuals	20	3.647	0.182		
TOTAL	**26**	**9.754**			

Error: within rats

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
treatment (tr)	1	2.425	2.425	14.265	0.000
quadrant (quad)	3	1.086	0.362	2.128	0.099
tm:tr	6	1.841	0.307	1.804	0.102
tm:quad	18	2.454	0.136	0.802	0.695
tr:quad	3	2.025	0.675	3.970	0.009*
tm:tr:quad	18	3.620	0.201	1.183	0.283
Residuals	140	23.805	0.170		
TOTAL	**189**	**37.256**			

Table 5.18: Analysis of variance of treatment, quadrant distribution and time interactions. (* = significant.)

This shows that the treatment: quadrant interaction is the only significant interaction (P=0.009), and that there are not significant differences between the quadrants overall (Table 5.19), only differences in how they react to the treatment (Table 5.20).

TIME	QUADRANT			
	Buccal	Mesial	Palatal	Distal
15 min	0.744	0.933	0.733	0.600
1 hr	0.711	0.411	0.633	0.711
4 hrs	0.789	1.033	0.611	0.678
24 hrs	0.589	0.778	0.433	0.422
1 wk	0.489	0.322	0.211	0.344
4 wks	0.611	0.722	0.500	0.722
8 wks	0.333	0.222	0.111	0.222
Mean	**0.650**	**0.679**	**0.508**	**0.545**
SEM	**0.037**	**0.037**	**0.032**	**0.033**

Table 5.19: Mean mast cell counts in each quadrant for each time group.

TOOTH	QUADRANT			
	Buccal	Mesial	Palatal	Distal
Left	0.885	0.840	0.547	0.535
Right	0.416	0.519	0.469	0.556
R:L	**0.47**	**0.62**	**0.86**	**1.04**
SE (R:L)	**0.06**	**0.07**	**0.11**	**0.13**

Table 5.20: Treatment:Quadrant interaction indicating the mean mast cell counts for each quadrant for treated (right) and control (left) teeth.

Table 5.20 shows that the major treatment effect is in the buccal and mesial quadrants, but that there is no evidence of a change in the palatal and distal quadrants.

5.5.3.4 Interaction Of Quadrant By Vertical Level

There was an interest in finding any interaction between the quadrant and vertical distribution of the mast cells. This is because of our accepted model of tooth movement where buccally directed pressure on the crown of a tooth results in compression of the periodontal ligament in the coronal-buccal area and the apical- palatal area (Figure 5.23).

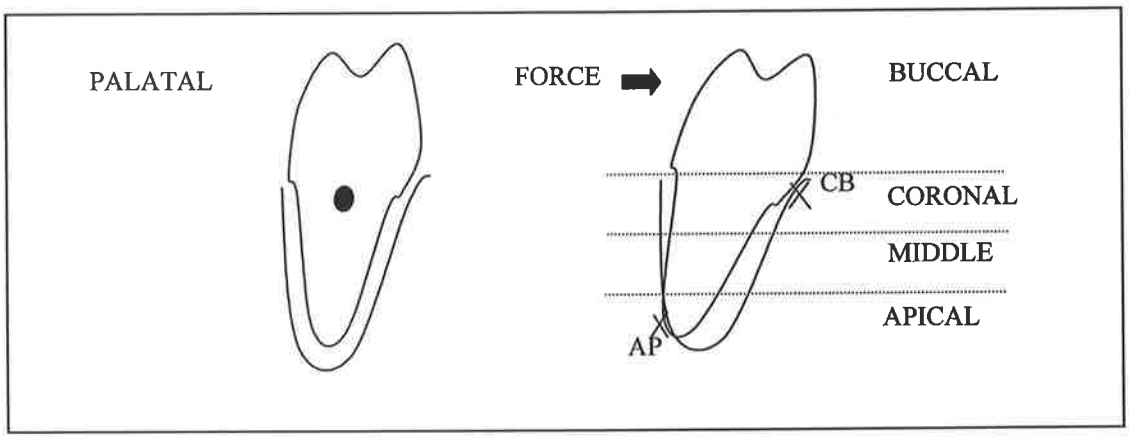


Figure 5.23: Model of tooth movement in the periodontal ligament space with buccally directed force.

The following tables (5.21 and 5.22) consider the mean mast cell counts, averaging over all rats and over the 3 positions. Each entry is the average of 81 readings:

LEFT (CONTROL)

VERTICAL LEVEL	QUADRANT			
	Buccal	Mesial	Palatal	Distal
Coronal	1.901	1.630	0.272	0.123
Middle	0.062	0.074	0.259	0.444
Apical	0.691	0.815	1.111	1.037

RIGHT (EXPERIMENTAL)

VERTICAL LEVEL	QUADRANT			
	Buccal	Mesial	Palatal	Distal
Coronal	0.568	0.321	0.185	0.074
Middle	0.037	0.457	0.370	0.395
Apical	0.642	0.778	0.852	1.198

Table 5.21: Mean mast cell counts for each quadrant and vertical level.

and the ratios for the right:left are:

VERTICAL LEVEL	QUADRANT			
	Buccal	Mesial	Palatal	Distal
Coronal	0.299*	0.197*	0.680	0.602
Middle	0.597	6.176*	1.429	0.890
Apical	0.929	0.955	0.767	1.155

with standard errors:

VERTICAL LEVEL	QUADRANT			
	Buccal	Mesial	Palatal	Distal
Coronal	0.050	0.042	0.228	0.311
Middle	0.436	2.719	0.407	0.216
Apical	0.179	0.168	0.123	0.172

Table 5.22: Ratios for Right:Left teeth for each quadrant and vertical level plus their standard errors. * = significant.

Note that the large ratio of 6.176 is in fact significant, despite the high standard error of 2.719. The approximate formula for the standard deviation does not work well when the denominator is so small, and in fact a Chi-square test for the difference R:L at (Mesial, Middle) gives a Chi-square of 22.3 on one degree of freedom ($P<0.001$). Thus, the starred entries in the table above indicate cases where there is a significant departure from 1, i.e. the Right tooth differs significantly from the Left.

Therefore (Buccal, Coronal) and (Mesial, Coronal) numbers go down in the Right teeth whilst (Mesial, Middle) goes up. The others do not differ sufficiently to detect any change.

For buccal-coronal Chi-square test :

Left: 1.901 x 81 = 154 mast cells

Right: 0.568 x 81 = 46 mast cells

	LEFT	RIGHT	
observed	154	46	200
expected	100	100	

Table 5.23: Buccal-Coronal Chi-square test

$$\sum \frac{(O-E)^2}{E} = 58.32$$

For mesial-coronal Chi-square test :

Left: 1.630 x 81 = 132 mast cells

Right: 0.321 x 81 = 26 mast cells

	LEFT	RIGHT	
observed	132	26	158
expected	79	79	

Table 5.24: Mesial-Coronal Chi-square test

$$\sum \frac{(O-E)^2}{E} = 71.114$$

For mesial-middle Chi-square test :

Left: 0.074 x 81 = 6 mast cells

Right: 0.457 x 81 = 37 mast cells

	LEFT	RIGHT	
observed	6	37	43
expected	21.5	21.5	

Table 5.25 Mesial-Middle Chi-square test

$$\sum \frac{(O-E)^2}{E} = 22.349$$

5.5.4 THE CONTROL DATA

In the experimental setup there were two controls. Firstly, there were the control rats which did not have orthodontic appliances placed - therefore both left and right teeth provided control data. Secondly, there were the control teeth (the left teeth) of the experimental rats which acted as a basis for comparison for the experimental teeth (the right teeth).

A number of comparisons could be made using this data. The first would be to compare left and right teeth in experimental animals (discussed previously in Results Section 5.5.1). This would give a valid measure of change, except it must be noted that the left teeth may be affected by movement of the right teeth. A second comparison would be to compare left teeth between control and experimental animals, and a third would be to do the comparison on right teeth between control and experimental groups.

Since the differences between rats are not large relative to the differences within rats, most comparisons have similar accuracies and hence the following tables can be presented. In these tables the mast cell numbers have been averaged over time because there does not appear to be a dramatic change due to treatment over time, as evidenced by the failure of any time x treatment interaction to appear (discussed previously in Results section 5.5.2).

5.5.4.1 Horizontal Distribution

The mean mast cell counts for the experimental rats (N=27) was obtained by averaging the 324 readings for the 27 rats over 4 quadrants and 3 vertical levels. The mean mast cell counts for the control rats (N=11) was obtained by averaging the 132 readings for the 11 rats over 4 quadrants and 3 vertical levels.

Table 5.26 includes the right:left ratios for the experimental and control rats as well as their corresponding standard errors. This illustrates that there is a significant difference (*) between left and right teeth in the experimental rats in the bone and mid thirds of the ligament. However, there is no difference between the left and right teeth in the control rats for the same thirds, as the R:L ratios do not significantly differ from 1.

For the tooth third of the ligament neither the experimental nor the control rats show a significant difference between the left and right teeth because the mast cell counts were too low and the standard errors were too high.

BONE THIRD

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	1.775	1.321	0.74	0.05*
Control Rats	1.568	1.621	1.03	0.10

MID THIRD

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	0.275	0.111	0.41	0.08*
Control Rats	0.212	0.273	1.29	0.32

TOOTH THIRD

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	0.056	0.037	0.67	0.24
Control Rats	0.068	0.023	0.34	0.22

Table 5.26: Mean mast cell counts for each horizontal third for left and right teeth for controls and experimental rats.

To see whether the left teeth in the experimental animals differ from the left teeth in the controls a similar ratio could be performed down the left tooth column in each table, however, it is obvious that there are no significant differences.

5.5.4.2 Vertical Distribution

The mean mast cell counts for the experimental rats was obtained by averaging the 324 readings for the 27 rats over 4 quadrants and 3 horizontal thirds. The mean mast cell counts for the control rats was obtained by averaging the 132 readings for the 11 rats over 4 quadrants and 3 horizontal thirds.

Table 5.27 indicates that there is a significant difference between left and right teeth in the experimental rats in the coronal (high significance) and middle (marginal significance) levels of the ligament, whilst there is no change in the apical levels (the R:L ratio is essentially 1). There is no difference between the left and right teeth in the control rats for the coronal and apical levels, however there does appear to be a difference in the middle level (R:L = 0.58). Given that the level of the test is 5% and many such tests have been done, it is likely that this is an artefact, an example of the 1 in 20 cases where we find a significant result when there is no real difference present. Also

the mast cell numbers in the middle level are much smaller than the coronal and apical levels and therefore more difficult to measure changes accurately.

CORONAL

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	0.981	0.287	0.29	0.03*
Control Rats	0.795	0.985	1.24	0.16

MID

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	0.210	0.315	1.50	0.23*
Control Rats	0.318	0.182	0.58	0.15

APICAL

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	0.914	0.867	0.95	0.08
Control Rats	0.735	0.750	1.02	0.15

Table 5.27: Mean mast cell counts for each vertical level for left and right teeth for controls and experimental rats.

5.5.4.3 Quadrant Distribution

The mean mast cell counts for the experimental rats were obtained by averaging the 243 readings for the 27 rats over 3 horizontal thirds and 3 vertical levels. The mean mast cell counts for the control rats were obtained by averaging the 99 readings for the 11 rats over 3 horizontal thirds and 3 vertical levels.

Table 5.28 indicates that the experimental rats show a significant deviation from 1.0 in the R:L ratio for the buccal and mesial quadrants indicating a treatment difference in these quadrants. On the other hand, the palatal and distal quadrants are close to 1.0 in their R:L ratio and therefore indicate no change. The control rats show a reasonable change from 1.0 in the R:L ratios in the buccal quadrant and a smaller change in the palatal and distal quadrants perhaps indicating that the mast cell distribution in this plane of space is more random normally than the vertical and horizontal (i.e. bone, mid, tooth) planes of space.

BUCCAL

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	0.885	0.416	0.47	0.06
Control Rats	0.566	0.949	1.67	0.28

MESIAL

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	0.840	0.519	0.61	0.07
Control Rats	0.788	0.848	1.07	0.17

PALATAL

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	0.547	0.469	0.85	0.11
Control Rats	0.657	0.444	0.67	0.13

DISTAL

	Left Tooth	Right Tooth	R:L Ratio	S.E.
Experimental Rats	0.535	0.556	1.03	0.13
Control Rats	0.455	0.313	0.68	0.16

Table: 5.28: Mean mast cell counts for each quadrant for left and right teeth for controls and experimental rats.

5.5.5 MAST CELL NEAR BLOOD VESSELS

When counting the mast cells it was noted that a high proportion appeared to be close to blood vessels. It was therefore decided to record whether or not the mast cells counted were within a certain distance from any blood vessels. This was an arbitrary distance measured using the millimetre graticule in the eyepiece of 3mm using the 25X objective. This distance was later quantified to be 12.5 μm using a millimetre graticule on a slide viewed at 25X.

To do this analysis the statistician counted the total number of mast cells seen for each tooth and then the total number of mast cells within the required distance (12.5 μm) of a blood vessel.

The results have been presented in the following tables (Table 5.30) for experimental animals and (Table 5.31) for control animals. The experimental animals are age-matched to the control animals as follows (Table 5.29):

Experimental		Control	
Rat No.	Time	Rat No.	Time
1 - 5	15 min	29 - 32	Zero
6 - 10	1 hr		
11 - 15	4 hrs		
16 - 20	24 hrs		
21 - 25	1 week	33 - 36	1 week
26	2 weeks	37	2 weeks
27	4 weeks	38	4 weeks
28	8 weeks	39	8 weeks

Table 5.29: List of experimental periods with corresponding controls

EXPERIMENTAL RATS					
Time	Rat	Total MCs Left	Left MCs Near BV	Total MCs Right	Right MCS Near BV
15 min	1	35	21	40	26
	2	51	32	20	14
	3	60	30	34	26
	4	39	28	53	15
	5	36	28	22	16
1 hr	6	27	14	26	23
	7	16	14	29	22
	8	32	17	36	20
	9	43	28	34	24
	10	51	32	35	29
4 hrs	11	57	31	34	31
	12	72	13	32	27
	13	36	24	29	25
	14	39	21	28	23
	15	82	44	42	36
24 hrs	16	31	12	13	11
	17	42	15	26	21
	18	44	26	17	16
	19	37	28	14	13
	20	38	23	20	17
1 week	21	5	0	11	11
	22	11	5	7	2
	23	39	26	22	18
	24	31	19	9	7
	25	32	18	21	16
2 weeks	26	0	0	37	34
4 weeks	27	27	18	27	20
8 weeks	28	22	19	10	8
TOTAL		**1035**	**586**	**728**	**551**

Table 5.30: Total mast cell counts for each tooth and the total number of mast cells near blood vessels for the experimental animals.

CONTROL RATS					
Time	Rat	Total MCs Left	Left MCs Near BV	Total MCs Right	Right MCS Near BV
Zero	29	14	13	13	11
	30	42	22	53	25
	31	47	20	56	32
	32	32	3	29	6
1 week	33	42	38	36	22
	34	26	18	36	28
	35	29	11	39	11
	36	28	22	30	27
2 weeks	37	26	9	64	13
4 weeks	38	39	20	32	23
8 weeks	39	28	16	31	15
TOTAL		**353**	**192**	**419**	**213**

Table 5.31: Total mast cell counts for each tooth (column 3 and 5) and the total number of mast cells near blood vessels (column 4 and 6) for the control animals.

Note that zero time in the control animals is equivalent to the 15 min, 1 hr, 4 hrs and 24 hrs experimental animals. Also note that rat 26, a two week experimental animal, had all data missing for the left tooth, hence the numbers are recorded as zero for that side. Rat 26 was therefore excluded from the following analysis (Table 5.32).

	est	sd	t
Side	0.909	0.240	3.78

(Dispersion Parameter for Binomial family taken to be 2.11)
Null Deviance: 270.7777 on 53 degrees of freedom
Residual Deviance: 115.7637 on 26 degrees of freedom

	Df	Deviance Residual	Df	Residual Deviance
NULL			53	270.778
Rat	26	87.784	27	182.994
Side	1	67.230	26	115.764

Table 5.32: Analysis of deviance.

Table 5.32 indicates that there is a strong difference due to "side" with a t-value of 3.78 on 26 degrees of freedom. On the logit scale the effect is about 0.91 units with a standard deviation of 0.24. This effect can be seen by studying the "empirical logits" in the following tables (Table 5.33) for experimental animals and (Table 5.34) for control animals.

EXPERIMENTAL RATS				
Time	**Rat**	**Left**	**Right**	**Diff (R-L)**
15 mins	1	0.394	0.603	0.209
	2	0.511	0.802	0.292
	3	0.000	1.137	1.137
	4	0.908	-0.910	-1.817
	5	1.210	0.932	-0.278
1 hr	6	0.072	1.904	1.833
	7	1.758	1.099	-0.659
	8	0.121	0.217	0.096
	9	0.609	0.847	0.238
	10	0.511	1.513	1.002
4 hrs	11	0.173	2.197	2.024
	12	-1.483	1.609	3.093
	13	0.673	1.735	1.062
	14	0.150	1.452	1.302
	15	0.145	1.726	1.581
24 hrs	16	-0.445	1.526	1.971
	17	-0.573	1.363	1.937
	18	0.359	2.398	2.039
	19	1.099	2.197	1.099
	20	0.416	1.609	1.193
1 week	21	-2.398	3.136	5.533
	22	-0.167	-0.789	-0.621
	23	0.675	1.414	0.739
	24	0.445	1.099	0.654
	25	0.244	1.099	0.855
2 weeks	26	0.000	2.288	2.288
4 weeks	27	0.667	1.006	0.339
8 weeks	28	1.718	1.224	-0.494
			MEAN	**0.976**
			S.D	**1.392**
			S.E.M.	**0.268**

Table 5.33: Empirical logits for mast cells near blood vessels for left teeth (control) and right teeth (experimental) and their differences for experimental animals.

CONTROL RATS				
Time	**Rat**	**Left**	**Right**	**Diff**
zero	29	2.197	1.526	-0.671
	30	0.093	-0.111	-0.204
	31	-0.294	0.283	0.576
	32	-2.132	-1.285	0.846
1 week	33	2.147	0.439	-1.707
	34	0.778	1.210	0.432
	35	-0.475	-0.908	-0.432
	36	1.242	2.061	0.820
2 weeks	37	-0.611	-1.339	-0.728
4 weeks	38	0.050	0.906	0.856
8 weeks	39	0.278	-0.063	-0.340
			MEAN	**-0.050**
			S.D	**0.828**
			S.E.M.	**0.250**

Table 5.34: Empirical logits for mast cells near blood vessels for left teeth and right teeth and their differences for control animals.

From Tables 5.33 and 5.34 it can be seen that there is a definite effect of orthodontic movement with regards to mast cell proximity to blood vessels. In Table 5.33 of the experimental rats there is a shift of almost 1 unit on the logit scale (0.976) in the proportion of mast cells within the defined distance of a blood vessel, being higher in the experimental side.

On the other hand, Table 5.34 of the control rats there is almost no shift on the logit scale (-0.050) in the proportion of mast cells close to blood vessels. Figure 5.24 shows the effect of this as a function of time. In this plot time is across the x-axis and the R-L difference in logits (which are the figures in the last column of Tables 5.33 and 5.34) on the vertical axis.

This graph shows that most of the differences in logits are scattered around zero for both experimental and control rats, however, all of the values for the 4 and 24 hour experimental rats are above zero.

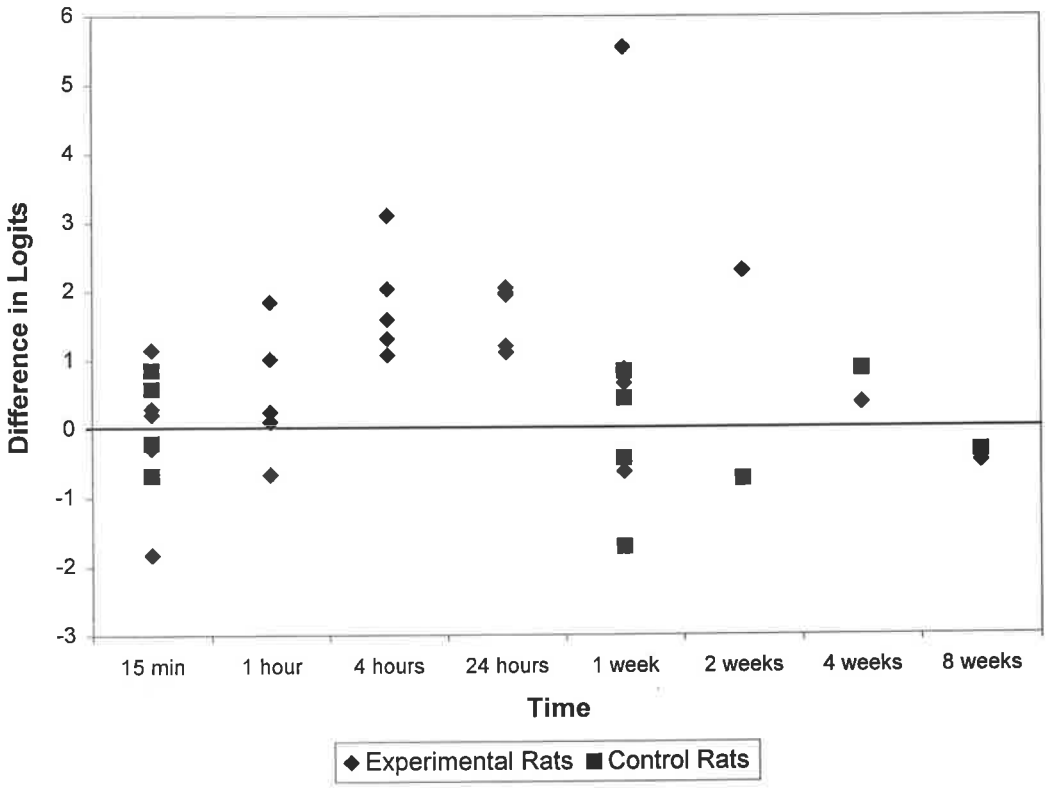


Figure 5.24: Difference in logits (R-L) for closeness to blood vessels.

SUMMARY

HORIZONTAL DISTRIBUTION

- There were statistically significant differences between bone, mid and tooth thirds of the ligament ($p \leq 1 \times 10^{-7}$).
- For control teeth, the greatest number of mast cells were found in the bone third, followed by the mid third with the least found adjacent to the tooth.
- For experimental teeth, there was a drop in mast cell numbers in each third, however, the distribution stayed the same i.e. bone > mid > tooth.

VERTICAL DISTRIBUTION

- There were statistically significant differences between coronal, middle and apical levels of the ligament ($p \leq 1 \times 10^{-7}$).
- For control teeth, the greatest number of mast cells occurred in the coronal and apical levels, with considerably less found in the middle level.

- For experimental teeth, this distribution changed such that the coronal mast cell numbers dropped dramatically with essentially no change of the mast cell numbers in the middle and apical levels.

QUADRANT DISTRIBUTION

- Although there were differences between the quadrants of the ligament, these were not statistically significant ($p=0.127$).
- For control teeth, there were nearly twice as many mast cells in the buccal and mesial quadrants as in the palatal and distal quadrants.
- For experimental teeth, this distribution changed such that the buccal and mesial quadrants reduced markedly (by approximately half) whilst the palatal and distal quadrants remained essentially the same.

MAST CELLS NEAR BLOOD VESSELS

- For control teeth (of both experimental and control animals), more than half (54.8%) of the mast cells were located near blood vessels.
- For experimental teeth, more than three-quarters (75.7%) of the mast cells were located near blood vessels.
- There was a significant increase in the number of mast cells located near blood vessels at 4hrs and 24hrs after starting orthodontic tooth movement.

CHAPTER SIX: DISCUSSION

6.1 THE EXPERIMENTAL ANIMALS

6.1.1 SEX

The Sprague-Dawley rats used in this study were all males. A review of the literature revealed that there may be sex differences in mast cell numbers as secretion from mast cells has been shown to be augmented by estradiol or triggered by progesterone (Theoharides, 1990). In the future it may be interesting to repeat this study using female rats. This may identify a sex difference with reference to orthodontic tooth movement and mast cell numbers in the periodontal ligament.

6.1.2 AGE

The rats at the time of appliance insertion were 83 or 84 days old. This age was chosen as it corresponds to twelve or thirteen years of age for humans - a time when many teenagers are seen for orthodontic treatment. The choice of age was felt to be important as previous studies have revealed age-related differences in the presence of mast cells in different locations. Enerbäck and Wingren (1980) reported a higher number of mast cells in the dermis and tongue and a lower number in the intestinal mucosa in one month old rats than in six month old rats. A study by Matsson (1993) confirmed the results of the study undertaken by Enerbäck and Wingren (1980) when he found that the mast cell numbers in the tongue, buccal mucosa and gingival mucosa were significantly higher in one month old rats than in six month old rats. In the intestine, however, Matsson (1993) found the younger rats showed a significantly lower number of mast cells. An age related difference has also been reported in humans by Abdel-Aal *et al.* (1976) who reported an increase in the number of human dermal mast cells from five to fifteen years of age, followed by a gradual decrease.

6.1.3 ORTHODONTIC APPLIANCES

The orthodontic appliances used in this study differed from those found in the literature, in both direction and level of orthodontic force used.

6.1.3.1 Direction Of Force

In previous studies most of the appliances moved the first maxillary molar in a mesial direction. The designs varied in sophistication from an elastic band placed between the first and second molar (Yamasaki *et al.*, 1982b; Ki, 1990; Tanaka *et al.*, 1990; Lee, 1990 and Saito *et al.*, 1991) to a constructed appliance. These included an extended closed spring between an incisor band and an attachment to the first maxillary molar (Mohammed *et al.*, 1989; Kvinnsland and Kvinnsland, 1990; Row and Johnson, 1990; Brudvik and Rygh, 1991; King *et al.*, 1991; Brudvik and Rygh, 1993 and Brudvick and Rygh, 1994) to a mesially directed spring on the occlusal surface of the first maxillary molar (Rygh *et al.*, 1986 and Bowling and Rygh, 1988).

One of the appliances which moved one first maxillary molar buccally was designed by Rygh and consisted of a band on the maxillary incisors to which was soldered an 0.028" diameter sectional arch. An active spring (0.010" diameter) was coiled and welded to the sectional arch to provide a force to the palatal side of the molar (Rygh, 1972a, 1972b, 1973, 1974, 1976 and Rygh and Selvig, 1973). Another unilateral appliance was designed by Takano - Yamamoto *et al.* (1992) and consisted of a 0.36mm round wire coiled into a spring and bonded to the upper incisors using orthodontic adhesive resin. The remaining two buccally directed appliances found in the literature were bilateral. The first, by Hellsing and Hammarström in 1991, was similar in design to a W-arch and comprised of 0.011" Wilcock wire welded to wire mesh which was bonded on the occlusal surfaces of both first molars. The second, by Zahrowski and Turley in 1992, was a premaxillary expansion device consisting of bands on both central incisors connected to a helical expansion spring.

The reason for using buccal movement as opposed to mesial movement in this study was to simulate what orthodontists do each and every day - placing in expanded archwires. The pendulum between extraction and non-extraction seems to be swinging towards non-extraction orthodontic therapy. The only methods available in an non-extraction case to create any space required include expansion, interproximal stripping and distalisation of buccal segments (or a combination of these). Of these, expansion is the main method used. Even in extraction cases expanded archwires are used to prevent lingual/palatal tipping of the buccal segments. Hence, it was felt that studying buccal movement in this research project would reflect more what orthodontists do clinically than mesial movement would. In addition, it is intended to use this material for future study, in particular the bone reaction to buccal tooth movement.

6.1.3.2 Level Of Force

Most of the orthodontic appliances in the literature used much greater forces than that used in this study (Table 6.1). Yamasaki *et al.*, (1982b); Ki (1990); Tanaka *et al.* (1990); Lee (1990) and Saito *et al.* (1991) did not measure the force levels their elastic bands produced and many did not state the diameter of the elastic, so even an approximation is impossible. The forces used for buccal movement varied from 5-50g; for mesial movement 30 - 60g and for extrusive movement 100g. Even the median value of 30g is huge when a consideration is given to the size of the tooth being moved - the force per millimetre of root surface would have been very high.

Force Direction	Force Level	Reference/s
buccal	5, 10, 25g	Rygh, 1972a, b; 1973; 1974; 1976.
buccal	5, 10, 25g	Rygh and Selvig, 1973
buccal	5, 20g	Takano-Yamamoto *et al.*, 1992
buccal	15g	Hellsing and Hammarström, 1991
mesial	30g	Rygh *et al.*, 1986
mesial	30g	Bowling and Rygh, 1988
mesial	30g	Row and Johnson, 1990
buccal	30 - 50g	Norevall *et al.*, 1995
mesial	30 - 50g	Kvinnsland and Kvinnsland, 1990
mesial	50g	Brudvick and Rygh, 1991, 1993, 1994
mesial	20, 40, 60g	King *et al.*, 1991
mesial	20, 40, 60g	Keeling *et al.*, 1993
mesial	60g	King and Collier, 1986
mesial	60g	Mohammed, 1989
mesial	F= ?(elastic)	Yamasaki *et al.*, 1982b
mesial	F= ?(elastic)	Ki, 1990
mesial	F= ?(elastic)	Tanaka *et al.*, 1990
mesial	F= ?(elastic)	Lee, 1990
mesial	F= ?(elastic)	Saito *et al.*, 1991

Table 6.1: Previous studies of orthodontic tooth movement indicating direction and magnitude of forces used.

It was felt that as there is a trend toward using lighter forces in orthodontics now it would be prudent to reflect this in the present study.

The difficulty in using the low force in this study, 5 grams, was accuracy of measurement. A GEC tension gauge[1] was used, however, it would be more accurate to state the force used was in the range of 4 - 7 grams. To try to improve the accuracy all the springs were made the same length and when activated these were initially adjusted to sit passively in the middle of occlusal surface of the right first maxillary molar (Mathiesen, 1973b).

When examined histologically the control teeth demonstrated the periodontal ligament to be evenly spaced around the MB root (Figure 5.7A). In comparison the experimental teeth demonstrated the MB root to be displaced buccally within the periodontal ligament (Figure 5.7B). This compression of the periodontal ligament without evidence of cell death was typical of the orthodontically moved teeth and would indicated the force applied during tooth movement was generally within physiological limits.

Of the experimental teeth only one demonstrated hyalinization of the PDL (Figures 5.8A and B). According to Rygh *et al.* (1986) this indicates that the forces applied during this experiment, other than on this tooth, were within physiological limits. Rygh *et al's* study in 1986 used forces of 30g and they felt that this was a "tolerable" force since only 9 out of 45 pressure areas (20%) showed hyalinization.

6.1.3.3 Sham Appliances

Mohammed *et al.* (1989) included a sham group in their study. This group had inactive springs in control animals. One concern using a sham group is if the springs are distorted during mastication and do become active.

6.1.3.4 Ligatures

Perhaps the control teeth (in both control and experimental animals) should have had ligatures placed around them in this study. The rationale for placing the ligatures in controls would be to simulate the ligature placed on the experimental teeth to hold the spring in place. It would exclude the possibility of periodontal induced changes in the mast cell population due to plaque accumulation and local irritation. The rationale against was that the effect of tying the ligature in controls may cause mesial tooth movement due to pressure between the first and second molars and therefore the

[1] GEC tension gauge = General Electric Company of England. British Patent No. 323282 441164, range 4-24g.

controls would be subject to orthodontic movement. It was therefore decided not to place ligatures around the control teeth.

6.2 TISSUE PREPARATION

6.2.1 FIXATION

The choice of fixative for this study was difficult as different studies in the literature cited the use of different fixatives. Formaldehyde based fixatives were not considered because of studies indicating a reduction in mast cell counts when comparing formol-saline with Carnoy's fixed tissue. This did not just relate to mucosal mast cells as the highest reduction in mast cell numbers were noted in atria (75.4%), then kidney (75.0%), lung (71.0%), intestine (63.0%) and finally mesentery (53.0%) and lung (47.0%) (Ghanem *et al.*, 1988).

This was also found in a study of human dermal tissue by Marshall *et al.*, (1987) when they compared 10% neutral buffered formalin with Carnoy's fixative. Sections were then stained with either toluidine blue or alcian blue/safranin O. With both stains approximately 50% of the mast cells observed in the Carnoy's fixed tissue could be visualised in the formalin fixed tissue. The structural integrity of MC granules depends on the ionic linkages between the glycosaminoglycans and proteins. The type of fixative, pH and ionic concentration affect the cationic dye-binding properties of the granules. Aldehyde fixation may precipitate a protein shell, shielding the glycosaminoglycan and its dye-binding groups. The effect of formalin fixation can be overcome by mild proteolytic digestion of fixed sections or by prolonged staining (5 - 7 days) with toluidine blue (Marshall *et al.*, 1987 and Ghanem *et al.*, 1988).

Carnoy's fixative was chosen for the present study because it can be used as both a connective tissue and mucosal mast cell fixative. IFAA can also be used for both CTMC and MMC and is reported to be superior in preserving structural detail. Ghanem *et al.*, (1988) reported, however, that mast cells were extremely well preserved with Carnoy's fixative. Carnoy's is recommended for fixation of IgE and mast cell antigens and in case these were to be used in later studies of this tissue it was felt that it was preferable to use Carnoy's (Ghanem *et al.*, 1988).

All the tissue appeared to be adequately fixed and there were no signs of tissue degeneration in the sections studied.

6.2.2 DECALCIFICATION

EDTA is a chelating agent which combines with calcium ions to form soluble, non-ionised compounds, hence no gas bubbles are formed and the disruption of tissues by these is avoided. Because this solution is effective at neutral pH, staining generally is excellent and vastly superior to that obtained after any other decalcifying agent.

Of different decalcification solutions tested by Bjurholm *et al.* (1989) EDTA cacodylate gave best preservation of antigenicity for immunohistochemical staining of neuropeptides.

Tissue is not hardened after decalcification in EDTA; on the contrary, some microtomists believe that bone is easier to cut after this than after acid decalcifying solutions. Hilleman and Lee (1953) advocated the use of EDTA for dental tissues and this reagent may be used with success for bone, teeth or any other calcified tissue.

6.3 SECTION PREPARATION AND STAINING

6.3.1 ZERO LEVELS

A common starting point (zero) was needed to be identified for all the blocks to determine the staining run for mast cell counting. After staining every tenth section for a quarter of the blocks with haematoxylin and eosin it was found that the mesial of the first maxillary molar was usually the first area to demonstrate a gingival attachment. Therefore, the mesial site was chosen to find zero.

Next it was necessary to find an anatomical landmark that was easy to locate in this mesial area. For the quarter of samples previewed three anatomical landmarks were considered:

1. The first cementum appearance (approximately the CEJ).
2. The first epithelial attachment to the tooth.
3. The first connective tissue attachment to the tooth.

The first cementum appearance was difficult to locate in the transverse sections used in this study. The cementum was a thin purple band on pink dentine and difficult to see in the CEJ region. The cementum was easier to identify further down the tooth as it was thicker.
The first epithelial attachment was also a little difficult to accurately locate. Since the decalcifying medium dissolved the enamel completely this left an enamel space between the epithelium and the

dentine. It was difficult to know if the area viewed was near the bottom of the sulcus prior to the epithelial attachment or if it was the epithelial attachment itself.

The first connective tissue attachment to the tooth was the most easily reproducible anatomical landmark identified and therefore chosen as zero. Therefore, the first connective tissue attachment on the mesial of the first maxillary molar was used as zero (Figure 5.3).

One perceived problem with using the first connective tissue attachment as zero is the possible loss of attachment encountered due to the presence of orthodontic springs and ligatures, particularly in long-term experimental animals.

6.3.2 MAST CELL STAINING REGIME

The staining regime for the study of mast cell populations was decided on after studying the work done by Andersson *et al.* (1987) on root resorption. This paper describes the use of one 5μm section per 100μm. It was found that a sample of 10 to 17 sections per tooth provided sufficient data.

As there were, on average, 600 5μm thick sections per tooth the average length of the rat maxillary first molar was 3000μm, including the crown. It was estimated that the root length would be in the vicinity of 2000μm. Studying every 20th section, that is every 100μm, would result in twenty levels per tooth. This would give more than the required 10 to 17 levels as determined by Andersson *et al.* in 1987.

6.3.3 TOLUIDINE BLUE STAINING

The toluidine blue stain employed was buffered to pH 0.5. This was to enhance the specificity of staining of mast cell granules and to decrease background staining (hence the difficulty in seeing bone and cementum clearly). Churukian and Schenk (1981) developed the acidified toluidine blue stain in an effort to minimise background staining, enhance nuclear clarity, and maintain the distinctive metachromasia of the mast cell granules. They did report, however, a loss of granular staining unless the sections were pretreated with potassium permanganate and potassium metabisulfite solutions.

McKenna *et al.*, (1990) observed good staining of the granules with acidified toluidine blue both in the presence and absence of pretreatment and explained this was due to their prestaining with Villaneuva osteochrome bone stain.

In the present study neither pretreatment with potassium permanganate and potassium metabisulfite nor prestaining with Villaneuva bone stains were used, however, granular detail was well preserved. This can be seen in Figure 5.6 which shows a mast cell degranulating.

McKenna *et al.*, (1990) did note that the use of acidified solution contributed to loss of bone detail and this was found to be the case in the present study (Figures 5.4 and 5.5). McKenna *et al.*, (1990) and McKenna (1994) recommend that if bone detail is important then prior staining with Congo red ameliorates this problem. It must be noted, however, that these studies used undecalcified bone sections.

6.3.4 HRP-AVIDIN STAINING

The reason for the failure of staining with HRP-avidin are inconclusive. Initially it was felt that one of the reagents was inactive. However, the same tissue was unsuccessfully stained by researchers at the Queen Elizabeth Hospital using their own proven reagents.

Another possible reason is an adverse reaction with Carnoy's fixative. However, it has been previously reported that avidin peroxidase is not sensitive to methods of fixation.

As HRP-avidin binds to the heparin in mast cell granules one reason could be that the mast cells in the periodontal ligament do not contain heparin. If this is the reason it may mean that the mast cells in the ligament are not typical CTMC and may be MMC (or another subtype altogether!).

6.3.5 ANTI-MAST CELL TRYPTASE ANTIBODY

An anti-human mast cell tryptase antibody (Code No. M7052) has been developed by Dako Corporation. This binds specifically to the secretory granules of mast cells in a range of human tissues including those of the respiratory and gastrointestinal tracts, skin, conjunctiva and brain. Immunocytochemical procedures with this antibody consistently provide more sensitivity in the demonstration of mast cells than standard metachromatic staining techniques. The antibody shows strong reactivity in both Carnoy's and formalin-fixed paraffin embedded tissue, resin embedded tissue, and in cytocentrifuge preparations (Dako fact sheet, 1995).

On enqiry with representatives of Dako it became apparent that this antibody would probably not work on rat tissue. However, the fact that this antibody has been developed for human mast cells could mean that an antibody for rat mast cells will follow which would be useful in later research.

6.4 HISTOLOGICAL AND OTHER OBSERVATIONS

6.4.1 MAST CELL HETEROGENEITY

The discovery that large numbers of free mast cells could be obtained by simple lavage of the peritoneal cavities of the rat (Uvnäs, 1974), coupled with the fact that such cells may be readily purified to near homogeneity, has meant that the vast majority of our knowledge of mast cell physiology and pharmacology has been derived from studies of only one mast cell type ie. the peritoneal mast cell (Barrett and Metcalfe, 1984). However, the existence of mast cell heterogeneity, and of functional heterogeneity in particular, has implications in understanding the role of mast cells in orthodontic tooth movement. The properties of rat peritoneal mast cells do not necessarily parallel those of the rat periodontal ligament mast cells (and more so human periodontal ligament mast cells). From the more limited studies on human mast cells it is at least apparent that they vary from those in the rat according to granular morphology (Barrett and Metcalfe, 1984, and Dvorak, 1986).

6.4.2 ROOT RESORPTION

In the present study root resorption was seen not only in experimental teeth but also in control teeth of both experimental and control animals (Figures 5.10 - 5.12).

According to Brezniak and Wasserstein (1993a and b), who compiled a literature review on root resorption, up to 90.5% of non-orthodontically treated teeth displayed microscopic lesions of root resorption. These papers and the present study support the view that surface root resorption is a normal occurrence, to some degree.

The root resorption seen in this study was not measured. However, this material will be used in future studies to quantify the root resorption discovered and relate it to the tooth movement performed.

6.4.3 BONE RESORPTION

Reitan (1985) stated that light orthodontic forces resulted in frontal or direct resorption of bone on the pressure side proceeding directly at the adjacent lamina dura. Increasing load causes cessation of blood flow in the compression zone resulting in sterile necrosis - referred to as hyalinization because of its histological appearance (Per Rygh, 1989). Undermining or indirect resorption is associated with hyalinization whereby bone resorption on the compressed side occurs from the underside of the lamina dura adjacent to the hyalinized zone (Reitan and Rygh, 1994).

In the present study one animal demonstrated hyalinization within the periodontal ligament and concomitant undermining resorption (Figures 5.14 A-C and 5.15 A and B for higher power views) supporting the contention that the forces applied were gentle, but sufficient to induce tooth, bone and PDL responses.

6.4.4 MAST CELLS AND BLOOD VESSELS

Close proximity of mast cells to the vascular bed has been observed in several reports (Tharp, 1985; McKenna and Frame, 1985; Keller *et al.*, 1989; and Rakusan and Campbell, 1991) and has been noted in the present study (Figures 5.16 and 5.17).

There are at least two possible explanations for this preferential location. Firstly, their presence close to blood vessels is simply a reflection of their original transport by the blood. If this is the case, one would expect their position to be mainly in the distal portions of the terminal vascular bed, i.e., venular portions of capillaries and venules (Rakusan and Campbell, 1991).

Secondly, the proximity of mast cells to the vascular walls is a reflection of their function ie. to modulate the vessels and their reactions by local release of mast cell granules containing heparin, histamine, chymotrypsin, serotonin and platelet activating and chemotactic factors. If this is the case, one would expect a predilection for an upstream location, in arteriolar portions of capillaries (Rakusan and Campbell, 1991).

Rakusan and Campbell (1991) stained arteriolar and venular portions of coronary capillaries using the differential staining method described by Lojda (1979) and compared the density of mast cells in these two regions. Dipeptidyl peptidase IV stained the venular portion of capillaries red whilst alkaline phosphatase stained the arteriolar portion blue. These authors found a significantly higher

percentage of mast cells close to arteriolar portions of coronary capillaries and stated this preferential location reinforced the role of mast cells in influencing the function of the vascular wall. Since the PDL vasculature is predominantly venular: postcapillary venule, venule and collecting venule, it is likely the mast cells are adjacent to venular vessels. This fact and also the occasional finding of mast cells in blood vessels in the present study (Figure 5.17) may cast doubt on Rakusan and Campbell's hypothesis and lend support to the transport theory. However, it may be interesting in the future to stain the blood vessels according to their techniques in order to determine whether the vessels next to mast cells in the PDL are arteriolar or venular.

6.4.5 MAST CELLS AND NERVES

A number of observations support the concept of complex interactions between mast cells and nerves (Tharp, 1985, McKenna and Frame, 1985; Keller *et al.*, 1989; Theoharides, 1990; Hukkanen *et al.*, 1991; Domeij *et al.*, 1991 and Valent and Bettelheim, 1992). Interactions between nerves and mast cells are made more plausible by observations suggesting that neuropeptides such as vasointestinal polypeptide (VIP) or substance P can be found in mast cells, basophils and neutrophils (Bienenstock *et al.*, 1986).

In the study by Stead *et al.*, (1989) normal human gastrointestinal mucosa revealed many mast cells adjacent to nerve fibres and membrane-to-membrane contact between axon-like processes and mast cells. These authors documented that one half to two thirds of human intestinal mucosal mast cells are closely apposed to nerves. This is in contrast to the study by Ruokonen *et al.* (1993) which showed no such spatial associations in normal human buccal mucosa.

Ferrante *et al.* (1990) examined functional relationships between nerve terminals and mast cells, and found removal of the superior cervical ganglion caused an impairment of the morphology of a large percentage of mast cells and a reduction in their 5-hydroxytryptamine fluorescence. Conversely, electrical stimulation of the superior cervical ganglion caused an increase in 5-hydroxytryptamine fluorescence. This would suggest a functional association between mast cells and nerve fibres in the dura mater, at least.

Both substance P (SP) and calcitonin gene-related peptide (CGRP) immunoreactivity has been demonstrated in nerves apposed to mast cells (Stead *et al.*, 1989). Substance P is thought to mediate both vasodilatation and to augment vascular permeability. Substance P also induces mast cell degranulation in vitro and in vivo (Theoharides, 1990; Domeij *et al.*, 1991; Ruokonen *et al.*, 1993 and Valent and Bettelheim, 1992).

SP induced release of histamine from human (skin) mast cells is accompanied by an increase in intracellular calcium but (in contrast to IgE-dependent release reactions) is independent of the presence of extracellular calcium. SP-induced release from mast cells also differs from IgE-dependent release by the failure to promote synthesis of larger amounts of prostaglandin D_2. SP failed to induce histamine release from human lung or gut mast cells, supporting the concept of mast cell heterogeneity (Valent and Bettelheim, 1992).

CGRP causes a persistent local erythema and has been implicated in modulation of vascular permeability and also prolongs the effects of SP by inhibiting degradation of SP (Domeij *et al.*, 1991 and Ruokonen *et al.*, 1993). CGRP has also been found to degranulate mast cells (Domeij *et al.*, 1991) though is less effective than SP (Valent and Bettelheim, 1992).

Domeij *et al.* (1991) demonstrated a large or moderate number of mast cells only where there were numerous SP/CGRP containing nerve fibres. Even though true synaptic contacts between the mast cells and nerves were not observed the distances between them were small (within 100nm) which is within the range that neurons may exert their action on effector cells. Whether the actions of the peptides in inflammation are mainly mediated via direct actions on blood vessels or indirectly via the release of mediators from mast cells (and/or other cell) or via other effects remains to be established (Baraniuk *et al.*, 1990; Domeij *et al.*, 1991).

Yano *et al.* (1989) demonstrated that mast cell-deficient mice did not develop ear inflammation after injection of SP unless they were reconstituted with mast cells, which suggests mast cells act as an intermediary between nerves and blood vessels. This is in contrast with a study by Baraniuk *et al.*, (1990) which found neuropeptides released from sensory nerves acting directly on postcapillary venules (and mast cell degranulation was not involved). The latter study measured responses within minutes whilst Yano *et al.*'s study (1989) measured responses two hours later indicating that mast cells appear to be involved in later stages of neurogenic inflammation.

6.4.6 MAST CELL MITOSIS

In all of the sections viewed in the present study one mitotic figure was observed (Figure 5.18). This was in the periodontal ligament of a control animal (90 days of age).

Selye (1965) documented that mature mast cells appear to undergo mitosis during reactions which elicit their in vivo accumulation. Tizard (1995) agrees that irritation may cause local proliferation.

This mitotic figure was found in a control animal and therefore was not stimulated (at least not by orthodontic movement).

Galli *et al.* (1984) and Galli (1986) felt that mast cells retained a latent or limited proliferative capacity which declined during maturation. Dvorak (1991) agreed that cell division occurred rarely.

6.5 STATISTICAL RESULTS

6.5.1 OVERALL EFFECT OF TREATMENT ON MAST CELL COUNTS

Observation One: . There were quite large treatment differences between control and experimental teeth (Table 5.1).

The analysis of variance of treatment effect (Table 5.1) indicates that there were quite large treatment differences between the left (control) and right (experimental) teeth for each rat (Mean Sq = 21.83 and Pr = 0.002). This table, though it indicates a difference related to tooth movement, does not identify the direction of the change, i.e. whether the control numbers increased or the experimental numbers decreased.

Yamasaki *et al.* (1982b) found a decrease in the toluidine blue stained mast cells incident to orthodontic tooth movement so it is expected that in the present study the experimental numbers have decreased instead of an increase in the control numbers.

Observation Two: Although control mast cell numbers were generally higher than experimental numbers, treatment differences were highly significant at 4 hrs and 24 hrs.

Studying the overall mean mast cell counts (Table 5.2) showed that generally the experimental teeth had lower mast cell counts than the control teeth. This treatment difference of experimental < control was highly significant at 4 hours and 24 hours as shown by Figure 5.19 and also the paired *t*-test below it. This trend, however, was reversed in the one hour time group where three rats demonstrated more mast cells in the experimental teeth. This may not be significant though, because in Figure 5.19 which plots the left right differences the measurement at 1 hour is within one standard error of the mean from zero.

A possible explanation for the changes in mast cell counts could be:

At 15 min: Initial degranulation of mast cells in the experimental teeth resulting in C>E.

At 1 hour: An influx of mast cells from the surrounding tissues causing a reversal of the general trend so at least E=C or possibly even E>C.

At 4 hours and 24 hours: Secondary degranulation of mast cells in experimental teeth resulting in C>>E.

At 1 week: Start of regranulation of degranulated mast cells in experimental teeth resulting in C>E.

At 2 weeks: No results - Rat 26 has been excluded.

At 4 weeks and 8 weeks: Results are not significant as there has only been one animal per time group used in this study. It may be possible to hypothesise that the differences between experimental and controls be close to zero as the error bars of both overlap this area. If this does occur it could suggest complete regranulation of the degranulated mast cells in the experimental teeth.

Yamasaki *et al.* (1982b) found a significant drop in mast cell numbers at 15 minutes following the onset of orthodontic movement. There was a return to normal levels at 1 hour through to the end of the experiment (including measurements at 3 hrs, 6 hrs, 12 hrs, 24 hrs, 3 days and 5 days).
Interestingly, we both found a drop in mast cell numbers at 15 minutes and a return to normal levels at 1 hour. However, the present study demonstrated a second drop in mast cell numbers which was more significant (than the drop at 15 minutes) at 4 and 24 hours and a much more gradual return to normal levels.

A few studies of recovery from degranulation indicate that the gradual increase in mast cell numbers in the one week sample could be due to regranulation. Fawcett (1955) found that 18-20 hours following intraperitoneal injection of compound 48/80 the mast cell numbers returned to nearly

normal, though they were smaller in size. This time frame of recovery was similar to Weill and Renoux (1982). They found by 24 hours 60% of mast cells stimulated by compound 48/80 had recovered their granules and that by 48 hours 90% had done so. Dvorak *et al.*, (1987) found that mast cells stimulated with anti-IgE had recovered at 24 hours, though the number of granules was reduced compared with controls.

In view of the studies cited above it is unlikely that the return to normal levels at one hour found in Yamasaki's study (1982b) and the present study are due to regranulation. It is probable that this quick increase in stainable mast cells is due to repopulation from the surrounding tissues.

In the present study following the second drop in mast cell numbers (at 4 and 24 hours) there was a gradual return to normal levels. It seems that most of the recovery of granules after tooth movement has occurred some time between 24 hours and one week. This would indicate a recovery from degranulation which is slower than in the studies cited above.

6.5.2 POSITIONAL EFFECTS OF TREATMENT ON MAST CELL COUNTS

This refers to the effect of orthodontic treatment on the mast cell distribution within the ligament. The relative importance of the positions noted (by vertical level, quadrant and horizontal distribution) have been shown in Table 5.4 which shows that the horizontal distribution is the most significant followed by level and then quadrant.

6.5.2.1 Horizontal Distribution

Observation Three: There were huge differences between bone, mid and tooth thirds of the ligament.

This can be seen in the analysis of variance of positional effect (Table 5.4) where Mean Sq = 444.094 and $P \leq 1 \times 10^{-7}$ for horizontal distribution.

Observation Four: The greatest number of mast cells are in the bone third, followed by the mid third with the least found adjacent to the tooth.

In Yamasaki *et al's* study (1982b) there was no subdivision of the periodontal ligament into horizontal thirds. However, the photographs depicted in the paper show most mast cells in the bone side of the ligament.

In the present study the overall means including left and right teeth for each third are as follows:

Bone - 1.548±0.049 (86.6%)
Mid - 0.193±0.017 (10.8%)
Tooth - 0.046±0.008 (2.6%)

This could be explained by the mast cell distribution mirroring the blood vessel distribution in the ligament. Freezer (1984) found that blood vessels were distributed in the PDL as follows:

HORIZONTAL THIRD	SURFACE AREA (μm)^2	VOLUME (%)
Bone	189.3	67%
Mid	115.9	28%
Tooth	58.4	5%

Freezer, 1984

A similar blood vessel distribution has been confirmed by Cameron (1995) who looked at PCV numbers. PCV comprise 88% of mouse PDL vascular volume (Freezer and Sims, 1987). Most PCV occur in the bone third of the ligament with a lesser number in the mid third and the least in the tooth third for both young and old mice (Cameron, 1995).

	Young mice	Old mice
Bone	77.2%	73.8%
Mid	22.5%	21.6%
Tooth	0.3%	4.6%

Adapted from Cameron, 1995

Observation Five: There were more mast cells in each horizontal third on the control side than on the experimental side (refer to Table 5.5 and Figure 5.20).

This can be seen in Table 5.5, Figure 5.20 and Table 5.10. This can be explained by degranulation of mast cells on the experimental side.

Interestingly, when comparing left and right sides, the greatest absolute drop in mast cell numbers is in the bone third. However, the greatest percentage drop occurs in the mid third.

Absolute Mean Change:		**Percentage Change:**	
L-R:		(L-R)÷Lx100%:	
Bone	0.454	Bone	25.58%
Mid	0.164	Mid	59.63%
Tooth	0.019	Tooth	33.92%

The treatment: horizontal distribution interaction (Table 5.10) is significant on the log scale (Table 5.7) ($P = 0.017$) and is therefore not a proportional interaction. This represents an effect of the orthodontic tooth movement and illustrates a reduction in mast cell numbers in the experimental teeth. The greater proportionate drop in mast cell numbers in the mid third ($R:L = 0.41$) as compared to the bone third ($R:L = 0.71$) is interesting because with the higher mast cell numbers in the bone third one would expect the reverse to occur. One possible explanation for this is that as most blood vessels are found in the bone third there may actually be replacement of some of the degranulated mast cells in the bone third with newly recruited mast cells resulting in a smaller proportionate drop in this third.

6.5.2.2 Vertical Distribution

Observation Six: Anova for tooth:vertical level gave a $P \leq 1x10^{-7}$ indicating a difference between these levels for control and experimental teeth, but not specifying which direction.

Observation Seven: A large difference was evident between coronal levels in control and experimental teeth (control > experimental).

The mean mast cell count in the coronal level averaged over the time groups was 0.981 for control teeth and 0.287 for experimental teeth in the experimental animals (Table 5.11). This can be seen graphically in Figure 5.21.

Observation Eight: Data from control teeth indicate that both coronal and apical values were much higher than mid-values.

The mean mast cell count in the coronal and apical levels averaged over the time groups for the control teeth were 0.981 and 0.914 respectively as compared to 0.210 in the middle level (Table 5.11). This vertical distribution of mast cells in the control teeth can be seen graphically in Figure 5.21.

Therefore, on the control side the greatest number of mast cells occurred in the coronal and apical levels with numbers considerably less in the middle level.

This is consistent with the blood vessel distribution found in young mice (35 days old). There is a steady reduction in PCV numbers (the most common vessel type) from the alveolar crest to a minimum at the mid point of the PDL followed by a steady increase in PCV numbers to the apex (Cameron, 1995).

This distribution changed in the experimental teeth such that the coronal mast cell numbers drop dramatically with essentially no change of the mast cell numbers in the middle and apical levels. Refer to Table 5.6 and Figure 5.21.

This phenomenon could be explained in two ways. Firstly, the application of force is on the crown of the tooth which results in more pressure on the coronal third of the ligament and hence more degranulation in this area. Tipping movements concentrate compression on a small periodontal area in the coronal region with corresponding tissue changes seen apically on the opposite side (Rygh and Moyers, 1988). It is characteristic that a tipping movement nearly always results in the formation of a hyalinized zone slightly below the alveolar crest (Reitan, 1960; Rygh and Moyers, 1988) which may indicate a greater magnitude of force in the coronal region than the apical region. Secondly, the supporting ligature tied around the neck of the tooth to prevent dislodgement of the spring in the experimental animals may be causing a periodontal inflammatory response and hence more degranulation in the coronal third of the teeth.

6.5.2.3 Quadrant Distribution

Observation Nine: On the control side there are generally more mast cells in the buccal and mesial quadrants than in the palatal and distal quadrants.

On the control side there were nearly twice as many mast cells in the buccal and mesial quadrants (B=0.885, M=0.840) than in the palatal and distal quadrants (P=0.547, D=0.535). Refer to Table 5.17 and Figure 5.22.

Analysis of variance of treatment, quadrant distribution and time interactions (Table 5.18) indicate that the treatment:quadrant interaction is significant (P = 0.009). The fact that this interaction is significant but that quadrant is not significant (P = 0.099) illustrates that there are not significant

differences between the quadrants overall (Table 5.19), only differences in how they react to treatment.

Observation Ten: A large difference was evident between the buccal and mesial quadrants in the control and experimental teeth (control > experimental) indicating a large treatment effect.

This distribution changed in the experimental teeth such that the buccal and mesial quadrants reduced markedly (B=0.416, M=0.519) whilst the palatal and distal quadrants stayed essentially the same (P=0.469, D=0.556). Refer to Table 5.7 and Figure 5.22.

The treatment:quadrant interaction (Table 5.20) confirms this with a drop in the mast cell numbers in the experimental teeth for the buccal and mesial quadrants (the largest being in the buccal) with essentially no change in the palatal and distal quadrants (R:L ratios close to 1.00).

This can be explained by the fact that even though the intention was to move the right teeth in a buccal direction there is histologic evidence that the teeth were actually moved in a mesio-buccal direction. This could have been due to the spring having a slight mesial component of force and/or the ligature tied around the experimental teeth could have been putting pressure between the first and second molars and moving the first mesially. The increased pressure in these areas has perhaps caused increased degranulation of the mast cells in these two quadrants.

This finding has also been reported by Yamasaki *et al.* (1982b) who found a greater drop in mast cell numbers on the pressure side of the ligament than on the tension side.

6.5.3 MAST CELLS NEAR BLOOD VESSELS

Yamasaki *et al.* (1982b) noted a number of mast cells around the blood vessels of the periodontal ligament, though they did not quantify them.

From Tables 5.30 and 5.31 it can be seen that a high proportion of mast cells are located "near" (i.e. within 12.5μm) a blood vessel in both the experimental and control animals.

From Tables 5.33 and 5.34 it can be seen that there is a definite effect of orthodontic movement with regards to mast cell proximity to blood vessels. In Table 5.33 of the experimental rats there is a shift of almost 1 unit on the logit scale in the proportion of mast cells close to blood vessels, being

higher on the experimental side. On the other hand, Table 5.34 of the control rats there is almost no shift on the logit scale. This can be seen illustrated in Figure 5.24 where the control data obtained from Table 5.34 is scattered around zero. In comparison the experimental data of the 4 hour and 24 hour time groups show a definite increase in the mast cells proximity to blood vessels and a possible increase at 1 hour.

The 4 and 24 hour time groups were significant in terms of absolute mast cell numbers as there was a significant drop in numbers at this time (Refer to Table 5.2 and Figure 5.19). There could be two possible explanations:

Firstly, the effect of tooth movement may cause the mast cells to migrate towards the blood vessels where they degranulate (mostly at 4 hours and 24 hours) so their vasoactive mediators are released where they are most potent, near blood vessels.

Secondly, as mast cell degranulation is highest at 4 and 24 hours the mast cells we are seeing near the blood vessels may in fact be newly recruited mast cells to temporarily replace degranulated mast cells.

It would be of considerable interest to count degranulated mast cells in order to give more of an idea as to what is occurring at these two timepoints.

SUGGESTED AREAS OF FUTURE RESEARCH

- As this study used male Sprague Dawley rats, an evaluation and comparison with female Sprague Dawleys may indicate the influence of sex variation.

- As the study only involved one force value (approximately five grams) carrying out parallel experiments with varying force levels may indicate differing effects on the mast cell population.

- As the mast cell counts were generally low (zero or near zero) it may be advisable to repeat this experiment with larger experimental groups (N=8-10) and also possibly study more levels for each tooth (30-40) to improve the accuracy of the results obtained.

- The use of antibodies to cell membrane markers may make it possible to identify degranulated mast cells as well as intact mast cells.

- Stain the blood vessels in the ligament adjacent to mast cells to confirm if they are arteriolar or venular.

- Use immunohistochemistry to see if substance P (SP) and calcitonin gene-related peptide (CGRP) in the periodontal ligament are linked to the mast cell distribution.

CHAPTER SEVEN: CONCLUSIONS

The principal aim of this study was to test the null hypothesis that there would be no change in the number of stainable mast cells subsequent to orthodontic tooth movement. The metachromatic reaction of mast cells for toluidine blue is due to the acid mucopolysaccharides present in the granules. Stimulation of the mast cell causes extrusion of these materials, which, in turn, results in the mast cell losing its stainability for toluidine blue. A decrease in the number of mast cells in toluidine blue sections would therefore suggest activation of mast cells.

- The Sprague Dawley rat proved to be an adequate experimental model for the analysis of mast cell population changes in the periodontal ligament incident to orthodontic tooth movement.

- The orthodontic appliances proved to be successful over the time frame studied.

- The periodontal tissues were well preserved using Carnoy's fixative.

- The first connective tissue attachment to the tooth proved to be a reliable and easily identifiable landmark as zero point.

- The toluidine blue stain employed proved to be a reliable stain for mast cells with minimal background staining.

7.1 TREATMENT EFFECTS

- Analysis of variance indicated there were large treatment differences between the left (control) and right (experimental) teeth within the experimental animals.

- Mean mast cell counts throughout the PDL indicated that the control teeth generally had higher mast cell counts than the experimental teeth, except this trend was reversed in the one hour time group. Therefore, orthodontic tooth movement causes mast cells to degranulate in the experimental teeth, hence the higher numbers in the control teeth.

- A split-plot analysis was used to see if there was an interaction of treatment and time. This analysis suggested:

    - There was a highly significant difference between time groups irrespective of treatment but this difference was not important because of the low F value (5.583).

    - There was a highly significant effect related to treatment (ie. left vs right) which was important (F = 17.498).

    - There was no relationship between time and treatment.

7.2 POSITIONAL EFFECTS

- Analysis of variance which specified the effect of position (by level, quadrant and horizontal distribution) indicated that the horizontal position (ie. bone, mid and tooth thirds) was most significant. Level (ie. coronal, middle and apical) was the next important position with quadrant (ie. mesial, buccal, palatal and distal) being the least important.

7.2.1 Horizontal Distribution

- The greatest number of mast cells were found in the bone third, followed by the mid third with the least found adjacent to the tooth.

- There were more mast cells in each horizontal third on the control side than on the experimental side.

7.2.2 Vertical Distribution

- On the control side the greatest number of mast cells occurred in the coronal and apical levels with considerably less found in the middle level.

- This distribution changed on the experimental side such that the coronal mast cell numbers dropped dramatically with essentially no change of the mast cell numbers in the middle and apical levels.

7.2.3 Quadrant Effect

- On the control side there were nearly twice as many mast cells in the mesial and buccal quadrants as in the palatal and distal quadrants.

- This distribution changed on the experimental side such that the mesial and buccal quadrants reduced markedly whilst the palatal and distal quadrants stayed essentially the same.

This project supported the hypothesis that the number of stainable mast cells decreased following orthodontic tooth movement, however, it is interesting to note this decrease was not uniform throughout the ligament. When considering the horizontal distribution across the PDL all thirds demonstrated a drop in mast cell numbers; when considering the vertical distribution only the coronal mast cell numbers dropped; when considering the quadrant distribution only the mesial and buccal mast cell numbers dropped.

7.3 MAST CELLS AND BLOOD VESSELS

- For control teeth more than half of the mast cells were located near blood vessels:

Experimental rats	-	left teeth :	586/1035	(56.6%)
Control rats	-	left teeth :	192/353	(54.4%)
Control rats	-	right teeth :	213/419	(50.8%)

This averaged out at 991 mast cells near blood vessels out of a total of 1807 mast cells i.e. 54.8%.

- For experimental teeth more than three quarters of the mast cells were located near blood vessels:

Experimental rats	-	right teeth :	551/728	(75.7%)

- There was a significant increase in the number of mast cells located near blood vessels at 4hrs and 24hrs after starting orthodontic tooth movement.

Analysis of variance indicated there were large treatment differences between the left (control) and right (experimental) teeth within the experimental animals (Mean Sq = 21.83, P = 0.002).

The present study demonstrated a change in the mast cell numbers when comparing experimental (orthodontically moved) and control teeth. The mean mast cell counts throughout the PDL indicated that the control teeth generally had higher mast cell counts than the experimental teeth, except that this trend was reversed in the one hour time group.

This change in mast cell counts was not universal throughout the ligament and was highly affected by position (ie. vertical distribution, quadrant and horizontal distribution). Analysis of variance indicated that the horizontal distribution (ie. bone, mid and tooth thirds) was the most significant followed by the vertical distribution (i.e. coronal, mid and apical levels) with the least significant being the quadrant (buccal, mesial palatal and distal).

The results obtained were statistically significant and therefore the experimental design was adequate to achieve a result. However, it can be noted that for analysis of variance it is necessary that the data be roughly "normal". This was not true for this study as a large proportion of the data was zero, and the rest of the data generally were low counts. Another difficulty is that the occasional high value would adversely affect the statistical analyses.

CHAPTER EIGHT: APPENDICES

8.1 MAST CELL MEDIATORS

8.1.1 PRE-FORMED MEDIATORS

Histamine:

A major component of mast cell granules, accounting for about 10% of the granule weight. It is stored, preformed, in the granules bound to sulphated proteoglycans (heparin or chondroitin sulphate)(Stevens *et al.*, 1986). Rat mast cells contain 20 - 30 pg of histamine per cell (Login *et al.*, 1991. Histamine is produced from the amino acid L-histidine by the cytoplasmic enzyme histidine decarboxylase. Histamine is a CNS neurotransmitter which is stored in many sites in the body and has many pharmacological effects. The biologic effects of histamine release are mediated by three receptor subtypes: H_1, H_2 and H_3 receptors. These receptors have different tissue distributions and mediate different effects when they bind histamine. Stimulation of H_1 receptors results in contraction of non-vascular smooth muscle (bronchiolar, urinary bladder, GI tract) and endothelial cells (increasing permeability), prostaglandin production, and eosinophil chemotaxis[1]. Activation of H_2 receptors also increases vasopermeability through endothelial cell contraction, and also augments gastric acid secretion, promotes mucous production, inhibits the release of histamine from basophils (but not mast cells), inhibits lymphokine and neutrophilic-enzyme release and reduces T-lymphocyte-mediated cytotoxicity. The function of the most recently discovered histamine receptor, H_3, is currently under investigation. (Tharp, 1985; Bona, 1990; Kuby, 1994; Siraganian, 1992 and Paul, 1993).

Serotonin:

Also known as 5-hydroxytryptamine (5-HT). Human mast cells do not contain serotonin, however, in addition to histamine, serotonin is also present in the mast cells of other species eg. rodents. Serotonin is formed by decarboxylation of the 5-hydroxytryptophan (5-HTP) molecule (Lagunoff and Benditt, 1959). In rodents it causes oedema and plays an important role in anaphylactic reactions (Siraganian, 1992 and Paul, 1993). Serotonin in mast cells can be the result of synthesis

[1]At micromolar concentrations, histamine is selectively chemotactic for eosinophils while at higher concentrations it appears to inhibit eosinophil migration.

of 5-HT from 5-HTP by the cells or be the result of uptake of 5-HT from extracellular fluid (Enerbäck and Gustafsson, 1981).

Proteoglycan:

Heparin is comprised of a protein core to which glycosaminoglycan side chains are attached. Due to its high negative charge density, the heparin molecule appears to function as a storage matrix in the mast cell granule for histamine which is positively charged as well as for some chemotactic factors and enzymes. In addition to its anticoagulation effects, heparin may inhibit complement activation at several steps along the cascade (Tharp, 1985). Evidence indicates that it may moderate the reaction in part by inhibiting the generation of complement fragments or their effective association (Bona, 1990; Abbas *et al.*, 1994).

Chondroitin sulphate is another proteoglycan that may be present which is different from heparin in its carbohydrate linkage. The characteristic metachromatic staining of mast cells is due to the presence of proteoglycans in their granules: heparin stains red and chondroitin sulphate stains blue with the combined alcian blue/safranin O stain (Siraganian, 1992).

Neutral Proteases:

More than half the protein in the mast cell granules consists of trypsin or chymotrypsin-like neutral proteases including tryptase, chymotryptase and carboxypeptidase. These proteases can destroy nearby cells and also cleave complement components yielding C3a and C5a (anaphylatoxins). Tryptase may activate the Hageman factor and possibly cleave kininogen to facilitate bradykinin formation. The generation of kinins has been associated with the onset of pain, increased vascular permeability, vasodilation and smooth muscle contraction. Activation of the Hagemen factor could potentially stimulate the clotting, fibrinolytic, and complement cascades (Tharp, 1985; Tizard, 1995 and Roitt *et al.*, 1996).

C3a induces mast cell and basophil degranulation, smooth muscle contraction (which may be secondarily mediated by histamine) and an increase in mucus secretion. C5a has the same effects and is also chemotactic for phagocytic cells and increases vascular permeability. (Bona, 1990 and Siraganian, 1992).

Other Enzymes:

The human and rodent mast cell has a number of granule-associated enzymes. These enzymes are not limited to the corrective tissue mast cell; however, their release during mast cell-degranulation may have potentially important local and systemic effects. These enzymes include ß-glucosaminidase, ß-hexosaminidase, ß-glucuronidase, ß-D-galactosidase, tryptase, arylsulfatase A, superoxide dismutase and peroxidase and are released from the secretory granules (Tharp, 1985 and Siraganian, 1992).

Some mast cell enzymes appear to have potential anti-inflammatory effects. Arylsulfatase A has been reported to inactivate leukotrienes while superoxide dismutase, which facilitates the conversion of superoxide to hydrogen peroxide, may protect tissues from potentially toxic molecules (Tharp, 1985).

Tharp (1985) reports that the overall relevance of these granule-associated enzymes following mast cell secretion remains to be established. McKenna and Frame (1985) state that these enzymes are capable of metabolising the ground substance mucopolysaccharides and proteins derived from the circulation.

Eosinophil Chemotactic Factor:

The mediator eosinophil chemotactic factor of anaphylaxis (ECF-A) is stored in granules as a mixture of four tetrapeptides (Tharp, 1985 and Tizard, 1995). Clinically, variable numbers of eosinophils are often observed in tissue sections from patients with urticaria and mastocytosis (Webb *et al.*, 1982). Eosinophils neutralise the effects of the released mediators; histamine is diffused by histaminase, SRS-A by aryl sulphatase and platelet activating factor by phospholipase D. As a result, eosinophils modulate the reactions consequent upon mast cell degranulation (Roitt and Lehner,1980).

Neutrophil Chemotactic Factor:

This is a high-molecular-weight protein ($M_r > 160,000$). As its name implies it is chemotactic for PMNs (Bona, 1990).

Platelet Activating Factor:

Platelet activating factor (PAF) is synthesized by acylation of lysoglyceryl ether phosphorylcholine, which is derived from a membrane phospholipid by phospholipase A_2 release of arachidonic acid. PAF makes platelets aggregate and release their vasoactive molecules, especially serotonin, and synthesize thromboxanes. PAFs promote neutrophil aggregation, degranulation, chemotaxis and the release of oxygen radicals. PAFs are major activators of eosinophils (Tizard, 1995). PAF may also cause contraction of pulmonary vascular and bronchial smooth muscle. Whether this is a direct effect or is mediated through prostaglandins is not clear. PAF also produces wheal and flare reactions, although the exact mechanism is unknown (Bona, 1990; Abbas, 1994).

Interleukin 3:

Interleukin 3 (IL-3) is secreted by activated T cells and by mast cells following IgE-induced degranulation. IL-3 induces differentiation of pluripotent stem cells in the bone marrow by inducing responsiveness to colony stimulating factors. IL-3 also increases production of granulocytes and lymphocytes and, in conjunction with IL-4, promotes growth of mast cells. The action of IL-3 is mediated (at least in part) via an increase in histamine production in the bone marrow. IL-3 may also activate mature granulocytes and macrophages. IL-3 is encoded by a single gene on chromosome 5 in a locus containing genes encoding several other cytokines. IL-3 receptors are found on bone marrow cells and peripheral blood monocytes (Bona, 1990).

Interleukin 4:

Interleukin 4 (IL-4) has M_r=20,000 and is produced by stimulated T cells, mast cells, and bone marrow stromal cells. IL-4 increases the surface density of MHC class II antigens on resting B cells; it promotes both the development of immature pre-B cells to mature B cells, as well as activation of mature B cells. IL-4 is necessary (but not sufficient) for mature B cell entry into S phase upon activation. It also causes an increase in B cell size and the acquisition of locomotor capacity. IL-4 also has effects on T cells: it induces antigen-specific cytotoxic T cell function, and, like IL-2, can induce the proliferation of $T_{h/i}$ and T_c cells. Additional effects of IL-4 include synergism with IL-3 in promoting growth of mast cells and haemopoietic stem cells; also enhancement of neutrophil phagocytosis and the respiratory burst. IL-4 is encoded by a single gene on chromosome 5. IL-4 receptors have been found on lymphoid and myeloid cells, as well as on hepatocytes, and fibroblasts (Bona, 1990). In addition, through its selective induction of endothelial

VCAM-1 and an eosinophil-selective chemotactic substance, it is responsible for selective recruitment of eosinophils (Abbas *et al.*, 1994 and Paul, 1993).

Interleukin 5:

The major activity of interleukin 5 (IL-5), produced by T_h cells, is to stimulate the differentiation of eosinophils. IL-5 also stimulates B cell proliferation. IL-5 is a protein of M_r=21,000 and is encoded by a single gene on chromosome 5. Two types of IL-5 receptor with different affinities have been identified on a B lymphoma cell line (Bona, 1990).

Interleukin 6:

Interleukin 6 (IL-6) is a polypeptide with M_r=19-32,000 and has many biological activities. IL-6 induces immunoglobulin secretion by B cells, but not their proliferation; it increases IgM, IgG, and IgA secretion by mitogen-stimulated B cells; it influences the differentiation of hemopoietic stem cells; it induces differentiation of $T_{h/i}$ and T_c cells; it promotes growth of malignant plasma cells (plasmacytomas); and it also has activity similar to that of nerve growth factor. Its most important physiologic role may be the induction of the acute phase reaction (hepatic synthesis of acute phase proteins and decreased albumin synthesis). Several activities once attributed to IL-1 are now thought to be mediated by IL-6.

IL-6 is produced by T cells, B cells, macrophages, mast cells, fibroblasts, keratinocytes, endothelial cells, bone marrow stromal cells, mesangial cells and brain astrocytes. IL-6 is encoded by a single gene located on chromosome 7.

IL-6 increases the rate of cell division of plasmacytomas *in vitro*, and addition to the culture of antibodies binding IL-6 reduces the rate of cell cycling. Abnormal production of IL-6 occurs in some haematologic malignancies, and other disorders. Castleman's disease is a localised or systematic anomalous proliferation of polyclonal plasma cells in lymph nodes or other lymphoid tissue. Overproduction of IL-6 in germinal centers has been implicated in the etiology of this disease. Autoimmune phenomena are frequently associated with a cardiac myxoma (a type of connective tissue tumor); some myxomas produce IL-6. The synovial fluid of patients with rheumatoid arthritis contains elevated levels of IL-6. Both synovial cells and infiltrating T cells are stained with labelled antibodies binding IL-6. Kidney mesangial cells may also produce IL-6 and patients with mesangial proliferative glomerulonephritis have increased amounts of IL-6 in their

urine. Renal adenocarcinoma (also called hypernephroma or Grawitz tumor) has also been shown to secrete IL-6.

IL-6 receptors are distributed widely in the body. They are found on hemopoietic precursors, resting or activated B cells, resting T cells, myeloid cells and hepatocytes. Receptors will probably be found in other tissues as well. As with several other cytokines, there appear to be high-affinity and low-affinity receptors (Bona, 1990).

GM-CSF:

Granulocyte-monocyte colony stimulating factor (GM-CSF). This may act in concert with IL-3 and IL-5 to activate eosinophils and basophils, as these three cytokines cross-react on receptors because the receptors share a common β chain (Paul, 1993).

TNF:

Tumour necrosis factor (TNF) is a cytokine which is stored by mast cells. This increases the expression of cell-adhesion molecules (ICAM-1) on venular endothelial cells, thus facilitating the build up of neutrophils, eosinophils and monocytes (Kuby, 1994; Abbas *et al.*, 1994).

8.1.2 NEWLY SYNTHESIZED MEDIATORS

A number of lipid mediators are generated after mast cells are stimulated. During secretion, arachidonic acid is released from phospholipids due to the activation of phospholipase enzymes. The arachidonic acid is then metabolised either along the cyclo-oxygenase pathway with the formation of the prostaglandins and thromboxanes, or by the lipoxygenase pathway with the formation of the leukotrienes (Siraganian, 1992 and Paul, 1993).

Prostaglandins:

The principal product of cyclo-oxygenase activity is prostaglandin D_2 (PGD_2), but other prostaglandins may be generated in smaller quantities as well as prostacyclins and thromboxanes (McKenna and Frame, 1985; Tizard, 1995). PGD_2 mimics many of the local and systemic effects of histamine; yet, its action on various tissues is mediated independently of the histamine receptors (Bona, 1990). PGD_2 binds to receptors on smooth muscle cells and acts as a vasodilator and as a

bronchoconstrictor. It is chemotactic for eosinophils and neutrophils and also inhibits platelet aggregation (McKenna and Frame, 1985; Abbas *et al.*, 1994).

Note: PGE_2 suppresses the immediate hypersensitivity reaction. Neutrophils and macrophages synthesize PGE_2 which inhibits mediator release from mast cells (Bona, 1990).

Thromboxanes:

Thromboxane is a minor mast cell product generated by the cyclo-oxygenase pathway of arachidonic acid metabolism. It activates platelets and also induces bronchoconstriction.

Leukotrienes:

As secondary mediators, the leukotrienes are not formed until the mast cell undergoes degranulation and its plasma membrane is broken down. Therefore, it takes longer for their biologic effects to become apparent. Their effects are more pronounced and longer-lasting, however, than those of histamine. Nanomole levels of leukotrienes are as much as 1000 times more potent as bronchoconstrictors than histamine and are also more potent stimulators of vascular permeability. Lipoxygenase activity results in slow-reacting substance of anaphylaxis (SRS-A), a leukotriene (LT) comprised of three distinct sulfidopeptide leukotrienes (LTC_4, LTD_4, and LTE_4) (McKenna and Frame, 1985). Leukotrienes C (LTC_4) and D (LTD_4) have been identified in human lung mast cells. Both compounds are potent bronchoconstrictors, LTD_4 being approximately 100-fold more active than LTC_4. LTC_4 constricts skin blood vessels while LTD_4 relaxes cutaneous vasculature (Tharp, 1985). Some important actions of the leukotrienes are the stimulation of phagocyte chemotaxis and the release of neutrophil lysosome contents (Bona, 1990 and Kuby, 1994).

8.2 FIXATION

Carnoy's Fixative

Formula:

Absolute alcohol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

8.3 DECALCIFICATION

EDTA In Cacodylate Buffer

Formula:

EDTA	74.44 gm	
Cacodylate buffer (0.06M)	1800 ml	(see below)
25% gluteraldehyde	200 ml	

Dissolve EDTA in cacodylate buffer by gently heating.

When cool, add the gluteraldehyde.

pH to 6 at 4°C using the pH meter (remember to change the temperature).

Shelf Life: 7 days at 4°C.

0.6m Cacodylate Buffer (Arsenic)

Formula:

Sodium Cacodylate	23.11 gm
DD water	1800 ml

pH to 7.4 at 20°C

Shelf Life: 7 days at 4°C

8.4 PARAFFIN WAX EMBEDDING

Routine paraffin wax embedding can be discussed under the following headings:

1. Dehydration
2. Clearing
3. Infiltration
4. Embedding

8.4.1 DEHYDRATION

Paraffin wax does not penetrate tissues in the presence of water so dehydration is an essential preliminary step in this process. This is effected by immersion of the tissue in increasing concentrations of ethyl alcohol (Drury and Wallington, 1967)

A commonly used range of dehydrating solutions is one change in each of 70%, 80%, and 90% ethanol and three changes of absolute alcohol. This regime was used in this study.

8.4.2 CLEARING

Alcohol is not miscible with paraffin wax and so after dehydration it is necessary to treat tissue blocks with a reagent that mixes with both substances and which may in turn be eliminated in the process of wax impregnation. This step has come to be known as clearing because all the reagents used for this purpose raise the refractive index of tissue rendering it more or less transparent. This is incidental to the main purpose of replacement of the alcohol by a wax solvent. (Drury and Wallington, 1967).

The most common clearing agents in use are chloroform, benzene, xylene, toluene, carbon tetrachloride and cedar-wood oil. The clearing agent used in this study was Clearene, a select blend of terpenes (Surgipath - Grayslake, Illinois).

8.4.3 INFILTRATION

Molten wax for infiltration and embedding is kept in an electrically heated temperature controlled oven. The temperature of the oven should be maintained at 2-3°C. above the melting point of the wax (Drury and Wallington, 1967).

The function of wax infiltration is to remove the clearing agent from the tissues and for them to be completely permeated by the paraffin wax which is subsequently allowed to harden to produce a block from which sections may be cut. The duration and number of changes of wax necessary for this varies with the size and consistency of the specimen, but as the temperature of the wax oven causes shrinkage and hardening of tissue, the time in the oven should be kept to the minimum period which still allows thorough impregnation (Drury and Wallington, 1967).

The melting point of wax to be used depends on personal preference, the type of tissue and the general temperature of the laboratory; the most popular melting point for routine work is 54°C which gives support for hard tissues and yet allows ribboning of sections. The wax used in this study was blue ribbon infiltrating medium (MP: 56-57°C) which is manufactured by Surgipath - Richmond, Illinois.

Evaporation of clearing agent, impregnation with paraffin wax and removal of trapped air in specimens occurs more quickly and completely if carried out at reduced pressure. This reduced pressure is obtained by use of a vacuum embedding oven. A reduced pressure of 300-500mm Hg is adequate for most purposes (Drury and Wallington, 1967).

8.4.4 EMBEDDING

Having been completely impregnated with wax, it is necessary to obtain a solid block containing the tissue. This was done by filling a mould of suitable size with molten paraffin wax, orientating the specimen in the mould such that the occlusal surfaces of the molars were sectioned first. The mould was cooled in a freezer to promote solidification. The embedding wax used in this study (MP: 56-57°C) was manufactured by Surgipath - Richmond, Illinois.

If properly fixed, dehydrated, cleared and impregnated tissues embedded in paraffin wax will retain their cutting and staining characteristics indefinitely. Blocks should be stored in a cool place, such as in a refrigerator or freezer. If tissue is intended for microscopical examination at some future date it keeps better in the form of a paraffin block than in any fixative or preserving fluid (Drury and Wallington, 1967).

8.5 SLIDE COATING PROCEDURE

Subbing Solution

Formula:

3-APT*[4]	4 ml
Absolute alcohol	196 ml

*[4] 3-APT = 3-aminopropyltriethoxysilane manufactured by Sigma Chemical Co. - St. Louis, USA

Procedure:

1. Soak slides in Pyroneg for at least 24 hours.
2. Rinse slides in running water for 12 hours.
3. Rinse in several changes of distilled water.
4. Agitate in absolute alcohol for 30 secs.
5. Agitate in absolute alcohol for 30 secs.
6. Soak in 2% 3-aminopropyltriethoxysilane for 10 secs.
7. Agitate in absolute alcohol for 30 secs.
8. Agitate in absolute alcohol for 30 secs.
9. Rinse in distilled water for 60 secs.
10. Allow slides to dry in 37°C oven.

8.6 HAEMATOXYLIN AND EOSIN STAINING

Haematoxylin

Formula:

Haematoxylin	5 gm
Ammonium alum *	50 gm
Glycerol	300 ml
Distilled water	700 ml
Sodium Iodate	1 gm
Glacial Acetic Acid	20 ml

* Aluminium ammonium sulphate

Dissolve the haematoxylin in about 20 ml of absolute alcohol.

Dissolve the alum. in some of the distilled water by heating. Allow to cool.

Mix in other ingredients and filter into bottle; label and date.

Eosin - Stock Solution

Formula:

Eosin	10 gm
Potassium dichromate	5 gm
Saturated Picric acid	100 ml
Absolute alcohol	100 ml
Distilled water	800 ml

For working solution, dilute 1:9 with distilled water.

i.e. 25 mls Eosin + 225 mls distilled water.

Dilute Alkali Formula

Formula:

Ammonium hydroxide (NH_4OH)	42ml
Absolute alcohol	200ml
Distilled water	550ml

Procedure For Mayer Lillie Haematoxylin And Eosin Staining Method

Remove Wax (deceration)

1. Xylene*[5] 5 minutes
2. Xylene*[5] 5 minutes
3. Absolute alcohol 2 minutes
4. Absolute alcohol 2 minutes

Check for Incomplete Removal of Wax and Xylene

5. Dip in tap water

Staining

6. Haematoxylin (Mayer Lillie) 10 minutes
7. Wash approximately 1 minute in running tap water

Differentiation

8. 0.5% HCI Hydrochloric Acid - one dip

Blue

9. Running water 10 minutes
10. Dilute Alkali 2 dips (see below)
11. Wash in running water 1 minute

Eosin

12. 10% stock solution 30 seconds

Dehydration and Clearing

13. Absolute alcohol 2 minutes
14. Absolute alcohol 2 minutes
15. Xylene*[5] 2 minutes
16. Xylene*[5] 2 minutes

Mounting with Coverslips

17. Mount with DPX*[6]

*[5]Xylene - General purpose reagent manufactured by Merck Pty Ltd - Kilsyth, Victoria, Australia.

*[6]DPX - Neutral mounting medium manufactured by Ajax Chemicals - Auburn, New South Wales, Australia.

8.7 STAINING METHODS FOR GRANULAR GLYCOSAMINOGLYCANS

Most of the following methods described are compatible with fixation in formalin and paraffin embedding according to standard procedures. In some instances Carnoy's fixation or fixation in IFFA (Enerbäck, 1966a) give improved results. The latter fixative contains 0.6% formaldehyde and 0.5% acetic acid, prepared from reagent-grade formaldehyde solution (37%) and glacial acetic acid. Tissues are usually fixed for 12-24 hr followed by immersion in 70% ethanol overnight, dehydration, and paraffin embedding according to standard procedures.

Since this tissue contained mineralised tissue it had to be rehydrated following fixation before being placed in the EDTA solution. After decalcification the tissue was dehydrated before being processed and embedded.

8.7.1 TOLUIDINE BLUE

Metachromatic Staining at pH 0.5

Procedure:

Remove Wax (deceration)

1. Xylene*[5] 5 minutes
2. Xylene*[5] 5 minutes
3. Absolute alcohol 2 minutes
4. Absolute alcohol 2 minutes

Check for Incomplete Removal of Wax and Xylene

5. Dip in tap water

Staining

6. 0.5% toluidine blue at pH 0.5*[7] (0.5N HCI) for 30 minutes
7. Wash 1 minute in running tap water

Dehydration and Clearing

8. 70% alcohol 15 seconds
9. 96% alcohol 15 seconds
10. Absolute alcohol 15 seconds
11. Xylene*[5] 2 minutes
12. Xylene*[5] 2 minutes

Mounting with Coverslips

13. Mount with DPX*[6]

*[5]Xylene - General purpose reagent manufactured by Merck Pty Ltd - Kilsyth, Victoria, Australia.

*[6]DPX - Neutral mounting medium manufactured by Ajax Chemicals - Auburn, New South Wales, Australia.

*[7]pH - measured by Activon digital pH/mV meter.

Note: For 0.5N HCI
= 0.5M HCI add 4.3ml stock HCI (36%) to DDW and make up to 100ml.

Metachromatic Staining at pH 4

Procedure:

1. Deparaffinize, and hydrate to water.
2. Stain in 0.5% toluidine blue solutions at pH 4 (McIlvaine's citric acid-disodium phosphate buffer) for 45 sec.
3. Rinse in water, and dehydrate quickly through 70%, 96%, and absolute ethanol (each step, 15 sec) to xylene, and mount in synthetic resin.

Result: Mast cells stain metachromatically violet or purple. Cell nuclei stain blue at pH 4.

Long Toluidine Blue (Wingren and Enerbäck, 1983)

Procedure:

1. Deparaffinize, and hydrate to water.
2. Stain in 0.5% toluidine blue at pH 0.5 for 5 days (filter dye solution every other day to avoid precipitates).
3. Optional: Rinse in water, and counterstain for 20 sec in 1% eosin in 70% ethanol.
4. Rinse in water, and dehydrate quickly through an ethanol series (as above) to xylene, and mount in synthetic resin.

Result: Mast cell granules stain dark blue; background is unstained or red with eosin counterstaining.

Combined Staining of Mast Cells and Eosinophils

Procedure:

1. Deparaffinize, and hydrate to water.
2. Stain with 0.5% toluidine blue at pH 0.5 for 5 days.
3. Rinse in water.
4. Stain with eosin at pH 10 for 1 min (1% eosin gelblich, C.I. 45380, dissolved in a solution of 2.5g disodium tetraborate in 100 ml distilled water adjusted to pH 10 with 10M NaOH).
5. Rinse quickly in water, and dehydrate in 70% ethanol for 3 min, 96% ethanol for 1 min, absolute ethanol for 1 min, clear in xylene, and mount in synthetic resin.

Result: Mast cell granules stain dark blue, granules of eosinophil granulocytes stain red, and nuclei stain weakly red or are unstained.

8.7.2 COPPER PHTHALOCYANINS

Alcian Blue at pH 0.2 (After Bloom and Kelly, 1960.)

Procedure:

1. Deparaffinize, and hydrate to water.
2. Stain for 10 min in 0.1% Alcian blue (Alcian blue 8GX, G.I. 74240) in 0.7 M HCl, pH 0.2.
3. Rinse in 0.7 M HCl for 10 min.
4. Dehydrate through ethanol to xylene, and mount in synthetic resin.

Result: Mast cell granules stain blue; background is unstained.

Alcian Blue-Safranin O (Enerbäck, 1987)

Procedure:

1. Deparaffinize, and hydrate to water.
2. Stain for 30 min in 0.5% alcian blue in 3% acetic acid.
3. Wash in water for 3 min.
4. Stain for 30 sec in 0.25% safranin O (C.I. 50240) in 0.125 M HCl.
5. Dehydrate quickly through ethanol to xylene, and mount in synthetic resin.

Result: Mast cell granules usually stain red but at some sites stain blue. A modification of this staining sequence involves staining with alcian blue at pH 0.2 and rinsing for 10 min in 0.7 M HCl, followed by staining with 0.5% safranin O in 0.125 M HC1, to visualise differences between rat mucosal (blue) and connective tissue mast cells (Enerbäck, 1966b).

Critical Electrolyte Concentration (CEC) Staining with Alcian Blue (Scott and Dorling, 1965)

Procedure:

1. Deparaffinize, and hydrate to water.
2. Stain overnight in 0.05% alcian blue in 0.025 M sodium acetate buffer at pH 5.8 with stepwise addition of $MgCl_2$ to an electrolyte concentration at which the staining is extinguished (CEC).
3. Rinse in water, dehydrate through ethanol to xylene, and mount in synthetic resin.

Result: In formaldehyde-fixed tissue specimens, CEC for rat connective tissue mast cells is above 1.4 M, and, for intestinal mucosal mast cells, 0.8 M (Miller and Walshaw, 1972), and for human mast cells, 0.8 M $MgCl_2$ (Scott and Dorling, 1965).

8.7.3 BERBERINE FLUORESCENCE

Fixation in IFAA is preferred in order to reduce fluorescence of nucleoprotein. Formaldehyde fixation is less satisfactory (Enerbäck, 1974; Dimlich *et al.*, 1980).

Procedure:

1. Deparaffinize, and hydrate to water in which the sections are left for 10 min.
2. Stain for 20 min in 0.02% berberine sulphate (Fluka, AG) in distilled water acidified by addition of 1% citric acid to pH 4.
3. Rediffusion in distilled water, acidified to pH 4 by citric acid, for 5 min.

4. Mount in glycerol or, for permanent preparations, in Entellan after a quick dehydration in an alcohol series. Examine in fluorescence microscope with violet and blue activation and fluorescence barrier filter at 500 nm.

Result: Intense yellow fluorescence of mast cell granules is seen; background has low fluorescence intensity.

8.8 DEMONSTRATION OF GRANULE PROTEIN

8.8.1 BIEBRICH SCARLET

Fix tissue in Carnoy's fluid (Appendix 8.2,). Aldehyde fixatives block the staining (Spicer, 1962, Enerbäck, 1987).

Procedure:

1. Deparaffinize, and hydrate to water.
2. Stain for 30-90 min in 0.04% Biebrich scarlet in Laskey's glycine buffer adjusted to pH 9.5 by NaOH.

Result: Mast cell granules stain orange.

8.8.2 CHLOROACETATE ESTERASE

Fixation in 4% neutral buffered formaldehyde or IFAA (resulting in shorter incubation time) (after Burstone, 1957).

Procedure:

1. Deparaffinize, and hydrate to water.
2. Incubate sections at room temperature in a medium containing:

Naphthol-AS-D-chloroacetate (in 0.5 ml dimethylformamide)	5 mg
Distilled water	25 ml
0.2 M TRIS buffer (pH 7.1)	25 ml
Fast garnet GBC	30 mg

Incubate for 15-60 min (depending on fixative).

Note: If pH exceeds 7.1, spontaneous substrate breakdown may occur.

3. Rinse in water.
4. Counterstain nuclei in Mayer's carmalum for 10-15 min.
5. Wash in water, and mount in glycerol.

Result: Sites of esterase activity in mast cells are red; nuclei stain dark blue.

8.9 STAINING METHODS FOR MAST CELL AMINES

8.9.1 HISTAMINE (ENERBÄCK, 1969)

Procedure:

1. Fix small tissue pieces (up to 4x4x4 mm) in Carnoy's fluid at room temperature for 2 hr.
2. Dehydrate in ethanol for 3 hr and ethyl benzene for 1 hr.
3. Embed in paraffin in a vacuum for 30 min.
4. Flatten paraffin sections on dry prewarmed (60°C) slides to melt the paraffin.
5. Treat the sections with 1% *o*-phthalaldehyde in ethylbenzene at 100% relative humidity in a Petri dish for 2 min.
6. Mount in tetrahydrofurfuryl alcohol and examine in fluorescence microscope with violet and blue activation and fluorescence barrier filter at 500 nm.

Result: Yellow fluorescence is confined to mast cell granules; background fluorescence is green. Diffusion of histamine results in yellow fluorescence in cell nuclei.

8.9.2 MONOAMINES

The monoamines can be demonstrated by formaldehyde-induced fluorescence according to Falck *et al.*, 1962 or with immunohistochemical methods. Serotonin may be demonstrated in mast cells containing high concentrations of the amine after formalin fixation and paraffin embedding by standard procedures. The chromogenic material formed under such conditions can be demonstrated by yellow fluorescence, diazo coupling, or reducing properties (argenataffin reaction or ferric ferricyanide reaction). (Enerbäck, 1987)

8.10 HORSERADISH PEROXIDE-AVIDIN STAINING

Procedure:

1. Xylene*[5] 5 minutes.
2. Xylene*[5] 5 minutes.
3. Absolute alcohol 2 minutes.
4. Absolute alcohol 2 minutes.
5. Rinse in tap water 1 minute.
6. 0.5% hydrogen peroxide (H_20_2) 30 minutes.
7. Rinse in running tap water 1 minute.
8. Several quick washes in distilled water.
9. Outline sections with a "PAP" pen*[8].
10. Phosphate buffered saline (PBS) pH 7.4 5 minutes.
11. PBS pH 7.4 3 minutes.
12. PBS pH 7.4 3 minutes.
13. Horseradish peroxidase-Avidin D (HRP - Avidin D)*[9] 60 minutes.
14. PBS pH 7.4 3 minutes.
15. PBS pH 7.4 3 minutes.
16. Diaminobenzidine (DAB)*[10] solution (1 tablet in 1 mL of distilled water) for 20 minutes.
17. Wash in PBS pH 7.4.
18. Absolute alcohol 1 minute.
19. Absolute alcohol 1 minute.
20. Xylene*[5] 2 minutes.
21. Xylene*[5] 2 minutes.
22. Mount in DPX*[6].

*[5]Xylene	-	General purpose reagent manufactured by Merck Pty Ltd - Kilsyth, Victoria, Australia.
*[6] DPX	-	Neutral mounting medium manufactured by Ajax Chemicals - Auburn, NSW, Australia.
*[8] "PAP" pen	-	to localise the reagents. Manufactured by Dakopatts, USA.
*[9] HRP-Avidin D	-	Manufactured by Vector Laboratories.
*[10] DAB	-	Manufactured by Sigma Chemical Company. Cat No. D5637.

8.11 MICROSCOPIC PROCEDURE FOR COUNTING MAST CELLS

An Olympus BH-2 light microscope was used to identify the mast cells in the periodontal ligament (PDL) of the mesiobuccal root of the maxillary first molar in the following manner:

- The 4X objective was used to locate the correct root.
- The 20X objective was used to scan the PDL twice to identify mast cells.
- The 40X objective was used to positively identify any questionable cells.

If mast cells were present:

- The 4X objective was used to align the template (Figure 4.3).
- The 10X objective with the same template orientation was used to specify the locations of the previously identified mast cells (using the 20X and 40X objectives) into quadrants.
- The 20X objective in conjunction with the mm ruler graticule in the eyepiece was used to specify the position of the mast cells within each third.

CHAPTER NINE: BIBLIOGRAPHY

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