# ASPECTS OF THE CELLULAR IMMUNE RESPONSE IN PERIODONTAL DISEASE

By

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# DECLARATION

This thesis is the original work of the author.

Ourania Koulouri

To my parents

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# SUMMARY

Studies on the kinetics of inflammatory/ immune cells have been undertaken in our laboratory and these investigations were confined to the inflamed gingival tissue obtained from adult periodontitis patients. Furthermore, it has been reported that studies of the superficial gingiva are probably not sufficient to give a clear picture of the inflammatory and immune processes of severe periodontitis (Moskow & Polson, 1991). In an attempt to acquire a better knowledge of the dynamics and complex interactions among the immune cells in periodontitis sites, deeper granulation tissue was examined and compared with superficial gingival tissue. The highly vascular and heavily infiltrated granulation tissue was obtained from adult periodontitis and early onset periodontitis patients during surgery and the biopsies were taken from the most apical part of the periodontal pocket. A thorough investigation of the periodontal granulation tissue has not been performed so far. Therefore, the detection of this type of tissue may lead to interesting findings.

Two techniques were employed throughout the studies comprising this thesis; immunohistochemistry and *in situ* hybridisation were used to assess immune cells' kinetics and synthetic activity and to examine possible differences within the two types of tissue and the two forms of periodontal disease. Phenotypic analysis of cell surface markers and qualitative and

quantitative assessment of the cellular infiltrate (macrophages, B cells, T cells, T cell subsets i.e. memory, helper and suppressor) were carried out using immunohistochemistry. Combination with *in situ* hybridisation offered the opportunity to further elucidate synthetic capacity, proliferation and apoptosis in these cells by detecting their messenger RNA (mRNA) content *in vivo.* In order to examine the local antibody production and to determine their relative proportions in granulation tissue compared to gingiva, complementary oligonucleotide probes against specific mRNA sequences of IgG, IgA, IgM and IgE subclasses were utilised. Secretory IgA and IgM were evaluated in an attempt to assess their probable origin and destination and to compare their relative numbers within the two tissues. Furthermore, comparison was made between AP and EOP granulation tissue specimens for all the variables mentioned above in order to give an insight in the histopathology of the two forms of periodontal disease.

When cellular synthetic activity was examined with the oligo d(T) and 28S rRNA probes, it was found that the staining intensity of epithelial and plasma cells was strong. Slight to moderate staining was observed in fibroblasts, macrophages, giant cells, endothelial cells and lymphocytes with no difference in the numbers of any of the cell types among the tissue groups. Further localisation of the mononuclear cells, according to their CD antigen markers, revealed a mixed reaction, ranging from a few strongly stained through weakly positive to negative. It was concluded that plasma cells are

synthetically active in both tissue groups, but lymphocytes do not exhibit a major synthetic capacity within the actual tissue sites.

Histone probe and anti-Ki-67 monoclonal antibody were used for the detection of proliferating cells and it was revealed that epithelium, mononuclear cells and fibroblasts stained positive, but the overall numbers of dividing cells (excluding epithelium) in the gingival and granulation connective tissues were small. It was also noted that CD positive cells were stained negative for the proliferation markers. An interesting finding was that the numbers of proliferating cells were significantly higher in the granulation compared to gingival tissue biopsies, and this could be accounted for by the numbers of fibroblasts. Fibroblasts could proliferate in an attempt to rearrange the borders of the actual inflamed area. Collectively, it could be hypothesised that leukocytes do not actively proliferate or synthesise proteines in the periodontally affected tissues. The possibility exists that already differentiated and committed B and T cells arrive in the inflamed tissues and that this differentiation occurs in more distant sites i.e. lymph nodes. The recruitment of cell clones which are specific for some plaque-derived antigens, occurs by migration rather that by proliferation.

Apoptosis was not found to be a major feature in the inflamed gingival and granulation tissues. Some epithelial cells, fibroblasts and possibly PMNs were stained positive, but neither lymphocytes nor macrophages exhibited any apoptotic function. This could lead to the result that lymphocytes and

macrophages are long-lived cells within the periodontal gingival and granulation tissues and that they exert their effects for a long period of time. In conclusion, it seems that the actual turnover rate of leukocytes is very slow in the tissues studied.

Mononuclear cells were further differentiated and their occurrences were studied. Monoclonal antibodies to CD3 and CD45RO were used to identify pan T cells and memory T cells, respectively. Around 60-90% of the T cells present in the lesions were potentially memory T cells. CD20 and CD68 positive cells (specific for B or pre-plasma cells and macrophages, respectively) were also assessed. It was found that T cells ($CD3^+$) were significantly more abundant in the sections taken from early onset periodontitis (EOP) than in those from adult periodontitis (AP) patients. Additionally, a trend towards higher numbers of B cells was noted in AP sections, although not significant. The B : T cell ratio was significantly increased in AP sections compared to EOP, and this seemed to be brought about by an increase in the occurrence of T cells in EOP sections. The relative distributions of B and T cells to the combined total of CD20, CD3 and CD68 followed the same pattern, with higher proportions of T cells in EOP and higher proportions of B cells in AP. This inverse relationship may lead to the conclusion that AP appears to be a more mature chronic inflammatory lesion and it has been established for a longer period of time, whereas EOP is a younger lesion.

Monoclonal antibodies to CD4 and CD8 (specific for T helper and T cytotoxic cells, respectively) were used to detect the functional T cell subsets. The ratio of CD4 : CD8 seemed to be within the normal range in all tissue groups, suggesting no abnormal immune response, although a wide individual range was noted among the biopsies.

The synthetic activity of plasma cells was further detected. Plasma cells were categorised into different groups according to their imunoglobulin class and subclass mRNA expression. IgG was the predominant class followed by IgA and IgM, whereas only traces of IgE were detected. The distribution of IgG subclass mRNA-bearing plasma cells was similar in both gingival and granulation tissue biopsies, with $IgG_1$ being predominant and $IgG_2$, $IgG_3$ and $IgG_4$ being detected in a descending scale.

$IgA_1$ mRNA-expressing plasma cells were predominant in both gingival and granulation tissue groups. Significantly higher $IgA_1$ positive cells were detected in granulation compared to gingival tissues, whereas an inverse relationship was noticed with respect to $IgA_2$ mRNA expressing plasma cells. $IgA_2$ mRNA expression was significantly higher in gingival tissues. When plasma cells were evaluated for J chain mRNA expression, it was revealed that the majority J chain positive cells were located adjacent to the epithelium with relatively few cells in the deeper lamina propria or the more apical granulation tissue. Furthermore, staining of serial sections for J chain and immunoglobulin expression demonstrated that the vast majority of J

chain mRNA expressing plasma cells were also expressing the mRNA encoding $IgA_2$. Very few of the $IgA_1$ or IgM expressing plasma cells were positive for J chain mRNA. The fact that the proportion of J chain/ $IgA_2$ plasma cells was significantly higher in the gingiva and that IgM mRNA expression was found to be significantly higher in the deep granulation tissue, suggests that from all the Ig expressing plasma cells, $IgA_2$ mRNA positive cells are more likely to be destined for secretion in the periodontal gingival tissue. This study has demonstrated the simultaneous presence of a systemic (IgG) and a mucosal (IgA) immune response in the periodontal tissues. The various immunoglobulin class and subclass mRNA expression could reflect the variety of antigenic stimuli which may induce a specific immune response and a switch towards one or another Ig class or subclass.

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

Periodontal disease has affected the human race since early times. Descriptions about periodontal diseases were found in ancient Chinese and Egyptian writings of more than 4000 years ago (Page, 1995). Nowadays, periodontal disease still appears to be one of the most prevalent examples of a chronic inflammatory process afflicting humanity. Furthermore, with an increase in the average life span, and a decrease in the prevalence of tooth loss due to caries, more teeth are lost through periodontal disease. Much effort has been expended to increase the knowledge of the pathogenesis of the disease. In spite of the rapidly expanding knowledge of the aetiological factors which interact with disease progression, the role of the complex immune and inflammatory responses during the development of periodontitis has yet to be fully elucidated.

The work comprising this thesis is designed broadly to evaluate the role of the cellular immune response in periodontal disease with respect to:

a. homing of specific inflammatory cells to target tissues;

b. proliferative capacity and synthetic activity of immune cells within the periodontal lesion and;

c. the immunoglobulin subclasses of periodontal plasma cells.

The introduction section has therefore been designed to provide a background to these topics. It starts with an introduction of the basic histology and moves on through disease definition through to the pathology of this condition and the background to the techniques adopted for this study. The following literature review initially introduces some basic definitions and general aspects of periodontology.

## 1.2 Anatomical and histological appearance of the "normal" periodontium

Periodontal disease is a general term for inflammatory reactions affecting the periodontium. The term "periodontium" has its origin in the Greek words "peri" meaning "around" and "odontos" meaning "tooth". It consists of the supporting tissues of the tooth, including gingiva, periodontal ligament, root cementum and alveolar bone, whose architecture is partially or completely disrupted by the inflammatory mechanisms of the disease. Since the periodontium comprises tissues of different origin, a large range of systemic conditions with periodontal consequences could be included under this heading (Kinane & Davies, 1990). However, in the present context, this term will only be used to denote gingivitis and periodontitis, i.e. dental plaque-induced inflammatory processes of the periodontium.

### 1.2.1 The oral mucosa

The oral mucosa can be divided into three zones:

**1.** the masticatory mucosa i.e. the gingiva and the covering of the hard palate;

**2.** the specialised mucosa i.e. the mucosa covering the *dorsum* of the tongue and;

**3.** the oral mucous membrane, lining the remaining of the oral cavity.

Clearly the masticatory mucosa, particularly the gingiva, is relevant to any discussion of periodontal disease and is hereby discussed.

### 1.2.2 The gingiva - structure and function

The gingiva is the part of the oral mucosa that covers the alveolar processes of the jaws and the cervical portions of the teeth. It is anatomically divided into marginal, attached and interdental areas. In about 50% of cases, the marginal gingiva is demarcated from the adjacent attached gingiva by a shallow linear depression, called the free gingival groove (Ainamo and Löe, 1966). The free gingival groove is positioned at a level corresponding to the level of the cemento-enamel junction (CEJ). In fully erupted teeth the gingival margin is located on the enamel approximately 0.5 to 2.0 mm coronal to the CEJ.

In human teeth the gingival margin, also known as marginal gingiva or free gingiva, seldom forms a knife-edged termination against the tooth. Instead, it is rounded and this creates the orifice of the gingival sulcus. The gingival sulcus is a shallow crevice around the tooth bound to the surface of the tooth on the one side and the epithelium lining the free margin of the gingiva on the other. In fully developed teeth the gingival sulcus is lined coronally with sulcular epithelium, the non keratinised extension of the oral epithelium; additionally, the bottom of the sulcus is formed by the coronal surface of the junctional epithelium. The junctional epithelium unites the gingival connective tissue with the enamel surface of the tooth. The length of the junctional epithelium rarely exceeds 2 to 3 mm.

The attached gingiva is continuous with the free gingiva and its apical extension leads to the mucogingival junction. This is where it becomes continuous with the relatively loose and movable alveolar mucosa, although the attached gingiva is tightly attached to the underlying alveolar bone, and, therefore, demonstrates a comparative immobility in relation to the underlying tissue. The width of the attached gingiva differs among different teeth (Bowers, 1963). It is greater in the incisors' region, while it is less in the posterior segments; attached gingiva of the least width is located in the first premolar area (Ainamo & Löe, 1966).

The interdental gingiva occupies the interproximal space beneath the area of tooth contact. The interdental gingiva can be pyramidal i.e. one papilla's tip

is immediately beneath the contact point, or have a "col" shape i.e. a valley-like depression that connects a facial and a lingual papilla and conforms to the shape of the interproximal contact (Cohen, 1959).

### 1.2.3 Gingival epithelium

The marginal gingiva comprises three areas of epithelium:

a. the oral epithelium, which faces the oral cavity;

b. the sulcular epithelium, which faces the tooth without any contact with tooth surface and;

c. the junctional epithelium, which is in contact with tooth surface (Figure 1.1).

The keratinocyte is noted to be the principal cell type of the gingival and oral epithelia. Other cell types included are clear cells or non-keratinocytes, which comprise Langerhans cells (LCs) (DiFranco *et al.*, 1985), Merkel cells (Ness, Morton & Dale, 1987) and melanocytes (Schroeder, 1969).

The keratinocytes constitute more than 90% of gingival epithelium. According to Schroeder (1981), the process of keratinisation involves a sequence of biochemical and morphologic events that occur in the cell during its migration from the basal layer towards the surface. Gingival epithelium is able to express three types of surface differentiation:

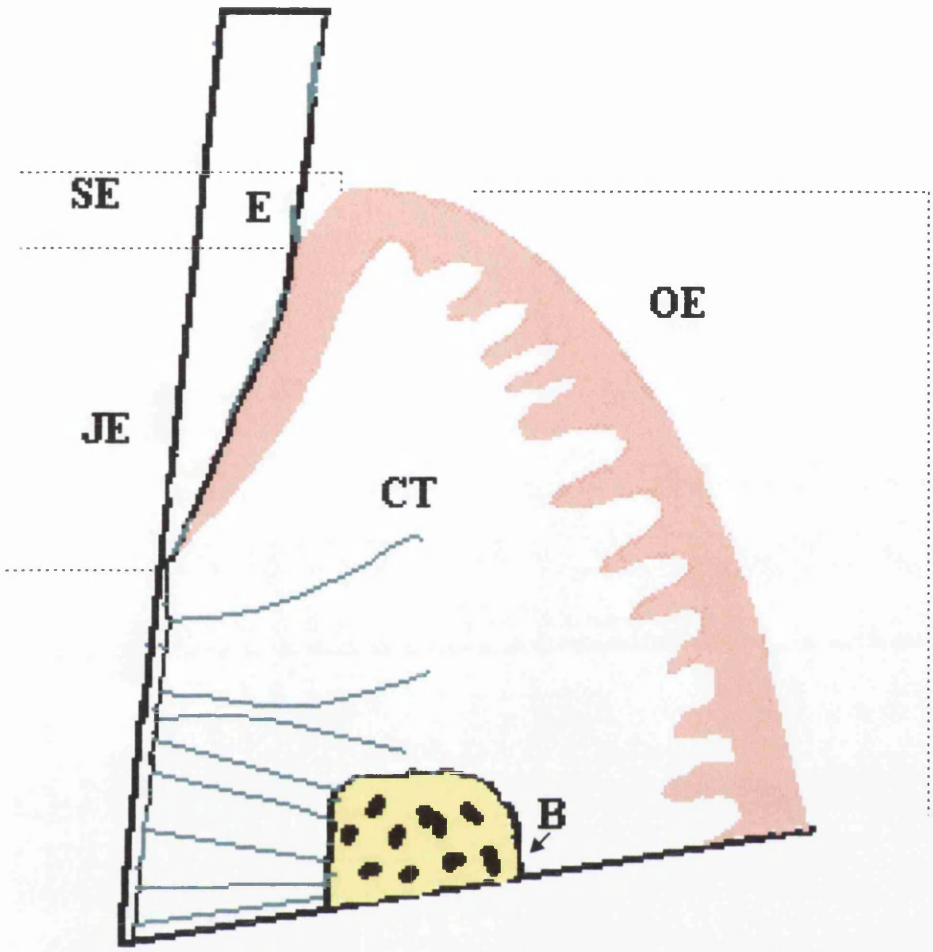



**Figure 1.1:** Schematic drawing of the gingiva which comprises three areas of epithelium and connective tissue.
E: enamel; OE: oral epithelium; SE: sulcular epithelium; JE: junctional epithelium; CT: connective tissue; B: alveolar bone

a. keratinisation i.e. the surface cells form scales of keratin and lose their nuclei;

b. parakeratinisation i.e. the cells of the superficial layers retain their nuclei but no granular layer is present and;

c. non keratinisation i.e. the cells of the surface layers preserve their nuclei and signs of keratinisation are absent.

### 1.2.3.1 The oral epithelium

The oral epithelium of the gingiva covers the crest and outer surface of the marginal gingiva as well as the surface of the attached gingiva. It is of stratified squamous type and it exhibits keratinisation or parakeratinisation or even a combination of both forms. It is bound to the underlying connective tissue by a *basal lamina* (Stern, 1965). In normal, non inflamed gingiva, the border between oral epithelium and connective tissue is characterised by deep epithelial ridges (*rete pegs*) which are separated from each other by connective tissue papillae.

The oral epithelium can be divided into four different layers from the base towards the surface:

a. Basal layer (*stratum basale or stratum germinativum*). Cells composing this layer are adjacent to the connective tissue, from which they are separated by a basement membrane (basal lamina).

b. Spinous cell layer (*stratum spinosum*).

c. Granular cell layer (*stratum granulosum*). Cells' cytoplasm characteristically displays keratohyalin granules and

d. Keratinised cell layer (*stratum corneum*). This layer consists of flattened cells which have lost their nuclei and most other organelles and have become keratinised (Schroeder & Theilade, 1966).

### 1.2.3.2 The sulcular epithelium

The sulcular epithelium, which lines the gingival sulcus, is a thin, non keratinised stratified squamous epithelium, displaying *rete pegs*. It extends from the coronal limit of the junctional epithelium to the crest of the gingival margin.

### 1.2.3.3 The junctional epithelium

The junctional epithelium, also known as dento-gingival epithelium, consists of a collar-like band of stratified squamous epithelium. The number of its layers increases with age from 3-4 to 10 or even 20, and its length ranges from approximately 0.25 to 1.35 mm. The junctional epithelium is attached to the tooth surface (epithelial attachment) by means of a *basal lamina*(basement membrane) (Listgarten, 1966). The *basal lamina* is comparable to that which attaches epithelium to the connective tissue

elsewhere in the body (Listgarten, 1986). The *basal lamina* consists of a *lamina densa* (adjacent to the enamel) and a *lamina lucida* to which hemidesmosomes are attached. Unlike the oral and sulcular epithelium which display the characteristic morphological feature of *rete pegs,* junctional epithelium seems to lack these structures (Lindhe & Karring, 1989).

### 1.2.4 The gingival connective tissue

The connective tissue (*lamina propria*) of the gingiva is primarily composed of collagen fibres, cells, nerve processes and blood vessels embedded in dense and intact collagenous matrix. The major component of the connective tissue is a prominent system of collagen fibre bundles with a distinct orientation, called gingival fibres, which are arranged in four groups: circular, transeptal, dentogingival and dentoperiosteal (Lindhe & Karring, 1989).

The main cell is the fibroblast, which synthesises the basic elements of the connective tissue i.e. the collagen fibres. Other cells detected include undifferentiated mesenchymal cells, mast cells (Shelton & Hall, 1968) and macrophages.

### 1.2.5 Vessels within the periodontium

Two main types of vessels are described in the gingival connective tissue: blood and lymphatic vessels.

The blood supply of the gingival tissues is derived mainly from supraperiosteal vessels originating from the lingual, mental, buccinator and palatine arteries. These vessels give off branches along the facial and oral surfaces of the alveolar bone (Karring & Löe, 1967).

The blood vessels of the gingiva are arterioles, capillaries and small veins. Thin capillaries terminating immediately below the basement membrane provide the nutritional supply for the gingival epithelium. On the one hand, the oral epithelium of the free and attached gingiva are supplied by the *subepithelial plexus* which leads to several capillary loops to each of the connective tissue papillae. On the other hand, the *dentogingival plexus* is located in the area beneath the sulcular and junctional epithelia and extends under the epithelial surface from the gingival margin to the apical part of the junctional epithelium (Egelberg, 1966); no capillary loops occur in "clinically healthy" gingiva (Lindhe & Karring, 1989).

The lymphatic drainage of the periodontal tissues involves the lymphatics of the connective tissue papillae. The lymph progresses into the collecting

network of larger lymph vessels and is drained into the regional lymph nodes of the head and the neck, before entering the blood circulation. Lymphatics just beneath the junctional epithelium extend into the periodontal ligament and accompany the blood vessels.

## 1.3 Clinical and histological appearance of "normal" periodontium

In "normal" periodontium, gingiva is clinically characterised by its pale pink colour, firm consistency, scalloped outline of the gingival margin; furthermore, it does not bleed on gentle probing and fills the entire space between adjacent teeth (Ainamo & Löe, 1966; Wennström, 1988).

In health, the depth of gingival sulcus is minimal at the microscopic level. In a normal periodontium the alveolar bone is located 1 mm apical to the cementoenamel junction (Eliasson *et al.*, 1986). The oral surface is covered with keratinised oral epithelium that fuses with the junctional epithelium and shows a firm hemidesmosomal attachment to the tooth. The junctional epithelium is relatively weakly attached to the tooth surface via hemidesmosomes. Both oral and junctional epithelia are supported by the collagenous structures of the underlying connective tissue, while immediately below the junctional epithelium a microvascular plexus, containing numerous venules, is located (Egelberg, 1967). Histologically, no inflammatory exudate is present and only a few discrete polymorphonuclear leukocytes (PMNs) and

macrophages can be seen within the connective tissue and the junctional epithelium. Some isolated neutrophilic granulocytes and mononuclear cells may be present around some vessels in more distant locations (Attström *et al.*, 1975). Normal gingiva is, therefore, free from inflammation at both the clinical and microscopical level. Thus, this kind of normal gingiva that finds itself in a super healthy state, may be better described as "pristine" gingiva (Kinane & Lindhe, 1997). These conditions are, however, rarely met in typical human subjects (Taichman & Lindhe, 1989). However, histologically normal gingival tissue can be found in germ-free animals (Listgarten & Beneghan, 1971) or even in animals whose gingivae have been kept meticulously clean (Lindhe & Rylander, 1975).

### 1.3.1 Clinically healthy periodontium

Although in the normal periodontium the alveolar bone is located 1 mm apical to the cementoenamel junction (Eliasson *et al.*, 1986), a clinically healthy periodontium may show tissue recession and reduced height of the alveolar bone. Biopsies of clinically healthy gingival tissue exhibit inflammatory cell infiltration under microscopic observation. These inflammatory cells appear to be important in the day-to-day host defence against bacteria and other substances to which the gingivae are exposed. This small but definite infiltrate can be detected in the junctional epithelium and the coronal portion of the connective tissue; it usually contains lymphocytes, predominantly T cells with

a few B cells or even plasma cells (Page & Schroeder, 1976; Seymour *et al.*, 1983a, 1983b) along with PMNs, monocytes and macrophages. This infiltrate comprises 3-5% of connective tissue volume contiguous with the junctional epithelium. Furthermore, the classical microscopic picture of the "normal" periodontium has recently been an issue of debate. Investigators have found that an inflammatory infiltrate, comprising of both polymorphonuclear leukocytes and small round mononuclear cells are always present in gingival biopsies from clinically healthy gingivae (Seymour, Powell and Aitken, 1983; Brecx *et al.,* 1987). The inflammatory cells, predominantly PMNs, travel across the junctional epithelium (Cattoni, 1951; Grant & Orban, 1960; Thurre *et al.*, 1984) to the gingival sulcus where they accumulate and are eventually lost within the oral cavity. Even at this stage, collagen depletion and an increase in vascular structures within the infiltrated area are noted. Gingival crevicular fluid (GCF) is created by exudative and transudative fluid and plasma proteins that arrive in the gingival crevice region from the vessels and through the tissues (Cimasoni, 1983). Although the ideal condition of "pristine" gingiva can only be established in humans after supervised daily plaque control for several weeks, which is probably not close to reality, clinically healthy gingivae can be attained by patients who manage to be in a satisfactory oral hygiene level. Therefore, for the above reasons the term "clinically healthy" compared to "normal" periodontium seems preferable (Wennström, 1988), because it is the type of healthy periodontium that is encountered in everyday situations.

## 1.4 Diseased periodontium

### 1.4.1 Histopathological and inflammatory features of the diseased periodontium - The classical model

When the balance between the pathogenic micro-organisms and the defensive mechanisms is upset, the microbial products give the signal for the initiation of an inflammatory response that leads to the formation of a lesion.

Following the establishment of an experimental link between gingival inflammation and plaque accumulation in the landmark study of Löe *et al.* (1965), the histopathologic changes associated with gingivitis and periodontitis were the subject of a series of investigations conducted in the 70's. Page & Schroeder (1976) reviewed clinical and histopathologic evidence of periodontal disease and proposed four distinct states in the pathogenesis of periodontitis, ranging from initial to advanced disease. The stages within this classical picture were the initial lesion, the early lesion, the established lesion and the advanced lesion. The initial PMN infiltrate was postulated to be rapidly replaced within 48 hours by a lymphocytic lesion. This lesion could then stabilise as the early lesion unless further challenged by plaque. The initial and early lesions were thought to reflect the histopathology of clinically "acute" or early stages of gingivitis, while the established lesion was referred to as "chronic" gingivitis. With further plaque challenge, however, the cellular

infiltrate was then thought to transform into a plasma cell dominated lesion. The description of the advanced lesion was considered to reflect the progression from gingivitis to periodontitis. At this stage tissue destruction including bone resorption was thought to occur.

However, Page & Schroeder's model has been brought into serious question. The factors contributing to this were: 1) the inability to test the validity of the model with true longitudinal studies, 2) recent changes in ideas on periodontal disease progression; and 3) the fact that not all postulated stages have been observed histologically (Zachrisson, 1968; Payne *et al.*, 1975; Seymour, Powell & Aitken, 1983; Seymour *et al.*, 1983a; Brecx *et al.*, 1987). Moreover, these descriptions are based on information obtained mostly from animal biopsy material which is not totally applicable to the human situation.

The Page & Schroeder model and the amendments outlined by Kinane & Lindhe (1997) will be described for each stage in the pathogenesis of periodontal disease.

#### 1.4.1.1 The initial gingival lesion

The initial lesion is localised around the gingival sulcus and usually emerges after 2-4 days of plaque build-up, although vascular changes beneath the junctional epithelium can be seen after 24 hours of plaque accumulation.

Histopathological alterations of the *dentogingival plexus* are evident. Dilation of the arterioles, capillaries and venules occurs and hydrostatic pressure within the microcirculation increases. The elevated permeability of the microvascular bed to fluids and proteins permits them leakage into the tissues This results in a rise in gingival crevicular fluid (GCF) flow that may function in a defensive and protective way. These changes have been detected in both animal and human models (Payne *et al.*, 1975; Schroeder *et al*, 1975). GCF flow helps to dilute bacterial products both in the tissue and the crevice and to flush them from the sulcus, and supplies antibacterial serum components such as antibodies, complement and protease inhibitors. The volume of the GCF may be used to estimate the extent and the severity of the gingival inflammation (Fine & Mandel, 1986). Furthermore, the concentrations of its various components in the gingival sulcus, such as plasma proteins, tissue proteases, break-down products and leukocyte enzymes are currently being examined in an attempt to elucidate their relative importance to the aetiopathogenic process and the state of the disease. Several GCF molecules have been considered to be very useful biochemical indicators and markers of the inflammatory process, or potential diagnostic markers of periodontal disease (Page, 1992; Lamster, Smith & Celenti, 1994; McCulloch, 1994; Kinane, 1997).

As the vascular changes occur numerous neutrophils, monocyte/ macrophages as well as lymphoid cells begin to migrate from the dentogingival microvascular plexus. They travel through the connective tissue and the majority seem to accumulate in the junctional epithelium and the gingival

sulcus; by 2 to 4 days this cellular response appears to be well established. (Lindhe *et al.*, 1973; Listgarten & Ellegaard, 1973; Payne *et al*, 1975; Schroeder *et al.*, 1975; Brecx *et al.*, 1987). The migration of leukocytes, preferentially neutrophils, to the crevice is enhanced by the adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1) which are uniquely expressed on the junctional epithelial cells (Moughal *et al.*, 1992). These adhesion molecules act by mediating leukocyte binding to the post-capillary venules and thus, helping the cells to escape from the blood vessels. Host and microbial chemotactic factors are also responsible for further assisting the movement of the inflammatory cells.

As mentioned before, clinically healthy gingivae in humans comprise a small but definite inflammatory infiltrate which is not very different to the one described above. This observation may lead to the assumption that Page & Schroeder's initial lesion could be considered the normal physiological state in humans, since it shares many common histopathological features with the typical clinically healthy gingivae (Kinane & Lindhe, 1997). In fact, this stage contributes one of the main problems in elucidating the pathogenesis of periodontitis. Investigators have been unable to make a clear distinction between normal and pathologically altered tissue. The crucial point at which disease commences has not yet been determined. Since definitive evidence on this point is lacking so far, a hypothesis has been proposed, stating that the features of the initial lesion merely reflect enhanced levels of the host defences

occurring within the gingiva as part of normal surveillance procedures (Attström *et al.*, 1975; Payne *et al.*, 1975; Schroeder *et al.*, 1975; Page & Schroeder, 1976).

The role of animal models in studying the development of human gingival and periodontal inflammation has been challenged by many researchers. In human models the initial lesion contains no plasma cells in the connective tissue area subjacent to the junctional epithelium (Payne *et al.*, 1975; Brecx *et al.*, 1988). This is in contrast to observations on animal models where plasma cells are found to predominate apically to the gingival sulcus (Listgarten & Ellegaard, 1973). It has been suggested that, although neutrophils and macrophages accumulate in the coronal part of the connective tissue and the junctional epithelium in order to defend the host against the microbial attack, they could also be the cause of much of the damage detected in this area (Lehner, 1972; Page & Schroeder, 1973; Genco *et al.*, 1974; Borton, Oppenheim & Mergenhagen, 1974). A feasible mechanism provoking this destructive process could be the activity of collagenase and other enzymes released by the infiltrating neutrophils during the initial stage (Attström & Schroeder, 1979; Schroeder & Attström, 1979). A discrepancy, however, exists in the development of inflammation at this initial stage with respect to humans, as such collagen loss is not observed to the same extent (Brecx *et al.*, 1987).

### 1.4.1.2 The early gingival lesion

The early lesion evolves from the initial lesion after approximately 7 days of plaque accumulation (Schroeder *et al.*, 1973; Payne *et al.*, 1975), although in humans there is a greater variability of the time required for the establishment of this stage. This is due to subject and site variation caused by either differing plaque retentive capacities or systemic factors such as differences in hormonal levels (Kinane & Lindhe, 1997).

The vessels of the coronal portion of the *dentogingival plexus* remain dilated (Egelberg, 1967; Lindhe & Rylander, 1975) and an increasing number become apparent due to an ongoing distention of previously inactive capillary beds. Additional plaque accumulation gives rise to a more pronounced infiltration of PMNs and monocyte/ macrophages within the dentogingival epithelium, compared to the initial lesion. The junctional epithelium now contains increased numbers of transmigrating neutrophil granulocytes and infiltrated mononuclear cells, especially lymphocytes (Listgarten & Elegaard, 1973; Schroeder *et al.*, 1973; Payne *et al.*, 1975; Schroeder *et al.,* 1975). The inflammatory cell infiltrate may at this stage comprise approximately 10-15% of the connective tissue volume of the free gingivae (Schroeder *et al.*, 1973) and it contains small and medium sized lymphocytes, many of which are T cells, while others are B cells (Seymour *et al.,* 1983b). During the early stage only a few plasma cells are noted within the lesion (Listgarten & Elegaard, 1973; Payne *et al.*, 1975; Seymour *et al.*,

1983; Brecx *et al.*, 1987). Within the lesion, the fibroblasts degenerate (Simpson & Avery, 1973, 1974; Page *et al.*, 1975), while fibroblasts in adjacent non inflamed tissue appear to have normal ultrastructural features (Simpson & Avery, 1973, 1974). The fibroblasts become pathologically altered as evidenced by swollen mitochondria, vascularisation of the *endoplasmic reticulum* and rupture of the cell's membrane. The altered cells have also been shown to be intimately associated with activated lymphocytes (Simpson & Avery, 1974) and presumably to be injured by cytotoxic products of lymphoid cells (Granger & Williams, 1968; Schectman *et al.*, 1972; Walker & Lucas, 1972). Takahashi, Poole & Kinane (1995) have suggested that degeneration of fibroblasts probably occurs by apoptosis and, therefore, their removal from the area functions as a mechanism which permits more leukocyte infiltration. Collagen destruction is present and is additionally necessary in order that tissues create space to accommodate the infiltrating cells. Furthermore, the basal cells of the junctional and sulcular epithelium have proliferated and epithelial *rete pegs* can be seen entering the coronal part of the infiltrate (Schroeder, 1970; Schroeder *et al.*, 1973; Horton *et al*, 1974; Simpson & Avery, 1974). Clinically, the early lesion may exhibit symptoms of gingivitis such as swelling and oedema. The GCF flow and the numbers of transmigrating leukocytes reach their maximum values between 6 and 12 days after the onset of clinical gingivitis (Lindhe *et al.*, 1973).

The duration of the early lesion in humans has not been determined. According to studies such as by Seymour *et al.* (1983a, 1983b), where biopsies from individuals undergoing a 21-day period of experimental gingivitis were examined, the lymphoid cell infiltrate (of which around 70% consists of T cells) changes in size but not in composition. In general, the early lesion may persist for a longer time than previously thought and the variability in time required to produce an established lesion may be due to the susceptibility status of certain individuals, as discussed in the following section.

### 1.4.1.3 The established gingival lesion

This stage, as defined by Page & Schroeder, 1976, is hypothesised to be dominated by plasma cells. Several investigators have supported this concept by stating that only 3-4 weeks of plaque accumulation are needed for the formation of a plasma cell-dominated lesion (Zachrisson, 1968; Payne *et al.*, 1975). However, Brecx *et al.* (1988) in their long-term experimental gingivitis study, demonstrated that even after six months of oral hygiene neglect, plasma cells comprised only 10% of the total cellular infiltrate and they were not the predominant type. Thus, the exact period of time required for the established lesion to mature into plasma cell dominated in humans is still unknown. In contrast, according to Schroeder & Lindhe (1975), within 18 months periodontitis can be initiated in otherwise healthy dogs by

withdrawing oral hygiene procedures. Therefore, with the passage of time, the established lesion characterised by a predominance of plasma cells and B lymphocytes progresses from the early lesion, probably in conjunction with the creation of a small pocket lined with pocket epithelium.

Generally, continuing exposure to plaque enhances further fluid exudation from the microvasculature along with leukocyte migration into the tissues and the gingival crevice. In the classical established lesion, features seen in the earlier stages are still present but to a more marked degree (Mulvihill *et al.*, 1967; Simpson & Avery, 1974) and clinical signs of "established" gingivitis are present. Large numbers of mature plasma cells are seen (Freedman, Listgarten & Taichman, 1968; Simpson & Avery, 1974). They are situated primarily in the coronal part of the connective tissue as well as around vessels. Collagen destruction continues in both lateral and apical directions as the inflammatory cell infiltrate expands, resulting in collagen-depleted areas radiating deeper into tissues (Page & Schroeder, 1973). The dentogingival epithelium continues to proliferate and the *rete pegs* extend into the connective tissue infiltrate trying to form a barrier to microbial invasion. The pocket epithelium is not attached to the tooth surface and is heavily infiltrated with PMNs , macrophages, lymphocytes and plasma cells. Most of the PMNs migrate across the epithelium into the gingival pocket. The pocket epithelium is more fragile and more permeable compared to the original junctional epithelium (Thilander, 1964) and may in places show ulcerations. At this stage, the lesion is still reversible after removal of plaque

and healing occurs by repair and not by resolution, as seen in the earlier stages (Mackler *et al.*, 1977; Okada, Kida & Yamagami, 1983).

#### 1.4.1.3.1 Progression of the established lesion

Two types of established lesion appear to exist, some remaining stable and not progressing for months or years (Lovdal, Arno & Waerhaug, 1958; Suomi, Smith & McClendon, 1971; Lindhe, Hamp & Löe, 1975; Page *et al.*, 1975), while others become more active and convert to progressive destructive lesions. Controversy surrounds the nature of this conversion. Seymour, Powell & Davis (1979) have hypothesised that a change from T-cell to B-cell dominance induces the conversion from stability to activity involving aggressive destruction. This view is supported by the study of Walsh *et al.* (1987), who have suggested that the non progressive lesions of chronic gingivitis in children are T cell dominated.

However, Page (1986) has disagreed with this view; a recent study has shown B-cell infiltrate mainly associated with stable, non-progressive lesions in childhood gingivitis (Gillet, Cruchley & Johnson, 1986). Controversies surrounding this question continue to exist, as shown by a recent study by Liljenberg *et al.* (1994). Plasma cell densities were compared in sites with active progressive periodontitis and in sites with pathological pockets but without significant attachment loss over a 2-year period. The results showed

that the subfraction of plasma cells in active sites was significantly increased in density (51.3%) while plasma cells in inactive sites were less numerous (31.0%).

#### 1.4.1.4 The advanced gingival/ periodontal lesion

The final stage of the lesion is known as the *advanced lesion.* Several studies have focused on the investigation of the histopathological and ultrastructural features of this stage (Freedman *et al.*, 1968; Page *et al.*, 1975). Continuous plaque growth results in further pocket deepening, while the junctional epithelium extends and proliferates in a further apical direction, leading to marked attachment loss. The advanced lesion shares many characteristics with the established lesion but it also differs in that some additional features appear, including alveolar bone loss and fibrosis of the gingiva with widespread manifestations of inflammatory and immunopathological tissue damage (Freedman *et al.*, 1968; Garant & Mulvihill, 1972; Simpson & Avery, 1973). The lesion is no longer localised and the inflammatory cell infiltrate extends laterally and apically into the connective tissue. It is now generally accepted that plasma cells are the dominant cell type in the advanced lesion with noted presence of macrophages, lymphocytes and PMNs (Freedman *et al.*, 1968; Garant & Mulvihill, 1972; Simpson & Avery, 1973). However, experimental periodontitis studies should not be performed in humans due to ethical reasons (Brecx *et al.*, 1988), thus the validity and the possible application of the animal

experimental results to the human situation is further questioned. Therefore, alternative models could possibly be used i.e. cultured human cells extracted from diseased tissues to mimic the human condition.

### 1.4.2 Clinical features of the diseased periodontium

The inflammatory and immune reactions that result from the host's response to the plaque microflora and its products, are visible both microscopically and clinically in the affected periodontium.

Periodontal disease is broadly classified into two distinct entities, depending upon the topographic involvement of the inflammatory response: gingivitis and periodontitis. Gingivitis refers to pathological inflammatory changes which are confined to the gingivae, and are clinically manifested by a change in color (redness), texture and appearance (swelling) of the gingivae, together with an increased tendency to bleeding on gentle probing and gingival sensitivity (Löe *et al.*, 1965). Periodontitis affects the deeper attachment apparatus (alveolar bone, periodontal ligament and cementum), resulting in loss of periodontal support. It is frequently associated with the presence of periodontal pockets and bleeding on probing. Bone loss is the pathognomonic feature.

Gingivitis can be subdivided into the following forms, according to secondary factors which appear to modify the clinical characteristics of the disease: 1) Plaque-induced gingivitis, 2) Acute necrotising ulcerative gingivitis, 3) Hormonally-related gingivitis, 4) Drug-induced gingival overgrowth and 5) Desquamative gingivitis.

The World Workshop in Clinical Periodontology (1989), followed by the review of Papapanou (1994), classified different forms of periodontitis as follows: 1) Adult periodontitis; 2) Early-onset periodontitis which includes: a) Prepubertal periodontitis (localised and generalised), b) Juvenile periodontitis (localized and generalised), and c) Rapidly progressive periodontitis; 3) Periodontitis associated with systemic disease; 4) Necrotising ulcerative periodontitis; and 5) Refractory periodontitis. However, there is considerable overlap within the classification defined above. For example, the presence of a systemic disease such as diabetes mellitus may contribute to the development of a non-responsive so-called refractory periodontitis. HIV-associated periodontitis could be classified as periodontitis associated with systemic disease, although it is usually manifested by features of necrotising ulcerative periodontitis. Furthermore, separation of the generalized form of juvenile periodontitis from rapidly progressive periodontitis adds confusion. Finally, the age of onset is sometimes difficult to be determined retrospectively, since patients are quite often not aware of the presence of the disease until informed by their dentist.

Alternatively, in the 1st European Workshop on Periodontology, Attström & van der Velden (1994) have proposed a classification of periodontal diseases based on aetiological and host response factors. The existing difficulty in defining the clinical situation and the extensive overlap among the different diagnostic categories led the authors to present the following classification:

1) Early onset periodontitis,

2) Adult periodontitis and

3) Necrotising periodontitis.

This basic classification could be further differentiated according to subject-specific factors, such as distribution within the dentition, rate of progression, response to treatment, relation to systemic disease, microbiological findings, ethnic group and other factors. Periodontitis is a multifactorial and complex chronic disease. Clearly, a better understanding of histopathological and epidemiological characteristics of the disease is needed before more clear-cut and well-defined classifications become available.

### 1.4.3 Characteristics of the different forms of periodontal disease

#### 1.4.3.1 Gingivitis

Plaque-associated gingivitis is the most common form of periodontal disease. Through several studies using the experimental gingivitis model, it has been revealed that plaque development is characterised by a gradual increase in mass

and a thickening of bacterial plaque at the gingival margin. Analysis of the composition of the bacterial plaque has demonstrated that it is predominantly comprised of Gram-positive bacteria which are normally associated with health (Socransky, 1977), although in chronic or long-standing gingivitis 25% of the plaque micro-organisms are identified as being Gram-negative (van Palenstein-Helderman, 1975).

### 1.4.3.2 Acute necrotising ulcerative gingivitis (ANUG)

Acute necrotising ulcerative gingivitis refers to an acute gingival infection of complex aetiology. It is characterised by necrosis of the tips of the gingival papillae, spontaneous bleeding and pain (Glossary of Periodontic terms, 1986). ANUG rarely occurs in a generalised form and may be very advanced in the anterior segment, whereas the rest of the teeth may not be implicated. Disease is caused by a fusospirochetal complex of micro-organisms in the presence of a number of predisposing factors. Among these poor oral hygiene, smoking and stress may be the prime suspects involved in the initiation of the disease (Sabiston, 1986). Recurrent episodes of this form of gingivitis can develop into a chronic condition which may lead to a periodontitis type lesion (Silver, Southcott & Wade, 1974) with marked interproximal bony craters.

#### 1.4.3.3 Hormonally-related gingivitis

This form of gingivitis may be associated with pregnancy, puberty, birth control medication, steroid therapy and, in general, with any condition that can alter the hormone balance in the human body (Löe, 1965; Samant *et al.*, 1976; Kalkwarf, 1978). The clinical appearance is characterised by an apparently exaggerated response to plaque, reflected by intense inflammation, redness, oedema and enlargement.

#### 1.4.3.4 Drug-induced gingival overgrowth

Drug-induced gingival overgrowth or, alternatively, gingival hyperplasia is caused by drugs used for immunosuppresion i.e. cyclosporin, for treating epilepsy i.e. phenytoin, or by calcium channel ion blockers (reviewed by Seymour & Heasman, 1988). The clinical characteristic of the entity are the excessively hyperplastic gingivae that bleed readily and result in the formation of pseudopockets.

#### 1.4.3.5 Desquamative gingivitis

Sloughing of the gingival epithelium that leaves an intensely red surface is the main clinical feature. Several assumptions have been made for the cause of this type of lesion. Allergic reactions have been considered as responsible

(Perry, Deffner & Sheridan, 1973). Furthermore, desquamative gingivitis may be presented as one of the clinical manifestations of oral dermatoses (McCarthy & Shklar, 1980; Greenberg, 1984).

#### 1.4.3.6 Adult periodontitis (AP)

Adult periodontitis may have its onset in adolescence but it is not usually clinically manifested until the mid-thirties or early forties. Ageing is a possible risk indicator, there is no sex bias and the local tissue destruction is a slow process following a rather continuous pattern (Russel, 1967; Löe *et al.*, 1978, 1986; Listgarten, 1986; Jeffcoat & Reddy, 1991). Although most forms of periodontal disease, including adult periodontitis, are plaque-associated disorders and only by removal of the primary aetiologic factor successful treatment effect can be established in humans (Löe *et al*, 1965; Ramfjord *et al.*, 1968; Hellden & Lindhe, 1973; Lindhe & Nyman, 1975, 1984), the overriding influence is probably dependant upon the individual host susceptibility. Fifteen to thirty percent of individuals are susceptible to severe periodontal destruction (Jenkins & Kinane, 1989) and the inflammatory and immune processes are likely to be crucial factors in this individual predisposition. The lesions are not confined to any certain teeth and the amount of microbial deposits i.e. dental plaque may be consistent with the severity of the lesions (Page *et al.*, 1982).

### 1.4.3.7 Early onset periodontitis (EOP)

Early onset periodontitis encompasses a number of different clinical entities distinguished from periodontitis in adults by age of onset, distribution of lesions and rapid rate of connective tissue attachment and alveolar bone loss. Early onset forms of periodontitis include prepubertal periodontitis (localised and generalised), juvenile periodontitis (or localised EOP) and rapidly progressive periodontitis (or generalised EOP).

### 1.4.3.8 Prepubertal periodontitis

Prepubertal periodontitis is a rare form of periodontitis that occurs as a generalised or localised disease (Page *et al*, 1983; Waldrop *et al*, 1987) and is often associated with nutritional and/ or systemic host immune deficiencies. Target periodontal pathogens are implicated in the pathology of both the localised and the generalised forms. The most strongly associated among them are *Actinobacillus actinomycetemcomitans (A.a.), Capnocytophaga sputigena, E. corrodens, P. intermedia and Porphyromonas gingivalis* (*P.g.*) (Watanabe, 1990; Lopez, 1992; Ram & Bimstein, 1994). Generalised prepubertal periodontitis begins with the eruption of primary teeth and affects both primary and secondary teeth. It is characterised by severe gingival inflammation, rapid bone loss, increasing mobility and, finally, tooth loss. PMN and mononuclear leukocyte defects are implicated (Page *et al*, 1985) and a molecular basis for

the functional abnormality in PMNs has been suggested (Page, Beatty & Waldrop, 1987). Subjects may also have increased susceptibility to skin and upper respiratory tract infection. Localised prepubertal periodontitis affects some of the primary teeth in a lesser extent, is less aggressive and may be related to either PMN or mononuclear leukocyte deficiency, but not to both.

### 1.4.3.9 Localised EOP or Juvenile periodontitis (JP)

Juvenile periodontis is usually detected in localised form, which is characterised by a molar/ incisor pattern of alveolar bone loss with usually a symmetrical distribution of lesions (Baer *et al.*, 1971). The rate and severity of distribution is not consistent with the relatively sparse plaque and the lack of severe clinical signs of inflammation (Baer, 1971). *Actinobacillus actinomycetemcomitans* (*A.a.*) is aetiologic in some but not necessarily all cases of juvenile periodontitis. According to the criteria for implicating bacteria as being important in the aetiology of periodontal diseases (Socransky, 1977), *A.a.* may be considered as strongly associated with the causation of localised juvenile periodontitis (Zambon, 1985; De Graaff *et al.*, 1989; Socransky & Haffajee, 1990). In fact, there have been proposals to change the nomenclature for cases of periodontitis associated with this pathogen from a traditional clinically descriptive terminology such as "localised juvenile periodontitis" to one reflective of the bacterial aetiology, such as "Actinobacillary periodontitis" or "Actinobacillus-associated periodontitis"

(Mombelli *et al*, 1994a, 1994b). There is overwhelming evidence that *A. actinomycetemcomitans* is in high prevalence of 68% of early onset periodontitis patients while it has been detected in a lower percentage (24%) in adult periodontitis subjects. It tends to infect subgingival sites in younger subjects (Slots *et al.*, 1990; Rodenburg *et al.*, 1990; Nakagawa *et al.*, 1994) and it is highly prevalent in subjects under 25 years of age (De Graaff, Van Winkelhoff & Goene, 1989; Capelli, Ebersole & Kornman, 1994). Zambon, in his review of 1985, stated that *A. a.* was an important organism in the aetiology of JP because of its increased prevalence in LJP patients and their families, and increased antibody responses to this organism.

Juvenile periodontitis appears to have a racial bias with more blacks affected than whites (Burmeister *et al.*, 1984). The onset of juvenile periodontitis is rapid and the attachment loss rate has been calculated 3 to 5 times higher when compared with the loss occurring in adult periodontitis subjects (Waerhaug, 1977).

### 1.4.3.10 Generalised EOP or Rapidly progressive periodontitis (RPP)

Rapidly progressive periodontitis (RPP) occurs in young adult populations from their early twenties through to mid-thirties. This form of the disease has been reported by Page *et al.* (1983) to be generalised in nature, rapidly progressing with loss of connective tissue attachment and alveolar bone

support within a short period of time. It is characterised by an acute florid inflammation of the gingival tissues. The progression of this entity was proposed to be episodic with rapid destruction of periodontal tissues occurring during periods of acute exacerbations (Page *et al.*, 1983). Patients affected by RPP did not seem to differentiate from healthy, age matched groups when comparison was made between them in terms of humoral immunoglobulin and complement levels in a study by Ranney *et al.* (1981). Furthermore, Tew *et al.* (1981) failed to show any significant differences in relation to the parameters detected between the two groups; blastogenic response of blood-derived lymphocytes, production of lymphocytic inhibition factor and tests for phagocytic or killing capacity, neither of them seemed to follow different patterns. The bacterial species associated with RPP appear similar to that found in adult periodontitis. Kamma *et al.* (1995) have reported that *P.gingivalis, F.nucleatum, Streptococcus intermedius* and *B.forsythus* are in high prevalence in subjects affected by RPP.

#### 1.4.3.11 Periodontitis associated with systemic disease

Systemic conditions may modify the normal host defence by altering the individual's immune response; thus leading to a predisposition to specific forms of periodontitis. For example, reduction in numbers or change in function of PMNs results in increased rate and severity of periodontal

breakdown (Kinane & Davies, 1990). In such cases periodontal disease could be considered as a symptom of the pre-existing disorder.

### 1.4.3.12 Necrotising ulcerative periodontitis

This form of periodontitis results from ANUG after recurrent multiple episodes not only confined to the gingival tissues but further progressing to involve deeper portions of the periodontium.

### 1.4.3.13 Refractory periodontitis

Refractory periodontitis is seen as an enigmatic condition in clinical periodontology. According to the World Workshop in Periodontics (1989), it refers to cases having multiple sites which continue to exhibit attachment loss despite appropriate management. The resistance to treatment that such sites show is presumably due to the fact that periodontal pathogens still persist and can not be totally eliminated (Haffajee *et al.*, 1988; Van Dyke *et al.*, 1988). However, whether a particular site is truly refractory or refractory periodontitis exists as a separate disease entity is a critical question. It s quite clear that several forms of periodontal disease can become resistant to treatment. Therefore, it seems more realistic to use the term *refractory* to sites which do not respond to treatment rather than to the whole clinical entity (Adams, 1992).

### 1.4.4 Epidemiological features of the diseased periodontium

Early reports on the prevalence of periodontitis suggested that destructive periodontal disease affected a majority of the adult population above the age of 35-40 (Belting *et al.,* 1953; Sandler & Stahl, 1954; Marshal-Day *et al.,* 1955; Bossert & Marks, 1956; Russel, 1957; Schei *et al.,* 1959). Furthermore, it was widely accepted that once gingivitis was initiated it would inevitably develop to periodontitis (Schrep, 1964). Moreover, the fate of untreated periodontitis, whose prevalence increased from early middle age and beyond, was a linear progression until tooth loss occurred (Greene, 1963).

During the 1980's a new epidemiological picture emerged, as a result of new surveys, using more well-defined criteria in screening the patients. The new findings were not consistent with the old concepts. Generally, it was found that a small percentage of the population over the age of 40 were affected by periodontal disease (Baelum, Fejereskov & Manji, 1988; Youneyama *et al.,* 1988; Jenkins & Kinane, 1989; Brown, Oliver & Löe, 1990; Hugoson, Laurell & Lundgren, 1992). Ranney (1982) even suggested that gingivitis, at least as detectable histologically, has not been ruled out as a necessary precursor of periodontitis and that it does not invariably progress to periodontitis. Pilot & Miyazaki (1991) evaluated periodontal conditions in Europe and concluded that generalised periodontal destruction was rarely seen among adults aged around forty years and that there was a probable shift from 35-40 years to 50-60 with respect to tooth loss caused by periodontal disease. Moreover,

longitudinal studies on periodontitis populations have revealed that a very small fraction of sites undergo attachment loss if left untreated and these are present in only a small number of the whole sample (Lindhe, Haffajee & Socransky, 1983; Haffajee, Socransky & Goodson, 1983a, 1983b; Jenkins, MacFarlane & Gilmour, 1988). Papapanou *et al.* (1988) reported that over a 10 year period, only 7% of subjects over 25 years of age showed greater than 3 mm attachment loss. Consistently, Lindhe *et al.*, (1989) revealed that 12% of the subjects monitored over a 2-year period had deteriorated by 3 mm or more and this occurred in 70% of the sites followed. Furthermore, Löe *et al.* (1986) conducted a 15 year study on a population who never practised oral hygiene measures. It was found that rapid progression of periodontal disease occurred in 8% of the total population. These results are in agreement with those observed by Baelum *et al.* (1986) and Reddy *et al.* (1986), who conducted their studies in remote regions of Tanzania and South Africa, respectively. Although the subjects had limited access to dental care, a percentage of around 10% or less showed evidence of attachment loss exceeding 6 mm. Collectively, the recent studies indicate that a subfraction not exceeding 10-20% of the whole populations show evidence of extensive periodontal disease (Brown *et al.*, 1990; Brown & Löe, 1993).

Recent epidemiological and clinical data suggest that gingivitis does not always result in destructive periodontitis and that gingivitis may persist at certain individuals and even at certain sites for many years without significant loss of periodontal attachment and destruction of periodontal ligament and

supporting bone. Realisation of this fact gives rise to the question of susceptibility of a proportion of a population to periodontal disease. Investigators focus on risk factors and markers of this disease in order to determine the identity and characteristics of the susceptible groups. Over the years many risk factors have been proposed. These include host factors and environmental factors. Host factors could either be local (e.g. anatomic and plaque retentive factors) or systemic factors.

Although it has been documented that with increasing age, the prevalence of periodontal disease increases (Marshall-Day *et al.,* 1955), age *per se* may represent a past history of exposure to the disease rather than an actual current susceptibility. Abdellatif & Burt (1987) have suggested that although the prevalence of periodontitis may increase with age, age alone in a healthy adult does not lead to progression of periodontal disease. Therefore, it can also be concluded that age may be a correlate rather than a risk factor for periodontal destruction. Moreover, the increased progression of periodontal disease in the elderly may be due to the association of ageing with cumulative attachment loss (Haffajee *et al.,* 1991; Grossi *et al.*, 1994) or recession (which results in exposed roots with a greater tendency for plaque retention) (Kinane & Davies, 1990).

### 1.4.4.1 Environmental factors affecting the periodontium

Among the environmental factors the presence of some micro-organisms, which are referred to as putative periodontopathogens, have been reported to have strong associations with periodontal disease (reviewed by Haffajee & Socransky, 1994). This matter will be further discussed in section 1.4.5.

Additionally, smoking has been shown to affect various aspects of host immune response. Smoking may have an adverse effect on fibroblast function (Raulin *et al.*, 1988), chemotaxis and phagocytosis by neutrophils (Kenney *et al.*, 1977; Kraal *et al.*, 1977), immunoglobulin production (Holt, 1987; Johnson *et al.*, 1990) and induction of peripheral vasoconstriction (Clarke, Shephard & Hirsch, 1981). Epidemiological evidence indicates that cigarette smoking is a stronger risk indicator for the presence of periodontal disease than any of the 5 most suspected periodontal pathogens i.e. *A.a., P.g., Prevotella intermedia (P.i.), Eikenella corrodens (E.c.)* and *Fusobacterium nucleatum (F.n.)* (Stoltenberg *et al.*, 1993).

Stress may affect the host response by increasing the level of corticosteroids. Furthermore, it may also affect the patients' oral hygiene habits and therefore may be a risk indicator.

Race or ethnicity are also considered to have importance as risk indicators (Hughes *et al.*, 1982). It was revealed that there was a higher prevalence of severe periodontitis among American Blacks, especially older males, than among Caucasians.

Furthermore, the fact that periodontal disease is known to be a multifactorial and complex disease led investigators to assess the consequence of an individual having multiple risk indicators. In the model used by Beck *et al.* (1990), the likelihood of getting or having periodontal disease was expressed in terms of odds ratios. When odds ratios were 1.0, there was no association between the suspected indicator and the disease but when the values exceeded 1.0 a positive correlation existed, which became even stronger with higher values. In this model, patients having any three of the four conditions (risk indicators) studied had about 160 times the odds of developing serious periodontal conditions compared with persons who had none of these conditions. The values obtained from this test have clearly demonstrated that risk indicators act in a synergistic and cumulative way and that some of them are more powerful than others. Collectively, these tests may be important as risk indicators of periodontitis to identify subsets of the population who are at high risk of severe periodontitis.

### 1.4.5 Aetiology of plaque-associated periodontal disease

The classification of periodontal diseases shown in section 1.4.2 underlines the fact that there seems to be a variety of aetiologic factors, immunological reactions and clinical manifestations in the periodontal tissues undergoing any kind of resolution or destruction. In the 1960's and 1970's there was an accumulating body of evidence which suggested that periodontal disease, as a general term, was an "infectious" disease process in animals and humans, caused by bacteria (Jordan & Keyes, 1964; Keyes & Jordan, 1964; Löe *et al.*, 1965; Lindhe *et al.*, 1975). Loesche (1976), described the non-specific plaque hypothesis and the specific plaque hypothesis. According to the former, caries and periodontal disease resulted from the elaboration of noxious substance by the entire plaque microflora. This idea was replaced by the specific plaque hypothesis which suggested that only a part of plaque caused the disease. This was due to the presence of one or more pathogens and/ or a relative rise in the numbers of certain indigenous plaque micro-organisms. In fact, periodontal pockets may contain more than 400 different bacterial species with different disease-inducing potentials. It appears that different combinations of bacteria rather than a single species, can provoke progression from gingivitis to destructive periodontitis (Theilade, 1986) through alterations in their relative proportions and interactions among them over a long period of time. Periodontal disease has been referred to as a "mixed bacterial infection" (Mayrand, 1985) and thus, it is dissociated from most infectious diseases which

are caused by only one specific micro-organism and the state of the disease can be identified by the presence or absence of the pathogen. Between 10 and 30 species of subgingival plaque bacteria have been implicated to one extent or another in the aetiology of periodontitis in humans (reviewed by Haffajee & Socransky, 1994). *A.a.* and *P.g.* are very strongly associated with periodontitis, and *Bacteroides forsythus, Prevotella intermedia, Campylobacter rectus, Eubacterium nodatum* and *Treponema* species follow. In general, investigators agree that periodontally healthy sites seem to harbour a sparse plaque of Gram-positive coccal forms, gingivitis sites comprise increased *Actinomyces* and reduced *Streptococci* species, while Gram-negative black-pigmented *Bacteroides* species and motile forms are more prominent in severe periodontitis sites (Savitt & Socransky, 1984; Genco *et al.*, 1988).

The initiation and progression of periodontal disease are strongly associated with the presence of a multitude of plaque microorganisms with a virulence potential of either favouring bacterial adherence and colonisation or mediating host tissue destruction (Socransky & Haffajee, 1991). Host defence mechanisms which operate to control and eliminate the microbial attack include: (a) the gingival crevicular fluid flow which may remove microorganisms and their products; (b) the antimicrobial effect of antibodies; and (c) the phagocytic function of neutrophils and macrophages. The aspects of the host immune response will be further discussed in section 1.7.

### 1.4.6 Genetic factors in periodontal disease susceptibility

A significant association has been shown between a number of disorders with a genetic basis and periodontitis, e.g. cyclic neutropenia (Cohen & Morris, 1961), chronic idiopathic neutropenia (Charon *et al.*, 1985), Papillon-Lefevre syndrome (Gorlin, 1964), trisomy 21 (Cohen *et al.*, 1961) and Chediak-Higashi syndrome (McKusick, 1990).

In early-onset forms of periodontitis (EOP), however, only local disease occurs. Segregation analysis is a method of studying family trees (pedigrees) to formulate hypotheses concerning the mode of inheritance of a trait. By this method it has been established that there is a significant genetic component to some forms of EOP displaying autosomal dominant transmission (Marazita *et al.*, 1994). However, this is still problematic since other modes of inheritance cannot be excluded given the probable heterogeneity of EOP, and the difficulty of deducing mechanisms explaining the purely local disease manifestations. Therefore, conflicting views still exist as to whether the transmission of the trait is X-linked dominant (Page *et al.*, 1985; Spektor *et al.*, 1985), autosomal recessive (Boughman *et al.*, 1988) or autosomal dominant (Marazita *et al.*, 1994).

Although there is no clear evidence linking adult periodontitis to genetic factors, there are data suggesting fundamental differences in individual responses to the same bacterial stimulus reflecting underlying genetic

differences. For example, Garrison & Nichols (1989) have demonstrated that endotoxin-stimulated monocytes from periodontal disease-susceptible patients secrete more $PGE_2$ than do endotoxin-stimulated monocytes from periodontal disease-resistant patients. Offenbacher *et al* (1993) describe a $PGE_2$ host response model as an alternative paradigm of periodontal disease that places the emphasis on host response rather than bacterial aetiology. This is not to say that bacterial virulence properties are not important, but that host response may be the key factor in susceptibility.

A variety of immunologic and endocrine disorders are associated with impaired host response and thus, may be associated with increased susceptibility to plaque induced periodontal break-down (reviewed by Kinane & Davies, 1990). Systemic diseases such as diabetes mellitus, leukocyte adherence deficiency syndrome, Down syndrome, neutropenia and Papillon-Lefèvre syndrome are known to enhance significantly the susceptibility to severe periodontitis (reviewed by Page & Schroeder, 1982). Most of these are likely to be genetically based and individuals suffering from such diseases directly become members of a high-risk group. Michalowicz, *et al* (1991) in their studies on twins attempted to assess the role of heredity in susceptibility along with a probable genetic predisposition to early-onset forms of periodontitis and adult periodontitis. According to their results, around half of the variance related to susceptibility to severe periodontitis could be accounted for by genetics alone, without consideration of microbes. Furthermore, studies such as that by Baelum *et al.* (1986) have supported the idea that although bacteria are

essential for the development of periodontitis, they are insufficient alone because there must be a susceptible host.

## 1.5 Periodontal disease activity

Clinically, periodontal disease in adults has been shown to manifest itself as at least two distinct entities (Hirschfeld & Wasserman, 1978; McFall, 1982). One form is relatively stable and does not endanger the life of the dentition. Patients suffering from this form of the disease have been called "well maintained" (Hirschfeld & Wasserman, 1978), while the lesion itself has been termed the "stable lesion" (Seymour *et al*, 1979). The second form of the disease is characterised by periodic or cyclical patterns of periodontal breakdown (Goodson *et al.*, 1982). This type of lesion has been referred to as the "progressive lesion" (Seymour *et al.*, 1979), while the patients exhibiting such symptoms have been called "downhill" (Hirschfeld & Wasserman, 1978).

Moreover, three models have been suggested by Socransky *et al.* (1984) to explain destruction of the periodontal supporting tissues: a. *the continuous paradigm*, b. *the random burst theory* and c. *the asynchronous multiple burst hypothesis.*

*The continuous paradigm* implies slow, constant and progressive destruction and is supported by cross-sectional studies (Marshall-Day *et al*, 1955; Russel,

1967; Löe *et al*, 1978). Pooling data from groups, subjects and sites in the study by Löe et al, 1978, could give the impression that progression has been slow and continuous. Moreover, longitudinal monitoring of sites not responsive to treatment (Badersten, Nilveus & Egelberg, 1985) has given evidence of a more continuous pattern.

*The random burst theory* has proposed short periods of destruction separated by periods of remission, occurring randomly through time and at random sites within an individual (Socransky *et al*, 1984).

Finally, in *the asynchronous multiple burst hypothesis* the destruction occurs within a defined time frame followed by a remission stage (Socransky *et al*,. 1984). In other words, many sites would show bursts of activity over a limited period of time and the sites would subsequently become inactive for an indefinite period.

Presently available data have not established or refuted any of these proposed paradigms (Haffajee *et al*., 1988). On the contrary, more recent studies have strongly supported the idea that the progression of the disease may follow a more continuous pattern with brief episodes of localised exacerbation and remission and that detection of "bursts" may be due to the poor resolution of clinical measurements (Jeffcoat & Reddy, 1991).

## 1.6 Pathogenic mechanisms of periodontal disease

Antigens and other virulence factors, and in some cases whole invading bacteria, comprise the microbial challenge, and the host responds by initiating a series of immediate inflammatory and immune processes. Although it is now clear that periodontitis is not a single homogeneous disease but rather consists of a family of closely related diseases, a common underlying chain of events is shared by all forms of the disease. Host responses triggered by bacterial infections include an immunological response but the role of this process in the pathogenesis of periodontal disease is still unclear. The host response results in the production of various molecules, such as cytokines and immunoglobulins, and other inflammatory mediators i.e. kinins, complement activation products and matrix metaloproteinases, which may interact and cause connective tissue and bone destruction. A variety of immune cells are implicated in these processes, mainly T and B cells, macrophages and neutrophils among others. Nevertheless, the exact role of each cell type and the mechanisms accounting for the cell patterns in the histopathogenetic images of periodontal disease are still unknown.

The following sections will discuss some basic immunological concepts in relation to the cells of the immune system before reviewing their inter-relationships and participation in the pathogenesis of periodontal disease.

### 1.6.1 Cells of the immune system

The immune system has evolved to protect the host against pathogenic micro-organisms and other foreign substances. Recognition of these foreign molecules, functionally called antigens, is carried out primarily by lymphocytes. Lymphocyte-mediated recognition of antigen shows specificity as well as memory. Basically, the commencement of the immune response takes place once the antigen is taken up by the antigen-presenting cell i.e. macrophages, dendritic cells, Langerhans cells of the skin and mucous membrane, and B-lymphocytes. The antigen is processed and a peptide fragment is presented in association with self antigen to the T-lymphocyte and an immune response is initiated.

Two types of lymphocytes exist: T-lymphocytes which develop in the thymus and B-lymphocytes, which originate from the bone marrow and subsequently develop into antibody-producing plasma cells.

#### 1.6.1.1 T cells

T cells can be mainly subdivided into helper T cells and cytotoxic T cells. Helper T cells produce helper factors i.e. cytokines to assist other cells of the immune system, while cytotoxic T cells stimulate the microbicidal and cytotoxic activity of other immune cells, such as macrophages. Following

antigen presentation to them, helper T cells become activated and produce factors which stimulate B cells to undergo differentiation into plasma cells and eventually produce antigen-specific antibodies. Stimulation of cytotoxic T cells and phagocytes (i.e. macrophages, neutrophils and other mononuclear phagocytic cells) also occurs. The success of the interaction between the helper T cell and antigen-presenting cell is dependent upon the ability of the helper T cell to recognise processed antigen in association with self antigen on the surface of the antigen-presenting cell. These self antigens are encoded in a set of clustered loci, known as the major histocompatibility complex (MHC). MHC as well as playing a major role in graft rejection is also implicated in antigenic peptide recognition. In humans, MHC is called human leukocyte antigen complex, or the HLA complex, and is further divided into two major classes I and II. In general, MHC class I antigens are found on most nucleated cells, while MHC class II antigens are mainly located on activated T cells and on B cells. Furthermore, all T lymphocytes express a receptor ,known as the T-cell receptor, which aids T cells to specifically bind antigen and the MHC molecule on the antigen-presenting cell. There are two types of T cell receptors described: the $\alpha\beta$ heterodimer and the $\gamma\delta$ heterodimer; with the $\alpha\beta$ T cell receptor expressed on 90% of peripheral blood T helper and T cytotoxic lymphocytes. MHC restricted antigen recognition is seen only with T lymphocytes, whereas B lymphocytes possess the ability to recognise antigen without been helped by any mediator.

### 1.6.1.2 Cluster determinant (CD) antigens

CD antigens represent a group of cell surface molecules which have been discovered by immunising rabbits, mice *etc.* with human lymphocytes. The antibodies produced recognise unique antigens designated CD antigens. The acronym "CD" is "cluster determinant" referring to monoclonal antibody reactivity patterns to surface proteins. Labelled monoclonal antibodies to CD antigens have been used in a number of immunohistochemical procedures to differentiate leukocytic populations. For example, the anti-CD3 monoclonal antibody is useful for distinguishing T cells from B cells; CD20 antigen is associated with B cells and CD68 antigen accounts for macrophage/ monocyte recognition. Furthermore, CD4 is present on the surface of T helper cells' membrane and is the co-receptor for class II MHC, whereas CD8 antigen is found on the surface of T cytotoxic cells and is the co-receptor for class I MHC antigens. Therefore, $CD4^+$ T helper cells recognise antigen in a MHC class II restricted fashion and $CD8^+$ T cytotoxic/ suppressor cells seem to be responsive in a MHC class I restricted fashion.

### 1.6.1.3 Types of T helper cells

Early in antigen presentation and T cell expansion, two distinct subsets of T helper cells emerge, Th1 and Th2 which secrete different patterns of cytokines. Th1 cells secrete both interleukin-2 (IL-2) and interferon-γ (IFN-γ) when

activated by certain types of T-dependent antigens, so that they can enhance cell-mediated responses. The Th2 subset of T helper cells produce interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10) and interleukin-13 (IL-13) and thereby promote the humoral immune response. Th2-derived cytokines can influence the immune response through helping antigen-specific B cells differentiate into antibody forming plasma cells as well as determining the class of immunoglobulin being produced. Moreover, human T lymphocytes within both the class I MHC responsive $CD8^+$ cytotoxic/ suppressor and class II MHC responsive $CD4^+$ helper/ inducer sublineages can be separated into two broad subpopulations. These two subsets are based on their expression of distinct isoforms of the common leukocyte antigen, the CD45 molecule (Salmon, Kitas & Bacon, 1989; Yamashita & Clement, 1989). T lymphocytes expressing the high molecular mass CD45RA molecule (Streuli *et al.* 1988) are postulated to represent a pool of virgin or naive T lymphocytes that have yet to encounter their respective antigen (Damle *et al.*, 1987). On the other hand, T lymphocytes carrying only the low molecular mass CD45RO molecule (Streuli *et al.*, 1988) have the ability to respond to previously encountered antigen. Hence the $CD45RO^+$ T cells are thought to represent a pool of primed or memory T cells (Smith *et al.*, 1986; Damle *et al.*, 1987; Akbar *et al.*, 1988; Yamashita & Clement, 1989) and are recognised by the UCHL-1 (University College Hospital London-1) monoclonal antibody (Smith *et al.*, 1986). Not only do differences between these two T cell subsets occur with respect to the maturation stage, but they additionally seem to possess distinct activation

requirements and profiles of lymphokine production (Byrne, Butler & Cooper, 1988; Sanders *et al.*, 1989; Salmon *et al.*, 1988, 1989).

### 1.6.1.4 B cells - Immunoglobulins

B cells take part in the humoral immune response by being transformed into plasma cells at a later stage of the inflammatory process. Plasma cells produce specific antibodies when activated by certain T cell subsets.

An antibody, or immunoglobulin, molecule is made up of four peptide chains, two identical heavy chains (about 450 amino acids) and two identical light chains (about 214 amino acids) held together by interchain disulphide bridges and noncovalent forces. Heavy chain genes map to the q32 band of chromosome 14 (Cox et al., 1982). They are encoded by four linked gene families, the variable ($V_H$), diversity (D), joining ($J_H$) and the constant ($C_H$) gene segments. Immunoglobulin G (IgG) can be split by proteolytic enzymes to produce fragments giving clues to their function. The Fab fragment is now thought to be primarily involved in the specific interaction with the antigen and the Fc, particularly of the heavy chains, carries the sites for other biological functions, e.g. complement binding. The Fab fragment, consisted of about 107 to 115 amino acids, contains considerable variability whereas the Fc fragment is relatively constant and consists of about 107 to 110 amino acids for the light chain and about 310 to 330 amino acids for the heavy chain. Analysis of

amino-acid variability has identified three hypervariable sequences on the heavy chain and three on the light chain in the Fab fragment. There are five different types of heavy chain existing in humans and these define the immunoglobulin classes, IgG, IgA, IgM, IgD and IgE. Nine functional genes determine the class and the subclass to which the immunoglobulin molecule belongs (Flanagan & Rabbitts, 1982).

### 1.6.1.5 Immunoglobulin G

IgG is the principal immunoglobulin produced in the secondary immune response making up about 80% of serum immunoglobulin. IgG diffuses more easily than the other immunoglobulins into the extracellular spaces where it has the predominant role in neutralising bacterial toxins and binding to micro-organisms to promote complement fixation followed by phagocytosis. It is considered the most important antibacterial and virus-neutralising antibody of the internal milieu. The unique biological function of different immunoglobulin classes is mediated by the Fc portion. In the case of IgG, monocytes and macrophages contain receptors for Fcγ (FcγRI). FcγRII is found on monocytes, neutrophils, eosinophils, platelets and B-cells. IgG complexes can bind to platelets causing thrombosis. Stimulation of B-cell Fc receptor leads to downregulation of IgG production, a possible mechanism whereby IgG and IgG immune complexes exert a negative feedback effect on antibody production. The Fcγ region of IgG is responsible for complement

activation via the classical pathway, binding to C1q and causing its activation. Another low-affinity Fc receptor, FcγRIII, which is found on NK cells, macrophages, PMNs and eosinophils, is thought to be responsible for mediating immune complex clearance by macrophages and other cells.

### 1.6.1.6 Immunoglobulin A

IgA is adapted to defend the exposed external and internal surfaces of the body against micro-organisms. It is found predominantly in sero-mucous secretions, e.g. lung secretions, saliva and secretions of the gastrointestinal and genito-urinary tracts. In these fluids it is present as a dimer which is stabilised against proteolysis by combination with the secretory component, which is synthesised by local epithelial cells. Dimerisation occurs intracellularly via a joining sequence (J-chain), to prevent association of monomers with differing specificity. Secretory IgA (s-IgA) is predominantly found in the polymeric form and is transported by a secretory component (SC)-dependent mechanism into external secretions (Brandtzaeg, 1981). Essentially, IgA antibodies coat micro-organisms and thereby inhibit their adherence to mucosal cells. Aggregated IgA can also bind PMNs through FcαR and can activate the alternative complement pathway. Monomeric IgA is also found in relatively high levels in the circulation indicating an additional systemic role for IgA.

### 1.6.1.7 Immunoglobulin M

IgM exists as a pentamer joined by a J-chain and interacts with secretory component (SC) in order to be transported into external secretions (Brandtzaeg, 1981). IgM antibodies, although of low affinity as measured against single determinants, can demonstrate relatively high avidity, i.e.. overall binding strength with molecules containing multiple epitopes. IgM is largely confined to the blood stream, only about 25% being distributed extravascularly; however, some mucosal sites may have a significant supply of this immunoglobulin due to local synthesis (Brandtzaeg, 1975). IgM appears in the primary immune response and it is quite rapidly replaced by IgG. This is due to T cell products which facilitate a switch in production from IgM to IgG or other classes, such as IgA or IgE. IgM is extremely efficient in agglutination and cytolysis of invading cells (isohaemagglutinins are IgM antibodies). As such it is thought to play a vital role in control of bacteraemia. IgM activates the complement system. Such activation induces phagocytosis and killing of microorganisms by neutrophils and macrophages.

### 1.6.1.8 Immunoglobulins D and E

IgD and IgE are present as monomers. IgD is found on the surface membrane of most B cells and it serves as a receptor to which specific antigen can bind. Only trace amounts of IgD are found in the serum.

IgE's affinity for the surface of mast cells and basophils explains its major contribution to immediate hypersensitivity of type I which is visualised by degranulation of the defence cells and release of vasoactive amines and certain enzymes. The action of IgE may on the other hand have a physiological part by increasing vascular permeability and stimulating inflammation. Furthermore, IgE may trigger local anaphylactic reactions (Type III hypersensitivity) by augmenting the deposition of IgG-antigen complexes at the reaction site (Benveniste, 1973).

### 1.6.1.9 J chain

The J chain is a low molecular weight glycoprotein linked with two heavy chains through disulfide bonds to extracellular polymeric IgA and IgM. It is synthesised in lymphoid cells at various stages of differentiation. Analyses of IgA and IgM structure have revealed that one J chain is incorporated per polymer molecule and the binding is independent of the size of the polymer (reviewed by Koshland, 1985). Early studies have suggested that J chain plays an essential role in polymer assembly of IgA and IgM (Della Corte & Parkhouse, 1973; Wilde & Koshland, 1978) and this was reinforced by studies of somatic cell hybrids (Laskov, Kim & Asofsky, 1979) and by *in vitro* experiments of pentamer IgM synthesis in human lymphocytes (Kutteh *et al.*, 1983). By a variety of techniques, J chain production has been detectable in cells that synthesise polymeric immunoglobulins, as well as in normal and

malignant lymphoid cells that contain IgG and IgD and in some pre-B lines (Kaji & Parkhouse, 1974; Brandtzaeg & Berdal, 1975; Mossman *et al.*, 1978; Mestecky *et al.*, 1980; Hajdu *et al.*, 1983). Therefore, evidence from the above mentioned studies underlines the fact that although J chain has been implicated as a necessary component in polymerisation of IgA and IgM (Koshland, 1975), it may also perform some general, as yet unidentified, function in immune response in relation to B cell differentiation (Koshland, 1985) and to the maturation of monomer Ig-secreting cells (Haber & Mestecky, 1984). For the purpose of the present study the localisation of J chain mRNA positive cells will be associated with polymer IgA and IgM mRNA bearing plasma cells' expression within chronically inflamed periodontal gingival and granulation tissues.

#### 1.6.1.10 Immunoglobulin subclasses

Immunoglobulins are further grouped into subclasses. IgG, for example, exists as $IgG_1$, $IgG_2$, $IgG_3$ and $IgG_4$, the difference being in the heavy chains which exist as $\gamma$ 1, 2, 3 and 4 respectively. These heavy chain differences give rise to differences in biological properties. $IgG_2$, for example, is relatively inefficient in placental transfer, whereas $IgG_3$ is a very avid binder of complement, and $IgG_4$ is a poor fixer of complement. $IgG_1$ and $IgG_3$ both promote strong binding of monocytes. Some of these differences between different IgG

subclasses may have an impact on the course of periodontal disease, and this will be discussed later.

Moreover, IgA contains two subclasses, $IgA_1$ and $IgA_2$. The IgA subclasses may have evolved as a result of gene duplication and are very closely related as evidenced by their similar nucleotide sequence (Flanagan & Rabbits, 1982; Flanagan *et al.*, 1984). Apart from the primary structure, they also differ in antigenic determinants of their heavy chains and susceptibility to microbial IgA proteases (Kilian, Mestecky & Russel, 1988). $IgA_1$ predominates in normal serum (about 80-90%), whereas various exocrine secretions have been reported to contain a large proportion of $IgA_2$ (about 30-50% of total IgA) (Delacroix *et al.*, 1982).

## 1.7 Immune responses in periodontal disease

The periodontal immune response, including both cell-mediated and humoral reactions, has been extensively examined. Elevations in the cell-mediated (Seymour, Powell & Davies, 1979) or humoral response to certain plaque-associated micro-organisms is noted with the presence and/ or increasing severity of periodontal disease.

T-helper cells bind to an antigen for which they are programmed when it is found in association with a class II MHC receptor on the surface of an infected

antigen presenting cell (macrophage or Langerhans cell). They then produce lymphokines, including interferon-γ (IFN-γ) and other macrophage activating factors, re-triggering the microbiocidal mechanisms of the macrophage which had been repressed by the intracellular pathogen. The lymphokines produced act in turn not only on macrophages but also on B cells and other T cells and they are responsible for inducing inflammation, tissue damage and, at a later stage, bone resorption. A second plaque-derived microbial attack finds the lymphocytes sensitised. They respond to the antigenic stimuli by proliferating and synthesising cytokines more rapidly. The cytokines act as signals that initiate either stimulation, or inhibition or even killing of other cell types. This type of response is similar to the delayed type hypersensitivity response (section 1.7.1), where in addition to sensitised lymphocytes, the lesion contains non-sensitised lymphocytes as part of the inflammatory cell infiltrate. Non-specific induction by plaque-derived mitogens or various inflammatory cells may lead to further release of cytokines.

Cytotoxic T-cells are also capable of producing IFN-γ. These actions of T-lymphocytes are referred to as cell-mediated immunity. T-cells are selected and activated by antigen and expanded by clonal proliferation to produce a large clone of activated T-cells and also a pool of quiescent memory cells.

### 1.7.1 Periodontal tissue damage and types of hypersensitivity

Until recently (Grant, Stern & Listgarten, 1988), discussions of the immunological mechanisms of tissue damage in periodontal diseases have referred to the four types of hypersensitivity reaction, as classified by Coombs & Gell (1975). However, their characteristics are not in good accordance with the clinical and histopathological signs of the disease.

Type I (anaphylactic), type II (cytotoxic) and type III (immune complex) reactions are all antibody-mediated whereas type IV (delayed hypersensitivity) is cell-mediated. The hypersensitivity mechanisms involved in periodontal disease have not been defined. However, since B-cells and plasma cells predominate in the later stages of periodontitis, evidence points to type II and III. Type IV could probably be present in the early lesion (Seymour, 1987).

Type I hypersensitivity reactions rely on IgE antibody bound to the surface of mast cells. Upon reaction with specific antigen, these cells release histamine and other inflammatory mediators. Mast cells have been demonstrated in the gingiva (Angelopoulos, 1973), as has IgE (Nisengard, 1974), but type I reactions are probably not important elements in the immunopathogenesis of periodontal disease, as reflected in the very low numbers of mast cells and IgE antibody in the periodontal tissues.

Type II reactions rely on complement-fixing IgG and IgM antibody. Damaged and lysing cells have been demonstrated in periodontitis (Cobb *et al.*, 1990). This type of reaction is probably essentially protective. However, a study by Reinhardt *et al* (1989) has shown a predominance of total $IgG_1$ and $IgG_4$ in active as opposed to stable periodontitis sites. Since $IgG_4$ is relatively inefficient in fixing complement, two hypotheses have been suggested. $IgG_1$ is basically protective and a preponderance of $IgG_4$ may promote disease progression by failure of antibody/ complement mediated bacterial destruction. Alternatively, a shift to $IgG_4$ production locally may constitute an attempt to limit the potentially damaging effects of complement-mediated activity. However, immunoglobulin synthesised by gingival plasma cells has been shown to react against non-oral bacteria (Berglund, 1971) or non-bacterial antigens (Mallison *et al.*, 1989), so the possibility exists that the response is not always specific to the plaque-derived micro-organisms.

Type III reactions involve complement fixation by antibody-antigen complexes. However, Genco *et al.* (1974) have suggested that soluble proteins rather than fixed immune complexes occur. Moreover, attempts to extract insoluble immune complexes from periodontal tissue have not been successful (Clagett & Page, 1978). Collectively, since studies of complement in periodontal disease have demonstrated that these components are easily washed out, this type of reaction is unlikely to be important in periodontal destruction.

Type IV reactions involve T-cell mediated direct cytotoxic effects on target cells. Antigen-activated T cells and activated macrophages accumulate in the lesion, and by release of lymphokines and lysosomal enzymes, respectively, they eliminate foreign antigens.

### 1.7.2 Autoimmunity in periodontal disease

Another possible mechanism in the immunopathogenesis of periodontal disease is that of autoimmunity. Serum antibodies against type I collagen have been found in higher levels in periodontitis than in control subjects (Hirsch *et al.*, 1989). A model has been presented whereby polyclonal activators initiate clonal expansion of autoreactive B-cells, This initial response leads to the production of autoantigens which, in turn, induce a specific response. (Tew, Engel & Mangan, 1989). However, there is a general dearth of evidence implicating autoimmune mechanisms in the pathogenesis of periodontal disease. Current research is focused on whether this system has been developed to protect from or contribute to the progress of the disease (reviewed by Anusaksathien & Dolby, 1991).

### 1.7.3 Macrophages within the gingival tissues

Macrophages participate in the initiation and development of cell-mediated hypersensitivity reactions. Upon being recruited into the inflammatory

lesions, macrophages become activated by lymphokines and other substances, such as endotoxins and bacterial cell walls. Activated macrophages are able to phagosytose and kill bacteria and they also acquire cytotoxic properties enabling them to attack and destroy target cells. Upon activation macrophages posses the ability to synthesise and secrete numerous products, such as hydrolytic enzymes, complement components, binding components (fibronectin), oxygen and arachidonic acid metabolites, as well as monokines (IL-1). The latter may have a number of effects on the growth and activation of T and B cells, fibroblasts and osteoclasts.

Topoll *et al.* (1989) analysed subsets of macrophages over a 19-day period of experimental gingivitis study. There were not any changes in the relative numbers of macrophages over the 19-day period, but an increase in the number of inflammatory over the non-inflammatory macrophages occurred. Inflammatory macrophages are antigen-presenting cells and by this action they can induce the initiation of an inflammatory/ immune response. Other investigators have shown that mononuclear phagocytes appear in association with osteoclasts at sites of active bone resorption (Rifkin & Heijl, 1979) and that endotoxin-stimulated mononuclear phagocytes produce soluble factors with bone resorbing activity (Gregory, 1988). Therefore, it could be suggested that macrophages have a significance in the pathogenesis of periodontal disease.

### 1.7.4 Immune response in adult periodontitis

It is now clear that the gingiva adjacent to periodontal lesions is heavily infiltrated with mononuclear leukocytes. Models used in the earlier years suggested that the initial lesion is composed mainly of T lymphocytes, with B cells and plasma cells predominating at a later stage (Mackler *et al.*, 1977; Seymour *et al.*, 1982; Seymour *et al.*, 1983b). However, later studies found that both T cells and B cells may predominate in lesions, but that there was considerable variability in proportions of plasma cells (Stoufi *et al.*, 1987b; Johannessen *et al.*, 1990). CD4 : CD8 cell ratios are decreased in periodontal lesions compared with those seen in peripheral blood of periodontitis patients or in healthy gingiva (Okada, Kida & Yamagami, 1982; Taubman *et al.*, 1984). Taubman *et al.* (1991) have demonstrated that many of the $CD4^+$ cells in periodontal lesions are in fact memory cells, and that $CD8^+$ cells suppress and macrophages promote immunoglobulin production by plasma cells in the gingiva. By these mechanisms, regulation of local immune responses by gingival cells is made possible.

Responsiveness of leukocytes to various antigens and mitogens has also been extensively studied in periodontal disease. Polyclonal B cell activation, has been shown to be increased in most periodontitis patients (McNulty *et al.*, 1985; Tew, Engel & Mangan, 1989). Polyclonal B cell activation involves hyper-reactivity of B-cells to bacterial products as indicated by the massive production of lymphokines and immunoglobulins.

Elevated monocyte response to LPS has also been demonstrated in periodontitis patients (Garrison & Nicholls, 1989; McFarlane, Reynolds & Meikle, 1990). An hyper-reactivity to LPS, as determined by an increased monocyte $PGE_2$ and IL-1ß production has been noticed. Therefore, it was suggested that these patients may be susceptible to disease progression because of this state of hyper-reactivity.

Degradation of soft tissue in periodontal disease can be effected by both bacterial and host enzymes, including the matrix metalloproteinases. These include collagenase which can be produced by both fibroblasts and PMNs. The fibroblast type degrades collagen type III, whereas the PMN type degrades collagen type I. The stromelysins have multiple functions and can also activate the collagenases. Gelatinase can degrade collagen types IV and V and also breaks down denatured collagen. Birkedal-Hansen *et al.* (1984) have shown that at least three suspected periodontopathogens, i.e. *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum,* produce factors that stimulate epithelial cells to degrade collagen fibrils. The ability of certain bacteria to induce tissue degradation, not only indirectly *via* cytokines, but also directly, further emphasises the crucial role of the host response in eliminating or limiting these pathogens.

Seymour *et al.* (1993) have proposed a model of the immunopathogenesis of chronic inflammatory periodontal disease. This suggests that susceptible subjects may have an increase in type 2 IL-4 producing T-cells which

selectively home to the gingiva and lead to B cell expansion. This may trigger periodontal destruction *via* IL-1 or protection *via* production of specific antibody by antigen-specific B-cells. Resistant subjects may have an increase in type 1 IL-2/IFN-α producing T-cells which selectively home to the gingiva resulting in B-cell suppression and a T-cell stable lesion with slow tissue destruction. Kinane *et al.* (1993) have suggested that T-cells localising in gingiva differ from those in skin and peripheral blood. They conclude that their findings are consistent with the existence of local immune systems composed of skin-homing and gingiva-homing memory T-cells. Shimauchi *et al.* (1993) demonstrated that a Th2 type *A. actinomycetemcomitans* specific $CD4^+$ clone would only accumulate in the gingival tissue of rats which had been infected with this organism. Thus, a system for the potential kinetics of T-cell entry into gingival tissues and their retention has been described for the first time.

Since the indications are that the periodontitis lesion in the susceptible subject is B-cell dominated, the immunoglobulins being produced by plasma cells at the site, their subclass distribution and relationship to immunoglobulins detected in the GCF are of relevance to local and systemic immunity.

From the above discussion it becomes clear that there is a relationship between immune function and the pathogenesis of periodontal disease. It has also been shown that different forms of periodontal disease show differences in their immunopathogenesis.

### 1.7.5 Immune response in early onset forms of periodontal disease

*A.a.* is known to be of relevance in the initiation and progression of juvenile periodontitis (JP) and *A.a.*-derived leukotoxin is an important virulence factor. A number of reports have confirmed the existence of a strong local and systemic antibody response to *A.a.* in patients affected with this type of periodontal disease (Listgarten, Lai & Evian, 1981; Ebersole *et al.*, 1982). It has been reported that PMN abnormalities exist in JP (Suzuki *et al.*, 1984; Page *et al.*, 1985) but not in every case (Kinane *et al.*, 1989a; Kinane *et al.*, 1989b). The initial rapid infection and progression may be due, in part, to the PMN dysfunction, which alters the defensive capability, and/ or to high virulence of the infecting micro-organisms (Rabie, Lally & Shenker, 1988). On the other hand, studies have indicated a negative correlation between antibody titer to *A.a.* and severity of disease in patients with EOP (Gunsolley *et al.*, 1987). Additionally, the predominance of IgG-producing plasma cells has been reported in the inflamed gingiva of patients affected with JP (Hall, Falkler & Suzuki, 1990). From all the above, it could be hypothesised that immune responses play a significant role in altering the clinical manifestations of JP and indicating subpopulations which are more susceptible to the disease than others.

There are indications suggesting that the generalised form of early onset periodontitis or rapidly progressive periodontitis (RPP) (Page *et al.*, 1983) may display some systemic immunological abnormalities. Studies have associated

severe generalised EOP with a hyper-responsiveness to B cell mitogens (Engel *et al.*, 1984). Additionally, lower or delayed spontaneous proliferation of peripheral blood lymphocytes in culture has been associated with generalised EOP or RPP (Evans, Mikulecky & Seymour, 1989). Farida *et al.* (1986) have found differences in antibody levels to several bacteria between localised and generalised cases of EOP, with the localised cases having high titers and the generalised ones exhibiting lower than normal titers. Kinane, Johnston & Evans (1989) have reported suppressed CD4 : CD8 ratios in the peripheral blood of patients with EOP or RPP. Collectively, it becomes apparent that abnormal immune response may be present in the severe forms of periodontitis, and research should be necessary to determine whether this abnormal function is the result or the cause of the disease.

## 1.8 Introduction to the methodology

Numerous inflammatory cells i.e. macrophages, lymphocytes, plasma cells and polymorphonuclear neutrophils (PMNs) comprise the cellular infiltrate in periodontal lesions. The role of the different cell types and their inter-relationships has been investigated by morphological and immunohistochemical studies. However, when cell synthetic activity or cell proliferation need to be investigated, morphological observation or phenotypic analysis of cell surface markers seem to be inadequate and not much information about the cell function *in vivo* is available, in the

periodontal lesion context. In studies which comprise this thesis, the synthetic capacity of cells *in vivo* within periodontal gingival and granulation tissue biopsies were evaluated by the technique of *in situ* hybridisation (ISH) (Pringle *et al.*, 1989; Talbot, Primrose & Pringle, 1989).

### 1.8.1 *In situ* hybridisation technique (ISH)

#### 1.8.1.1 Historical significance of *in situ* hybridisation

The earliest descriptions of the *in situ* hybridisation technique were given in 1969 by two groups working in the United Kingdom (John, Birnstiel & Jones, 1969) and the USA (Gall & Pardue, 1969). The technique's potential was quickly realised and within a few years it had been used for the demonstration of genomic sequences (Pardue & Gall, 1975), viral DNA (Orth, Jeanteur & Croissant, 1971), messenger RNA (mRNA) (Harrison *et al.*, 1973) and ribosomal RNA (rRNA) (Buongiorno-Nardelli & Amaldi, 1970). The identification of the nucleic acid sequences was successfully carried out on cryostat (Orth, Jeanteur & Croissant, 1971), paraffin wax (Buongiorno-Nardelli & Amaldi, 1970), electron microscopic (Jacob *et al.*, 1971) and chromosomal preparations (McDougall, Dunn & Jones, 1972).

The method was neglected for a period, due mostly to the technique's lengthy and technically demanding procedures. For example, difficulties in

probe preparation were realised, since neither pure nucleic acids could be prepared nor was there a routine method available for the *in vitro* incorporation of the radioactive label into the nucleic acid sequences. The problem of probe purity, however, was solved through the application of recombinant DNA technology (Hudson *et al.*, 1981). Moreover, after the introduction of the first effective non-radioactive label, a biotinylated nucleotide analogue of dTTP, biotin-dUTP (Langer, Waldrop & Ward, 1981) and the description of the nick translation labelling method (Rigby *et al.*, 1977) exploitation followed and *in situ* hybridisation became a major development area.

### 1.8.1.2 Probes used for *in situ* hybridisation technique

Two main categories of labelled probes are employed in *in situ* hybridisation technique: radioactive and non-radioactive. The most commonly used radioactive markers are phosphorus ($^{32}P$) and sulphur ($^{35}S$) or less frequently tritium ($^{3}H$). These have the advantage of being sensitive and having high hybridisation efficiency. The disadvantages of the radioactive probes are the long exposure times when compared to non isotopic labels (Warford, 1988; Asa, 1993). The long delay before results are available by autoradiography and the repeated requirement for preparation of radioactive probes before each experiment due to their unstability, were among the reasons for the increased current utilisation of non-radioactive probes. Comparison of $^{35}S$

and $^{3}H$ with non-isotopic methods has confirmed that optimised non-radioactive methods can give results of equivalent (Syrjanen *et al.*, 1988) or even enhanced sensitivity (Lo, 1986; Allan, Todd & Smyth, 1989). Biotin UTP has largely been responsible for the rapid development of non-radioactive *in situ* hybridisation; however, since biotin is present in active cells or as an endogenous component of several organs (Wood & Warnke, 1981; Banerjee & Pettit, 1984), non-specific binding often occurs and, therefore the signal is not always specific for the sequence under detection. In the present study, digoxigenin-11-dUTP was utilised as a non-radioactive label. It has been reported that digoxigenin UTP can be substituted directly for biotin UTP in labelling reactions (Herrington, *et al.*, 1989a, 1989b), because it is of equivalent sensitivity to biotin and has also no endogenous tissue distribution.

Various types of probes can be utilised, including double stranded DNA (cDNA), single stranded DNA (ssDNA), single stranded anti-sense RNA (ssRNA), synthetic oligoriboprobes and synthetic oligodeoxyribonucleotides ("oligonucleotides") (Ogilvie *et al.*, 1990). Each one has distinct advantages and disadvantages. Most workers agree that the optimal probe size for *in situ* hybridisation is between 50 and 250 bases (Cox *et al.*, 1984; Bressler & Evinger-Hodges, 1987) and that the best probe for hybridisation to mRNA is single stranded RNA (ssRNA), followed by single stranded DNA (ssDNA) and double stranded DNA (cDNA) (Diaz *et al.*, 1981; Cox *et al.*, 1984). Pringle *et al.* (1990) disputed this convention when he compared the

hybridisation efficiency and specificity of ssRNA and oligonucleotide probes. According to his study, oligonucleotides of 30 bases long showed increased hybridisation efficiency when used in the non-radioactive detection of mRNA in paraffin tissue sections. Numerous protocols have been developed that use either enzymatically synthesised, (more than 100 bases long), DNA or RNA probes or chemically synthesised, (less than 100 bases long), DNA "oligonucleotide" probes. The latter possess several advantages, including simple and rapid preparation of the probe and easy penetration of the shorter probes into tissues and cell preparations (Asa, 1993). Additionally, these probes can be designed in a way that regions of sequence homology among members of a family of genes' transcripts can be avoided (Young, III, 1992). In contrast, the most serious disadvantage of the use of the shorter oligos is their relative lack of sensitivity, which can lead to false positive results. The problem of sensitivity, however, may be overcome by using a cocktail of two or more oligos to different portions of the same transcript (Weiner, Levey & Brann, 1990). In the present study, the use of synthetic oligonucleotides was preferred. Oligos were prepared in a DNA synthesiser or purchased as "cocktails" of several labelled sequences.

### 1.8.1.3 Principles and significance of *in situ* hybridisation

The whole concept of *in situ* hybridisation lies on the following principle: a labelled nucleic acid, i.e. probe, hybridises through complementary base

pairing to an immobilised target sequence. By visualising the label, precise cytological localisation of the target sequence is obtained. Of all the molecular techniques available, perhaps *in situ* hybridisation offers most utility in examination of clinical disease at present, since not only can gene expression be studied, but foreign bacterial and viral nucleic acids in host cells can be identified *in vivo*. It can identify gene expression in a small population of cells or detect messenger RNA with a very low copy number (Multhaupt *et al.*, 1989). Unlike immunohistochemistry and immunocytochemistry which localise the gene product (protein or hormone), *in situ* hybridisation offers the possibility of detection of cells that are genetically inclined to synthesise proteins but can not store them in significant numbers, as well as cells that express a specific mRNA but fail to translate it into a protein product. In other words, it is a morphological method which can distinguish synthesis potential from uptake and storage. Moreover, when combined with immunohistochemistry or immunocytochemistry it can co-localise both the transcribed gene product (mRNA) and its translated protein in the same cell (Shivers *et al.*, 1986). Another type of this application is the "double target" *in situ* hybridisation which allows the simultaneous identification of two different mRNA sequences within the same cell.

### 1.8.2 Synthetic activity

The messenger RNA (mRNA) content of various cells *in vivo* can be assessed with poly-deoxythymidine (oligo d(T)) oligonucleotide probe to poly-adenosine (poly-(A)) sequences (Pringle *et al*, 1989; Danks *et al,* 1995). The total number and the distribution of mRNA molecules vary from one cell type to another and they depend on the level of the cell activity and its stage in the cell cycle. Furthermore, *in situ* hybridisation for 28S ribosomal RNA (rRNA) is a convenient method for localising hybridisable RNA in tissue sections, since rRNA is present in every cell that synthesises proteins (Maheshwari *et al.*, 1993; Yoshii *et al.*, 1995). As a conclusion, information about the relative concentrations of total mRNA and rRNA would be useful in order to assess the cellular synthetic activity within periodontal tissue sections. Studies aiming to reveal information about the synthetic capacity of the inflammatory infiltrate in periodontal gingival biopsies have been carried out in our laboratory using *in situ* hybridisation technique (Takahashi Lappin & Kinane, 1996). Still there has not been any attempt to evaluate this activity within deep granulation tissue from periodontal lesions. Therefore, it would be challenging to assess the cell and, furthermore, the tissue function *in vivo*.

### 1.8.3 Cell proliferation

The size and regulation of tissues and organs depend on the balance between cell proliferation and death. The investigation of the proliferative activity in inflammatory lesions is important because it gives information about normal cell turnover, clonal expansion of T and B lymphocytes and tissue development, regeneration or destruction. The incidence of S phase or "mitosis" has been regarded as an indication of proliferation in tissues (Quinn & Right, 1990; Hall & Coates, 1995). Histone represents a short-lived cytoplasmic protein which is produced in significantly high levels during the S phase of the cell cycle (Heintz, Sive & Roeder, 1983; Shah *et al.*, 1994; Alison *et al.*, 1994). Therefore, the presence of histone mRNA permits the assessment of proliferating cells in the tissues. In addition, Ki-67 is produced in the nuclei of cells during $G_1$, S, $G_2$ and M phases of the cell cycle, but it is absent in the $G_0$ (resting) phase (Hall & Coates, 1995). Mouse anti-human monoclonal Ki-67 antibody is regarded as a useful proliferation marker, which identifies cells undergoing active division (Brown & Gatter, 1990).

### 1.8.4 Apoptosis

Proper organisation and function of the tissues is also highly dependent on the process of cell death. Two types of cell death can be identified and

determined according to the cell morphology, as it appears under the microscope: necrosis and "programmed" cell death i.e. apoptosis. The main features in necrosis are the damage to the plasma and internal membranes, rapid cell swelling, disruption of ion gradients and autolysis. Necrosis is the critical "point of no return" of cell injury inflicted by severe circumstances of hypoxia, virus infection and exposure to toxins. It usually results in a local acute inflammatory reaction which may extend and damage the surrounding tissue (Duvall & Wyllie, 1986; Wyllie & Duvall, 1992).

However, apoptosis is associated with very different distinctive morphological changes in the cell; cell shrinkage, nuclear and cytoplasmic organelle condensation and DNA fragmentation are the cardinal signs of this process (Arends & Wyllie, 1991). Among other situations, apoptosis occurs physiologically to PMNs after the completion of their defensive mission or to T and B cells after cessation of stimulation by cytokines and is, therefore, an important factor in the termination of the inflammatory response. The effect in the neighbouring tissues is not as dramatic as it is in necrosis, since neither inflammation nor collapse of the tissue structure occurs. The apoptotic cell ends up being a target for phagocytosis by parenchymal cells or specialised phagocytes.

### 1.8.5 Immunolocalisation of leukocytes

Leukocytes can be identified and localised according to their CD antigens which are recognised by specific antibodies.

The CD3 molecule consists of five different polypeptide chains with molecular weights ranging from 16-28 kD. The five chains are designated gamma, delta, epsilon, zeta and eta. NCL-CD3-PS1 monoclonal of the $IgG_2$ subclass, is specific for the non-glycosylated epsilon chain of the CD3 molecule. The CD3 complex is closely associated at the lymphocyte cell surface with the T cell antigen receptor (TCR). It is believed that the CD3 complex is involved in signal transduction to the T cell interior following antigen recognition. Moreover, CD3 antigen is first detectable in early lymphocytes and its appearance probably represents one of the earliest signs of commitment to the T cell lineage. NCL-CD3-PS1 is a pan T cell marker which reacts with around 88% of the peripheral blood T lymphocytes and most of the thymocytes along with the majority of neoplastic cells of T cell origin (Beverley & Callard, 1981; Campana *et al.*, 1987; Chetty & Gatter, 1994). Anti- CD3 mAb has been proved to be effective on paraffin wax embedded tissue that has been microwave-treated with citrate buffer.

The DAKO anti-human T cell monoclonal antibody CD45RO recognises the 180 kD isoform of the CD45 common leukocyte antigen. This 180 kD

glycoprotein is found on most thymocytes, mature activated T cells and a subpopulation of resting T cells, whereas most normal B cells and NK cells are consistently negative for this isoform (Smith *et al.* 1986; Schwinzer, 1987).

Anti-CD4 mouse mAb (NCL-CD4-1F6) of $IgG_1$ subclass, reacts with a single chain transmembrane glycoprotein which represents the CD4 molecule (T4). This 59 kD antigen is located on a T cell subset (helper/ inducer) representing around 45 % of peripheral blood lymphocytes. It is also present on most thymocytes and at a lower level on monocytes and leukaemic T cells (Gay *et al.*, 1987; Petersen & Seed, 1988). Satisfactory results are found to be given with NCL-CD4-1F6 on paraffin wax embedded specimens that have been microwave treated with citrate buffer.

One of the antibodies used for the detection of the suppressor/ cytotoxic sub-population of T cells (T8) is the mouse anti-human monoclonal C8/144B of $IgG_1$ isotype. This antibody recognises satisfactorily the CD8 marker and is recommended for formalin-fixed paraffin embedded tissues that have been microwave treated with citrate buffer.

Mouse anti-human monoclonal CD20; L26 of $IgG_2$ isotype reacts with an intracytoplasmic epitope localised on the antigen designated CD20. More specifically, the antibody L26 recognises an intracellular epitope on the B cell-associated CD20 antigen (Mason *et al.*, 1990). Anti-CD20 mAb is a B cell

marker which detects both normal and neoplastic B cells and works well on the formalin-fixed paraffin-embedded tissues.

Finally, mouse anti-human mAb CD68; PG-M1 of $IgG_3$ isotype is used to detect human macrophages and monocytes (Falini *et al.*, 1993).

## 1.9 Aims of the study

Moskow & Polson (1991) have pointed out that most histopathologic studies of periodontal disease investigated only gingival tissue biopsies and, these studies of the superficial gingiva were probably not sufficient to give a clear picture on the inflammatory and immune processes of severe periodontitis. In an attempt to address this point, deeper periodontal granulation tissues from two different forms of periodontitis, AP and EOP, along with gingival biopsies for direct comparison were examined in the present study. The most apical part of the inflamed connective tissue, which is in close proximity to the advancing plaque front as well as to the alveolar bone, is referred to as "granulation tissue". In the present study, the highly vascular and heavily infiltrated granulation tissue was obtained from the most apical site of the periodontal pocket. Although it is established that granulation tissue should be removed during periodontal surgery, several studies (Lindhe & Nyman, 1985; Blomlöf *et al.*, 1989) have disputed this and, conversely, suggested that granulation tissue removal is not a critical measure for proper healing.

Gingival and granulation tissue biopsies were easily accessible and obtainable during routine surgical procedures, which did not interfere with normal periodontal treatment. This was considered as an advantage, as investigations were facilitated within the actual tissues where the inflammatory and immune processes took place (Figure 1.2).

Two techniques were employed throughout the studies comprising this thesis; immunohistochemistry and *in situ* hybridisation were used to assess immune cells' kinetics and activity and to examine possible differences within the two types of tissues and the two forms of periodontal disease. Phenotypic analysis of cell surface markers and qualitative and quantitative assessment of the cellular infiltrate (macrophages, B cells, T cells, T cell subsets i.e. memory, helper and suppressor) were carried out using immunohistochemistry. Combination with *in situ* hybridisation offered the opportunity to further elucidate synthetic capacity, proliferation and apoptosis in these cells by detecting their messenger RNA (mRNA) content *in vivo.* In order to examine the local antibody production and to determine their relative proportions in granulation tissue compared to gingiva, complementary oligonucleotide probes against specific mRNA sequences of IgG, IgA, IgM and IgE subclasses were utilised. Secretory IgA and IgM were evaluated in an attempt to assess their probable origin and destination and to compare their relative numbers within the two tissues. Furthermore, comparison was made between AP and EOP granulation tissue specimens for all the variables mentioned above in order to give an insight in the histopathology of the two forms of periodontal disease.

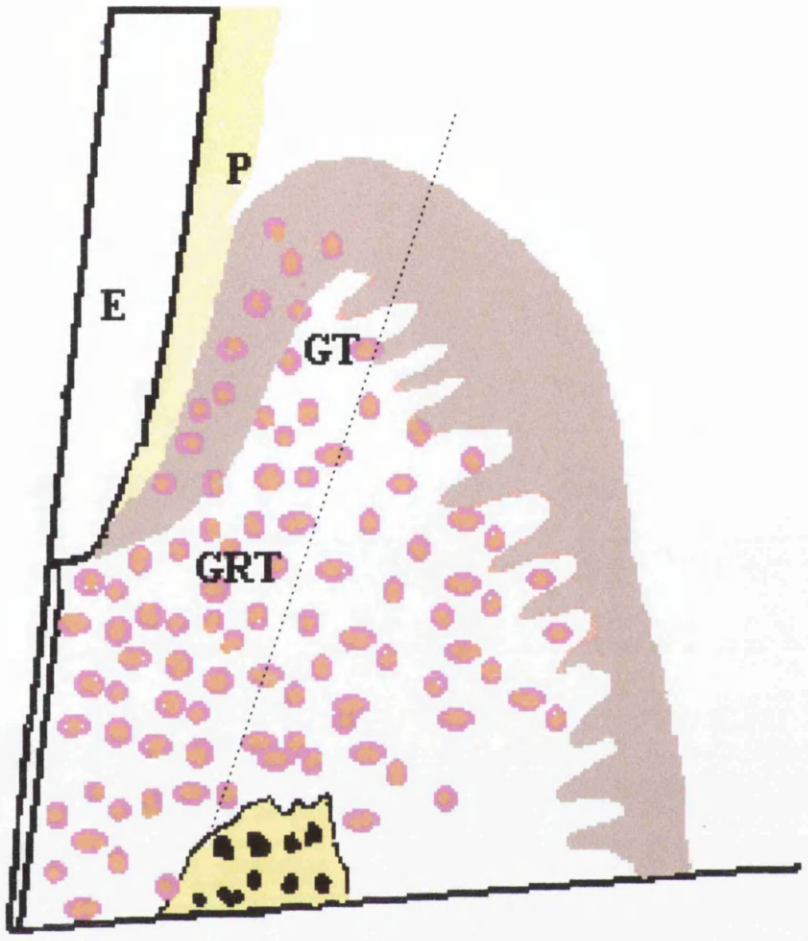



**Figure 1.2:** Schematic drawing of the diseased periodontium. The main features are: apical migration of the junctional epithelium, pocket formation, alveolar bone loss, and extensive inflammatory infiltrate seen in superficial gingiva and deeper granulation tissue.
E: enamel; P: microbial plaque; GT: gingival tissue; GRT: granulation tissue.

Generally, the scope of this study is to investigate some of the questions arising with respect to local inflammatory cell turnover and synthetic activity within the deep periodontal granulation tissue. Additionally, aspects of local cell-mediated and humoral immune responses are examined in order to provide information on the disease activity status. Inflamed periodontal granulation tissue is detected in relation to its inflammatory and immune cells' properties in order to find signs that may discriminate early onset periodontitis from adult periodontitis.

# CHAPTER 2

# SUBJECTS,

# MATERIALS AND METHODS

## Part I: Subjects

### 2.1 Subjects and biopsies

Nineteen patients in total with various forms of periodontal disease were enrolled in this study. Nineteen granulation tissue and eight gingival tissue specimens were analysed from patients undergoing periodontal therapy within the Periodontology Clinic, Glasgow Dental Hospital. The granulation tissue specimens consisted of nine biopsies obtained from nine patients having been diagnosed with adult periodontitis (AP) and ten biopsies from ten patients with early onset periodontitis (EOP). Of the nine individuals with AP, five were males and four females with a mean age of 50.9 years ranging from 42 to 62 years; while the EOP group consisted of four males and six females having a mean age of 37.1 years (range 24-39 years). The pocket depths of the sites biopsied ranged from 6.0 to 9.0 mm in AP group and 5.8 to 9.6 mm in EOP group. All the patients included in the study, showed advanced forms of periodontal diseases with the exception of one diagnosis of moderate to severe adult periodontitis (AP).

The eight sections of gingival tissue were taken from eight patients having been diagnosed with adult periodontitis (AP) and from sites where pockets exceeded 5.0 mm in depth.

A thorough periodontal examination and a complete radiographic series were performed on each patient in order to determine the clinical status of the gingivae and the extent of bone loss. After evaluation of initial therapy outcome, all sites showing persistent deep pockets were subjected to periodontal surgery (modified Widman flap). The tissues were removed under local anaesthesia and immediately fixed in 10% neutral buffered formalin at room temperature (RT). The tissues were cut from buccal to lingual gingiva in a plane parallel to the long axis of the teeth and oriented so that junctional epithelium, oral epithelium and connective tissue were present in the same section. Some tissue specimens, especially granulation tissue biopsies, contained very little or no epithelium.

.Biopsies were subsequently embedded in paraffin wax and 4μm thick serial sections were cut using a microtome. Sections were collected on to silane-(Sigma Chemical Co., St Louis, Mo)-coated glass slides and stored at RT. Serial sections were used for non-radioactive *in situ* hybridisation (NISH), immunohistochemistry and *in situ* end labelling techniques (ISEL).

Two sections of each sample were used for morphological examination which was based on haematoxylin and eosin (H&E) staining before the procedures.

## Part II: Materials

### 2.2 Materials: Buffers and reagents

#### 2.2.1 Buffers used during probe labelling and *in situ* hybridisation

**1. DEPC (Diethyl pyrocarbonate)-treated solutions: DEPC/ PBS (phosphate buffered saline) or DEPC/ DDW (double distilled water).** 1 ml DEPC was added per 1L of PBS (or DDW), mixed well, left to stand overnight and then autoclaved.

**2. Phosphate Buffered Saline (stock solution, x10 conc., PBS):** 80g NaCl, 2.0g KCl, 14.4g $Na_2HPO_4$ and 2.4g $KH_2PO_4$ were dissolved in 800 ml of DDW, pH was adjusted to 7.4 with 1M NaOH. The buffer was made up to 1L, autoclaved and stored at RT. The above described buffer was diluted 1/10 immediately pre-use and used mostly as a wash buffer.

**3. PBST (PBS/ 0.01% Tween 20):** after diluting x10 PBS 1/ 10 (**2**), 100 µl Tween 20 was added in 1L of PBS, pH 7.4. PBST was used as a wash buffer while it was fresh.

**4. Tris Buffered Saline (TBS):** 50 mM Tris/ HCl, 150 mM NaCl, pH 7.6. 6.05g NaCl and 8.76g Tris/Base were dissolved in 800 ml of DDW and pH

was adjusted to 7.6 with concentrated HCl. The buffer was made up to 1L in a volumetric flask, autoclaved, stored at RT and used for washings.

**5. Stock proteinase K:** 5 mg of PK enzyme were dissolved in 10 mls of DEPC/ DDW, then stored in 100µl aliquots at -20 °C.

**6. Proteinase K dilution buffer:** 0.1 M Tris/ 0.05 M EDTA, i.e. 0.88g Tris/ HCl, 0.53g Tris/ Base and 1.90g EDTA in were dissolved in 75 ml DEPC/ DDW, pH was ajdusted to 8.0 with HCl and the final volume was made up to 100 ml in measuring cylinder. Then filter sterilisation was performed and the dilution buffer was stored at room temperature (RT) for 1-2 months.

**7. TE buffer:** 10 mM Tris/ HCl, 10 mM EDTA. 0.88g Tris/ HCl, 0.53g Tris/ Base and 0.37g EDTA were dissolved in 800 ml of DEPC/ DDW, pH was adjusted to 7.5 at just under 1L, the solution was made up to a final volume of 1L in volumetric flask, followed by filter sterilisation.

**8. Denhardts x100:** 2% (w/ v) BSA, 2% (w/v) Ficoll 400 and 2% (w/ v) polyvinylpyrollidine (PVP). 2g BSA, 2g Ficoll 400 and 2g PVP were dissolved in 75 ml of DEPC/ DDW and then the final volume was adjusted to 100 ml.

9. **Tris (2.5M), pH 7.5:** 6.35g Tris/ HCl and 1.66g Tris/ Base, were dissolved in 15 ml of DEPC/ DDW, pH was adjusted with 1M NaOH and the solution was made up to 20 ml.

10. **Hybridisation buffer:** The following reagents were added in sequence as described:

| **Reagents** | **Quantity** | **Final concentrations** |
|---|---|---|
| a. x100 Denhardts | 2.5 ml | x12.5 |
| b. 2.5M Tris pH: 7.5 | 80 µl | 0.01M Tris |
| c. x20 SSC | 2.0 ml | x2 SSC |
| d. DEPC/ DDW | 4.420 ml | |
| e. 2g Dextran Sulphate | | 10% Dextran Sulphate |
| f. Salmon DNA (10 mg/ ml) | 500 µl | 0.25 mg/ ml |
| g. 20% SDS | 500 µl | 0.5% SDS |
| h. 100% Formamide | 10 ml | 50% Formamide |
| i. Triton-X-100 | 60 µl | 0.3% Triton-X-100 |

DEPC/ DDW was used to inhibit RNase's effect on tissue RNA. It was not easy to make up 50% Dextran Sulphate, therefore, it was recommended to mix all the above listed components except SDS. Then Dextran Sulphate was gradually added until all of it was dissolved and finally SDS was added. The hybridisation buffer was stored at 4 °C.

**11. Standard sodium citrate x20 (x20 SSC):** A stock solution of x20 SSC was prepared by adding 175.3g NaCl and 88.2g $Na_3$ citrate to 800 ml of DDW or DEPC/ DDW and the final volume was adjusted to 1L. The solution was autoclaved and stored at RT.

**12. Detection buffer:** 0.1 M Tris/ HCl, 0.1 M NaCl, 0.05 M $MgCl_2$.

1.52g Tris/ HCl, 10.94g Tris/ Base, 5.84g NaCl and 10.16g $MgCl_2$-$6H_2O$ were dissolved in 800 ml of DDW. A pH 9.0 solution was made up and the final volume was adjusted to 1L in volumetric flask. The detection buffer was autoclaved and stored at RT.

**13. Levamisole:** Levamisole solution was prepared by dissolving 180 mg of levamisole powder in 750 µl of detection buffer, pH 9.0 (**12**). The solution was stored in plastic tubes at 4 °C.

**14. NBT stock solution (nitro blue tetrazolium):** 750 mg of NBT were added to 10 ml of 70% Dimethylformamide to give the desired concentration and the solution was stored at 4 °C.

**15. BCIP stock solution (5-bromo-4-chloro-3-indolyl phosphate):** 500 mg of BCIP were dissolved in 10 ml of 100% Dimethylformamide and the solution was stored at 4 °C covered with aluminium foil.

**16. NBT/ BCIP solution (nitro-blue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate):** 33 µl NBT, 25 µl BCIP and 7.5 µl levamisole solution (**13**) were added in 7.5 ml of detection buffer. The solution was used immediately after preparation.

**17. Neutral red (0.2%):** 2g of neutral red were dissolved in 100 ml $H_2O$.

### 2.2.2 Reagents used during probe labelling and *in situ* hybridisation

All chemicals were analytical grade, most of them obtained from BDH Chemicals Ltd., Poole, Dorset UK, unless otherwise stated.

All reagents utilised during probe labelling and *in situ* hybridisation technique are described in Appendix 1.

### 2.2.3 Buffers and reagents used during immunohistochemistry assays

All chemicals were analytical grade, most of them supplied from BDH Chemicals Ltd., Poole, Dorset, UK, unless otherwise stated. The buffers and reagents employed in immunohistochemistry assays throughout this entire study, except where specifically mentioned in former or later sections, were as follows:

1. **Deperoxidising solution:** 10 ml of 30% $H_2O_2$ were added to 90 ml of methanol to give 3% methanolic hydrogen peroxide which was stored at 4°C.

2. **Tri-sodium citrate buffer (0.01M, pH 6.0):** 2.94g of tri-sodium citrate were dissolved in 800 ml of DDW. 1.0 M hydrochloric acid (HCl) was used to adjust pH to 6.0 and then the final volume was adjusted to 1L in measuring flask and the solution was stored at RT.

3. **Stock proteinase K:** as described in section 2.2.1.

4. **Proteinase K dilution buffer:** as described in section 2.2.1.

5. **EDTA solution (1 mM, pH 8.0):** A 1 mM EDTA solution was prepared by dissolving 0.37g of EDTA in 800 ml of DDW. pH was adjusted to 8.0 with NaOH and the solution was made up to a final volume of 1L with DDW.

6. **Blocking serum:** Horse serum was obtained from Vector Laboratories, Peterborough, UK as part of an ABC kit. Blocking serum was prepared by adding three drops of this horse serum to 10 ml of PBS, pH 7.4, to obtain the working solution.

7. **Biotin-conjugated secondary antibody:** Biotin-conjugated horse anti-mouse IgG was obtained from Vector Laboratories, Peterborough, UK, as part of the proprietary ABC kit (VECTASTAIN *Elite* ABC-POD Kit). Working

solution was prepared prior to use by adding one drop of the secondary antibody to 10 ml PBS/ 3 drops horse serum (**6**).

**8. ABC-peroxidase (ABC-POD) complex:** ABC reagent was obtained from Vector Laboratories, Peterborough, UK. The solutions were in two bottles: reagent A (Avidin DH); and reagent B (biotinylated horseradish peroxidase H). Two drops of reagent A were added to 10 ml of PBS (pH 7.4) and mixed, this was followed by two drops of reagent B , the solution was again mixed. The complex was prepared 30 minutes before use and was stored at 4 °C.

**9. Peroxidase substrate - DAB:** A DAB substrate kit for peroxidase was obtained from Vector Laboratories, CA, USA. The kit contained four solutions. The working solution of 3, 3′ diaminobenzidine (DAB) was prepared as follows: immediately before use, two drops of Buffer stock solution were added to 5.0 ml of DDW and mixed, then four drops of DAB stock solution were also added and again mixed. Lastly, two drops of the Hydrogen peroxide solution were mixed to give the working solution which yielded a reddish brown stain after 2-10 minutes. The fourth solution was a Nickel solution which, if added to the above described DAB substrate solution, resulted in a black stain.

**10. Mayer's haematoxylin (0.1%):** haematoxylin (1g) was dissolved in 1L DDW; 50g of aluminum alum ($AlNH_4 (SO_4)_2 . 12 H_2O$) and 0.2g sodium iodate ($NaIO_3 . H_2O$) were added and the solution boiled for 5 minutes. After

overnight incubation, 50g of chloral hydrate ($CCl_3$ . $CH(OH)_2$) and 1g of citric acid were added and the solution was shaken for the reagents to dissolve. The 0.1% Mayer's haematoxylin solution was filtered before use.

**11. Gurrs eosin:** 0.5% solution of $CaCl_2$ (0.5g / 100 ml) was prepared in DDW and 1g eosin powder was added to make a 1% eosin solution (1g/ 100ml).

### 2.2.4 Probes used during *in situ* hybridisation (ISH)

Oligonucleotide probes were chosen to be utilised in the present study, as follows:

To detect messenger RNA (mRNA), digoxigenin-labelled oligo d(T) oligonucleotide (30-mer) cocktail was purchased from R & D Systems Europe Ltd (Abingdon, UK). For the detection of rRNA, 28S rRNA oligonucleotide probe (34-mer) was used. As described by Yoshii *et al.* (1995), 28S rRNA probe was synthesised using the β-cyanoethyl phosphoramidite methodology on an applied biosystems automated DNA synthesiser. Revelation and detection of synthetic capacity of inflammatory infiltrate within periodontal granulation and gingival tissue was attempted by using the above mentioned oligonucleotide probes.

Fluorescein-labelled histone oligonucleotide (NCL-HISTONE) cocktail was purchased from Novocastra Laboratories Ltd (Newcastle upon Tyne, UK). Histone probe was utilised for the detection of histone H2b, H3 and H4 mRNA sequences. The synthesis of histone mRNA is closely coupled with DNA replication. During the S phase of the cell cycle the level of histone mRNA increases over fifty fold and then rapidly disappears at the start of $G_2$ phase (Heintz, Sive & Roeder, 1983; Stein *et al.*, 1984). These changes are produced by rapid mRNA degradation as well as modulation of transcription. Therefore, the presence of high quantities of histone mRNA provides a molecular marker of a cell population in cycle (Alison *et al.*, 1994) and assesses the S phase proliferation index in normal and neoplastic cells. In contrast, anti-Ki-67 antibody, which was also used against the Ki-67 proliferation marker in the present study, recognises dividing cells in all phases of the cell cycle, except in $G_0$ phase (section 2.2.5).

Additionally, for the detection of plasma cells expressing different immunoglobulin classes and subclasses synthetic digoxigenin labelled anti-sense human oligonucleotide probes were prepared.. The sequences of the anti-sense probes composed for each of the four IgG subclasses were as follows (Ellison, Berson & Hood, 1982, Ellison & Hood, 1982; Sideras *et al.*, 1991):

**1. $IgG_1$:** 5′-CCC AGG AGT TCA GGT GCT GGG CAC GGT GGG CAT GTG TGA GTT TTG TC-3′, 47-mer.

2. **$IgG_2$:** 5′-TGG GCA CGG TGG GCA CTC GAC ACA ACA TTT GCG CTC AAC TGT-3′, 42-mer.

3. **$IgG_3$:** 5′-TGG GCA CCG TGG GCA TGT GTG AGT TGT GTC ACC AAG TGG GGT TTT GAG CTC-3′, 51-mer.

4. **$IgG_4$:** 5′-AA CTC AGG TGC TGG GCA TGA TGG GCA TGG GGG ACC ATA TTT GGA-3′, 44-mer.

Synthetic digoxigenin-labelled oligonucleotide anti-sense human $IgA_1$ and $IgA_2$ gene were used:

1. **$IgA_1$:** 5′-CC GGA TTT TGA GAG GGT GGC GGT TAG CGG GGT CTT GGA CTC GGG GTA GGC-3′, 50-mer.

2. **$IgA_2$:** 5′-CC GGA TTT TGT GAT GTT GGC GGT TAG TGG GGT CTT CAA CTC GGG GTG GGC-3′, 50-mer.

The absence of cross-hybridisation of the two probes has been reconfirmed by Islam *et al.* (1992). The problem in identifying the four IgG and two IgA subclasses lies also in the high homology in their gene sequences (about 95%) (Takahashi *et al.*, 1982; Flanagan, Lefranc & Rabbitts, 1984). However, there is substantially less homology (<60%) in the exons coding for their hinge regions (Takahashi *et al.*, 1982) and it was from this area that sequences were chosen for the mRNA probes.

Furthermore, synthetic digoxigenin-labelled anti-sense human IgM (5′-CTC CCG GGC TGG TGG CAG CAA GTA GAC ATC GGG CCT GTG CAG GGC C-3′; 46-mer), (Word *et al.*, 1989) and IgE (5′-GAC TGT AAG ATC TTC ACG GTG GGC GGG GTG AAG TCC CTG GAG TAG ACG CTG AAG G-3′, 55-mer) (Max *et al.*, 1982) oligonucleotide probes were utilised in the present study.

Lastly, fluorescein isothiocyanate (FITC)-labelled J chain oligonucleotide cocktail for the detection of J chain mRNA sequences was purchased from Novocastra Laboratories Ltd (Newcastle upon Tyne, UK). J chain mRNA can be demonstrated in plasma cells secreting polymeric IgA and IgM antibodies. Therefore, according to Harper *et al.* (1992), the demonstration of J chain mRNA in plasma cells, identified immunochemically as containing IgA heavy chain immunoglobulin, has been used to discriminate between the presence of monomeric and dimeric IgA.

The oligonucleotide probes were titrated and different concentrations were checked in a preliminary experiment. The optimal concentrations which gave a satisfactory staining are shown in Table 2.1.

| Probe | Probe concentration | Result |
|---|---|---|
| **Oligo d(T)** | 1 : 20 | +++ |
| **28S rRNA** | 1 : 20 | +++ |
| **$IgG_1$** | 1 : 160 | +++ |
| **$IgG_2$** | 1 : 40 | +++ |
| **$IgG_3$** | 1 : 20 | +++ |
| **$IgG_4$** | 1 : 20 | +++ |
| **$IgA_1$** | 1 : 80 | +++ |
| **$IgA_2$** | 1 : 20 | +++ |
| **IgM** | 1 : 20 | ++ |
| **IgE** | 1 : 10 | ++ |
| **J chain** | | +++ |
| **Histone** | | ++ |

+++: satisfactory hybridisation signal;
++: less satisfactory hybridisation signal

**Table 2.1:** Probes and optimal concentrations as used during *in situ* hybridisation technique. J chain and histone probes were already diluted and were used according to the manufacturer's guidelines.

### 2.2.5 Primary antibodies used during immunohistochemistry assays

DAKO Ltd (Glostrup, Denmark) was the source for the mAb which labels proliferating cells (against Ki-67 antigen). This mouse anti-human monoclonal Ki-67 antibody was utilised to label proliferating cells in all human tissues. It reacts with cells in all stages of the cell cycle (late $G_1$, S, M and $G_2$ phase) but not with cells in $G_0$ phase. In other words, it serves as an indicator of the growth fraction, i.e. the proportion of cells undergoing active division (Brown & Gatter, 1990; Sawhey & Hall, 1992). Therefore, two strategies were used for the re-evaluation of proliferating cells in the present study, as mentioned in section 2.2.4; on the one hand, immunohistochemical detection of the possibly existing dividing cells was performed using anti-Ki-67 antibody. On the other hand, the *in situ* hybridisation technique using the fluorescein-labelled histone oligonucleotide cocktail was suitable for detecting proliferating cells.

Mouse monoclonal antibody (mAb) against human pan T cells (CD3; NCL-CD3-PS1) and antibody reacting with CD4 (NCL-CD4-1F6; helper/ inducer T cells) were purchased from Novocastra Ltd, Newcastle-Upon-Tyne, UK. The UCHL-1 antibody, which recognises CD45RO positive memory T cells along with antibodies detecting suppressor/ cytotoxic T cells (CD8; C8/144B), B cells (CD20; L26) and macrophages (CD68; PG-M1) were purchased from DAKO Ltd, Glostrup, Denmark (Table 2.2).

| mAb | Antigen | Source | Optimal dilution | Reference |
|---|---|---|---|---|
| **anti-Ki-67** | Ki-67 | DAKO | 1/50 | Brown & Gatter, 1990<br>Cattoretti *et al.*, 1992 |
| **NCL-CD3-PS1** | CD3 | Novocastra | 1/100 | Beverley &Callard, 1981<br>Campana *et al.*, 1987 |
| **UCHL-1** | CD45RO | DAKO | 1/100 | Smith *et al.*, 1986 |
| **NCL-CD4-1F6** | CD4 | Novocastra | 1/40 | Gay *et al.*, 1987 |
| **C8/144B** | CD8 | DAKO | 1/50 | |
| **L26** | CD20 | DAKO | 1/100 | Mason *et al.*, 1990 |
| **PG-M1** | CD68 | DAKO | 1/100 | Falini *et al.*, 1993 |

**Table 2.2:** Monoclonal antibodies and optimal dilutions as used during immunohistochemistry procedure. The source of the monoclonal antibody and the antigen it is directed against are indicated.

## Part III: Methods

### 2.3 Siliconised cover slips

Cover slips (22 x 22 mm, Shandon) were immersed in Sigmacote® liquid (Sigma) at RT for 30 minutes and were subsequently air dried on paper towel. They were then washed in DDW for more than 30 minutes, twice so that excess coating could be removed. The cover slips were then air dried and stored.

### 2.4 Haematoxylin and eosin sections (H&E)

Tissue sections were thoroughly air dried and then placed in Mayer's haematoxylin (section 2.2.3) for ten minutes, rinsed under running water, then placed in 0.1% eosin for ten minutes. Sections were finally dehydrated in a range of alcohols and mounted under xylene.

### 2.5 Probe labelling

Initially the probe concentrations were adjusted following spectrophotometric measurement of their absorbance at 260 nm. The probes were then analysed by

polyacrylamide gel electrophoresis and the resulting bands were visualised using ethidium bromide labelling and ultraviolet photography.

The oligonucleotide probes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) according to the manufacturer's guidelines. 1-2 μg probe was 3'-end labelled with a mixture containing 1.0 μl digoxigenin-11-dUTP, 4μl x5 reaction buffer (30 mM Tris base, 140 mM potassium cacodylate,1 mM cobalt chloride, pH: 7.2/ x5 TdT buffer, Gibco BRL, UK) and 2.0 μl terminal deoxynucleotidyl transferase (TdT enzyme, 15 U/μl, Gibco BRL, UK). The labelling reaction was done at 37°C for 3-5 h. Thereafter, the labelled probes were separated from unincorporated material and purified through a Sephadex column (DNA grade, Pharmacia Biotech, Uppsala, Sweden). Digoxigenin-labelled oligonucleotide probes were detected following the manufacturer's instructions and probe labelling was confirmed with dot-blot analysis.

## 2.6 Dot- blot analysis

Ten 200μl fractions of serial dilutions were collected, 1μl of each was spotted onto nitro-cellulose filters (Gelman Sciences, MI). The filters were air dried and prepared in the UV-cross linker (Anachem, Luton, UK) and then washed in phosphate buffered saline (PBS)/ 0.01% Tween 20 (Sigma Chemical Co., St. Louis, MO) containing 5% non-fat dry milk (PBSTM; pH:

7.4) for 30 min at room temperature (RT). Digoxigenin-3'-end labelled control oligonucleotide (Boehringer Mannheim) was used as standard control. Bound oligonucleotides were then detected with the method described above and their sensitivity compared. The fractions containing labelled probes were precipitated with ethanol and the pellets dissolved in TE buffer (0.01M Tris/HCl, 0.01M EDTA, pH: 7.5, section 2.2.1) and stored at -20 °C.

## 2.7 Description of *in situ* hybridisation methods

### 2.7.1 Pretreatment

All solutions were prepared with 0.1% diethyl pyrocarbonate (DEPC, Sigma)-treated double distilled water (DDW) or phosphate buffered saline (PBS, pH: 7.4).

Slides were deparaffinised in xylene, hydrated through descending isopropyl alcohol concentrations (99% and 95%) and immersed in double distilled water (DDW). The slides were washed in PBS twice for 5 min and then digested with proteinase K (Sigma) in dilution buffer (0.1 M Tris/ HCl, 50 mM EDTA, pH: 8.0) for 30 min at 37°C. Preliminary experiments on the concentrations of proteinase K for granulation and gingival tissues were carried out. The concentrations checked were 5, 10 15, 30 and 50 μg/ml. Granulation tissue

specimens were detected to give a satisfactory staining when treated with 15 µl/ mg of proteinase K and gingival tissue biopsies with a higher concentration of 50 µl/ mg. The slides were washed twice for 5 min in PBS, and at this stage, pretreatment with RNase A type 1A (Sigma) at 100 µg/ml in 2 x SSC/ 10 mM $MgCl_2$ at 37°C for 1 h was performed as a negative control (section 2.7.5). Post-fixation was performed by incubation in 4% paraformaldehyde at 4°C for 5 min, followed by washing twice for 5 min in PBS. The slides were immersed in prehybridisation buffer (x2 standard saline-citrate, SSC and 50% formamide) for 2 h at 37 °C. This procedure was carried out for all the probes used in this study, except for histone and J chain probes. Sample preparation for histone and J chain oligonucleotide probes followed the manufacturer's protocol (Novocastra Laboratories Ltd, UK, section 2.7.4).

### 2.7.2 *In situ* hybridisation

Hybridisation mixtures were prepared and hybridisation was performed as described in a report from our laboratory by Takahashi, Poole & Kinane (1995). Hybridisation mixtures were composed of 50% formamide, 0.25% bovine serum albumin (BSA), 0.25% Ficoll 400, 0.25% polyvinyl pyrrolidine-360 (BDH), 0.01% M Tris-HCl (pH: 7.5), x2 standard saline-citrate 10% dextran sulphate, 0.5% sodium dodecyl sulphate and 250 µg /ml denatured salmon-sperm DNA (section 2.2.1).

After draining, sections were hybridised with 20 µl volumes of the mixtures containing either a digoxigenin-labelled oligo d(T) or 28S rRNA probe at a concentration of 0.25-2.5 ng/µl, in hybridisation buffer. For synthetic immunoglobulin oligonucleotide probes the optimal concentration was found to be 0.16-0.65 ng/µl. Slides were covered with dimethyldichlorosilane-(BDH, UK)-coated coverslips that were sealed with rubber cement, and incubated at 37 °C for 2h for poly A and 28S rRNA probes, while Ig mRNA probes remained in the incubator overnight.

The slides were then rinsed in x4 standard saline-citrate (SSC), sequentially, in x2 SSC at room temperature twice for 20 min, x0.1 SSC at 37 °C twice for 20 min, and finally washed in x2 SSC for 10 min at room temperature. *In situ* hybridisation with fluorescein-labelled histone and J chain probe cocktails was performed according to the manufacturer's guidelines (Novocastra Laboratories Ltd, UK, section 2.7.4).

### 2.7.3 *In situ* detection

The immunological detection of the digoxigenin-labelled oligonucleotide-mRNA complex was performed as follows: slides were placed for 3 h in PBS containing 0.01% Tween 20 (Sigma, PBST) and 3-5% non-fat dry milk (PBSTM) including alkaline phosphatase (AP)-conjugated sheep anti-digoxigenin (Boehringer Mannheim, Germany) at room temperature (RT).

The optimal concentrations of the antibody for oligo(d)T and 28S mRNA probes were 1:100, while for Ig probes were 1:500. Alkaline phosphatase (AP)-conjugated anti-FITC antibody (Novocastra Laboratory Ltd) was utilised for the detection of histone and J chain probes at RT, according to the manufacturer's guidelines (section 2.7.4).

After thorough rinsing in a PBST buffer and 5 min preincubation in an alkaline phosphatase substrate solution (detection buffer 3, pH: 9.0), the alkaline phosphatase complex was revealed with a freshly prepared solution of nitro blue tetrazolium (NBT, Sigma) and 5-bromo-4-chloro-3-indolylphosphate (BCIP, Sigma) containing levamisole (Sigma) in an alkaline buffer solution (pH: 9.0, section 2.2.1). Optimal development time for poly A and 28S mRNA probes was 30-60 min, whereas for Ig mRNA probes was set in 8h to overnight in dark at RT. Sections were then washed in distilled water (DDW) three times. Finally, slides were counterstained with 1% neutral red, washed in DDW three times and mounted in aqueous mounting medium (DAKO). The dark blue/black precipitate, an indication of the presence of specific mRNA, was assessed by bright-field microscopy.

### 2.7.4 *In situ* hybridisation protocol for histone and J chain probes

Fluorescein isothiocyanate (FITC)-conjugated histone and J chain probes were detected as described in the report by Takahashi, Lappin & Kinane (1996), and

developed for 24-48 h, as preliminary checks on the development time revealed.

Histone and J chain-expressing cells were detected by *in situ* hybridisation as described in the protocol of Novocastra NCL-HISTONE and NCL-J-CHAIN oligonucleotide probe data sheet and ISH Detection Kit (Code NCL-ISH-D) with minor modifications.

All solutions were prepared with 0.1% diethyl pyrocarbonate (DEPC, Sigma)-treated DDW. Sections were dewaxed in xylene, hydrated through descending ethanol and immersed in DDW twice for 3 minutes. Enzymatic digestion was performed with proteinase K with a concentration of 15 μl/ mg for granulation tissue specimens and a higher one of 50 μl/ mg for gingival tissues. Sections were covered with 200 μl of proteinase K in proteinase K dilution buffer (section 2.2.1) and incubated for 30 minutes at 37 °C. They were washed twice in DDW, dehydrated through ascending ethanol concentrations and left to air dry for 2 minutes.

After drying, the sections were hybridised with 20 μl volumes of the mixtures containing fluorescein isothiocyanate (FITC)-conjugated histone or J chain probes for 2 hours at 37 °C in a humid chamber. The slides were then washed in Tris buffered saline (TBS, pH 7.6, section 2.2.1) containing 0.1% Triton-X-100 for 3 x 3 minutes.

Sections were then covered with 200 µl of normal rabbit serum diluted 1.5 in TBS containing 3% BSA and 0.1% Triton-X-100 and incubated at RT for 10 minutes. The blocking solution was tipped off and slides were covered with diluted (1:200) rabbit F(ab) anti-FITC/ alkaline phosphatase conjugated secondary antibody for 30 minutes at RT. The dilution buffer was the above described in the blocking step. After immersing in TBS for 2 x 3 minutes, the slides were rinsed in detection buffer, pH 9.0 (section 2.2.1) for 5 minutes. Alkaline phosphatase activity was demonstrated by incubating the slides at RT in the dark with the following solution:

| | |
|---|---|
| Detection buffer | 10 µl (section 2.2.1) |
| BCIP | 80 µl (section 2.2.1) |
| NBT | 80 µl (section 2.2.1) |
| Levamisole (1M) | 10 µl (section 2.2.1) |

Development time varied between 24 to 48 hours according to the signal given by each probe after microscopic observation. After washing in running water for 5 minutes, the sections were counterstained with Mayer's haematoxylin for 5-10 seconds and mounted in aqueous mounting medium (DAKO).

### 2.7.5 Controls for *in situ* hybridisation

As a negative control, sections were incubated with hybridisation buffer only or sense probe. To determine the specificity of probes binding to tissues

RNAs, sections were digested with RNase A prior to hybridisation as a further negative control.

Another set of specificity controls was performed by carrying out the hybridisation reactions with the labelled probe together with an unlabelled probe for each of the different Ig subclasses simultaneously. The concentration of the former was as above, but the latter was in ten-fold excess. As an example, labelled $IgA_1$ probe along with ten-fold non-labelled $IgA_2$ were used to check the specificity of the $IgA_1$ probe and vice versa. The same procedure was carried out to determine the specificity of $IgG_{1-4}$ probes.

## 2.8 Immunohistochemistry technique

### 2.8.1 Pretreatment

Tissue specimens were deparaffinised through xylene and graded ethanol, as described in section 2.7.1 and immersed in distilled water (DDW). For UCHL-1, L 26 and Ki-67, inhibition of endogenous peroxidase followed, by incubating the sections with 3% methanolic hydrogen peroxide at 4 °C for 15min. For CD45RO, CD20 and Ki-67 positive cells' detection, deparaffinised sections were placed in a plastic box filled with a 0.01M tri-sodium citrate solution, pH 6.0 (section 2.2.3) and heated in a conventional microwave oven at 650W to unmask the antigen. Different time schedules

were employed for microwave heating and it was revealed that 4 x 4.5 min were optimal for the granulation tissue specimens, whereas 20 min (4 x 5 min) microwave heating treatment resulted in satisfactory staining of the gingival tissues processed (Cattoretti *et al.*, 1992). While undergoing microwave processing, slides were always covered with solution. After heating, slides were permitted to cool down to room temperature over a period of 30-60 min. For the detection of macrophages, proteinase K digestion was preferred and it was performed as described in section 2.7.1.

Moreover, unmasking of antigen for CD3, CD4 and CD8 positive cells was performed using a Prestige stainless steel pressure cooker. Sections were positioned into metal staining racks, lowered into the pressure cooker and well immersed in 0.01M sodium citrate buffer, pH 6.0 for CD3 detection and in 1 mM EDTA, pH 8.0 for CD4 and CD8. Sections were washed in TBS buffer, pH 7.6 (section 2.2.1) and at this stage were followed by inactivation of endogenous peroxidase with incubation for 30 min at 4 °C in 3% $H_2O_2$ in methanol. After washing in PBS (pH 7.4) and blocking, slides were incubated with monoclonal antibodies (mAb).

### 2.8.2 Immunological detection

Monoclonal antibodies were reacted for 1 h at 37 °C for UCHL-1, L26 and PG-M1, whereas overnight incubation at 4 °C with Ki-67 was performed. After

washing with PBS, they were sequentially incubated with biotin-labelled goat anti-mouse IgG secondary antibody (Vectastain *Elite* ABC-POD kit; Vector Lab, CA), section for 30 min. After washing with PBS, they were treated with avidine-biotin peroxidase complex for 30 min, then treated with DAB substrate kit (Vector lab) for 2-7 minutes under microscopic observation. Preparation of the above mentioned working solutions was described in detail in section 2.2.3. After washing with DDW, they were finally counterstained with Mayer's haematoxylin, rinsed in DDW three times and mounted in aqueous mounting medium (DAKO).

## 2.9 DNA *in situ*-end labelling assay (ISEL)

The labelling of DNA fragments *in situ* was performed according to the *in situ*-end labelling method (ISEL), described by Gavrieli *et al.* (1992), which is based on the fact that DNA fragmentation occurs as an early event in apoptosis. The modified method used in this study, was as follows:

Deparaffinisation and rehydration was done by transferring the slides through the following solutions: twice to xylene bath for 5 min and then through graded ethanol (twice in 99% for 5 min and 95% for 5 min) and double distilled water (DDW). Endogenous peroxidase was inactivated by covering the sections with 3% $H_2O_2$ in methanol for 15 min at 4 °C and rinsed twice with DDW for 5 min. Sections were incubated with 15 µg/ ml (or 50 µl/mg for gingival tissues)

proteinase K for 30 min at 37 °C, washed in DDW and immersed in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT (0.3 e.u./ µl) and biotinylated dUTP (biotin-16-dUTP, Boehringer Mannheim, Germany) were then added to cover the sections. Tissues were incubated in humid atmosphere at 37 °C for 60 minutes. The reaction was terminated by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 minutes at RT. The sections were then washed twice in PBST and ABC-POD (VECTASTAIN® *Elite* ABC Kit, Vector) was utilised.

ABC-POD was prepared at least half an hour prior to use, according to the manufacturer's instructions: two drops of each reagent A and B were added to 5 ml of PBST and mixed well before they were left to stand for at least 30 minutes prior to use (section 2.2.3). Sections were incubated for 1-2 hours at RT, washed with PBST for 5 minutes, twice and covered with 200 µl biotinylated-anti-avidin D-conjugate (Vector) diluted 1:100 in PBST for 30 minutes at RT. Tissues were rinsed with PBST for 5 minutes, twice and incubation with ABC-POD was performed again for 30 minutes at RT. They were immersed in PBST for 5 minutes, twice and stained with DAB substrate buffer for 2-10 minutes in the dark at RT. After desired stain intensity was developed, sections were washed in DDW for 5 minutes, counterstained with Mayer's haematoxylin, washed again with DDW and mounted with DAKO glycergel.

### 2.9.1 Controls for DNA *in situ*-end labelling assay

As a negative control, sections were incubated without the enzyme (rTdT, Gibco, BRL), so that its specific binding could be determined. Two adjacent serial sections from each type of tissue (periodontal granulation and gingival tissue) were processed. The enzyme was added only to one of the pair of adjacent specimens, whereas the second section was incubated in a buffer which lacked the TdT enzyme. The same procedure was repeated using tonsilar tissue sections which were subjected to the ISEL assay and served as negative (without the enzyme) and as positive controls (with the enzyme).

## 2.10 Enumeration

### 2.10.1 Areas

In general, when counting the numbers of cells stained with each of the markers, twelve microscopic fields from each serial section of all 26 biopsies, were chosen. Haematoxylin and eosin sections were viewed under low magnification (x100 or x200) for the morphological characterisation of the cellular structures. When oral or junctional epithelium were present, usually in all gingival tissues, but not in all periodontal granulation tissue specimens, sections could be easily divided into the following well defined areas:

a. oral epithelium,

b. junctional epithelium,

c. connective tissue subjacent to the oral / junctional epithelium and

d. connective tissue subjacent to the junctional epithelium but also located in the deepest site of the periodontal pocket, representing the most apical extent of the inflammatory tissue (Figures 1.1 & 1.2).

Counting was performed using a meshwork eyepiece (0.172 mm x 0.172 mm; 0.0296 $mm^2$). The number of positive cells present in a given field were counted in duplicate and then the results were compared. Positive cells were also counted using the Kontron 300 Image Analysis System and KS300 software which were programmed to maximise sensitivity and specificity of positive cell detection (Kontron Elektronik Eching, Germany).

One major criterion was to have the same fields available for counting for each compared staining procedure: thus, each area included was present in all of the series of sections, otherwise the total number of common undamaged fields available was analysed. The minimum number of fields available in any section included in the study was five. Great care and considerable effort was expended to identify the corresponding areas in the series of the sections. The efficiency of the method for relocating the same areas in serial sections was tested, as follows: the cells of a specific area were counted, then the slide was removed from the microscope, reinserted after the slide holder had been repositioned and finally the area was relocated and the cells in the same area

were recounted. Kontron system was used to capture an image of the original field and subsequently relocate the same area again with precision. The results of replicate cell counts which differed by less than or equal to 5% were deemed acceptable.

## 2.11 Statistical analysis

All statistical analyses were performed on an IBM PC computer using the Minitab and SPSS/ PC statistical packages. The non parametric Mann Whitney U test was applied on cell numbers, whereas the Student t-test was chosen for the detection of differences on the percentages of immunoglobulin subclasses. The null-hypothesis was rejected at $p<0.05$.

# CHAPTER 3

# RESULTS

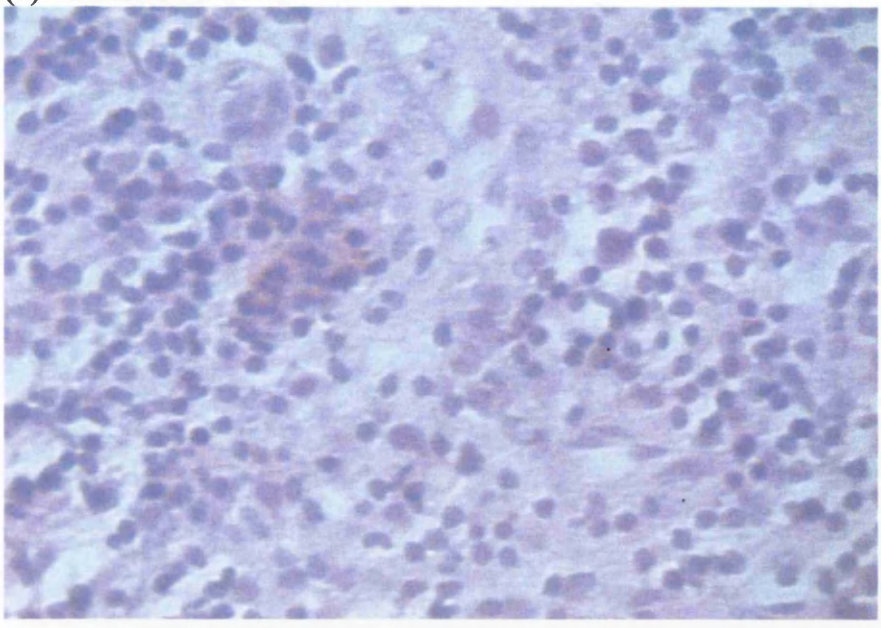
## 3.1 Haematoxylin and eosin sections (H & E)

Histologically, both the gingival and granulation tissue biopsies were well preserved. In the gingival tissue, oral epithelium, junctional epithelium and connective tissue were present in all sections. *Rete peg* proliferation along with loss of tissue integrity related to the disease process were noted. The cellular infiltrate comprised numerous inflammatory cells, such as few PMNs, macrophages, lymphocytes and plasma cells. Fibroblasts, endothelial and epithelial cells were also present (Figure 3.1b).

The granulation tissue was generally less intact than the epithelium. Some granulation tissue biopsies contained very little or no epithelium. The inflammatory infiltrate was very extensive and consisted of macrophages, lymphocytes, plasma cells, fibroblasts and few PMNs and giant cells (Figure 3.1a).

The characteristic morphological features of apoptosis, that is the shrinkage of cells, condensation of chromatin and apoptotic body formation were not clearly observed in H&E sections.

**(a)** Granulation tissue



**(b)** Gingival tissue

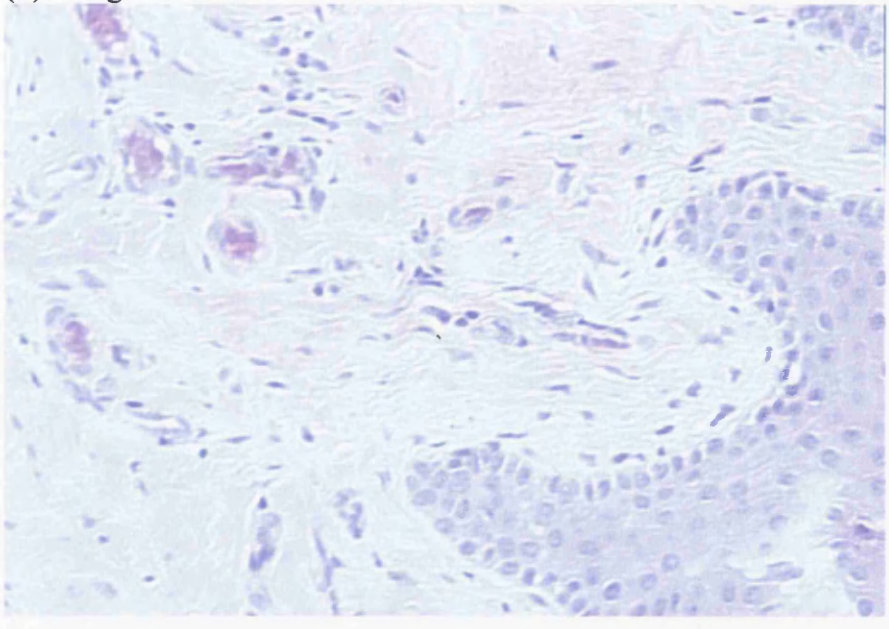


**Figure 3.1:** Haematoxylin and eosin (H&E) staining. **(a)** Granulation tissue exhibits a dense inflammatory infiltrate (magnification x400). **(b)** Gingival tissue shows good morphology: epithelium and connective tissue are present in the section (magnification x200).

### 3.2 Proteinase K digestion

Gingival tissue sections used in this study gave the strongest hybridisation signals with a proteinase K concentration of 30-50 μg/ml and showed good morphological detail at this concentration, while the proteinase K digestion at less than 10 μg/ml gave very weak or no signal. On the contrary, proteinase K concentrations of 15-30 μg/ml were considered as ideal for the granulation tissue.

### 3.3 Counterstaining

Counterstaining with 0.2% neutral red was preferred to Mayer's haematoxylin (blue color) with the alkaline phosphatase staining, as the precipitation colour of NBT / BCIP is blue black. When peroxidase detection system was used, which resulted in a brown colour, the contrast given with Mayer's haematoxylin (0.1%) was more satisfactory.

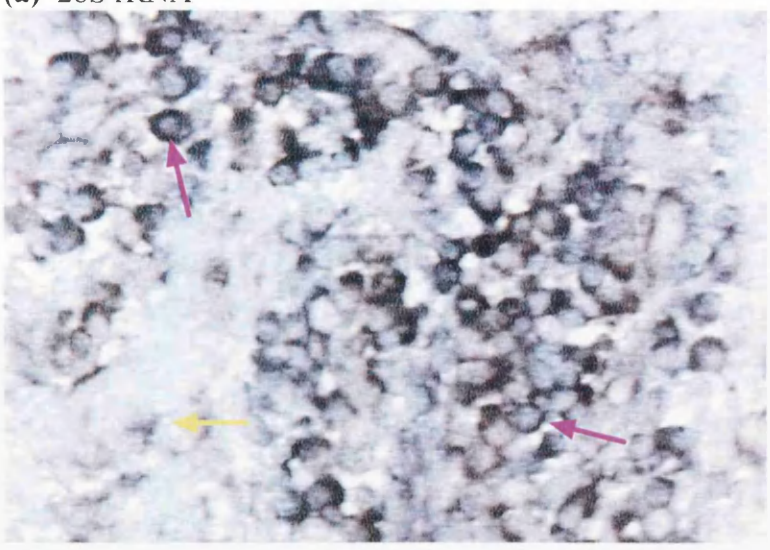
### 3.4 Cell synthetic activity

Sections were initially examined with the oligo d(T) and 28S rRNA probes for the detection of hybridisable total mRNA and 28S rRNA, which was found in all sections tested. Strong staining was observed in plasma cells and

epithelium. Slight to moderate staining were seen in fibroblasts, macrophages, giant cells, endothelial cells and lymphocytes (Figures 3.2a & 3.2b). Almost all PMN were negative although a few positive PMN were found to be located around blood vessels. *In situ* hybridisation with the 28S rRNA probe gave similar results to that of the oligo d(T) probe (Figures 3.2a & 3.2b). Close examination of adjacent serial sections stained with both markers confirmed that they could be detected in the same cells.

The strongest signals were frequently seen in plasma cells within connective gingival tissue and granulation tissue. With respect to epithelial cells within the epithelium the staining intensity appeared to decrease from the base towards the superficial epithelial layers. Plasma cells and basal epithelial cells always stained positively, when present, acting as an in-built positive control. Fibroblasts in the connective tissue sites were surrounded by bundles of collagen fibrils. The majority of the fibroblasts in many areas were moderately stained by both probes (Figures 3.2a & 3.2b), whereas in other sites almost all fibroblasts were poorly stained although the cell shapes were similar. Some giant cells were also moderately stained , whereas other cells were negative (data not shown). Endothelial cells were stained slightly to moderately (data not shown). In contrast, lymphocytes close to the epithelium also gave a mixed reaction, ranging from a few strongly stained through weakly positive to negative.

**(a)** 28S rRNA



**(b)** Oligo d(T)

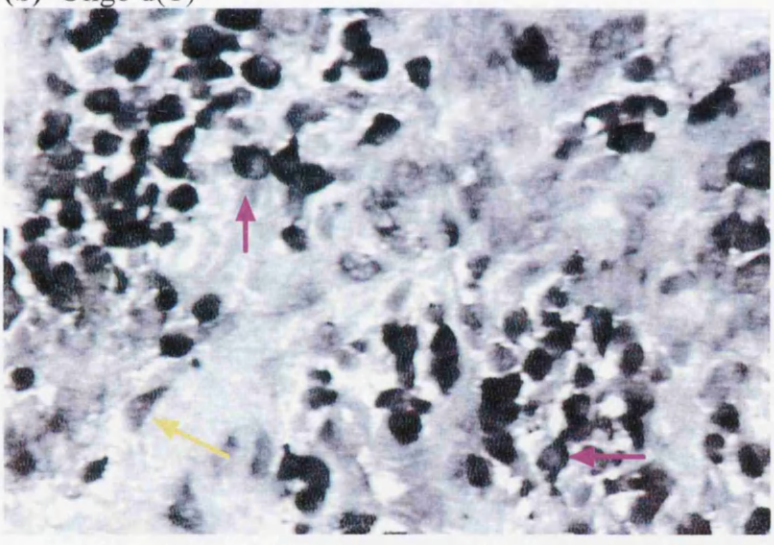


**Figure 3.2:** Hybridisation signals for 28S rRNA **(a)** and oligo d(T) **(b)** in the periodontal granulation tissue from corresponding fields in adjacent sections. Strong staining can be seen in plasma cells (pink arrows) and slight to moderate cytoplasmic staining can be seen in fibroblast "like" cells (yellow arrows, magnification x400)

There were no significant differences in the number of synthetic cells, as assessed by either 28S rRNA or oligo d(T) probes, between any of the tissue groups (Table 3.1 b). However, greater numbers of 28S rRNA positive cells were noted in AP gingival tissues compared to AP granulation tissues (Table 3.1 a).

Almost all control sections of the periodontal tissues without probes showed no background signal, suggesting that probes could specifically bind to the target sequences (Figure 3.3a). Total mRNA was not detected in the RNAse-treated control sections (Figure 3.3b).

### 3.5 Cell proliferation

A few histone mRNA-expressing cells were detected in basal and suprabasal epithelial cells and/or mononuclear cells in only 4 out of 20 cases (in 2 AP and 2 EOP granulation tissue sections) of granulation tissue specimens and in none of the gingival tissue sections tested and their reactivity was weak (Figure 3.4b, positive cells are indicated by black arrows).

In contrast, Ki-67 positive cells were also noted in epithelium (data not shown); mononuclear cells and fibroblasts were strongly stained in all samples (Figure 3.4a, positive cells are indicated by black arrows).

| Diagnosis | N | Probes | Cells per field | | |
|---|---|---|---|---|---|
| | | | Median | Interquartile range | |
| **AP Granulation Tissue** | 9 | | 128.4 | 96.0 | 148.9 |
| **EOP Granulation Tissue** | 10 | **28S rRNA** | 196.7 | 119.7 | 243.8 |
| **AP Gingival Tissue** | 8 | | 243.3 | 239.6 | 244.2 |
| **AP Granulation Tissue** | 9 | | 144.2 | 103.1 | 166.0 |
| **EOP Granulation Tissue** | 10 | **Oligo d(T)** | 186.7 | 109.4 | 251.9 |
| **AP Gingival Tissue** | 8 | | ND | ND | ND |

N: number of biopsies (1 biopsy per patient)
ND: not determined

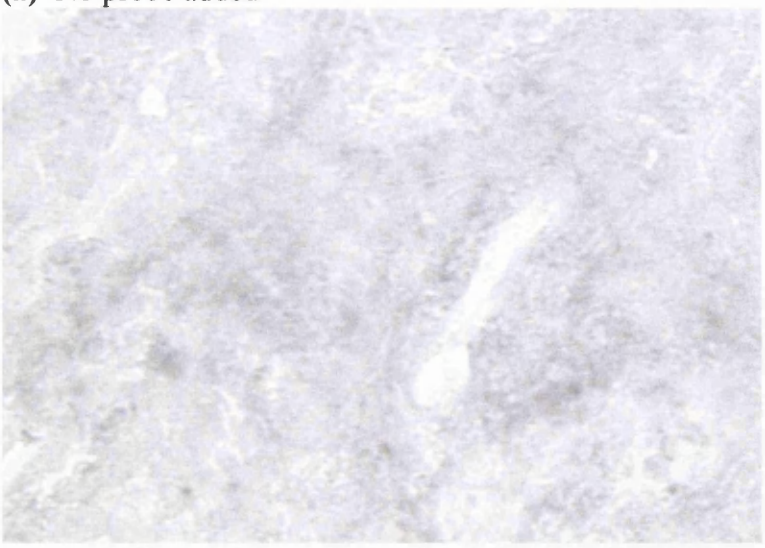
**Table 3.1 (a):** Cells per field stained positive for the two markers of synthetic activity used in the three tissue groups. The data are expressed as median values (Q2) including interquartile range (Q1-Q3).

| Variable | AP v EOP | AP v GING |
|---|---|---|
| **28S rRNA** | NS | NS |
| **p value** | p=0.247 | p=0.160 |
| **Oligo d(T)** | NS | ND |
| **p value** | p=0.393 | |

NS: non significant ; ND: not determined
AP: AP Granulation Tissue; EOP: EOP Granulation Tissue
GING: AP Gingival Tissue

**Table 3.1 (b):** Statistical results: Mann-Whitney U test.

**(a)** No probe added



**(b)** RNAse-treated section

**Figure 3.3:** Negative controls for *in situ* hybridisation. **(a)** No probe added and **(b)** RNAse-treated section with probe added. The control sections show no background staining which suggests that probes bind specifically to the target sequences.

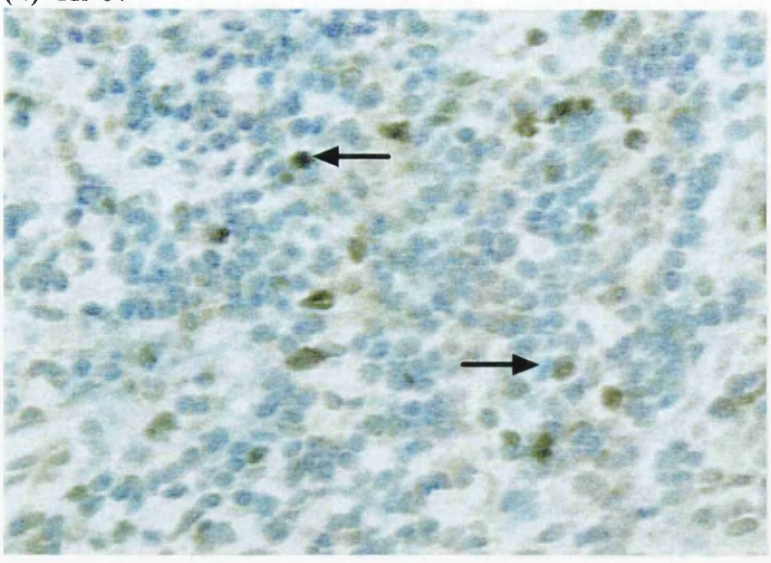
Microwave processing was used instead of proteinase K digestion for Ki-67 antigen. Fine tuning of the microwave treatment time required to totally unmask the antigen reduced it to 18 minutes (4 x 4.5 min) for granulation tissue specimens, however, there was still some physical disruption of some of the smaller pieces of tissue.

In all cases the number of Ki-67 positive cells was consistently and significantly higher (Mann Whitney U-test; $p<0.001$) than the number of histone mRNA-positive cells (data not shown). Significantly greater numbers of Ki-67 positive cells were observed in the granulation tissue sections from AP granulation tissue group than in the AP gingival sections (Table 3.2 b, Mann Whitney U-test, $p=0.0004$). The median value of cells per field in AP granulation tissue group was 43.6 and the interquartile range was 22.5 to 73.8. In the gingival sections the median was 5.0 cells per field and the interquartile range 4.7 to 5.3 (Table 3.2 a). No significant differences were noted between AP and EOP granulation tissue biopsies (Table 3.2 b, Mann Whitney U test, $p=0.842$).

### 3.6 Apoptotic cells

Very low numbers of cells undergoing apoptosis, or programmed cell death, were detected within AP gingival tissues and AP and EOP granulation tissue specimens. Apoptotic cells were seen to be located in the connective tissue.

**(a)** Ki-67



**(b)** Histone probe

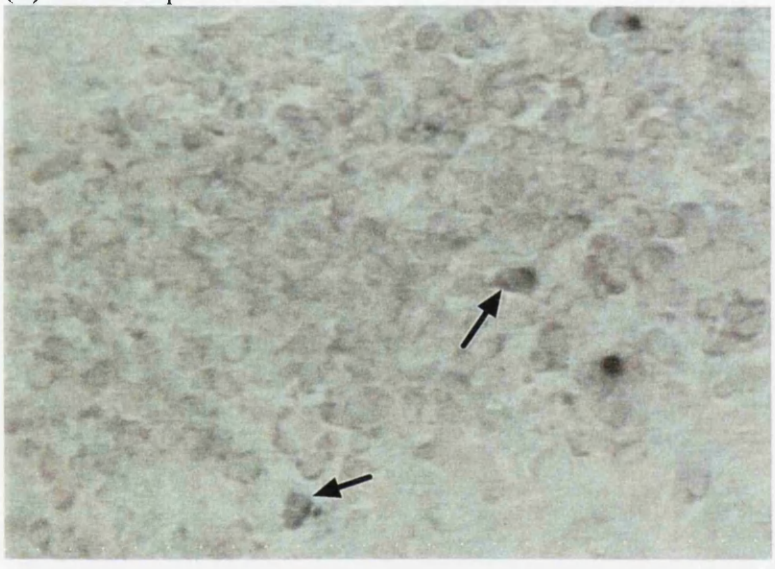


**Figure 3.4:** Proliferating cells within the granulation tissue. **(a)** Immunohistochemical staining using Ki-67 monoclonal antibody shows a few positive mononuclear cells. **(b)** Hybridisation signal for histone probe shows very sparse positive cells (magnification x400).

| Diagnosis | N | Marker | Cells per field | | |
|---|---|---|---|---|---|
| | | | Median | Interquartile range | |
| **AP Granulation Tissue** | 9 | | 43.6 | 22.5 | 73.8 |
| **EOP Granulation Tissue** | 10 | **Ki-67** | 52.8 | 24.6 | 118.7 |
| **AP Gingival Tissue** | 8 | | 5.0 | 4.7 | 5.3 |

N: number of biopsies (1 biopsy per patient)

**Table 3.2 (a):** Cells per field stained positive for Ki-67 in the three tissue groups. The data are expressed as median values (Q2) including interquartile range (Q1-Q3).

| | Diagnosis | | |
|---|---|---|---|
| **Variable** | **AP v EOP** | **AP v GING** | **Results** |
| **Ki-67** | NS | S | AP>GING |
| **p value** | p=0.842 | **p=0.0004** | |

S: significant; NS: non significant
AP: AP Granulation Tissue
EOP: EOP Granulation Tissue
GING: AP Gingival Tissue

**Table 3.2 (b):** Statistical results: Mann-Whitney U test.

No statistical test was applied, as there were very low numbers of positive cells detected in all tissue groups. Some epithelial cells, fibroblasts and possibly PMNs were stained positive, but lymphocytes did not exhibit any apoptotic function (Figure 3.5a).

As a positive control, sections of tonsilar tissue were used. Numerous positively staining apoptotic cells were noted within tonsilar tissues, suggesting that ISEL methodology was satisfactorily applicable for the detection of apoptotic cells (Figure 3.6). On the other hand, an absence of signal was observed on the tonsilar sections (data not shown), as well as on the granulation tissue sections (Figure 3.5a), which served as negative controls i.e. sections that had no enzyme added.

### 3.7 Immunohistochemical characterisation

Mononuclear cells were further differentiated by immunohistochemistry (IHC) into T, B cells and macrophages and these cells were detected in all samples. Close examination of adjacent serial sections revealed that many of the same cells were also positive for the oligo d(T) or 28S rRNA staining, although the signal was found to be moderate to weak. However, many CD positive cells displayed negative staining with both the oligo d(T) and 28S rRNA probes.

**(a)** Apoptosis: enzyme added

**(b)** Apoptosis: enzyme omitted

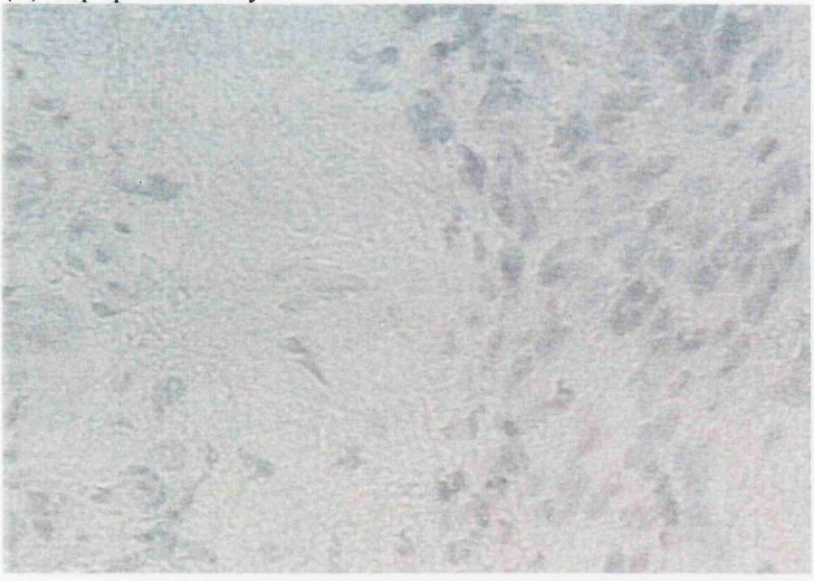


**Figure 3.5: (a)** Apoptotic cells detected by *in situ*-end labeling method within periodontal granulation tissue. **(b)** Apoptotic cells can not be detected when rTdT is not added (magnification x400).

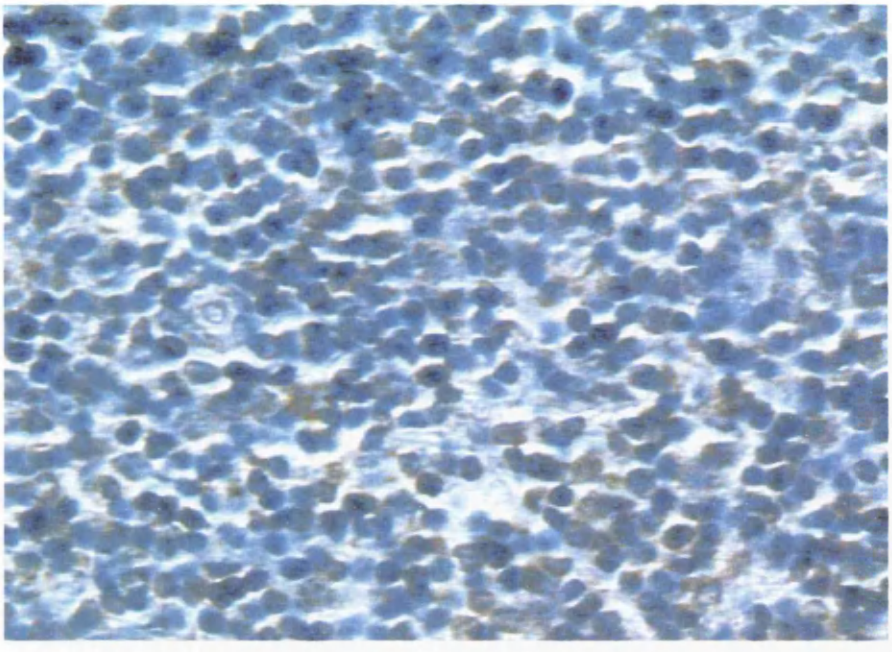


**Figure 3.6:** *In situ*-end labeling method yields numerous apoptotic cells in the tonsilar tissue which is used as a positive control (magnification x400).

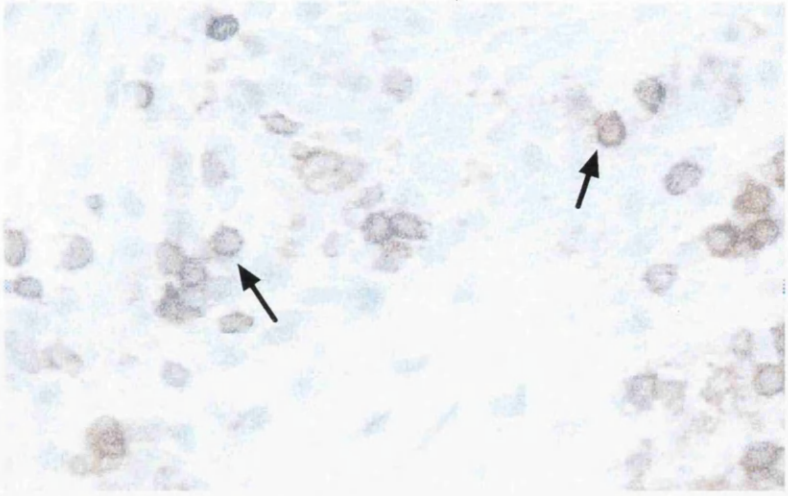
### 3.7.1 T cells

Mononuclear cells which stained positive for the pan T cell marker CD3 were detected in all sections. Monoclonal antibody UCHL-1 which was used to detect memory T cells, also gave positive results in all replicate sections. Both monoclonal antibodies gave comparable results, although the staining with the anti-CD3 monoclonal antibody appeared to be marginally better (Figures 3.7a & 3.7b, positive cells are indicated by black arrows). The absolute numbers of $CD3^+$ cells noted in each section were greater than those of $CD45RO^+$ cells in serial adjacent sections from the same subject.

When comparison for pan T cells was made between AP and EOP granulation tissue specimens, it was revealed that significantly greater numbers of T cells were observed in the EOP (median: 76.8 cells per field, interquartile range: 55.2 to 104.7) than in the AP sections (median: 47.3, interquartile range: 34.4 to 51.2) (Tables 3.3 a & 3.3 b, Mann Whitney U-test, $p=0.035$). However, greater variations in the numbers of CD45RO positive cells were noted within each group of sections and thus no significant differences in the numbers of CD45RO were observed among the groups (Table 3.3 b).

The numbers of memory T cells to pan T cells were compared for each group (AP *versus* EOP granulation tissue and AP granulation *versus* AP gingival tissue sections) and it was found that the CD45RO : CD3 ratio did not vary

**(a)** anti - CD3 monoclonal antibody



**(b)** anti - CD45RO monoclonal antibody

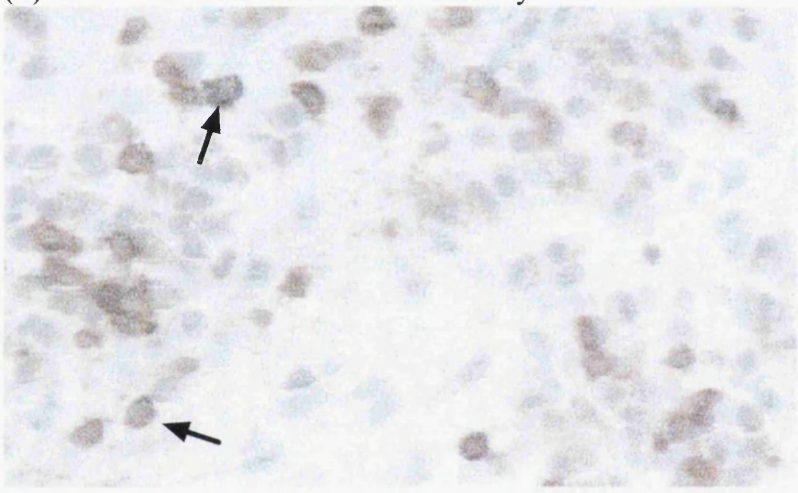


**Figure 3.7:** Immunohistochemical staining of T cells; **(a)** CD3 (pan T cells) and **(b)** CD45RO (memory T cells) in the corresponding fields in sections of granulation tissue biopsies (magnification x400).

| Diagnosis | N | Mab | Cells per field | | |
|---|---|---|---|---|---|
| | | | Median | Interquartile range | |
| **AP Granulation Tissue** | 9 | | 47.3 | 34.4 | 51.2 |
| **EOP Granulation Tissue** | 10 | **CD 3** | 76.8 | 55.2 | 104.7 |
| **AP Gingival Tissue** | 8 | | 58.2 | 48.5 | 59.4 |
| **AP Granulation Tissue** | 9 | | 41.2 | 36.5 | 45.2 |
| **EOP Granulation Tissue** | 10 | **CD 45RO** | 41.7 | 26.7 | 76.0 |
| **AP Gingival Tissue** | 8 | | 46.3 | 44.0 | 50.1 |
| **AP Granulation Tissue** | 9 | | 62.2 | 34.0 | 72.9 |
| **EOP Granulation Tissue** | 10 | **CD20** | 50.7 | 23.9 | 74.7 |
| **AP Gingival Tissue** | 8 | | 25.3 | 23.7 | 26.8 |
| **AP Granulation Tissue** | 9 | | 4.0 | 2.4 | 21.3 |
| **EOP Granulation Tissue** | 10 | **CD68** | 6.1 | 2.0 | 15.2 |
| **AP Gingival Tissue** | 8 | | 2.7 | 2.4 | 3.1 |

N: number of biopsies (1 biopsy per patient)

**Table 3.3 (a):** Cells per field stained positive for the CD3, CD45RO, CD20 and CD68 markers for the three tissue groups. The data are expressed as median values (Q2) including interquartile range (Q1-Q3).

| | Diagnosis | | |
|---|---|---|---|
| **Variable** | **AP v EOP** | **AP v GING** | **Results** |
| **CD 3** | **S** | NS | AP<EOP |
| **p value** | **p=0.035** | p=0.093 | |
| **CD 45RO** | NS | NS | |
| **p value** | p=0.528 | p=0.147 | |
| **CD20** | NS | NS | |
| **p value** | p=0.684 | p=0.056 | |
| **CD68** | NS | NS | |
| **p value** | p=0.719 | p=0.272 | |

S: significant; NS: non significant
AP: AP Granulation Tissue; EOP: EOP Granulation Tissue
GING: AP Gingival Tissue

**Table 3.3 (b):** Statistical results: Mann-Whitney U test.

significantly (Table 3.4 b). Therefore, about 60-90% of the T cells present in the lesions were potentially memory T cells. Many cells could be seen to stain positive for both CD3 and CD45RO markers when adjacent serial sections were compared (Figures 3.7a & 3.7b).

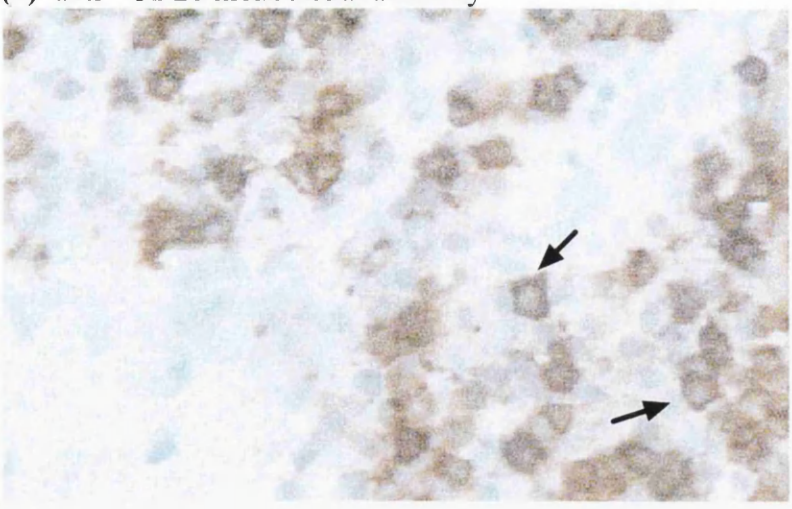
### 3.7.2 B cells

B cells were identified using the anti-CD 20 monoclonal antibody and were noted in all sections (Figure 3.8a, positive cells are indicated by black arrows). No statistically significant difference was observed in the numbers of B cells within AP and EOP granulation tissue sections as determined with the Mann Whitney U-test (Table 3.3 b, $p=0.684$). Furthermore, there were not significantly more B cells present in AP granulation tissue biopsies than in gingival sections. The statistical difference was marginal (Table 3.3 b; Mann Whitney U-test, $p=0.056$) so a trend could be easily observed with higher numbers of B cells in granulation compared to gingival tissues.

### 3.7.3 Macrophages

Macrophages were detected with the anti-CD68 monoclonal antibody (Figure 3.8b, positive cells are indicated by black arrows). They exhibited a variety of shapes and sizes, ranging from dendritic to round. There seemed to be higher numbers of CD68 positive cells in AP than in EOP sections, although

**(a)** anti - CD20 monoclonal antibody



**(b)** anti - CD68 monoclonal antibody

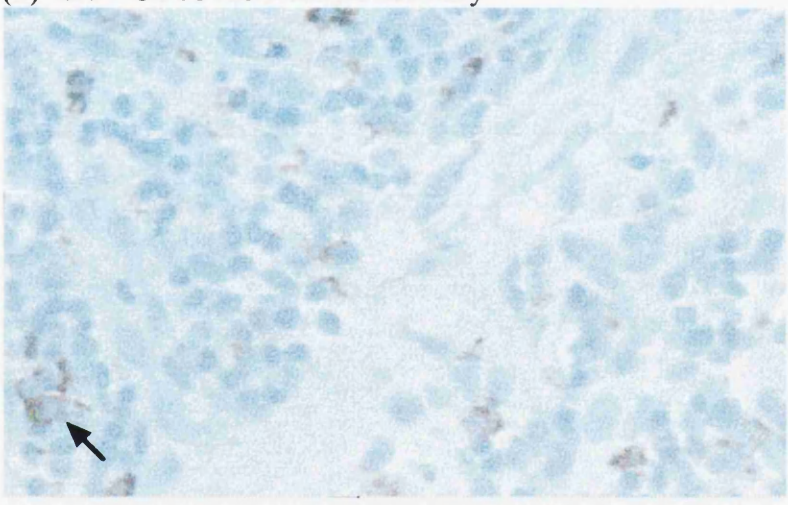


**Figure 3.8:** Immunohistochemical staining of **(a)** CD20 (B cells) and **(b)** CD68 (monocyte/ macrophages) in the corresponding fields in adjacent sections of granulation tissue biopsies (magnification x400).

there was no significant difference between them. Additionally, greater numbers were observed in AP granulation tissue sections compared to AP gingival sections but this failed to reach statistical significance (p=0.272, Table 3.3 b). Higher numbers of macrophages and a very wide range in cell counts were noted in the AP granulation tissue sections (median: 4.0, interquartile range: 2.4 - 21.3) than in the gingival tissues (median: 2.7, interquartile range: 2.4 - 3.1) (Table 3.3 a).

### 3.7.4 Relative distribution of B cells, T cells and macrophages

A further analysis of the data was to compare the relative ratios of memory T cells to pan T cells. No significant changes were noted within the tissue groups (Tables 3.4 a & 3.4 b). When AP and EOP granulation tissues were compared, the p value was 0.089 and when the comparison was made between AP granulation and AP gingival tissue the p value was 0.875. Interestingly, the comparison between B and T cells (CD20 : CD3), showed that the B : pan T cell ratio was significantly increased in AP sections compared to EOP (Table 3.4 b, p=0.002) and AP gingival sections (Table 3.4b, p=0.0002). The same trend was seen when the CD45RO marker instead of CD3 was used in the calculation (data not shown). When comparison was made for the macrophage : T cell ratio (CD68 : CD3) for the three different groups, an increase was detected in the CD68 : CD3 ratio in AP biopsies compared to EOP and gingival control sections (Table 3.4 a).

| Diagnosis | N | Marker | Ratio | | |
|---|---|---|---|---|---|
| | | | Median | Interquartile range | |
| **AP Granulation Tissue** | 9 | | 0.77 | 0.76 | 1.01 |
| **EOP Granulation Tissue** | 10 | **CD45RO/CD3** | 0.54 | 0.48 | 0.73 |
| **AP Gingival Tissue** | 8 | | 0.88 | 0.78 | 0.94 |
| **AP Granulation Tissue** | 9 | | 1.30 | 1.18 | 1.60 |
| **EOP Granulation Tissue** | 10 | **CD20/CD3** | 0.76 | 0.38 | 0.83 |
| **AP Gingival Tissue** | 8 | | 0.45 | 0.43 | 0.49 |
| **AP Granulation Tissue** | 9 | | 0.22 | 0.03 | 0.46 |
| **EOP Granulation Tissue** | 10 | **CD68/CD3** | 0.08 | 0.04 | 0.16 |
| **AP Gingival Tissue** | 8 | | 0.05 | 0.04 | 0.06 |

N: number of biopsies (1 biopsy per patient)

**Table 3.4 (a):** Ratios of memory Tcells/ pan T cells, B cells/ pan T cells and macrophages/ pan T cells for the three tissue groups. The data are expressed as median values (Q2) and interquartile range (Q1-Q3).

| Variable | AP v EOP | AP v GING | Results |
|---|---|---|---|
| **CD45RO/CD3** | NS | NS | |
| **p value** | p=0.089 | p=0.875 | |
| **CD 20/CD3** | **S** | **S** | AP>EOP |
| **p value** | **p=0.002** | **p=0.0002** | AP>GING |
| **CD68/CD3** | NS | NS | |
| **p value** | p=0.211 | p=0.224 | |

S: significant; NS: non significant
AP: AP Granulation Tissue; EOP: EOP Granulation Tissue
GING: AP Gingival Tissue

**Table 3.4 (b):** Statistical results: Mann-Whitney U test.

However, no significant changes were observed when either CD3 or CD45RO positive cells were used in the calculation (Table 3.4 b).

The relative numbers of B cells ($CD20^+$), pan T cells ($CD3^+$) and macrophages ($CD68^+$) as a proportion (%) of the combined total of CD20, CD3, and CD68 positive cells were calculated for each of the patients groups (Table 3.5 a). The results showed that T cells appeared to be of a significantly lower proportion when the AP granulation tissue sections were compared with either EOP granulation (Table 3.5 b, Mann Whitney U-test, $p=0.022$) or AP gingival tissues (Table 3.5 b, $p=0.005$). Unlike T cells, the proportion of B lymphocytes was higher in the AP granulation tissue sections than in the EOP granulation (Mann Whitney U test, $p=0.004$) or the AP gingival tissue sections ($p=0.0004$) (Table 3.5 b). No significant differences were noted for the proportions of macrophages among the tissue groups (Tables 3.5 a & 3.5 b).

### 3.8 T cell subsets

A full data was not available to compare $CD3^+$, $CD45RO^+$, $CD4^+$ and $CD8^+$ cell infiltration. This was due to the physical disruption of some areas of the tissues when harsh pressure cooker processing was carried out for CD4 and CD8. Only sections with common fields for all the above mentioned markers could be used. After microscopic examination, biopsies which had

| Diagnosis | N | Marker | Percentage | | |
|---|---|---|---|---|---|
| | | | Median | Interquartile range | |
| **AP Granulation Tissue** | 9 | | 44.67 | 42.49 | 49.01 |
| **EOP Granulation Tissue** | 10 | **%CD3/BTM** | 54.99 | 49.21 | 63.73 |
| **AP Gingival Tissue** | 8 | | 66.12 | 64.59 | 67.89 |
| **AP Granulation Tissue** | 9 | | 29.20 | 21.00 | 41.68 |
| **EOP Granulation Tissue** | 10 | **%CD45RO/BTM** | 34.65 | 25.34 | 41.53 |
| **AP Gingival Tissue** | 8 | | 58.35 | 51.99 | 60.73 |
| **AP Granulation Tissue** | 9 | | 47.24 | 44.67 | 49.29 |
| **EOP Granulation Tissue** | 10 | **%CD20/BTM** | 42.82 | 25.01 | 45.37 |
| **AP Gingival Tissue** | 8 | | 30.16 | 29.16 | 31.73 |
| **AP Granulation Tissue** | 9 | | 10.67 | 1.97 | 15.02 |
| **EOP Granulation Tissue** | 10 | **%CD68/BTM** | 3.96 | 1.46 | 9.16 |
| **AP Gingival Tissue** | 8 | | 3.57 | 2.96 | 3.84 |

N: number of biopsies (1 biopsy per patient)
BTM: total number of B, T cells and macrophages

**Table 3.5 (a):** Relative distribution of the leukocyte population within the three tissue groups. The data are expressed as median values (Q2) including interquartile range (Q1-Q3) and the % proportions have been calculated as follows:

$$\frac{x}{BTM} \times 100\ (\%),$$

x = number of pan T cells, memory T cells, B cells or macrophages.

| | Diagnosis | | |
|---|---|---|---|
| **Variable** | **AP v EOP** | **AP v GING** | **Results** |
| **CD3/BTM** | **S** | **S** | AP<EOP |
| **p value** | **p=0.022** | **p=0.0008** | AP<GING |
| **CD20/BTM** | **S** | **S** | AP>EOP |
| **p value** | **p=0.004** | **p=0.0004** | AP>GING |
| **CD68/BTM** | NS | NS | |
| **p value** | p=0.603 | p=0.066 | |

S: significant; NS: non significant
AP: AP Granulation Tissue; EOP: EOP Granulation Tissue
GING: AP Gingival Tissue

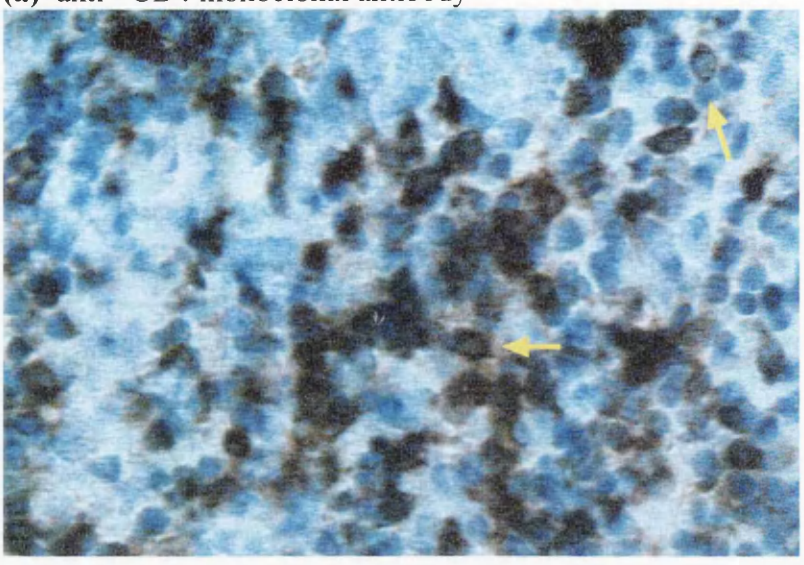
**Table 3.5 (b):** Statistical results: Mann-Whitney U test.

comparable fields for the four markers were chosen. As a result, AP and EOP granulation tissue and AP gingival tissue groups consisted of seven sections each (Table 3.6 a).

### 3.8.1 T cell subsets - T helper/ inducer cells

CD4 positive cells were detected in all granulation (Figure 3.9a, positive cells are indicated by coloured arrows) and gingival tissue specimens. Microscopic examination (low magnification x40) revealed that $CD4^+$ cells were widely dispersed. No specific localisation pattern was observed with respect to $CD4^+$ cells, although they did appear to cluster more than $CD8^+$ cells (section 3.8.2). There were discrete areas within individual sections that were relatively rich in CD4 or CD8 positive cells but these areas did not differ morphologically from each other nor did they contain any subtype exclusively. The median values calculated for $CD4^+$ cells per field were 23.2 for AP granulation tissue, 59.0 for EOP granulation tissue and 37.7 for AP gingival tissue specimens (Table 3.6 a). No statistically significant differences were observed in the numbers of $CD4^+$ cells among the tissue groups (Table 3.6 b). Furthermore, in AP granulation tissue sections, the subpopulation of CD4 positive cells comprised 64.22% (interquartile range: 59.66-66.84) of all CD3 positive cells detected (Table 3.7). Similar percentages were detected in EOP granulation tissue sections (60.98%,

**(a)** anti - CD4 monoclonal antibody



**(b)** anti - CD8 monoclonal antibody

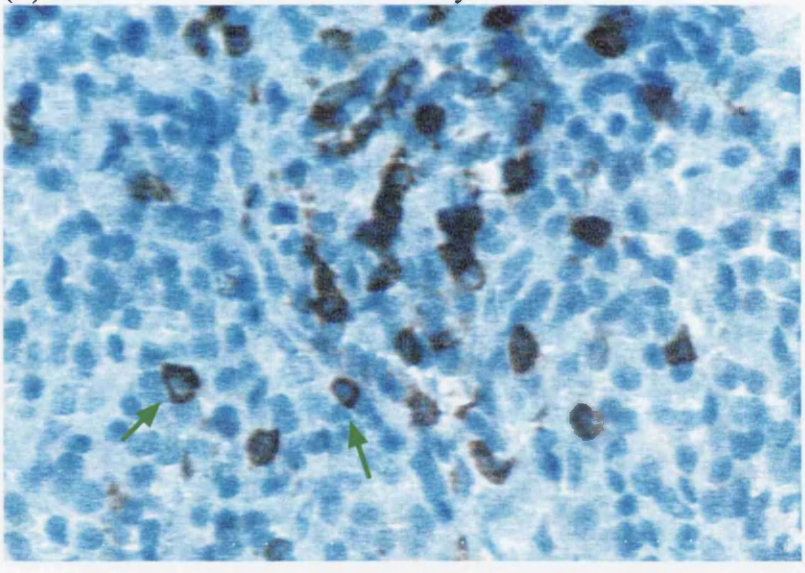


**Figure 3.9:** Immunohistochemical staining of **(a)** CD4 and **(b)** CD8 reveals more numerous T helper/ inducer than T cytotoxic/ suppressor cells in corresponding fields of adjacent sections of the granulation tissue biopsies (magnification x400).

| Diagnosis | N | Marker | Cells per field | | |
|---|---|---|---|---|---|
| | | | Median | Interquartile range | |
| **AP Granulation Tissue** | 7 | | 38.8 | 18.6 | 100.4 |
| **EOP Granulation Tissue** | 7 | **CD3** | 83.4 | 71.0 | 208.8 |
| **AP Gingival Tissue** | 7 | | 61.5 | 55.8 | 78.5 |
| **AP Granulation Tissue** | 7 | | 34.7 | 9.3 | 80.7 |
| **EOP Granulation Tissue** | 7 | **CD45RO** | 61.0 | 46.9 | 112.7 |
| **AP Gingival Tissue** | 7 | | 51.0 | 44.4 | 57.5 |
| **AP Granulation Tissue** | 7 | | 23.2 | 12.1 | 69.8 |
| **EOP Granulation Tissue** | 7 | **CD4** | 59.0 | 43.6 | 100.1 |
| **AP Gingival Tissue** | 7 | | 37.7 | 33.8 | 48.6 |
| **AP Granulation Tissue** | 7 | | 11.8 | 6.2 | 28.0 |
| **EOP Granulation Tissue** | 7 | **CD8** | 22.1 | 18.0 | 44.4 |
| **AP Gingival Tissue** | 7 | | 16.8 | 14.8 | 19.1 |

N: number of biopsies with common fields for all the markers (1 biopsy per patient)

**Table 3.6 (a):** Cells per field stained positive for the CD3, CD45RO, CD4 and CD8 markers in the three tissue groups. The data are expressed as median values (Q2) including interquartile range (Q1-Q3).

| | Diagnosis | |
|---|---|---|
| **Variable** | **AP v EOP** | **AP v GING** |
| **CD3** | NS | NS |
| **p value** | p=0.097 | p=0.376 |
| **CD45RO** | NS | NS |
| **p value** | p=0.165 | p=0.455 |
| **CD4** | NS | NS |
| **p value** | p=0.128 | p=0.382 |
| **CD8** | NS | NS |
| **p value** | p=0.165 | p=0.455 |

NS: non significant
AP: AP Granulation Tissue; EOP: EOP Granulation Tissue
GING: AP Gingival Tissue

**Table 3.6 (b):** Statistical analysis: Mann Whitney U test.

interquartile range: 59.10-64.26) and in AP gingival tissue biopsies (62.89%, interquartile range: 58.09-69.10) (Table 3.7).

### 3.8.2 T cell subsets - T cytotoxic/ suppressor cells

All 21 tissue specimens examined were found to contain CD8 positive cells. $CD8^+$ cells were detected in far lower numbers compared to $CD4^+$ cells in all sections (Figure 3.9b, positive cells are indicated by coloured arrows). The median values for $CD8^+$ cells were 11.8 for AP granulation tissue, 22.1 for EOP granulation tissue and 16.8 for AP gingival tissue (Table 3.6 a). Under microscopic observation (magnification x40) they seemed to be more widely dispersed than $CD4^+$ cells, although there were areas which were moderately rich in $CD8^+$ cells. When comparison was made among the tissue groups, no significant differences were observed in $CD8^+$ cell presence (Table 3.6 b). Moreover, 30.26% of all CD3 positive cells detected were also positive for the CD8 marker (interquartile range: 28.59-30.75) in AP granulation tissue biopsies (Table 3.7). Similar percentages were noted in EOP granulation tissue sections (27.32%, interquartile range: 23.57-29.91) and in AP gingival tissue biopsies (30.00%, interquartile range: 25.55-31.63) (Table 3.7).

| Diagnosis | N | Marker | Percentage | | |
|---|---|---|---|---|---|
| | | | Median | Interquartile range | |
| **AP Granulation Tissue** | **7** | | 61.94 | 59.86 | 82.99 |
| **EOP Granulation Tissue** | **7** | **%CD45RO/CD3** | 58.57 | 55.60 | 78.01 |
| **AP Gingival Tissue** | **7** | | 86.71 | 77.09 | 91.37 |
| **AP Granulation Tissue** | **7** | | 64.22 | 59.66 | 66.84 |
| **EOP Granulation Tissue** | **7** | **%CD4/CD3** | 60.98 | 59.10 | 64.26 |
| **AP Gingival Tissue** | **7** | | 62.89 | 58.09 | 69.10 |
| **AP Granulation Tissue** | **7** | | 30.26 | 28.59 | 30.75 |
| **EOP Granulation Tissue** | **7** | **%CD8/CD3** | 27.32 | 23.57 | 29.91 |
| **AP Gingival Tissue** | **7** | | 30.00 | 25.55 | 31.63 |

N: Number of biopsies (1 biopsy per patient)

**Table 3.7:** Percentages of memory T cells/ pan T cells, T helper cells/ pan T cells and T cytotoxic cells/ pan T cells for the three tissue groups. The data are expressed as median values (Q2) including interquartile range (Q1-Q3). The % proportions have been calculated as follows: $\frac{x}{CD3} \times 100\ (\%)$

x=number of memory T cells or helper T cells or cytotoxic T cells.

| Diagnosis | Marker | Ratio | | |
|---|---|---|---|---|
| | | Median | Interquartile range | |
| **AP Granulation Tissue** | | **1.97** | **1.94** | **2.50** |
| **EOP Granulation Tissue** | **CD4/CD8** | **2.51** | **2.42** | **2.69** |
| **AP Gingival Tissue** | | **2.21** | **1.98** | **2.49** |

**Table 3.8:** Ratio of CD4 / CD8 for the three tissue groups.

### 3.8.3 T helper: T cytotoxic cell ratio (T4:T8)

The numbers of $CD4^+$ to $CD8^+$ cells were compared for each group (AP and EOP granulation tissue and AP granulation tissue) and it was found that the CD4 : CD8 ratio did not vary significantly among the tissue groups (Table 3.8). The lower ratio was observed in AP granulation tissue group (median: 1.97), whereas the median values for EOP granulation and AP gingival tissue groups were 2.51 and 2.21 respectively. However, a wide range and an overlap was apparent among the groups.

## 3.9 Immunoglobulin isotypes

A full set of 9 AP granulation tissue, 10 EOP granulation tissue and 8 gingival tissue sections was available for the assessment of immunoglobulin mRNA-expressing plasma cells.

Preliminary experiments verified the specificity of the IgG and IgA subclass-specific probes. Specificity of the hybridisation signals was confirmed by the following control results. The negative control sections incubated with a hybridisation solution without probe (Figure 3.3a) or with a sense probe, showed a complete absence of signal in plasma cells and lymphocytes, in all cases. Further results of the hybridisation staining procedure following treatment with RNase A were negative, confirming that the method specifically

indicates the presence of RNA (Figure 3.3b). In addition, the hybridisation of the labelled probes was greatly reduced or abolished when the concentration of unlabelled probe against the same subclass was at a ten-fold excess. In contrast, the hybridisation of the labelled probes was unaffected when the concentration of unlabelled probe against another subclass was ten-fold in excess (data not shown).

In all biopsies, cytoplasmic staining was observed in plasma cells for IgG (Figures 3.10 & 3.11) and IgA subclass (Figures 3.12a & 3.12b) and IgM probes in serial sections (Figure 3.13). Positive cells are indicated by black or coloured arrows in the previously mentioned figures. Only a proportion of the tissues were positive (10/19 granulation tissues and no gingival tissues) for IgE (data not shown). The majority of immunoglobulin positive cells were observed in the lamina propria and subjacent to the epithelium.

The ratio of IgG: IgA: IgM was found to be approximately 72 : 21 : 3 for the granulation tissue biopsies, whereas it was 75 : 30 : 1 for the gingival tissue sections (Table 3.9). A statistically significant difference in the ratios of IgA-expressing cells was observed between AP granulation and AP gingival tissues (Table 3.9, $p=0.043$)with more cells being present in the gingival tissues. Conversely, granulation tissues comprised significantly more IgM-expressing plasma cells than gingival tissues (Table 3.9, $p=0.009$).

| Diagnosis | Ig class | Ratio | | |
|---|---|---|---|---|
| | | Mean | Standard deviation | Results |
| AP Granulation Tissue | IgG | 72 | 11 | NS |
| AP Gingival Tissue | IgG | 75 | 9 | |
| AP Granulation Tissue | IgA | 21 | 2 | S |
| AP Gingival Tissue | IgA | 30 | 3 | p=0.043 |
| AP Granulation Tissue | IgM | 3 | ND | S |
| AP Gingival Tissue | IgM | 1 | ND | p=0.009 |

S: significant; NS: non significant; ND: not determined

**Table 3.9:** Ratio of IgG : IgA : IgM for the AP granulation and AP gingival tissue groups. Statistical significant differences and p values are shown, as determined by Student t-test between the two tissue groups.

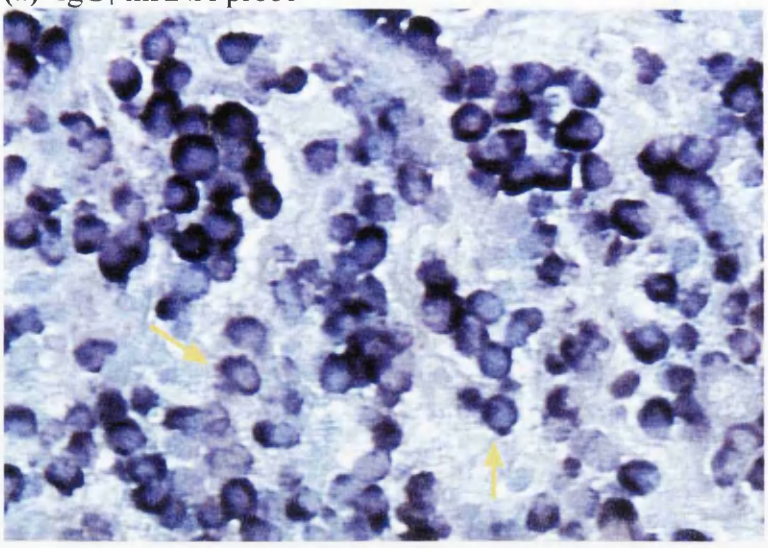
### 3.9.1 Immunoglobulin G subclasses

In the gingival tissue as well as in the periodontal granulation tissue specimens, the major IgG subclass was $IgG_1$ (mean values: 63.1% in the gingiva, 65.3% in the AP and 63.7% in the EOP groups) and followed by $IgG_2$ of total IgG mRNA bearing plasma cells (Figures 3.10a & 3.10b). $IgG_3$ and $IgG_4$ were present to a much lesser extent in fairly similar numbers (Table 3.10; Figures 3.11a & 3.11b). There were not any differences in the proportions of plasma cells expressing the IgG subclasses among the different tissue groups.

### 3.9.2 Immunoglobulin A subclasses

$IgA_1$ mRNA-expressing cells were the predominant IgA expressing plasma cell in the gingiva (68.7%) and the periodontal granulation tissue (AP: 81.4%; EOP: 81.6%) than $IgA_2$ mRNA-expressing cells (GING: 31.3%, AP: 18.6% and EOP: 18.4%) (Table 3.11a; Figures 3.12a & 3.12b). There was a statistical difference in the ratio of $IgA_1$ : $IgA_2$ expressing cells between the gingival tissue and the granulation tissues but not between the EOP and AP granulation tissue groups (Table 3.11b, $p=0.035$).

**(a)** $IgG_1$ mRNA probe



**(b)** $IgG_2$ mRNA probe

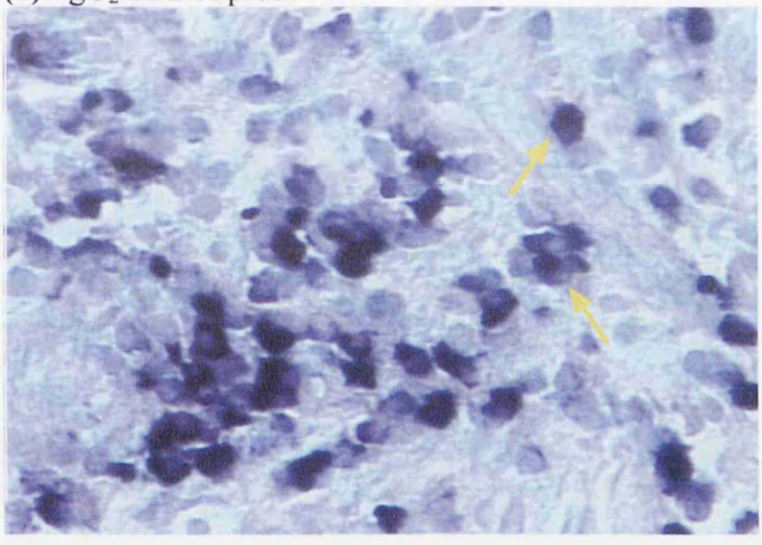
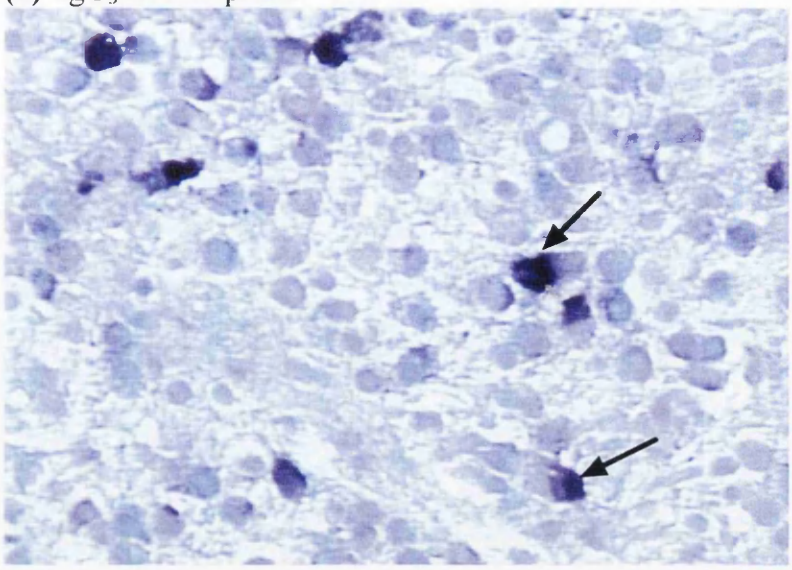


**Figure 3.10:** Expression of mRNA in adjacent sections of granulation tissue detected by *in situ* hybridisation using oligonucleotide probes for **(a)** $IgG_1$ and **(b)** $IgG_2$. Plasma cells exhibit strong cytoplasmic staining for both probes, but higher numbers of $IgG_1$ mRNA-bearing cells are evident in the lesion (magnification x400).

**(a)** $IgG_3$ mRNA probe



**(b)** $IgG_4$ mRNA probe

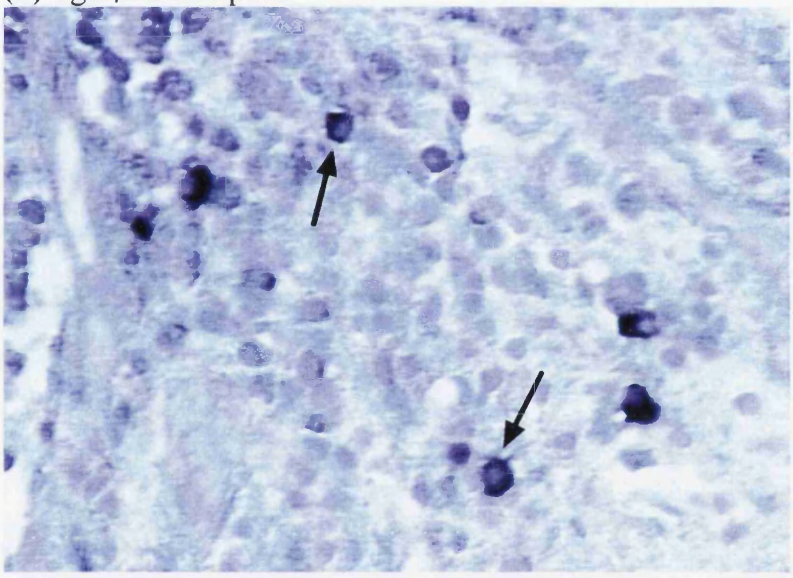


**Figure 3.11:** Expression of mRNA detected by *in situ* hybridisation using oligonucleotide probes for **(a)** $IgG_3$ and **(b)** $IgG_4$. Plasma cells exhibit strong cytoplasmic staining, although the expression of $IgG_3$ and $IgG_4$ mRNA is limited within the granulation tissue biopsies (magnification x400).

| Diagnosis | N | Subclasses | Percentage | |
|---|---|---|---|---|
| | | | Mean | Standard deviation |
| **AP Granulation Tissue** | 9 | | 65.3 | 4.7 |
| **EOP Granulation Tissue** | 10 | **% $IgG_1$** | 63.7 | 7.8 |
| **AP Gingival Tissue** | 8 | | 63.1 | 3.4 |
| **AP Granulation Tissue** | 9 | | 24.6 | 3.6 |
| **EOP Granulation Tissue** | 10 | **% $IgG_2$** | 25.5 | 6.8 |
| **AP Gingival Tissue** | 8 | | 22.8 | 4.2 |
| **AP Granulation Tissue** | 9 | | 5.21 | 2.4 |
| **EOP Granulation Tissue** | 10 | **% $IgG_3$** | 4.93 | 2.6 |
| **AP Gingival Tissue** | 8 | | 3.9 | 0.4 |
| **AP Granulation Tissue** | 9 | | 5.8 | 1.9 |
| **EOP Granulation Tissue** | 10 | **% $IgG_4$** | 5.7 | 2.4 |
| **AP Gingival Tissue** | 8 | | 9.8 | 1.6 |

N: number of biopsies (1 biopsy per patient)

**Table 3.10:** Relative distribution of the IgG subclass-producing plasma cells in the three tissue groups. The data are expressed as mean and standard deviation and the % proportions have been calculated as follows: $\frac{x}{(IgG_1+IgG_2+IgG_3+IgG_4)} \times 100$ (%)

x = $IgG_1$ or $IgG_2$ or $IgG_3$ or $IgG_4$ positive cell counts and
($IgG_1$+$IgG_2$+$IgG_3$+$IgG_4$) = total IgG bearing plasma cell counts.

### 3.9.3 Immunoglobulin M expression

IgM mRNA expressing plasma cells were found to be predominantly located in the relatively deep layers of the connective tissue in both tissue groups. It was also detected that the numbers of IgM mRNA bearing plasma cells were significantly higher in the AP granulation tissue sections compared to AP gingival tissue specimens (Table 3.9, section 3.9).

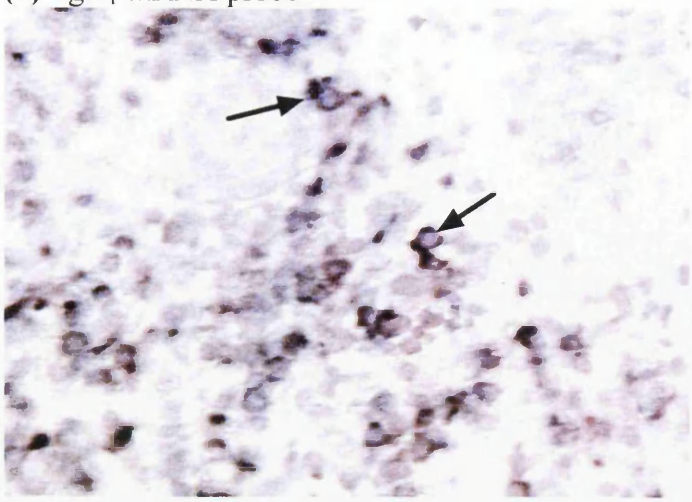
### 3.9.4 Immunoglobulin E expression

IgE mRNA positive plasma cells were only found in five out of twenty six tissue sections (5 AP and 5 EOP granulation tissue specimens). Additionally, when present, they were detected in very low numbers, lower than 5 cells per section. Since the vast majority of the sections were stained negative for IgE expression, no further analysis was carried out.

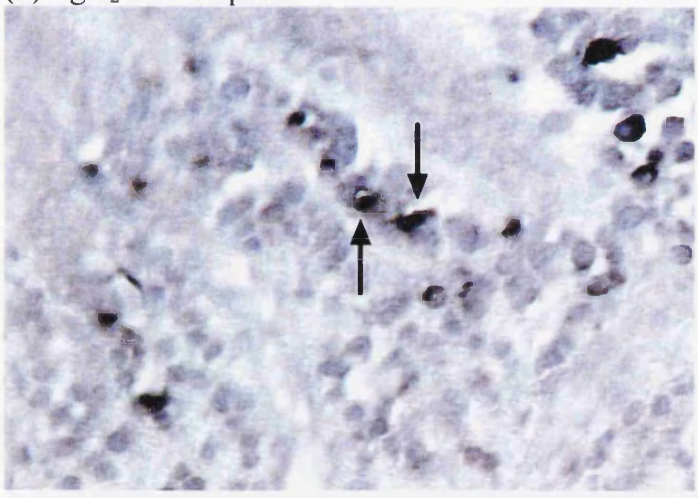
### 3.9.5 J chain expression

J chain mRNA positive plasma cells were noted in all 8 gingival tissue sections but only in 12 out of 19 granulation tissue sections stained for J chain expression. The vast majority of J chain expressing cells were found to be

**(a)** $IgA_1$ mRNA probe



**(b)** $IgA_2$ mRNA probe



**(c)** J chain probe

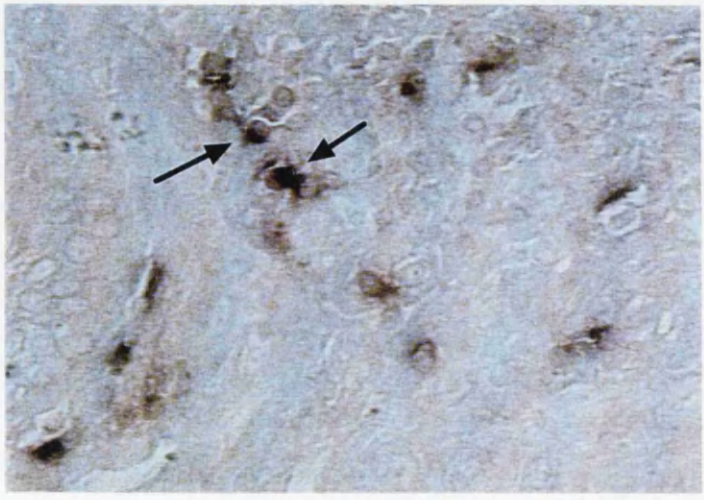


**Figure 3.12:** Expression of mRNA detected by *in situ* hybridisation in adjacent sections of granulation tissue using oligonucleotide probes for **(a)** $IgA_1$, **(b)** $IgA_2$ and **(c)** J chain (magnification x400).

| Diagnosis | N | Subclasses | Percentage | |
|---|---|---|---|---|
| | | | Mean | Standard deviation |
| **AP Granulation Tissue** | 9 | | 81.4 | 4.3 |
| **EOP Granulation Tissue** | 10 | **%$IgA_1$** | 81.6 | 4.4 |
| **AP Gingival Tissue** | 8 | | 68.7 | 1.3 |
| **AP Granulation Tissue** | 9 | | 18.6 | 4.3 |
| **EOP Granulation Tissue** | 10 | **%$IgA_2$** | 18.4 | 4.5 |
| **AP Gingival Tissue** | 8 | | 31.3 | 1.3 |
| **AP Granulation Tissue** | 9 | | 1.2 | 1.0 |
| **EOP Granulation Tissue** | 10 | **%J chain/IgA**[§] | 2.1 | 1.9 |
| **AP Gingival Tissue** | 8 | | 7.6 | 1.9 |
| **AP Granulation Tissue** | 9 | | 6.0 | 5.1 |
| **EOP Granulation Tissue** | 10 | **% J chain/ $IgA_2$**[§§] | 6.8 | 4.7 |
| **AP Gingival Tissue** | 8 | | 25.3 | 7.8 |

N: number of biopsies (1 biopsy per patient)
[§] expressed as a percentage of IgA
[§§] expressed as a percentage of $IgA_2$

**Table 3.11 (a):** Relative distribution of the IgA subclasses and J chain-producing plasma cells expressed in the three tissue groups. The data are expressed as mean and standard deviation and the % proportions have been calculated as follows:

$\frac{x}{(IgA_1 + IgA_2)} \times 100\ (\%)$,

x = $IgA_1$ or $IgA_2$ positive cell counts and

($IgA_1$ + $IgA_2$) = total IgA bearing plasma cell counts.

| Variable | AP v GING | Result |
|---|---|---|
| **%IgA1** | **S** | AP>GING |
| **p value** | **p=0.035** | |
| **%IgA2** | **S** | AP<GING |
| **p value** | **p=0.035** | |
| **%J/ IgA2** | **S** | AP<GING |
| | **p=0.019** | |

S: significant
AP: AP Granulation Tissue; GING: Gingival Tissue

**Table 3.11 (b):** Statistical analysis: Student t- test.

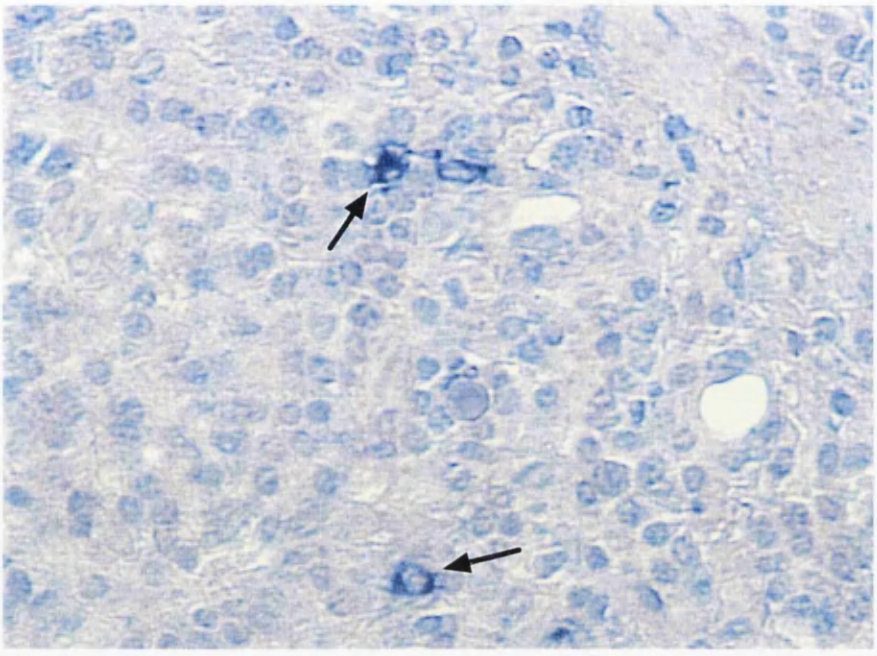


**Figure 3.13:** Expression of IgM mRNA detected by *in situ* hybridisation within granulation tissue biopsies. Very few IgM mRNA-expressing plasma cells are present in the lesions (magnification x400).

located adjacent to the epithelium with relatively few in the deeper lamina propria or the more apical granulation tissue.

Furthermore, the numbers of plasma cells expressing J chain were greater in the gingiva compared to those detected in either AP or EOP specimens. It was difficult to apply a statistical analysis to the differences in J chain expression between the gingival tissue and the AP or EOP tissues because not all of the granulation tissues were positive for the expression of this protein and this was due to the fact that epithelial cell layers were not always evident in the biopsies of granulation tissue. However, it was clear that when junctional epithelium was present in the biopsy, the J chain mRNA bearing plasma cells were detected in close proximity.

Interestingly, staining of adjacent serial sections for J chain and Ig expression demonstrated that the vast majority of J chain mRNA expressing plasma cells were also expressing the mRNA encoding $IgA_2$ (Figure 3.12c). Very few of the IgM or $IgA_1$ expressing plasma cells were positive for J chain mRNA and none of the IgG expressing cells demonstrated positive signals for the J chain mRNA staining.

The proportion of J chain expressing plasma cells, expressed as a percentage of the total IgA mRNA bearing cells was not significantly greater in the gingival sections, when they were compared with the areas subjacent to the junctional epithelium in AP tissue sections that contained the epithelial cell layers. In

contrast, the % proportion of J / $IgA_2$ was 25.3% in the gingiva, while it was only 6.0% in the granulation tissues, and this difference was significant (Table 3.11b, p=0.019).

# CHAPTER 4

# DISCUSSION

## 4.1 Introduction

The general aims of this thesis were:

a. to study the cell kinetics of the cellular infiltrate within periodontitis gingiva and periodontitis granulation tissues;

b. to examine the local distribution and the role of immune cells within these tissues;

c. to investigate the production by periodontal plasma cells of the main immunoglobulin classes and subclasses;

d. to assess possible differences in the immunopathology of AP and EOP with respect to their inflammatory cells' properties and;

e. to draw conclusions on the interactions and the roles played by the different variables analysed in these studies.

Studies published so far have tended to examine variables of the local cellular and humoral immune responses using gingival tissue biopsies. No attempts have been made to compare periodontitis gingiva and periodontitis granulation tissue from the same periodontitis site. The present study has focused on the typically studied periodontitis gingiva and the highly vascular granulation tissue, which is located in close proximity to the alveolar bone and the base of the periodontal pocket.

## 4.2 Cell synthetic activity

Two probes were used to assess the cell synthetic activity. Poly-deoxyribothymidine oligo d(T) was used to detect the poly-adenosine (poly-A) sequences of messenger RNA (mRNA) and 28S rRNA was targeted against the ribosomal RNA (rRNA). The number of total mRNA molecules and their species distribution depend on the level of the cell activity, including the synthesis of proteins and the stage in the cell cycle (Pringle *et al.*, 1989; Danks *et al.*, 1995). On the other hand, ribosomal RNA is present in every cell that synthesises proteins, in quantities related to the synthetic activity of cells (Yoshii *et al.*, 1995).

Endothelial cells and epithelium, especially basal epithelial cells, stained strongly with oligo d(T) and 28S rRNA probes, suggesting that these cells were actively synthesising proteins. Furthermore, plasma cells were also stained strongly with these probes in both tissue types. This finding is consistent with the results of a previous study by Takahashi, Lappin & Kinane (1996), who showed that the plasma cells were among the most active secretory cells in the gingiva. Moreover, this observation extends our knowledge and suggests that plasma cells function in a similar fashion in the deep granulation tissue.

Fibroblasts stained weakly to moderately with oligo d(T) and 28S rRNA in both tissue types. Given that fibroblasts display a functional heterogeneity regarding collagen I mRNA expression (Larjava, Sandberg & Vuorio, 1989), it could be suggested that synthetic activity of mRNA *in vivo* may be regulated by the different reactivities of the heterogeneous fibroblast. Furthermore, Takahashi, Lappin & Kinane (1996) have observed that the majority of fibroblasts found in close proximity to lymphocytes were not stained with these probes. Therefore, they do not actively synthesise proteins in inflammatory sites of the gingival connective tissue, while some activity was observed at non-inflammatory sites.

In contrast, most lymphocytes stained weakly or negatively in both tissue groups, indicating that lymphocytes in the granulation and gingival tissues did not actively synthesise proteins, as also suggested by Takahashi, Lappin & Kinane (1996). This finding emphasises the advantage of the ISH technique compared to immunohistochemistry, because not only can the phenotype of these cells be determined but also their active metabolism. The combination of *in situ* hybridisation and immunohistochemistry techniques form an ideal combination when both phenotype and function need to be considered in a study.

Both cell synthesis probes gave very satisfactory staining and specifically bound to the target sequences, as verified by the control sections. As a result, these probes were also utilised as positive controls showing the integrity and

the hybridisable RNA in tissue sections. Assessment of RNA integrity and hybridisability are essential for successful implementation of *in situ* hybridisation.

Although non-significant differences were noted in synthetic activity between any of the tissue types, it seemed that active cells of a higher proportion were found in gingival compared to granulation tissues. Since epithelial cells were not included in the calculations, it was assumed that the difference in the numbers of positive cells was accounted for by fibroblasts and plasma cells and possibly some lymphocytes.

### 4.3 Cell proliferation

Unlike the histone probe, the Ki-67 marker enabled the detection of proliferating cells in all tissue sections. However, the numbers of Ki-67 positive cells were low in both tissue types. Close examination of adjacent serial sections revealed that the majority of mononuclear cells that stained positive for CD markers were stained negative for Ki-67. These results suggest that the majority of T cells, B cells and monocytes/ macrophages were not proliferating and they were at the Go phase of the cell cycle in the periodontal granulation and gingival tissues.

Ki-67 positive cells were found to be reduced in gingival sections when compared with the granulation tissue biopsies. Granulation tissue consists of inflammatory cells, endothelial cells and fibroblasts (Clark, 1989). It is possible that during periodontal granulation tissue formation, a heterogeneous subpopulation of fibroblasts becomes activated by local inflammatory mediators and proliferate in the affected area. The second possibility could be that specific clones of fibroblasts within the actual inflammatory area are selected by local factors to differentiate into granulation tissue fibroblasts (Hakkinen & Larjava, 1992). Further investigation of the dynamics of fibroblasts in the periodontal granulation tissue could elucidate the kinetic behaviour of these cells.

### 4.4 Apoptosis

Kerr *et al* (1987) have underlined the difficulty in identifying *in situ* cells undergoing apoptosis by light microscopy. A complicating factor is that apoptotic bodies can be detected for only a few hours before they are phagocytised (Brusch *et al.*, 1990). Extensive cleavage of chromatin is the biochemical hallmark of apoptosis (Kerr *et al.*, 1987; Shi *et al.*, 1990) and techniques concentrating on the detection of nucleosomal DNA fragments have been developed (Gavrieli *et al.*, 1992; Wijsman *et al.*, 1993). In the present study, a modification of Gavrieli's technique was employed to detect apoptosis within periodontal granulation and gingival tissues. Apoptotic

cells were found very sparsely in both granulation and gingival tissue biopsies, most of them being fibroblasts and epithelial cells. The tonsil, a secondary lymphoid tissue that comprises numerous lymphocytes, and is well characterised with respect to its tissue dynamics (Bernstein *et al.*, 1994). Human tonsils show great B lymphocyte proliferative activity in their germinal centres as well as the appendix, Peyer's patches and lymph nodes (Soo *et al.*, 1995). In the control tonsilar biopsies, Ki-67 positive cells were detected in lymphocytes (B cells) in germinal centres (data not shown) and apoptotic cells were noted in great abundance. Conversely, in periodontal granulation and gingival tissues, it is apparent that few lymphocytes proliferate or undergo apoptosis.

## 4.5 Discussion on the kinetics of mononuclear cells in the periodontal granulation and gingival tissues

Interestingly, no clustering of B cells was seen, suggesting an absence of clonal expansion of these cells. Takahashi, Lappin & Kinane (1996) were able to detect proliferating lymphocytes (mostly B cells) within germinal centres in tonsilar tissues but not in gingival biopsies. These results support the hypothesis that B cells may be long-lived cells and that preferential immigration rather than preferential proliferation is responsible for their accumulation in the periodontal granulation and gingival tissue. It has been reported that the numbers of B cells that express the CD5 differentiation

marker are higher in inflamed gingival tissues than in peripheral blood of the same patients affected with periodontitis (Sugawara *et al.*, 1992). Additionally, CD5 positive cells do not actively proliferate (Abe, Tominaga & Wakasa, 1994). Furthermore, the presence of activated B cells was noted within periodontitis gingival tissues (Yamazaki *et al.*, 1993). The immune responses are usually induced within organised lymphoid tissues i.e. lymph nodes. It may be speculated that stimulated lymphocytes start to proliferate and differentiate in distant lymphoid tissues, such as lymph nodes, enter the lymphatic or blood circulation and specific clones arrive in the gingiva to react against the antigenic stimuli.

The present study shows that about 60-90% of the T cells present in the granulation and gingival tissues are potentially memory T cells. Moreover, several reports have indicated the presence of HLA-$DR^+$ T cells in periodontal gingival biopsies(Reinhardt *et al.*, 1988; Çelenligil *et al.*, 1990) and in gingival crevicular fluid (Takeuchi *et al.*, 1991), suggesting that they are *in situ* activated. Yamazaki *et al.* (1993) have reported a higher frequency of $CD4^+$ $CD45RO^+$ cells than $CD4^+$ $CD45RA^+$ cells in periodontitis lesions, confirming that T cells are found in an active stage in periodontitis gingiva, whereas T blasts as determined by transmission electron microscopy are very rare (Longhurst, Gillet & Johnson, 1980). Pan or memory T cell clusters were not observed in neither of the tissues examined, therefore, it could be hypothesised that T cells are activated, but do not proliferate.

T cell functions in periodontal granulation and gingival tissues can be elucidated by their cytokine synthesis profile (Yamamura *et al.*, 1991). Among other functions, interleukin-2 (IL-2) induces T cell proliferation (Genco, 1992). Controversial reports exist with respect to IL-2 expression in inflamed gingiva (Lundqvist *et al.*, 1994; Yamazaki, Nakajima & Hara, 1995). Furthermore, immunohistologically determined IL-4-producing cells in periodontal inflamed gingiva have been reported (Wassennaar *et al.*, 1995; Yamazaki, Nakajima & Hara, 1995), although Fujihashi *et al.* (1993) could not detect mRNA IL-4 in mononuclear cells extracted from periodontitis patients. In a recent report by Yamazaki *et al.* (1997), the mRNA expression of IL-4, IL-10, IL-12 and IL-13 was examined in tissue biopsies and peripheral blood from periodontitis patients using the reverse transcription polymerase chain reaction. They were able to detect very little IL-4 mRNA expression, since only a trace of this interleukin is found only 24 hours after activation of most T cell clones (Yssel *et al.*, 1992). Additional findings were the upregulation of IL-10 and IL-12 expression, as well as downregulation of IL-13 expression in the gingiva by comparison with peripheral blood. IL-10 is reported to be secreted by T cells and monocyte/ macrophages (de Waal Malefyt *et al.*, 1991) and along with IL-4 (Donnelly *et al.*, 1990), it inhibits activated macrophages to further produce other pro-inflammatory cytokines. In addition, IL-4 also regulates the proliferation of quiescent B cells. It is, therefore, clearly accepted that complex interactions exist regarding the local secretion and regulation of cytokines withiin periodontal inflamed gingiva.

Further studies are needed to elucidate functional T cell proliferative and synthetic activity with respect to their cytokine mRNA expressions.

The results of this study indicate that populations of fibroblasts become locally activated and proliferate in the area with the dense infiltration. They possibly possess the ability to preferentially synthesise proteins in less inflamed areas. The functional heterogeneity of gingival fibroblasts needs to be further elucidated with studies on their mRNA expression. As long as inflammation persists, periodontal granulation and gingival tissues may have a slow cell turnover rate. The occurrence of proliferation and programmed cell death should be a very rare event with respect to their leukocyte populations within the actual inflamed areas. Takahashi, Lappin & Kinane (1996), have hypothesised that specific clones of lymphocytes predominate in the periodontal inflamed gingiva and the possible mechanism for their actual presence could be selective homing to the tissue rather than proliferation. The results of this study support the model of the already differentiated and committed lymphocytes that migrate to the gingiva and also extend this hypothesis of the prevalent migration process to the deep granulation tissue.

## 4.6 Characterisation and relative distribution of mononuclear cells

It is well established that B lymphocytes and plasma cells predominate in advanced lesions of adult periodontitis (Seymour *et al.*, 1979; Page & Shroeder, 1982), whereas it has been reported that T cells are required for polyclonal activation and maturation of B cells into antibody-secreting cells (Seymour, 1987; Taubman *et al.*, 1991). More specifically, this process is regulated by $CD4^+$ T cells, which express the CD45RO antigen. Studies on the composition of the inflammatory infiltrate have revealed increased numbers of T cells as well as B cells and plasma cells in advanced periodontal lesions (Okada *et al.*, 1988; Johannessen *et al.*, 1990). However, a heterogeneity of the cellular infiltrates seems to be evident in several histological studies of gingival tissue (Seymour & Greenspan, 1979; Seymour, Crouch & Powell, 1981). These groups have limited their studies to the region adjacent to the gingival sulcus and have used superficial gingival tissue either taken in an axial (Johannessen *et al.*, 1986) or horizontal plane (Johannessen *et al.*, 1990) in relation to the tooth. In the present study, the periodontal inflamed tissue has been extended to include representational sites throughout the biopsy.

The comparison of the cell counts of B cells, T cells and macrophages present in the sections of gingival and granulation tissues, from patients with AP and EOP, revealed that there were differences in the mononuclear cell

profiles. The number of plasma cells and B cells were greater in the granulation tissue than in the gingival tissue sections.

In a study from our laboratory, higher numbers of T cells than B cells were detected in the gingival tissue obtained from patients affected with adult periodontitis (Takahashi, Lappin & Kinane, 1996). The findings of the present study showed a similar distribution pattern for T and B cells in the granulation tissues from patients with EOP and AP. In the EOP granulation tissue sections the T cells outnumbered B cells. In contrast, in the granulation tissue of patients with AP, B cells outnumbered T cells. The reduced B cell to T cell ratio observed in the EOP granulation tissue did not appear to be brought about by a reduction in the numbers of B cells but by an increase in T cells in the EOP granulation tissue sections. Active periodontal sites comprised more numerous T cells and T helper cells than healthy sites (Reinhardt *et al.*, 1988). Moreover, Modéer *et al.* (1990) found that in young subjects (12-19 years) with early signs of periodontitis, as determined by bleeding on probing, probing depth equal or greater than 5 mm and in some subjects alveolar bone loss, $CD3^+$ cells predominated within the lymphocyte infiltrate. In this respect, these results are in accordance with those of Yamazaki, Nakajima & Hara (1995). These investigators used the CD19 and CD3 markers to detect B cells and T cells, respectively. They compared the CD19 : CD3 ratios in three distinct sites of periodontal gingival tissue obtained from patients with advanced adult periodontitis. Elevated ratios were found in the three sites with elevated numbers of both B and T cells.

Although, there were not any significant differences in the numbers of CD45RO positive cells among the tissue groups, a greater variation in the numbers of CD 45RO positive cells were observed within each group of sections. Despite this variation, the ratio of memory T cells to pan T cells did not differ significantly between any of the tissue types. The present study showed that about 60-90% of the T cells present in the granulation and gingival tissues were potentially memory T cells. These results are thus in accord with the observation of Yamazaki *et al.* (1993) who stated that memory T cells greatly outnumbered naive T cells in periodontitis lesions. It has been reported that memory T lymphocytes ($CD29^+$ $CD45RO^+$) adhere to vascular endothelial cells and augment their permeability to macromolecules (Damle & Doyle, 1990). This functional ability of memory T lymphocytes to control endothelial permeability and to adhere to endothelial cells may be a dominant factor that contributes to the preferential migration of memory T cells into sites of chronic inflammation (Pitzalis *et al.*, 1988). Increased numbers of memory T cells have been shown in the skin of patients with systemic sclerosis (Rustin *et al.*, 1990). Afar *et al* (1992) have detected elevated levels of peripheral blood memory T cells ($CD45RO^+$) in patients suffering from periodontal disease. Moreover, Gemmell, Feldner & Seymour (1992) have demonstrated increased numbers of $CD4^+$ $CD45RO^+$ T cells in peripheral blood and in gingival cells extracted from AP lesions after stimulation in culture with *P. gingivalis* and *F. nucleatum.* It has been hypothesised that switching from the naive ($CD45RA^+$) to memory ($CD45RO^+$) pool does occur and that this switching is linked to the

maturation and activation state of the T cells, with an intermediate $CD45RA^+$ $CD45RO^+$ cell (Wallace & Beverly, 1992). The simultaneous dual expression of both CD45RA and CD45RO markers in a cell subpopulation has also been demonstrated by Yamazaki *et al.* (1993) in cells from periodontal gingival tissues, suggesting a conversion from naive T cells to primed memory T cells in the periodontal inflamed sites. An *in vitro* study by Kristensson, Borrebaeck & Carlsson (1992) have demonstrated that $CD4^+$ $CD45RA^+$acquire the lymphokine gene expression of $CD4^+$ $CD45RO^+$ T cells after activation, confirming that the $CD45RA^+$ T helper subpopulation represents the precursor of $CD45RO^+$ subset.

Studies supporting the view that a correlation exists between the severity of periodontal disease and the numbers of sensitised lymphocytes (Malberg *et al.*, 1992). This group have demonstrated that as gingival index (GI) scores increased so did the number of activated $CD4^+$ cells. The exact role of of naive and memory T cells has yet to be elucidated within the periodontal disease context. Investigation in terms of the different lymphokine profiles that these two T cell subsets express, may shed light on their functions in relation to disease status.

The results obtained with the CD3 monoclonal antibody clearly indicated that significantly fewer T cells were present in the AP than in EOP sections. Furthermore, the CD45RO marker is known to detect a proportion of granulocytes and monocytes/ macrophages as well as memory T cells.

Therefore, any analysis of B : T cell and macrophage : T cell ratios made with the CD45RO marker may be inaccurate.

Significant differences in the numbers of macrophages were not detected, although a trend towards greater numbers in the AP granulation than gingival tissue sections was evident.

In order to look at the relative abundance of the different cell types in the tissues, B cells (CD20), T cells (CD3) and macrophages (CD68) were analysed as a proportion (%) of the total of CD20, CD3 & CD68 positive cells, for each of the patient groups. These results showed that the percentage of B lymphocytes was greater in the AP granulation tissue sections than in the EOP granulation tissue or the gingival tissue biopsies. In that respect, Yamazaki *et al.* (1993) have shown that B cells were the predominant infiltrating cell in adult periodontitis lesions. The percentage of T cells was lower in the AP sections than in the other tissue types.

When the data were further analysed, by comparing the relative ratios of B to T cells (CD 20/ CD 3 and CD 20/ CD 45RO) and macrophages to T cells (CD 68/ CD 3 and CD 68/ CD 45RO), the results showed that there was a significant increase in the B : T cell ratio in AP sections compared to the other sections and an increase in the macrophage : T cell ratio in AP sections (but only when the comparison was made with CD 68/ CD 3), although not statistically significant.

These differences in the relative abundances of B cells, T cells and macrophages probably reflect a difference in the pathology of AP and EOP. The profile of the mononuclear cells present in AP compared to EOP may enable us to speculate that the AP lesion has been established for a longer period of time and it represents a more mature chronic lesion. In addition, the more rapid progression of the EOP when compared to AP lesions may be in part due to the increased number of cells of the T cell lineage in addition to the high numbers of B cells and plasma cells usually encountered.

Finally, the findings in this study support the hypothesis that the proportions of B and T cells may differ in various forms of periodontitis.

### 4.6.1 T helper /inducer- T cytotoxic/suppressor cells (T4-T8)

Previous phenotypic analyses of gingival T-cell subsets have revealed reduced (Okada *et al.*, 1984; Cole, Seymour & Powell, 1987; Stoufi *et al.*, 1987a; Stoufi *et al.*, 1987b) or unchanged (Johannessen *et al.*, 1986) CD4 : CD8 ratios in the periodontitis tissues compared with those in gingivitis tissues and the levels in peripheral blood. With the exception of the group of Johannessen *et al.* (1986), all the above mentioned investigators used cell-extraction techniques for the phenotypic analysis of T cell subsets. In the present study, an immunohistochemical method was used to identify the phenotypes of CD4 and CD8 positive cells in gingival and granulation tissue biopsies. The ratio of CD4 : CD8 T cells was 1.97 (range 1.94-2.50) for granulation tissues and 2.21 (range 1.98-2.49) for gingival tissues. There was not any significant difference between them. The results indicate that the proportion of $CD4^+$ was double that of $CD8^+$ T cells. Large individual variance existed however, in the two tissue groups and among patients in each group (data not shown) with respect to $CD4^+$ and $CD8^+$ cell counts. Gürses *et al.* (1996) examined three distinct areas of gingival tissues obtained from 25 adult periodontitis patients, one of them being beneath the pocket epithelium, near the base of the pocket. Our results confirmed these results in terms of a CD4 : CD8 ratio of approximatelly 2:1. They also grouped the patients according to their GI scores. They found that the CD4 :

CD8 ratio was positively correlated with the increase in GI score, especially in sites beneath the pocket epithelium.

Studies of peripheral blood T lymphocyte subsets in early onset forms of periodontal disease have revealed a wide variety of contradictory results with either depressed (Kinane, Johnston & Evans, 1989) or mixed (Katz *et al.*, 1988) CD4 : CD8 ratios, or even not correlated with the disease (Engel *et al.*, 1984). A number of studies have examined the distribution of T-cell subsets in gingival tissues of different forms of periodontal disease (Jully *et al.*, 1986; Okada *et al.*, 1988; Meng & Zheng, 1989). In the present investigation, the CD4 : CD8 ratio calculated from assessments made in AP and EOP granulation tissue samples were 1.97 (range 1.94-2.50) and 2.51 (range 2.42-2.69), respectively. These ratios were not statistically different.

Okada *et al.*, (1988) compared gingival biopsies taken from patients affected with gingivitis, adult periodontitis and advanced periodontitis in individuals younger than their early thirties. They examined the correlation between periodontal disease severity as determined by pocket depth and radiographic findings and the CD4 : CD8 ratio. However, they were not able to establish a relationship among the variables. Furthermore, Meng & Zheng (1989), reported similar or slightly elevated tissue CD4 : CD8 ratios in adult and juvenile periodontitis groups compared with healthy subjects. Our results agree with these observations and extend them in the deep inflammatory granulation tissue. The slightly higher ratio found in EOP biopsies, although

not significant, is due to the relative increase of $CD4^+$ T cells noted in EOP biopsies, although the number $CD8^+$ T cells was also elevated. However, a broad range of $CD4^+$ and $CD8^+$ T cells and, subsequently, of CD4 : CD8 ratios was exhibited in this particular sample in both tissue groups. Furthermore, it has been reported that the two T-cell functional states may change during the course of the disease and T-cell subsets may shift from the one state to the other (Pawelec, Schneider & Wernet, 1983). If changes in the lymphocyte population occur with time, only the phenotype of T cell infiltrate and the CD4 : CD8 ratio can barely discriminate different forms of the disease. Further investigation of possible differences in the cytokine profile of T-cell subsets in relation to disease activity may give information on the disease form and status within an actual time frame.

Taubman *et al.* (1984) reported a decreased ratio in tissues from adult and juvenile periodontitis patients (1.2 and 1.1, respectively) when compared with either gingivitis or peripheral blood ratios. The existing discrepancy between these results and our observations may be the result of different methodologies used to determine the lymphocyte subpopulations. Gingival cell extraction techniques are based on the actual recovery of cells from the soft tissue samples, thus, they do not provide information on the distribution of cells relative to the location within the tissue biopsies. In addition, cell surface markers may be disrupted during the procedure. Immunohistochemical studies are very important in that respect, because the various cells can be identified in well defined lesions in tissue sections. It

should be emphasised that, while Taubman and co-workers limited their study to the region adjacent to the gingival sulcus, our study was extended to include the deep granulation tissue.

### 4.7 Immunoglobulin isotypes

The present study extends the results obtained from our laboratory on the localisation of IgG and IgA subclass-specific mRNA-bearing plasma cells in periodontal gingival tissues (Takahashi *et al.*, 1997). Granulation tissue biopsies that exhibited a more dense infiltration of leukocytes and plasma cells than the inflamed gingiva along with gingival tissues from patients affected with periodontitis were selected to study the humoral immune response in periodontal tissues. The predominant immunoglobulin class was IgG followed by IgA, IgM and IgE, in this order, for all the tissue types detected. IgE mRNA-expressing plasma cells were almost absent in all biopsies tested. The ratio of IgG to IgA to IgM mRNA-expressing cells was around 75: 30: 1 in the gingiva, whereas the ratio was found to be 72: 21: 3 in the deep granulation tissues. Studies on Ig-producing cells assessed by enzyme-linked immunospot (ELISPOT) and by immunofluorescence have revealed that gingival mononuclear cells, isolated from inflamed tissues of adult periodontitis, contain predominantly IgG followed by IgA and IgM (Ogawa *et al.*, 1989; McGhee *et al.*, 1989). Similar results were found from tissue sections analysed by immunofluorescence (Kilian *et al.*, 1989). The

overall frequency of plasma cells was higher in granulation tissue in comparison with the gingival tissue.

IL-10, which has been reported to be upregulated in inflamed gingival tissues (Yamazaki *et al.*, 1997), is thought to induce activated B cells to produce IgG, IgA and IgM in high levels (Defrance *et al.*, 1992). Additionally, IL-4 and IL-6-producing cells were detected in periodontal gingival tissues by immunohistochemistry (Yamazaki, Nakazima & Hara, 1995). It has been reported that IL-4 induces B cells to switch to IgG and IgE producing cells in the murine system (Lutzker *et al.*, 1988; Lebman & Coffman, 1988) and to $IgG_4$ secreting cells in humans (Lundgren *et al.*, 1989). Furthermore, it has been shown that IL-6 plays a significant role in the terminal differentiation of B cells into plasma cells in mice and humans (Hirano *et al.*, 1986; Beagley *et al.*, 1989). IL-5 has been shown to induce the proliferation of B cells and Ig synthesis in mice (Karasuyama, Rolink & Melchers, 1988; Swain *et al.*, 1983). Our present data demonstrate the simultaneous presence of a systemic immune response, as IgG mRNA-expressing plasma cells are predominant in the lesion and a mucosal immune response, as secretory IgA mRNA-bearing plasma cells are located below the epithelial cell layers in the same specimens. It can also be suggested that interleukin-induced class switching is present in the inflamed periodontium. This could provide a basis for studying further the mechanisms of local B cell differentiation in biopsies from periodontitis sites with respect to the cytokine potential to induce such differentiation. It is still likely that the switching occurs in the local lymph

nodes and that committed B cells migrate to the gingival lesions, although the actual mechanisms are still unknown.

*P.gingivalis* has been reported to stimulate two distinct patterns of IgG and IgA response in chronically inflamed gingival tissues (Ogawa *et al.*, 1989). It may be suggested that the features of systemic and mucosal response noted in periodontal granulation and gingival tissues and the shift from one isotype to another are regulated by possible changes of microbial antigens during the course of the disease. Further studies are needed to investigate these points.

### 4.7.1 Immunoglobulin G subclasses

A significant difference in the relative distribution of IgG subclass mRNA-bearing plasma cells was not detected in the tissues tested. In both tissue groups the distribution pattern of IgG subclasses was $IgG_1$>> $IgG_2$> $IgG_3$=$IgG_4$. This subclass distribution profile is in accordance with normal systemic levels found in serum (French & Harrison, 1984). The major IgG subclass was $IgG_1$ for both tissue types. These results are in agreement with these of Ogawa *et al.* (1989), as well as with the findings from our laboratory (Takahashi *et al.*, 1997) and with levels detected in the synovium from patients with rheumatoid arthritis (Haber, Kubagawa & Koopman, 1985). The proportions $IgG_2$, $IgG_3$ and $IgG_4$ followed the same trend in the

granulation and the gingival tissues and this corresponds with the results of Takahashi *et al.* (1997).

The distribution of IgG subclasses is controlled by the nature of the antigenic stimuli. It has been reported that protein antigens elicit mainly $IgG_1$ antibody production with $IgG_3$ and $IgG_4$ in lower levels (Hammastrom & Smith, 1986). In contrast, carbohydrate antigens induce $IgG_2$ antibody production. The periodontopathic microflora displays a great variety in its microbial antigens and some of them are able to provoke a specific IgG subclass response (Mayumi *et al.*, 1983). Whitney *et al.* (1992) have indicated that the protein and lipid carbohydrate antigens of *P. gingivalis* stimulate an $IgG_2$ and $IgG_3$ response in the serum of rapidly progressive periodontitis patients. Nevertheless, our results did not demonstrate a high local production of these subclasses in the granulation tissue biopsies taken from EOP patients.

Our findings indicate a lower local $IgG_2$ production relative to gingival crevicular fluid (GCF) (Takahashi *et al.*, 1997) and systemic levels ((Jefferies *et al.*, 1985). It could be hypothesised that since $IgG_2$ production is induced by carbohydrate antigens, the involvement of this type of local response is decreased in the inflamed periodontium. Ogawa *et al* (1989, 1991) have reported an increased $IgG_4$ local production in the advanced stage of periodontal disease which may correlate with our slightly elevated, although not significant, findings with respect to local $IgG_4$ production. A similar observation has been made by Kiyono *et al.* (1989) who have reported that

$IgG_4$ producing cells are most heavily represented among gingival cells producing specific antibody to fimbriae from *P. gingivalis*. The hypothesis provided by these workers is that chronic antigenic exposure of gingival B cells could induce a shift from $IgG_1$ to $IgG_4$ production and thus, more protection from localised tissue damage. The fact that $IgG_1$ is known to fix complement and to form immune complexes, while $IgG_4$ does not, could raise speculations as to whether protective antibody switching mechanisms exist in the inflamed gingiva (Ogawa *et al.*, 1991; Kono *et al.*, 1991). Furthemore, as GCF levels of $IgG_4$ were shown to be reduced in the study of Takahashi *et al* (1997), the local secretion of $IgG_4$ could possibly be further studied, although its low numbers make analysis less reliable. Changes in the nature or the relative distribution of microbial antigens could alter the IgG subclass distribution pattern and induce a switch from one subclass to another, reflecting changes in the stage or the form of the actual disease.

### 4.7.2 Immunoglobulin A subclasses

The predominance of $IgA_1$ mRNA-expressing cells, within the population of IgA expressing plasma cells, was evident in all the sections tested. These results are not in accordance with these of Ogawa *et al.* (1989) who reported proportions up to 90% for $IgA_1$, similar to those detected in serum (Skvaril *et al.*, 1973). The possibility exists that the proportion was overestimated due to the high background staining often occurring during immunohistochemical

assays. The distribution pattern of IgA subclasses better corresponds with that seen in the report of Takahashi *et al.* (1997), who examined the IgA subclass distribution in gingival sections with *in situ* hybridisation technique and in GCF with ELISA assay from patients affected with periodontitis. It has been reported that protein antigens induce a specific $IgA_1$ response, whereas specific antibodies to LPS and lipoteichoic acids belong predominantly to the $IgA_2$ subclass (Mestecky & Russell, 1986; Moldoveanu *et al.*, 1988). Therefore, the occurrence of a relative increase in $IgA_1$-producing cells may give information on the nature of the antigens involved in the humoral immune response in periodontal disease. Differences in the relative IgA-subclass distribution were noted between the two tissue types. The predominance of $IgA_1$ in the deep areas of the periodontal tissue could reflect the type and the nature of specific microbial antigens which preferentially induce such an $IgA_1$ response. In the superficial gingiva, $IgA_1$ mRNA expression was decreased and so were $IgA_1$ levels in the GCF (Takahashi *et al.*, 1997). It is known that $IgA_1$ is less resistant than $IgA_2$ to cleavage by bacterial proteases (Frandsen *et al.*, 1995). It could be speculated that $IgA_1$ is degraded by periodontal pathogen-derived $IgA_1$ proteases in the way from the deep to the superficial tissue and to the crevice. An explanation could be that $IgA_1$ proteases exert their effect more efficiently in the superficial gingiva although there is no evidence confirming or refuting this hypothesis. In contrast, significantly more $IgA_2$-producing cells were observed in the gingival tissues compared to granulation tissues.

This could reflect a change in the nature of antigens inducing a specific $IgA_2$ response in different areas of the inflamed tissue.

It is not known how the expression of $IgA_1$ and $IgA_2$ is regulated. It has been shown that the two IgA subclasses are differentially regulated in different tissues (Kett *et al.*, 1986; Islam *et al.*, 1992). Further investigation of the kinetics of pre-plasma B cells, selective homing of specific B cell clones and antigen-induced local differentiation into specific plasma cells may give answers to the questions of the preferential induction of $IgA_1$ or $IgA_2$ responses in the human gingiva (Kinane *et al.*, 1993; Seymour *et al*, 1993).

### 4.7.3 J chain expression

$IgA_2$ mRNA expression was significantly elevated in the gingival over the deep granulation tissue specimens. In fact, the percentage of $IgA_2$ mRNA bearing plasma cells was higher in the periodontal granulation tissue than in serum (Skvaril *et al.*, 1973) or in periapical lesions (Takahashi, McDonald & Kinane, 1997). Additionally, J chain mRNA-expressing cells were mostly of $IgA_2$ subclass and were adjacent to the epithelial cell layers. It could be suggested that the majority of dimeric IgA destined for transportation by the secretory component-dependent mechanism and, finally, secretion consisted of $IgA_2$. This is supported by the study of Crago and co-workers (Crago *et al.*, 1984), who investigated the localisation of IgA subclasses and J chain in

various tissues using monoclonal antibodies. They concluded that higher numbers of $IgA_2$ than $IgA_1$-positive cells were stained for J chain. The fact that $IgA_2$ is predominant in the crevice fluid (Takahashi *et al.*, 1997) supports this observation. This finding supports the presence of a mucosal immune response in the superficial gingiva, which may show secretory tissue-like features. However, the presence of IgG-derived systemic response should not be underestimated. In conclusion, both features of a systemic and a mucosal immune response could be seen in the gingival tissue from periodontitis patients.

### 4.7.4 Immunoglobulin M expression

The numbers of of IgM mRNA expressing plasma cells were low in both gingival and granulation tissue biopsies. This observation is confirmed by several previous studies which have detected high numbers of IgG, followed by IgA and essentially no IgM-producing plasma cells in patients affected with periodontitis (Mackler *et al.*, 1977; Okada, Kida & Yamagami, 1983; McGhee *et al.*, 1989). However, some workers maintain that IgM plasma cells are most prevalent (Platt, Crosby & Dalbow, 1970). IgM mRNA-bearing plasma cells were predominantly located in the deep tissue layers and the majority seemed to be negative for J chain expression. However, it was noted that some IgM expressing cells were positive for J chain mRNA, although the staining intensity was weaker than that of $IgA_2^+$ J $chain^+$ mRNA

expressing cells. On the one hand, it becomes apparent that while IgM mRNA-bearing plasma cells are located in the deeper layers of the tissue and are mostly negative for J chain expression. On the other hand, many $IgA_2$ mRNA-expressing cells also contained the J chain mRNA, suggesting that they are likely to produce dimeric IgA in the superficial layers of the connective tissue.

## 4.8 General conclusions and future research

The kinetic behaviour of the mononuclear cells, which comprise the inflammatory cellular infiltrate within periodontal granulation and gingival tissue lesions, was examined in the first study of the present thesis. The results have indicated that it is most unlikely that the accumulation of leukocyte in the diseased periodontium occurs through proliferation in the actual inflamed sites. Since most of the T and B lymphocytes and macrophages have been stained negative for the proliferation markers, it is more feasible that differentiation of these cells takes place in more distant sites. For example, precursor B or plasma cells may divide and /or be activated in the lymph nodes and already differentiated and committed cell clones home to the gingiva to take part in the immune processes. Similar mechanisms could control the proliferation of T cells and macrophages. Further studies on the kinetics of mononuclear cells are needed, since it is still unclear where, how and under which circumstances leukocytes

proliferate and become activated, then migrate to and accumulate in the periodontal lesions. *In situ* hybridisation and other hybridisation techniques may elucidate further the actual proliferative capacity of these cells by molecular means.

Although the highly vascular inflamed granulation tissue shows epithelial and connective tissue turnover, it does not seem that the inflammatory cell turnover occurs at the same rate. Apoptotic cells were sparsely detected, mostly consisting of epithelial cells and fibroblasts, although some lymphocytes and degenerating plasma cells could also be seen. Apoptosis, or "programmed cell death" does not seem to be a major feature and this leads to the conclusion that the leukocytes examined may be long-lived cells.

Adult periodontitis and early onset periodontitis lesions comprise a large inflammatory infiltrate which is rich in B cells, T cells and plasma cells. The actual presence of B and T cells in periodontitis sites may have a significance in the degree of maturation of the lesion as well as in the rate of progression of the disease. T cells are found in higher numbers in the young lesion (such as EOP), whereas they are less abundant in the more mature chronic adult periodontitis lesion. Depressed ratio of T helper to T suppressor cells illustrates a deficiency in the immune process. This study demonstrates a balanced relationship in the immune system in both EOP and AP. Since controversies still exist regarding the composition of the inflammatory infiltrate and its changes during the course of the disease, it seems that

morphological and phenotypic analyses of T cells are not completely able to determine such a dynamic process. It should be stressed that observations regarding cell populations located in gingival tissues must be made with great care and with the recognition that even between sequential biopsies the inflammatory infiltrate may change. The immunoregulatory role of T cells is important in the progression of periodontal research and current studies focus on the functional abilities of their cytokine expression. Further studies should be undertaken in order to elucidate the importance of host response in the progression from a young to a more mature periodontal lesion. Central to this is the key role played by $Th_1$ and $Th_2$-derived cytokines in regulating leukocyte differentiation and activation and, therefore, in influencing the development of the disease.

The synthetic activity of plasma cells has been confirmed and further investigated by categorising them according to their immunoglobulin class and subclass mRNA expression. This study shows that IgG is the predominant class and is followed by IgA and IgM, whereas traces of IgE are detected. It becomes apparent that secondary systemic (IgG) and mucosal (IgA) immune responses are the main features of the advanced chronic inflammatory periodontal diseases. Neither IgM, which is associated with primary immune response, nor IgE do seem to play a role in the pathogenesis of the advanced periodontal lesion. This study demonstrates the simultaneous presence of a systemic and a mucosal immune response in periodontitis lesions. Although the occurrences of IgG subclass mRNA-

expressing plasma cells do not exhibit any significant difference between gingival and granulation tissues, IgA mRNA expression seems to be elevated in the superficial gingiva compared to the deep granulation tissue and so is $IgA_2$. Moreover, IgM mRNA-bearing plasma cells are significantly higher in the granulation tissue. In addition, the interesting finding of numerous J chain mRNA expressing cells located in the superficial layers beneath the epithelium, leads to the conclusion that $IgA_2$ may be the predominant secretory IgA subclass. It should be stressed that the local distribution of immunoglobulin and J chain mRNA-expressing cells may not always be correlated with the quantities of the antibody proteins that finally are secreted. A number of mechanisms are involved in the production of, immunoglobulins and complications in the translation, and secretion of protein and even protease-induced degradation of the secreted molecule will affect the final concentration in tissue fluids. Other mechanisms pertain to the transport of the secretory form of IgA across epithelial cell layers, i.e., the presence of the secretory component in epithelial cells. Studies need to be performed to obtain a more specific understanding of the role played by these mechanisms in the local immunoglobulin production. Nevertheless, it has been proposed that a combination of IgG and IgA subclass antibodies are found in GCF and these antibodies are likely to be both locally and serum derived (Takahashi *et al.*, 1997; Kinane, Takahashi & Mooney, 1997).

Studies so far have demonstrated that specific plaque-derived micro-organisms induce specific immunoglobulin isotype responses. Future studies

should focus on the nature of the antigenic microflora and the mechanisms that provoke such specific antibody responses *in vivo*. This could give answers as to whether the humoral immune response has a protective or damaging role in the pathogenesis of periodontal disease.

In conclusion, investigations into the humoral and cellular immune responses, occurring within periodontal lesions, may have various benefits and lead to a better understanding with respect to pathogenesis, progression and resolution of the disease.

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**Appendix 1:** List of reagents used during probe labelling and *in situ* hybridisation

| Description | Source |
|---|---|
| • ABC-alkaline Phosphatase Kit | Vector |
| • Acetone | Avondale Lab. Ltd |
| • Alkaline Phosphatase Avidin D | Vector |
| • Alkaline Phosphatase Substrate Kit | Vector |
| • Anti-DIG-mAb (mouse $IgG_1$) | B&M |
| • Anti-biotin-POD, Fab fragments | B&M |
| • Anti-biotin Ab (goat) | Sigma |
| • Anti-sheep/ goat IgG (donkey) | SAPU |
| • APAAP mouse monoclonal | DAKO |
| • Avidin D | Vector |
| • 5-bromo-4-chloro-3-indolylphosphate (BCIP)-Toludine salt | Sigma |
| • Biotin-16-dUTP (1mM) | B&M |

| Description | Source |
|---|---|
| • Biotinylated Alkaline Phosphatase | Vector |
| • Biotinylated anti-mouse antibody (γ-chain specific horse) | Vector |
| • Biotinylated anti-mouse antibody | B&M(sheep IgG) |
| • Biotinylated DNA/Hind III | GibcoBRL |
| • Biotinylated anti-avidin D | Vector |
| • Biotinylated Horseradish Peroxidase | Vector |
| • BSA-Fraction V | Sigma |
| • BSA-Fraction V Powder | Sigma |
| • Cacodylic Acid Sodium Salt | Sigma |
| • dATP (100 mM, pH 7.5) | Pharmacia |
| • Decon-90 | BDH |
| • Dextran Sulphate Sodium Salt | Sigma |
| • Diethyl Pyrocarbonate (DEPC) | Sigma |

| Description | Source |
|---|---|
| • DIG-3'-end labelled control oligonucleotide | B&M |
| • DIG-labelled control DNA | B&M |
| • Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIG-11-dUTP) | B&M |
| • Di-Sodium Hydrogen Orthophosphate -2-hydrate ($Na_2HPO_4$ $2H_2O$ | BDH |
| • N-N-Dimethylformamide | Sigma |
| • DNAse | Sigma |
| • Eosin yellowish | BDH,Gurrcertistaiprod. |
| • Ethanol | |
| • Ethylenediaminetetra-acetic acid disodium salt (EDTA) | BDH |
| • Extra Avidin-POD | Sigma |
| • Ficoll | Sigma |
| • Formamide | BDH |

| Description | Source |
|---|---|
| • Gel loading flat-ended pipette tips | Life Science Inter. |
| • Glass slides | |
| • Glycergel | DAKO |
| • Glycine | BDH |
| • Goat Biotinylated anti-mouse antibody | Vector |
| • Haematoxylin Sol | Sigma |
| • Haematoxylin | BDH Gurr prod. |
| • Hydrochloride (HCl) | |
| • ISH Detection Kit | DAKO |
| • Levamisole | Sigma |
| • Magnesium Chloride 6-hydrate (MgCl2.6H2O) | BDH |
| • NAP$^{Tm}$ -5 Column Sephadex G25 DNA Grade 20 columns | Pharmacia |
| • Neutral Red (1 % in dH2O) | DIFCO |
| • Nitro-cellulose Filter, Biotrace NT | Gelman Sciences, 20 cm x 20 cm |

| Description | Source |
|---|---|
| • Nitro-blue tetrazolium (NBT) | Sigma. |
| • Non-fat dry milk (commercial one) | |
| • Normal human serum (inactivated) | |
| • PAP (sheep/goat) | Scottish Antibody Production Unit, Carluke |
| • PAP pen | |
| • Paraformaldehyde | BDH |
| • Peroxidase Substrate Kit (DAB) | Vector |
| • Polyvinylpyrollidone (PVP) | Sigma |
| • Polysine™(silane-coated slide glass) | BDL |
| • Potassium dihydrogen orthophosphate | BDH ($KH_2PO_4$) |
| • Potassium chloride (KCl) | BDH |
| • Proteinase K | Sigma |
| • Rabbit anti-mouse Ig | DAKO |
| • Rabbit anti-mouse Ig/AP | DAKO |

| Description | Source |
|---|---|
| • RNase type 1-A | Sigma |
| • Rubber cement | Tip TopVulcanising fluid, Stahlgruber |
| • Salmon DNA | Sigma. |
| • 3-aminopropyltriethoxy-silane | Sigma |
| • Sigmacote® | Sigma |
| • Sodium chloride (NaCl) | BDH |
| • Sodium dodecyl sulphate (SDS) | BDH |
| • Sterile Tips | Labsystems LTD lemon tips autoclavable racks |
| • Sterile Tips | Labsystems LTD |
| • Blue tips autoclavable racks | |
| • Streptavidin-POD | B &M |
| • Terminal deoxynucleotidyl--Transferase (TdT) | Gibco BRL |

| Description | Source |
|---|---|
| • tri-Sodium Citrate ($Na_3C_6H_5O_7.2H_2O$) | BDH |
| • TRIZMA BASE | Sigma |
| • TRIZMA Hydrochloride | Sigma |
| • Triton X-100 | Sigma |
| • TRIzol ™ Reagent | GibcoBRL |
| • Tween 20 (polyoxyethylene-sorbitan monolaurate) | Sigma |
| • Xylene | |

# PUBLICATIONS

The following manuscripts are directly related to the studies presented in this thesis:

1. Lappin, D.F., Koulouri, O., Radvar, M., Hodge, P. & Kinane, D.F. (submitted) Relative proportions of mononuclear cell types in periodontal lesions analysed by immunohistochemistry.

2. Lappin, D.F., Koulouri, O., Buckley, A. & Kinane, D.F. (in preparation) Differential expression of J chain and immunoglobulin M and A & G subclasses mRNA by plasma cells in periodontal disease.

3. Koulouri, O., Lappin, D.F., Radvar, M. & Kinane, D.F. (in preparation) Cell division, synthetic capacity and apoptosis in periodontal lesions analysed by *in situ* hybridisation and immunohistochemistry

# Relative Proportions of Mononuclear Cell Types in Periodontal Lesions Analysed by Immunohistochemistry

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**Abstract**

In this study we investigated the relative proportions of infiltrating mononuclear inflammatory cells in sections of granulation tissue from periodontitis lesions in both adult periodontitis (AP) and early onset periodontitis (EOP) patients. We utilised a set of cluster of differentiation (CD) antigen specific monoclonal antibodies to detect different cell types within the tissues. These included anti-CD 20 (B cells), anti-CD 3 (pan T cells) and anti-CD 45RO (memory T cells), anti-CD 4 (helper T cells) anti-CD 8 (suppressor T cells) and anti-CD 68 (monocyte/macrophage). Biopsies of granulation tissue were obtained from 9 patients with adult periodontitis (AP), from 10 patients with early onset periodontitis (EOP) and for comparative purposes, biopsies of gingival tissue from 4 patients with AP. A significantly greater number of T cells ($p<0.05$) were observed in EOP and gingival sections than in AP sections. In addition, a greater number of B cells were observed in the granulation tissues than in the gingiva ($p<0.05$). The relative numbers of B cells (CD 20), T cells (CD 3) and macrophages (CD 68) were expressed as a percentage of their combined total, for each of the patient groups and indicated that the proportion of B

lymphocytes was greater in AP sections than in EOP or gingival sections ($p<0.02$). The proportion of T cells was lower in the AP periodontitis sections than in the EOP periodontitis sections ($p<0,05$). There were no significant differences in the proportion of macrophages between the three catagories of tissue specimens.

The relative ratios of B cells (CD 20) to T cells (CD 3) and B cells (CD 20) to memory T cells (CD 45RO) and macrophages (CD 68) to T cells (CD 3) and memory T cells (CD 45RO) were analysed and indicated that there was a significant increase in the B to T cell ratio in AP sections compared to EOP and gingival sections ($p<0.02$). There was also a significant increase in the macrophage to T cell ratio in AP sections asindicated by CD 68 to CD 3 ratios ($p<0.05$). There were no differences regarding the relative proportions of memory T cells or in the ratios of CD 4+ to CD 8+ T cells in the different disease categories. However, we observed differences in the distribution of the CD 4+ and CD 8+ cells in the tissue sections, where cells of each subtype appeared to present in different but overlapping areas of each section regardless of the tissue origin but as the localisation of these two subsets was not consistent we are unable to ascertain the significance

of this observation

In conclusion, these differences in the relative proportions of B cells, T cells and macrophages may reflect a difference in the immunopathology of AP and EOP.

## Introduction

Complex inflammatory and immune responses are involved in the progression of periodontitis. Tissue activity within the diseased periodontium comprises epithelial and connective tissue turnover and the cellular activity associated with the infiltrating inflammatory cells [1]. Previous work has suggested that B cells and T cells accumulate in large numbers in the periodontal tissues although until recently we have had little information on cellular synthetic activity and proliferation of these cells.

*In vitro* studies have demonstrated that T cells are involved in immunoglobulin synthesis [2,3,4]. However, the results of these studies do not easily extrapolate to *in vivo* situations where complex interactions between a variety of infiltrated inflammatory cells occur. It is also presumptive to assess the role of the different cell types and their inter-relationships in inflammatory sites from limited observations of morphology and/or phenotypic analysis of cell surface markers. Immunohistochemical methods have been used in the past for these assessments, e.g. lymphocyte subsets [5,6,7,8,9], but controversies still exist regarding these reports. The paradigms that "periodontitis is a B cell lesion"

and the "immunoregulatory role of T cells in periodontitis" have been proposed following immunohistochemical analysis although cell activity was not assessed [5,10,11,12].

Gemmell and Seymour [13] have shown an increased proportion of T cells with increasing size of infiltrate in diseased gingival tissues. Others [14] have shown an increase in T cell numbers in peripheral blood and it was suggested that homing of these cells to the gingiva may occur during the disease process. Reports from our laboratory [15] have revealed similarities in T cell gene rearrangement profiles between gingival biopsies from the same individual. It was suggested that selective localisation of different subsets of T cell clones may be occurring which would be consistent with the existence of gingiva-homing memory T cells. However, an alternative possibility exists, that specific T cells proliferate locally within certain tissues (in response to a local antigenic chalenge) giving rise to characteristic T cell clones for these regions. Clearly, information on leukocyte proliferation in the diseased gingival tissues may solve the debate as to whether specific leukocytes predominate in the gingival tissue through selective homing mechanisms or by proliferation. In our recent study on

periodontitis gingiva we demonstrated that the infiltrating lymphocytes were more likely to arise through selective homing than by local cell division [16].

The purpose of this study was to extend our investigations on the diseased periodontitis gingiva. The localisation patterns of T cells, B cells and monocyte/macrophage were also investigated by immunohistochemistry using specific monoclonal antibodies raised to cluster of differentiation (CD) antigens, known to be specific for these cell types. The AP and EOP sections were also compared for differences in their pathogenesis.

## Materials and methods

### Tissue preparation

The tissues used in this study consisted of 10 biopsies of granulation tissue from 9 patients (ages 42-62 yrs) with adult periodontitis (AP) and 10 biopsies of granulation tissue from 10 patients (ages 24-39 yrs) with generalised early onset periodontitis (EOP). the diagnostic criteria for EOP was those of Hart *et al.* [17] and briefly was as follows: patients under the age of Thirty five years of age, exibiting attachment loss (AL) of at least

5mm on at least eight teeth at least three of which were not first molars or incisors and at least one of which was a first molar. All the patients included in the study had advanced forms of periodontitis, with the exception of one patient with AP who had moderate to severe disease. The pocket depths of the sites biopsied ranged from 6.0-9.0 mm in patients with AP and 5.8-9.6 mm in patients with EOP. We also included for comparative purposes 6 sections of gingiva from 4 patients with AP all taken from periodontitis sites. The tissues were removed under local anesthesia during routine periodontal surgery within the Glasgow Dental Hospital and immediately fixed in 10% neutral buffered formalin at room temperature. They were embedded in paraffin wax and 5 μm serial sections were collected on silane-coated glass slides. Serial sections were used for *in situ* hybridization and immunohistochemistry. Two sections in each sample were used for morphological examination which was based on haematoxylin and eosin (H&E) staining before immunohistochemistry.

**Immunohistochemistry**

Briefly, slides were deparaffinised in xylene, hydrated through descending

isopropyl alcohol concentrations, and washed in DDW. The slides were washed in PBS twice for 5 min and immersed in DDW. To inhibit endogenous peroxidase, they were incubated with 3% $H_2O_2$ in methanol at 4°C for 15 min. (CD 3; NCL -CD 3 ) and the human helper T cell subset (CD 4; NCL-CD 4-IF6) were purchased from Novocastra LTD Newcastle Upon Tyne, UK . Monoclonal Antibodies against: human memory T cells( CD 45RO; UCHL-1), the human suppressor T cell subset (CD 8; 144/B), human B cells (CD 20; L26) and macrophages (CD 68; PG-M1) were purchased from Dako LTD (Glostrup, Denmark). For T, B cells detection, deparaffinised sections were placed in plastic box filled with a 0.01M tri-sodium citrate solution (pH 6.0) or 0.001 M EDTA pH=8.0 (for sections stained with anti -CD 4 IF 6), and heated in a conventional microwave oven for 18 min (4 x 4.5 min) at 650W to unmask the antigen []. While undergoing microwave processing, slides were always covered with the solution. After heating, slides were permitted to cool down to room temperature over a period of 30-60 min. For detection of macrophages proteinase K digestion (5 µg/ml) were performed for 30 min at 37 °C. After washing in PBS (pH 7.4) and blocking, slides were incubated with

monoclonal antibodies. Monoclonal Antibodies were reacted for 1 h for NCCD 3, UCHL-1, L26 and PG-M1, CD 4 IF 6, CD 8 144/B. After washing with PBS, slides were sequentially incubated with biotin-labelled goat anti-mouse IgG antibody (Vectastain *Elite* ABC-POD kit; Vector Lab, CA) for 30 min. After washing with PBS, they were treated with avidin peroxidase for 30 min, then treated with DAB substrate kit (Vector Lab) for 2-7 min under microscopic observation. After washing with DDW, slides were finally counterstained with hematoxylin and mounted in aqueous mounting medium.

**Cell Counting**

In counting the numbers of cells stained with each of the markers, twelve microscopic fields with the strongest cell infiltration, from each serial section of all 26 biopsies, were chosen for counting using a meshwork eyepiece (0.172 mm x 0.172 mm; 0.0296 $mm^2$. The number of positive cells present in a given field were counted in duplicate. Positive cells were also recounted as a check using the Kontron 300 Image Analysis System and KS300 software programmed to maximize sensitivity and specificity of

positive cell detection (Kontron Elektronik Eching, Germany).

In order to be confident we were analysing ncells in the same area, with different markers we adopted the following procedures. The major criterion was that each area included was present in all of the series of sections. Otherwise the total number of common undamaged fields available were analyzed. The minimum number of fields available in any section included in the study was five. Great care and considerable effort was expended to identify the corresponding areas in series of sections. We tested the efficiency of our method for relocating the same areas in serial sections. This was done by counting the cells in the same area after: removing the slide from the microscope, repositioning the slide holder and relocating the area after reinserting the slide. Capturing an image of the original field using the Kontron System enabled relocation of the same area again with precision. The results of repeated cell counts on the relocated fields differed by less than or equal to 5% and this was deemed acceptable.

## Results

IHC Cell Characterisation

Mononuclear cells were further distinguished by immunohistochemistry (IHC) into T, B cells and macrophages and these cells were detected in all samples. B cells were identified using the anti-CD 20 monoclonal antibody and were found in all of the sections (Figure 1). The numbers of B cells were similar in both EOP and AP sections and significantly greater than the numbers detected in gingival sections (Figure 2; Mann Whitney U test $p<0.05$).

Two monoclonal antibodies were used in replicate sections to detect T lymphocytes. These were anti-CD 45RO (memory T cells) and anti-CD 3 (pan T cells) monoclonal antibodies. Both monoclonal antibodies gave satisfactory results. The numbers of CD 3 positive cells detected in each section were greater than the numbers of CD 45RO positive cells. The results obtained with the CD 3 monoclonal showed that a significantly greater numbers of T cells (Mann Whitney U-test; $p<0.05$) were observed in the EOP and gingival sections than in the AP sections (Figure 3). However, greater variations in the numbers of CD 45RO positive cells were observed

within each group of sections and no significant differences in the numbers of CD 45RO positive cells were observed, between each of the groups.

Macrophages were detected by the anti-CD 68 monoclonal antibody and showed various shapes and sizes, ranging from dendritic to round. There were greater numbers of CD 68 positive cells in AP than in EOP lesions but this was not significant, but was significant when AP sections (Mann Whitney U-test; $p<0,05$) were compared to gingival sections from Ap patients (Figure ).

We also compared the relative numbers of B cells (CD 20), T cells (CD 3) and macrophages (CD 68) as a proportion (%) of the combined total of CD 20, CD 3 and CD 68 positive cells, for each of the patient groups (Figure ). The results showed that the proportion of B lymphocytes was greater in the AP sections than in the EOP or the gingival sections ( Mann Whitney U-test; $p<0.02$). The proportion of T cells was lower in the AP sections than in the other tissue types ( Mann Whitney U-test; $p<0.05$). No significant differences were noted for the proportions of macrophages in all three groups.

We then analyzed the data by comparing the relative ratios of B to T cells

(CD 20/ CD 3 and CD 20/ CD45 RO) and macrophages to T cells (CD 68/ CD 3 and CD 68/CD 45RO) for the different groups. That there was a significant increase in the B :T cell ratio in AP sections compared to EOP and gingival sections (Figure ; Mann Whitney U-test; $p<0.02$). There was also a significant increase in the macrophage :T cell ratio in AP sections compared to EOP and gingival control sections but, only when the comparison was made with CD 68/ CD 3. No significant changes were observed when CD 45RO positive cells were used in the calculation. We also compared the ratio of memory T cells to pan T cells for each group and found no significant differences. About 60-90% of the T cells present in the lesions were memory T cells, i.e. T cells positive with the CD 45RO marker (Figure).

There were significantly greater numbers of CD 4 positive cells in the EOP sections compared to AP sections (Mann Whitney U-test, $p< 0.05$). Although the CD 8 positive cells followed a similar trend the difference was not significant, possibly due to a lower number of CD 8 expressing cells being present in the sections. The ratio of CD 4: CD 8 positive cells did not differ between different groups of periodontitis tissues (table 1). On

average the ratio of CD 4/ CD 8 was 5: 2 with a wide range of ratios consisting of (from 4:5 to 6:1). There were discrete areas within individual sections that were relatively rich in CD 4 or CD 8 positive cells but these areas did not differ morphologically from each other nor did they contain any subtype exclusively..

**Discussion**

The results presented in this paper here extend our earlier study on periodontitis gingiva [16]. Moskow and Polson [18] have expressed doubt that experiments performed on the superficial gingiva were sufficient to give a clear picture of the disease, inflammatory and immune processes in severe periodontitis. In an attempt to address this point we have detected B cells, T cells and macrophages in the deeper periodontitis granulation tissue from two different forms of periodontitis, AP and EOP, and included gingival tissues for direct comparison. In addition to the qualitative approach to the histological data presented in our earlier paper we have extended the analysis and performed a quantitative analysis of the different cell types present in a set of serial sections. We have also looked at the cell

profiles in different diseased periodontitis tissues and within the different areas of the biopsies.

The results presented in this study support our earlier view that B cells in the gingiva are long lived cells and we extend our findings to show that B cells in the periodontal granulation tissues behave in a similar fashion. Observations, that: CD 5 positive B cells are present in the gingiva at higher proportions than in blood [9], activated B cells are present in the periodontium [19] and CD 5 positive B cells do not proliferate [20, 21], support the view that activated B cells and plasma cells home to the inflamed periodontium.

The above statements also appear to be true for T cells. There was no clustering of CD 3 or of CD 45RO positive cells in any of our sections of gingiva or granulation tissue. T blasts have been shown to be rare in periodontal tissue using transmission electron microscopy [22]. Furthermore, activation of T cells in periodontitis gingiva has been reported [6,7,].

When we compared the numbers of B cells, T cells and macrophages present in the sections of gingiva and the granulation tissues, from patients

with AP and EOP, we noticed that there were differences in the cell profile. The number of plasma cells and B cells were greater in the granulation tissues than in the gingival sections.

We have reported previously [16], that the numbers of T cells were greater than B cells in the gingiva . This was also true for the granulation tissues from patients with EOP. In contrast, in the granulation tissue of patients with AP, B cells outnumbered T cells. In this respect our results are similar to those of Yamazaki *et al* [23]. These authors used the CD 19 marker to detect B cells and compared the ratios of B cells to CD 3 positive T cells from three distinct sites in patients with advanced AP. The reduced ratio of B cells to T cells observed in the EOP did not appear to be brought about by a reduction in the numbers of B cells but in an increase in T cells in the EOP sections. Although, no significant differences in the numbers of CD 45RO positive cells were observed, between each of the groups, a greater variation in the numbers of CD 45RO positive cells were observed within each group of sections. Despite this variation we saw that the ratio of memory T cells to pan T cells did not differ significantly between any of the tissue types.

Like the study by Yamazaki *et al* [23], we found that memory T cells greatly out numbered naive T cells in the lesions. The results obtained with the CD 3 monoclonal clearly indicated that significantly fewer T cells were present in the AP sections. Furthermore, the CD 45RO marker is known to detect a proportion of granulocytes, and monocytes and macrophages as well as memory T cells. Therefore, any analysis of B to T cell and Macrophage to T cell ratios made with The CD 45RO marker may be inaccurate.

We were unable to see a significant difference in the numbers of macrophages. Although we saw a trend towards greater numbers in the AP sections. In order to look at the relative abundance of the different cell types in the tissue we also compared B cells (CD 20), T cells (CD 3) and macrophages (CD 68) as a proportion (%) of the sum total of CD 20, CD 3,& CD 68 positive cells, for each of the patient groups. These results showed that the percentage of B lymphocytes was greater in the AP sections than in the EOP or the gingival sections. The percentage of T- cells was lower in the AP sections than in the other tissue types. No significant differences were noted for the percentages of macrophages in all three

groups. When the data were analyzed further, by comparing the relative ratios of B to T cells (CD 20/ CD 3 and CD 20/ CD 45RO) and macrophages to T cells (CD 68/ CD 3 and CD 68/ CD 45RO), the results showed that there was an increased in the B :T cell ratio in AP sections compared to the other sections and an increase in the macrophage :T cell ratio in AP sections (but, only when the comparison was made with CD 68/ CD 3).

These differences in the relative abundances of B cells T cells and macrophages probably reflect a difference in the pathology of AP and EOP. The profile of the mononuclear cells present in AP compared to EOP may enable us to speculate that the AP lesion has been established for a longer duration and is a more mature chronic lesion.

In addition, the more rapid progression of the EOP when compared to AP lesions may be in part due to the increased number of cells of the T cell lineage in addition to the

high numbers of B cells and plasma cells usually encountered.

Finally, the findings in this study support the hypothesis that specific leukocytes predominate in the periodontitis tissues through selective

homing rather than by local proliferation and that the proportions of B and T cells may differ in various forms of periodontitis.

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Table 1. Ratio of CD 4 positive T cells to CD 8 positive T cells (The numbers of cells per field were compared).

| | | CD 4 : CD 8 | | |
|---|---|---|---|---|
| Tissue | n. | Median | Interquartile | range |
| EOP | 10 | 2.51 | 2.42 | 2.90 |
| AP | 9 | 2.05 | 1.97 | 2.50 |
| Gingiva | 8 | 2.21 | 1.98 | 2.49 |

**Legends**

Figure 1:

Serial sections of granulation tissue from a patient with Early-onset Periodontitis (EOP) showing: a) Immunohistochemical staining with a murine monoclonal antibody to Human CD 20 antigen, demonstrating a large number of B cells (x400). b) Immunohistochemal analysis with a murine monoclonal to the human CD 68 antigen, showing the presence of a few monocytes / macrophages (x400). Similar results were seen in the tissues of all the patients in the study.

Figure 2:

Serial sections from the same granulation tissue from the patient with EOP, as for figures 1&3, showing the corresponding fields and demonstrating : a) Immunohistochemical staining with a murine monoclonal antibody to Human CD 3 antigen, a pan-T cell marker, demonstrating a large number of T lymphocytes (x400)in the section. b) Immunohistochemal analysis with a murine monoclonal to the human CD 45RO antigen, a showing the presence of many memory T cells in the section. (x400). Similar results were seen in the tissues of all the patients in the study.

Figure 3:

Serial sections from the same granulation tissue from the patient with EOP, as for figures 1&3, showing the corresponding fields and demonstrating : a) Immunohistochemical staining with a murine monoclonal antibody to Human CD 4 antigen, a Helper T cell marker, demonstrating a large number of Helper T lymphocytes (x400) in the section. b) Immunohistochemal analysis with a murine monoclonal to the human CD 8 antigen, ashowing the presence of many suppressor T cells in the section. (x400). Similar results were seen in the tissues of all the patients in the study.

Figure 4:

The number of positively staining cells per field in serial sections of granulation tissues from patients with AP (n=10) and EOP (n=10), and sections of gingiva (Ging) from patients with AP (n=6) detected with: a) Monoclonal antibodies to CD 20 (B cells), CD 3 (pan-T cells), CD 45RO ( memory T cells). B) Monoclonal antibodies to CD 4 and CD 8 .The results are represented on the chart as the median value (black bar) surrounded by a box, the, the lower and upper limits of which show the $1^{st}$ and $3^{rd}$ quartiles of the data set, respectively. The fine lines projecting from the bottom and the top of the box and terminating with a bar represent the quartile range. Outliers are shown also.

Figure 5:

The ratios of cells stained in sections of granulation tissue from patients with AP (n=10) and EOP (n=10) and in sections of gingival tissue (Ging) from patients with AP (n=6), utilising the following markers: a) CD 20 (B cells), CD 45RO (memory T cells) and CD 68 (monocytes/macrophages) to the population of CD 3 positive cells (pan-T cells). b) CD 20 (B cells), CD 3 (T cells) and CD 68 (monocytes / macrophages) to the sum of the CD 20, CD 3 & CD 68 positive cells. The results are represented on the chart as the median value (black bar) surrounded by a box, the lower and upper limits of which show the 1$^{st}$ and 3$^{rd}$ quartiles of the data set, respectively. The fine lines projecting from the bottom and the top of the box and terminating with a bar represent the quartile range. Outliers are shown also.

**figure 1a** anti - CD20 monoclonal antibody

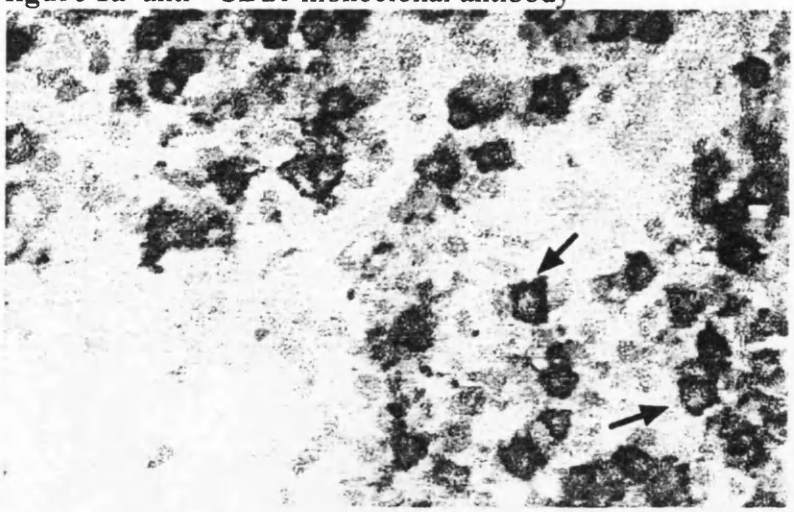


**Figure 1b** anti - CD68 monoclonal antibody

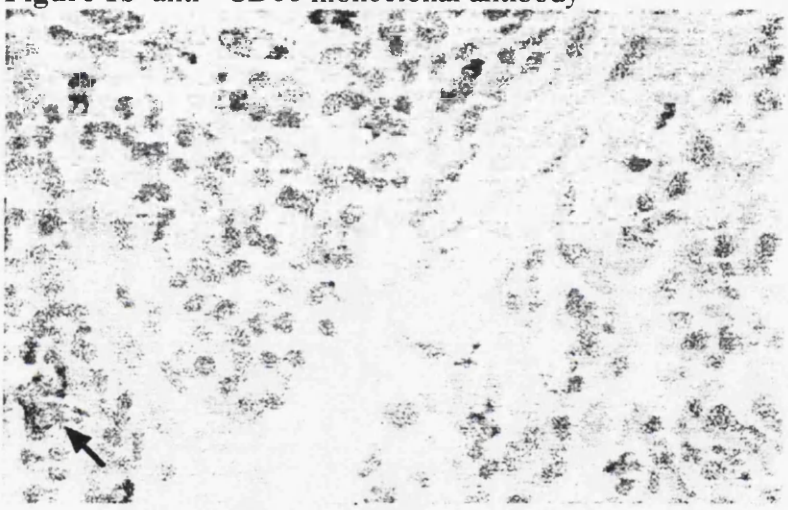


**Figure 1:** Immunohistochemical staining of **(a)** CD20 (B cells) and **(b)** CD68 (monocyte/ macrophages) in the corresponding fields in adjacent sections of granulation tissue biopsies (magnification x400).

**Figure 2a** anti - CD3 monoclonal antibody

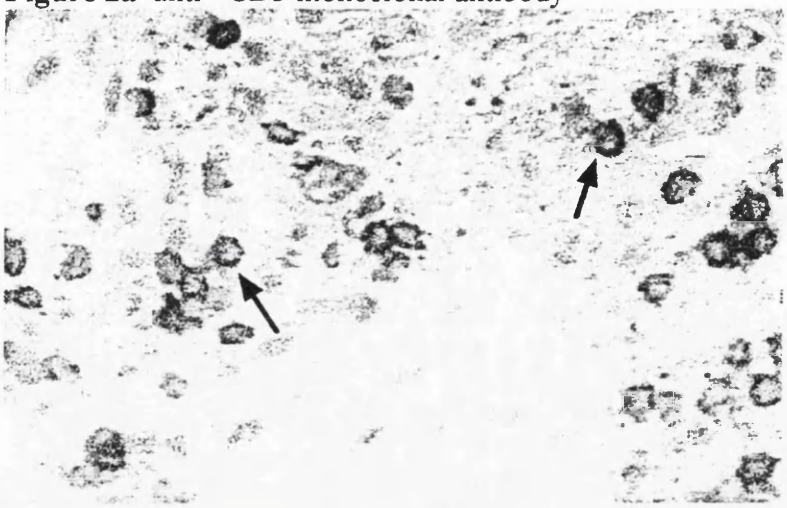


**Figure 2b** anti - CD45RO monoclonal antibody

**Figure 2** Immunohistochemical staining of T cells; **(a)** CD3 (pan T cells) and **(b)** CD45RO (memory T cells) in the corresponding fields in sections of granulation tissue biopsies (magnification x400).

**Figure 3a** anti - CD4 monoclonal antibody

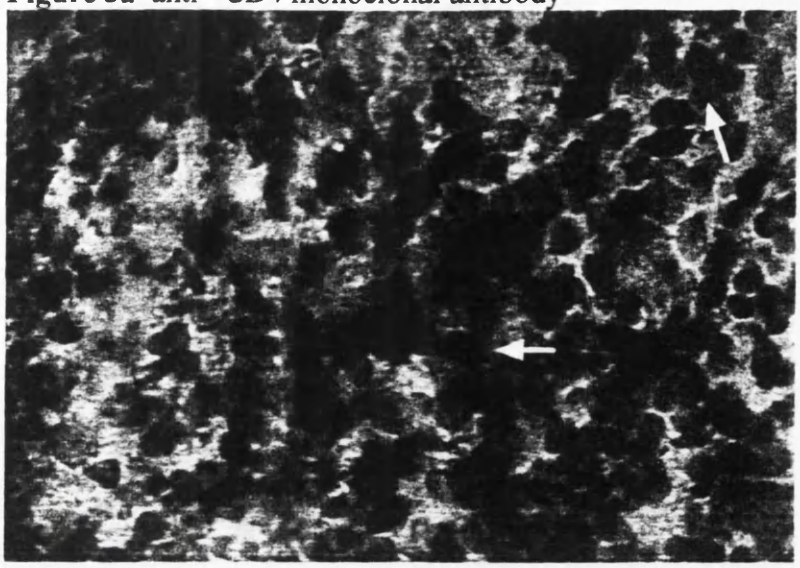


**Figure 3b** anti - CD8 monoclonal antibody

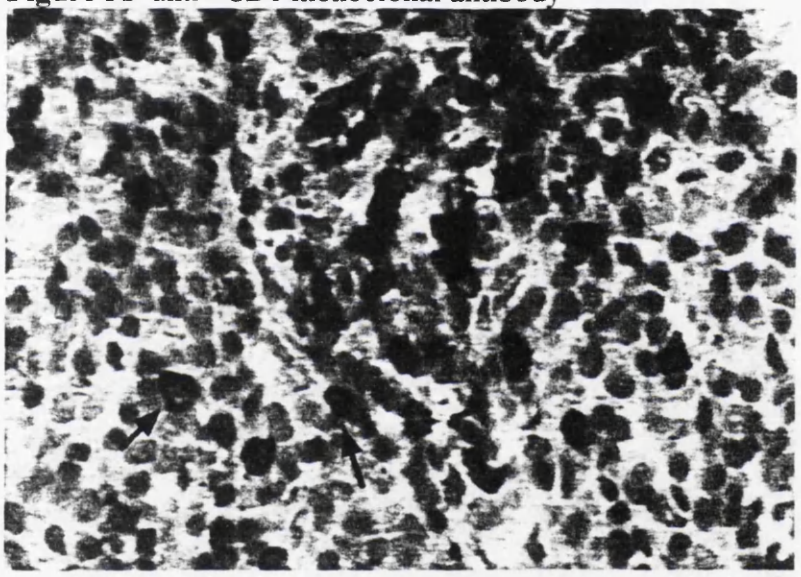
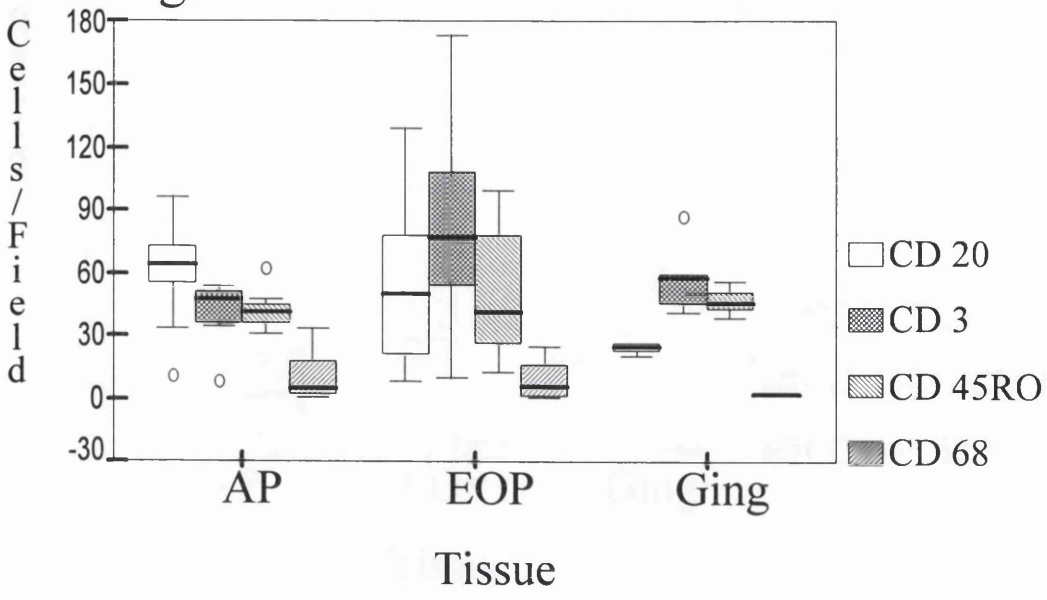


**Figure 3:** Immunohistochemical staining of **(a)** CD4 and **(b)** CD8 reveals more numerous T helper/ inducer than T cytotoxic/ suppressor cells in corresponding fields of adjacent sections of the granulation tissue biopsies (magnification x400).

Fig. 4a


Cells/Field
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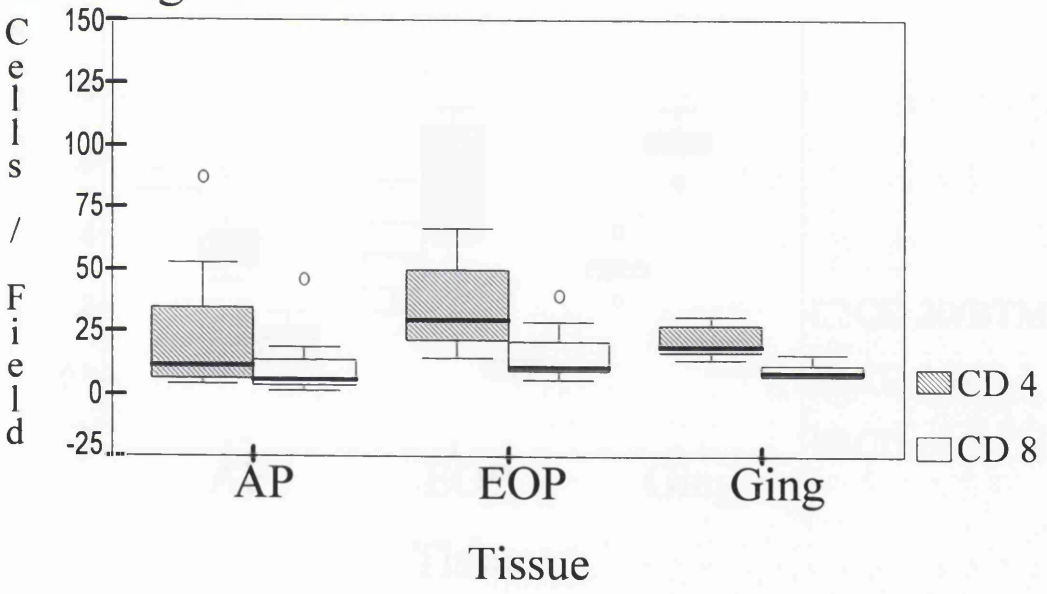
150
120
90
60
30
0
-30
AP
EOP
Ging
Tissue
CD 20
CD 3
CD 45RO
CD 68

Fig 4b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125
100
75
50
25
0
-25
AP
EOP
Ging
Tissue
CD 4
CD 8

Fig. 5a

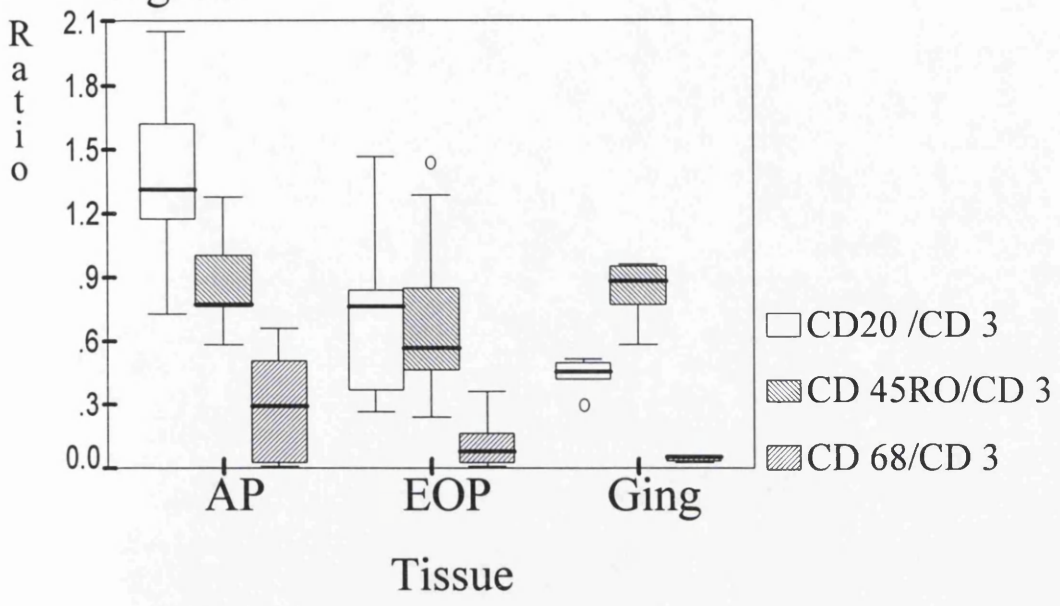
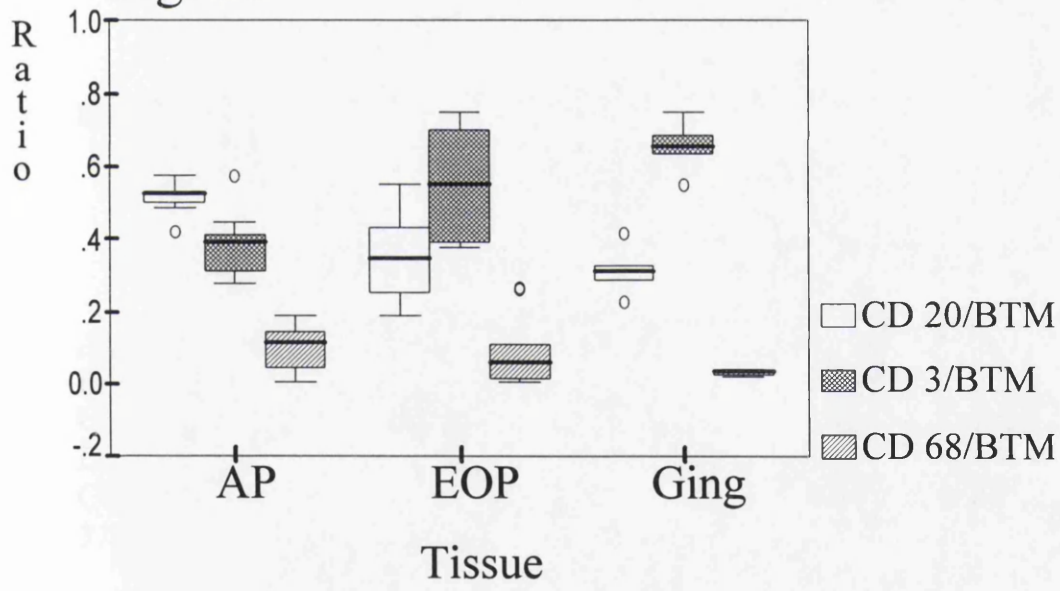

Ratio
2.1
1.8
1.5
1.2
.9
.6
.3
0.0
AP
EOP
Ging
Tissue
CD20 /CD 3
CD 45RO/CD 3
CD 68/CD 3

Fig. 5b


Ratio
1.0
.8
.6
.4
.2
0.0
-.2
AP
EOP
Ging
Tissue
CD 20/BTM
CD 3/BTM
CD 68/BTM

# Differential Expression of J Chain and Immunoglobulin M, and A & G Subclasses mRNA by Plasma Cells in Periodontal disease

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**Running title:** IgG and IgA subclass-specific mRNA in periodontitis

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**Key words:** IgG and IgA subclass mRNA, *in situ* hybridisation

**Abbreviations:** EDTA - ethylene diamine tetracetic acid; PBS - phosphate buffered saline; BSA - bovine serum albumin; Ig - immunoglobulin

**Abstract**

The humoral immune response, especially immunoglobulin G (IgG) and IgA, is considered to have a protective role in the pathogenesis of periodontal disease but the precise mechanisms are still unknown. In order to understand better the local Immunoglobulin production, we examined the local periodontal tissue expression of IgM, IgG, IgA, IgE and IgG and IgA subclasses and J chain. We detected plasma cells expressing mRNA encoding these proteins by *in situ* hybridisation, using digoxigenidigoxygenin and Biotin-labelled oligonucleotide probes in biopsies of periodontal granulation tissues. Granulation tissue biopsies from 9 patients with adult periodontitis (AP), 10 patients with early onset periodontitis (EOP) and for comparative purposes biopsies of gingival tissue from 8 of the patients with AP were used in this investigation. IgM, IgG subclass and IgA subclass mRNA-expressing cells were detected in all serial formalin-fixed/paraffin-embedded tissue sections sampled. Whereas, the cells expressing the mRNA encoding the J-chain were found in 13/19 granulation tissues and all 8 of the control gingival tissue biopsies, IgE was found in only 8/19 granulation tissues and 3/8 gingival tissues. Plasma cells showed strong cytoplasmic staining with a high contrast and a good retention of morphology with these probes. These data confirm that IgM, and IgG and IgA subclass proteins can be locally produced in the periodontitis granulation tissues. There was a reduced number of IgM expressing plasma cells with a sinificantly increased proportion of IgA expressing plasma cells in the gingiva ($P<0.01$) with plasma cells, the increase in IgA expression was largely made up of an increased proportion of IgA2 expressing plasma cells. Many of these cells also expressed the J chain mRNA. IgG1 mRNA-expressing cells were predominant in the granulation tissues and in the gingiva, constituting aproximately 65% of the total IgG expressing plasma cells, while cells that expressed IgG2 mRNA made up 24% and cells that expressed IgG3 mRNA or IgG4 mRNA were were present at aproximately 5% each, in the

granulation and gingival tissues.. The majority of the IgA expressing plasma cells in all of the tissue types expressed IgA1, but a greater proportion expressed IgA2 mRNA and J chain mRNA in the gingival tissues (30.5 % and 7.5% respectively) than in the periodontal granulation tissues (19% and 0-4% respectively), where most of the J chain expressing cells also expressed IgA2 mRNA. A higher proportion of IgA2 expressing plasma cells also expressed J chain mRNA in the gingiva (25.3%) than in granulation tissues (range 0-18%). The J chain expressing plasma cells were located mainly in the lamina propria, interdigitating between the Rete pegs and adjacent to the epithelial cells. This implies that IgA2 containing the secretory piece is produced mainly by gingival plasma cells i.e. those close to a mucosal surface or mucosal epithelium and that this tissue thus demonstrates features of a mucosal immune response.In contrast, deeper tissues more plasma cells that express IgM, and display a response which appears more akin to the systemic immune response. In conclusion this study demonstrates that immune mechanisms involved in pathogenesis of periodontitis may involve features of the mucosal and systemic immune systems depending upon the tissue location.

## Introduction

It has been suggested that the humoral immune response has a protective role in the pathogenesis of periodontal disease. Alterations in specific IgG and IgA responses both locally at inflammed sites and systemically have relevance in disease progression (Ebersole et al. 1992, Mooney et al. 1995), although the exact mechanisms are complex and poorly understood.

Previous work has shown that gingival crevicular fluid (GCF) levels of IgG may be reduced in active and deep periodontal pockets when compared to other sites in chronic periodontitis patients (Mooney et al. 1995). This finding, and more recent findings for IgA levels in GCF, suggest that GCF Igs may indicate "high risk" sites for periodontitis (Takahashi et al. 1997a). In these studies, the relative distribution of IgG and IgA isotypes was not investigated, nor was the relative contribution of local and serum antibodies to the GCF Ig profiles. GCF antibodies are both serum derived (Ebersole et al. 1986; 1987 ; Danielsen et al. 1993) and locally produced by the abundant plasma cells of the diseased periodontal tissue (Mackler et al 1978; Ogawa et al. 1989).

We have demonstrated that; there are numerous plasma cells in periodontitis gingiva and IgG-containing plasma cells predominate with lower numbers of IgA and a few IgM. as determined by *in situ* hybridisation (Takahashi et al 1997a). We have shown also the relative abundance of plasma cells expressing IgG and IgA subclass mRNA in gingival biopsies from periodontitis patients. To address the critisism raised by Moskow and Polson (1991); that experiments on superficial gingiva may not illustrate sufficiently the disease progression in the deeper granulation tissue. The present study, which is an continuation of our earlier study (Takahashi et al 1997a), was performed on on biopsies of gingival and periodontal granulation tissues, which contain a far greater number of infiltrating leukocytes and plasma cells than the inflammed gingiva. The presence of Ig specific plasma cells in periodontal granulation tissues determined by immunohistochemistry have

been reported (Mackler et al. 1978 ). However, it has been shown that the staining of surface and cytoplasmic Ig on B cells and plasma cells observed with fluorescence conjugated anti-sera could be confused with the binding of Ig complexes non-specifically via the Fc receptor (Winchester et al. 1975). Degradation or consumption of immunoglobulins and /or cross reactivity may also account for inaccuracies in protein detection.

Human IgG and IgA consists of four and two subclasses respectively, IgG1, IgG2, IgG3 and IgG4, IgA1 and IgA2, and the Ig heavy chain constant-region genes on chromosome 14 are 5'-:-*-(3-(1-"1-(2-(4-,-"2-3' (8, 9, 10, 19). Gamma genes exist linearly, whereas "1 and "2 genes are separated by considerable distances, with "2 being the most distal C gene from the 3' end. The exact mechanism by which human B cells class switch within IgG and IgA subclasses still remains unclear (Ellison et al 1982a; 1982b; Flanagan et al. 1982). Although extensive homology made it difficult to analyze the Ig subclasses both at protein and mRNA level, however, a number of recent studies have demonstrated that *in-situ* hybridisation techniques provide a method of IgG and IgA mRNA subclass detection without cross hybridisation (Islam et al. 1990; 1992; Sideras et al. 1991).

The anatomical location of the tissue suggests that either a mucosal, systemic immune response or a combination of both may occur. The gingival epithelium lays in close proximity to Gut associated lymphoid tissue (GALT) and Bronchus associated lymphoid tissue (BALT). However the gingival epithelium is also adjacent to skin, and has similarities in structure in that it is squamous and is partially keratinised. The study was carried out to determine the profile of immunoglobulin expression and determine whether the periodontal tissues demonstrated a mucosal or systemic humoral response or a combination of both.

## Materials and Methods

### Tissues

The tissues used in this study consisted of 10 biopsies of granulation tissue from 9 patients (ages 42-62 yrs) with adult periodontitis (AP) and 10 biopsies of granulation tissue from 10 patients (ages 24-39 yrs) with generalised early onset periodontitis (EOP). diagnostic criteria for EOP were according to Hart et al. (1991) and were as follows: Patients under the age of Thirty five years of age, exibiting attachment loss (AL) of at least 5mm on at least eight teeth at least three of which were not first molars or incisors and at least one of which was a first molar. All the patients included in the study had advanced forms of periodontitis, with the exception of one patient with AP who had moderate to severe disease. The pocket depths of the sites biopsied ranged from 6.0-9.0 mm in patients with AP and 5.8-9.6 mm in patients with EOP. We also included for comparative purposes 6 sections of gingiva from 4 patients with AP all taken from periodontitis sites. The tissues were removed under local anesthesia during routine periodontal surgery within the Glasgow Dental Hospital. The tissue sections were transferred to 10% neutral buffered formalin at room temperature and subsequently embedded in paraffin wax after which 5 μm serial sections were made onto silane-coated glass slides.. Serial sections were used for *in situ* hybridisation. Two sections in each sample were used for morphological examination which was based on haematoxylin and eosin (H&E) staining.

### Enzymatic pretreatment

All the solutions were prepared with 0.1% diethyl pyrocarbonate (Sigma) treated double distilled water or PBS. In brief, the slides were deparaffined in xylene, hydrated through descending ethanol, and washed in distilled water, then immersed in 0.2 M HCl at room temperature for 20 min to remove basic proteins. The slides were washed in PBS twice for 5 min and then digested

with proteinase K (0 - 50 μg/ml, Sigma) in 0.1 M Tris/HCl, pH 8.0, containing 50 mM EDTA for 30 min at 37 $^{o}$C. After immersing in 0.2% glycine/distilled water for 30 seconds, they were washed twice for 5 min in PBS. At this stage, pretreatment with RNase A type 1A (Sigma) at 10 μg/ml in 2 x standard saline-citrate (SSC)/ 10 mM $MgCl_2$ at 37 $^{o}$C for 1 h was performed as a negative control. Post-fixation was performed by incubation in 4% paraformaldehyde at 4$^{o}$C for 5 min, and then washed twice for 5 min in PBS. The slides were immersed in prehybridisation buffer (x2 SSC and 50 % formamide) for 2 h at 37 $^{o}$C.

**Probes**

Synthetic digoxigenin-labelled oligonucleotide anti-sense human IgG1, IgG2, IgG3 and IgG4 oligonucleotide probes were prepared (CCC AGG AGT TCA GGT GCT GGG CAT GTG TGA GTT TTG TC; TGG GCA CGG TGG GCA CTC GAC ACA ACA TTT GCG CTC AAC TGT; TGG GCA CCG TGG GCA TGT GTG AGT TGT AAG ATT TGG GCT C; AA CTC AGG TGC TGG GCA TGA TGG GGG ACC ATA, respectively) with reference to earlier literature (Elison et al 1982a; Flanagan et al. 1982; Islam et al. 1992; Sideras et al. 1991). Synthetic digoxigenin-labelled oligonucleotide anti-sense human IgA1 and IgA2 gene (CC GGA TTT TGA GAG GGT GGC GGT TAG CGG GGT CTT GGA CTC GGG GTA GGC: 50 bp long) were used. Underlined nucleotides show where the IgA1 probe differs from IgA2 probe. The absence of cross-hybridisation of the two probes was previously demonstrated by the Islam *et al.* (1990 ). The problem in identifying the four IgG and two IgA subclasses lies also in the high homology in their gene sequences (about 95%) . However, in the exons coding for the hinge regions of these proteins there is substantially less tah 60% homology (Takahashi et al. 1982).

**Labelling of oligonucleotides**

Initially the probe concentrations were adjusted following spectrophotometric measurement of their absorbance at 260 nm. The probes were then analyzed by polyacrylamide gel electrophoresis, and the resulting bands were visualized using ethidium bromide labeling and ultraviolet photography. The oligonucleotide probes were labelled with digoxigenin-11-dUTP or Biotin 16 dUTP (Boehringer Mannheim, Germany) according to the manufacturer's guidelines. Briefly, 1-2 μg probe was 3'-end labelled with a mixture containing 1.0 μl digoxigenin-11-dUTP or Biotin-16-dUTP, 4 μl 5 x reaction buffer (potassium cacodylate, 0.5 M pH 7.2; 10 mM $CoCl_2$ mM DTT), 2.0 μl 30 units terminal deoxynucleotidyl transferase (Gibco BRL, UK). The labelling reaction was done at 37 $^{o}$C overnight. Thereafter, the digoxigenin labelled probes were separated from unincorporated material using a Sephadex column (DNA grade, Pharmacia Biotech, Uppsala, Sweden). Ten 200 μl fractions were collected, 1 μl of each spotted on to nitrocellulose filters (Gelman Sciences, MI) and the filters were incubated in UV-cross linker (Anachem, Luton, UK). Digoxigenin labelled probes were detected following the manufacturer's instructions. Fractions containing labelled probes were precipitated with ethanol and the pellet dissolved in TE buffer and stored at -20 $^{o}$C. Biotinylated probes were detected using the Vecstain detection Kit (Vector Laboratories, Peterborough England).

***In situ* hybridisation**

Hybridisation mixtures were prepared and hybridisation was performed as described previously (Takahashi et al. 1995). After draining, the sections were hybridized with 20 μl volumes of the mixtures containing digoxigenin-labelled probes at a concentration of 0.16 - 0.65 ng/μl in the hybridisation buffer. The slides were covered with dimethyldichlorosilane-(BDH, UK)-coated coverslips that were sealed with rubber cement, and incubated at 37 $^{o}$C

overnight. The slides were rinsed in x4 SSC, sequentially, in x2 SSC at room temperature twice for 20 min, x0.1 SSC at 37$^{o}$C twice for 20 min, then washed in x2 SSC for 10 min at room temperature.

**Detection**

The detection of the digoxigenin labelled oligonucleotide - mRNA complex was performed as described previously (Takahashi et al. 1995). In summary, the sections incubated by Digoxgenin labelled probes were placed for 3 h in a PBSTM containing alkaline phosphatase (AP)-conjugated sheep anti-digoxigenin (1: 500; Boehringer Mannheim) at room temperature. After thorough rinsing in a PBST buffer and 5 min pre-incubation in an alkaline buffer solution, the AP complex was revealed with a freshly prepared solution of nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolylphosphate (Sigma) containing levamisole (Sigma) in an alkaline buffer solution for 8 h to overnight in a dark room at room temperature, and then washed in distilled water 3 times. Finally, the slides were counterstained with 1% neutral red, washed in distilled water 3 times and mounted in aqueous mounting medium (Dako). The dark blue/black precipitate, an indication of the presence of the target mRNA, was revealed with bright-field microscopy.

Section hybridised with Biotinylated probes were detected using the Vecstain detection Kit. The procedure was Briefly as follows; following high stringency washes the slides were incubated with A biotin avidin horseradish peroxidase (HRP) complex for 30 mins. Amplification was provided by incubating the slides with a biotinylated anti-avidin antibody for 60 mins. After which the HRP complex was added for a futher 30 minutes. The reaction was then developed with Diamino-benzidine substrate and hydrogenperoxide provided in the Kit.

**Controls for *in situ* hybridisation**

As a negative control, sections were incubated with hybridisation buffer only or sense probes. To determine the specificity of probe bound to tissues RNAs, sections were digested with RNAse prior to hybridisation. Another specificity controls were carried out by hybridizing with the labelled probe together with the unlabelled probe simultaneously. The concentration of the former was as above, but the latter was in 10-fold excess. For example, labelled IgA1 probe with 10-fold non-labelled IgA2 were used to check the specificity of the IgA1 probe and vice versa.

**Cell counts**

Briefly, five distinct microscopic fields which showed the strongest cell infiltration, from each serial section of all biopsies, were chosen for counting using a meshwork eyepiece (0.172 mm x 0.172 mm; 0.0296 $mm^2$). All the positively stained plasma cells were counted in each serial section. The percentage of each IgG subclass, IgA subclass and J chain as compared to the total IgG or IgA positive cells were also calculated (e.g. (IgG1/(IgG1 + IgG2 + IgG3 + IgG4)) x 100 %). The percentage of IgA1 subclass positive cells were also calculated (IgA1/(IgA1 + IgA2)) x 100 % . The percentage J chain expressing plasma cells were calculated as a percentage of the total IgA expressing plasma cells (J chain/(IgA1+IgA2)) x100 % and as a proportion of the IgA2 expressing plasma cells (J chain/ IgA2) x 100 %.

**Results**

Preliminary experiments verified the specificity of the IgG and IgA subclass-specific probes. These probes detected the presence of Plasma cells in tissue sections. The negative control sections incubated with a hybridisation solution without probe or with a sense probe, showed a complete absence of signal in plasma cells and lymphocytes, in all cases. Further results of the hybridisation staining procedure following treatment with RNase A were negative, confirming that the method specifically indicates the presence of RNA. In

addition, the hybridisation of the labelled probes was greatly reduced or abolished when the concentration of unlabelled probe against the same subclass was at ten-fold in excess. In contrast, the hybridisation of the labelled probes was unaffected when the concentration of unlabelled probe against another subclass was ten-fold in excess. (data not shown).

In all biopsy tissues, cytoplasmic staining was observed in plasma cells for IgG (Figure 1) and IgA subclass (Figure 2) and IgM probes in serial sections (Figure 3). Only a proportion of the tissues were positive for IgE (Figure 3), that is 3 of the 8 gingival sections and 8 of the 20 granulation tissues. All of the gingival tissues were positive for J chain expression (Figure 4), but only 13 of the 19 granulation tissues stained. The majority of immunoglobulin positive cells were observed in the lamina propria and subadjacent to the epithelium. In contrast, the vast majority of J chain expressing plasma cells were found adjacent to the epithelial cells with relatively few in the lamina propria or the deep granulation tissue (Figure 4).

In the gingival tissues as well as in the periodontal granulation tissues the major IgG subclass mRNA expressing cells was IgG1 followed by IgG2 of total IgG plasma cells and IgG3 and IgG4 were present to a much lesser extent in fairly similar numbers (Table 1). There were no differences in the proportions of plasma cells expressing the IgG subclass between all the tissue types (Table 2).

Significantly greater numbers of IgM ($p<0.01$) were observed in the AP granulation tissue than in the gingival tissue, but there were no significant differences in the total number of IgG or total number of IgA expressing plasma cells in these tissues. However there was a difference in the relative proportions of each plasma cell type beteen the AP granulation tissue and AP gingiva. The ratio of IgG mRNA to IgA mRNA to IgM mRNA positive plasma cells in AP granulation tissue and gingival tissue was approximately 71: 21 : 3 and 75: 30: 1 respectively. the change in the relative proportion of IgA expressing cells was because the numbers of plasma cells expressing IgA2 subclass and J chain were significantly greater in the gingiva ($p<0.01$)

than in deep AP granulation tissue. Epithelial cell layers were not always evident in the biopsies of granulation tissue. However, it is clear that when junctional epithelium was present in the biopsy that the J chain expressing plasma cells were found in close proximity. The double label detection of J chain and Ig expression confirmed that the vast majority of J chain mRNA expressing plasma cells were also expressing the mRNA encoding IgA2. None of the IgM or IgA1 expressing plasma cells were positive for J chain mRNA and non of the IgG expressing cells expressed the J chain mRNA (data not shown).

IgA1 mRNA-expressing cells were the predominant IgA expressing plasma cell in the gingiva (69%) and the periodontal granulation tissue (AP 81%; EOP 80%) than IgA2 mRNA-expressing cells.(Table 1) There was a statistical difference in the ratio of IgA1: IgA2 expressing cells between the gingival tissue and the AP granulation tissues ($p < 0.01$) but not between the EOP and AP groups. The proportion of J chain expressing plasma cells (expressed as a percentage of the total IgA or IgA2 expressing cells ) was no greater in the gingival sections, when they were compared exclusively with the areas adjacent to the junctional epithelium of AP tissue sections.

Gingival tissue sections used in this study gave the strongest hybridisation signals with a proteinase K concentration of 30-50 μg/ml and showed good morphological detail at this concentration, while the proteinase K digestion at less than 10 μg/ml gave very weak or no signal. In contrast proteinase K concentrations of 15-30 μg/ml gave the best results on the granulation tissues. Counterstaining with 1% neutral red gave better contrast than hematoxylin (blue color) with the alkaline phosphatase staining, as the precipitation colour of NBT with BCIP is blue black. Counterstaining with hematoxylin 1% was preferred with the the peroxidase detection, which gave a brown colour. When double label detection was performed no counterstaining was used to avoid masking the blue black, alkaline phosphatase or the brown peroxidase products.

**Discussion**

In this study we have detected immunoglobulin class and subclass-specific mRNA-expressing plasma cells in inflamed gingival tissues and granulation tissues from patients affected with periodontitis. We have demonstrated both features of a systemic immune response ,as IgG expressing plasma cells are predominant in the lesion, and a mucosal immune response, because plasma cells expressing the secretory form of IgA are present immediately below the epithelial cell layers. These results also suggest the that class switching pathways maybe induced at periodontitis sites and this finding may help to elucidate our understanding of the mechanisms of B cell differentiation at these sites. An alternative explanation would be that the switching occurs in local lymph nodes and then committed B cells home to gingival tissues, as we were unable to detect proliferating B cells in these lesions in our earlier study (Takahashi et al. 1997).

IgG1 mRNA-expressing plasma cells was the major subclass of IgG mRNA bearing plasma cell detected, which is similar to previous reports (Mackler et al 1978; Takahashi et al 1997a), and levels reported for normal tissues (Jeffries et al. 1985) and synovium from patients with rheumatoid arthritis (Haber et al. 1985). The subclass distribution of IgG antibodies produced as a consequence of bacterial infection varies according to the nature of the eliciting antigens. Protein antigens induce mainly IgG1 antibodies, with small amounts of IgG3 and IgG4 antibodies (Brandtzaeg et al. 1986; Hammarstrom & Smith 1986). In contrast, IgG antibodies reactive to carbohydrate antigens, including lipopolysaccharide (LPS), are predominantly IgG2. Since certain antigens elicit an Ig response largely confined to one IgG subclass (Mayuma et al. 1983), the distribution of IgG and IgA subclasses in gingiva may provide clues to pathogenic mechanisms. A major difference was observed in the relative proportions of plasma cells expressing the immunoglobulin classes. There were a relatively higher proportion of IgA mRNA expressing plasma cells in the gingiva than in the granulation tissues. Of the IgA expressing plasma cells a far higher proportion of IgA2 and J chain expressing plasma

cells was observed in the sub epithelial layers of the gingiva than in the granulation tissue, although IgA1 mRNA producing plasma cells were still the predominant IgA producing plasma cell in this region. Gingival tissue appears to have a similar IgA1 to IgA2 ratio to that seen in other mucosal tissues (Mestecky & Russell 1986; Moldoveanu et al. 1988). The proportion of IgA2 to IgA1 expressing plasma cells were greater in the gingival tissues than in the periodontal granulation tissues. The percentage of IgA2 expressing plama cells was still higher in the periodontal granulation tissue than in the circulation or in periapical lesions, where they accounted for less than 10% of the IgA expressing plasma cells (Takahashi et al 1997b). The expression of J chain mRNA was observed predominantly in IgA2 expressing plasma cells, and almost exclusively located in cells that were adjacent to the epithelial cell layers. The predominance of IgA2 subclass in the secretory form of IgA has been demonstrated by Brandtzaeg et al (1986). IgA2 expressing plasma cells that were present in the deeper tissue layers were essentially negative for J chain mRNA expression, the few examples of J chain mRNA expressing cells that were present in deeper tissues were found in blood vessels. We did not see any examples of plasma cells that expressed both IgA1 mRNA and J chain mRNA. However, that does not mean that plasma cells expressing IgA1 are negative for J chain expression, however, our results have confirmed previous observations (Brandtzaeg et al 1986). In a recent report from our laboratory it was shown that the numbers of J chain expressing plasma cells in periapical lesions was very low, although radicular cysts seemed to express higher levels than granulomas (Takahashi et al. 1997b). We did detect IgM mRNA expressing plasma cells in the tissues. These were found predominantly in the deep tissue layers and seemed to be mostly negative for J chain expression. It has been shown previously that a predominance of IgA1-producing cells (65.1%) are observed in periodontitis gingiva (Takahashi et al. 1997a). In that study IgA1-producing cells were detected in equal proportions to IgA2 in 5 out of the 24 sections, however it should be pointed out that 2 of these sections were from same subject. In the

present study the proportion of IgA2 plasma cells was only slightly, but not significantly less than the proportions observed in the earlier study.

The gingival tissues are exposed to a heavy bacterial load, and IgA2 appears to be resistant to several bacterial proteases which can cleave IgA1 (Frandsen et al. 1995). Therefore, increased IgA2 production would appear to be advantageous in the periodontitis gingiva possibly aiding the killing of bacteria in the GCF. Our present results although they agree with our earlier study (Takahashi et al 1997a) are not in accordance with the reports from other groups on the percentage of IgA1-producing cells determined by immunofluorescence (Kilian. et al 1989) or enzyme-linked immuno spot (ELISPOT) assay (Ogawa et al. 1989a; 1989b) which have reported proportions of more than 90%, similar to those seen in serum (Skvaril & Morell 1973). In an ELISPOT assay (Ogawa et al. 1989a), gingival mononuclear cells were isolated and cultured for 3h before the assay, their systems was unlikely to contain proteases which could degrade IgA1 produced in their *in vitro* system. Although we could not quantify the amount of IgA subclass mRNA expression in each cell We were able to count the cell numbers directly, the rate of IgA1 subclass secretion may be higher than that of IgA2. Immunohistochemical studies for Ig in inflamed sites are often unreliable due to high background staining. Furthermore, Fc receptors on the surface of cells may bind the complexes formed in the detection process unless Fab fragments have been used.

IgA antibodies specific for protein antigens are found predominantly in the IgA1 subclass, while specific antibodies to LPS and lipoteichoic acids are predominantly of the IgA2 subclass (Mestecky & Russell 1986; Moldoveanu et al.1988). Therefore the distribution of IgA1- and IgA2-producing cells in inflamed periodontium may reflect a difference in the predominant type and nature of antigens involved in the induction of the local humoral immune response in periodontium. The relative abundance of IgA1 or IgA2 expressing cells in inflammed periodontal tissues may be due to differences in the original precursors of IgA plasma cells destined to populate the

periodontium, selective homing of specific B cells and/or the local expansion of specific B cell clones by various antigens that induce preferential IgA1 or IgA2 responses ( Semour et al 1993).

There is a possibility that IgG producing plasma cells from the systemic circulation arrive at the gingiva and undergo a class switching event to become IgA plasma cells. We have no evidence to confirm or refute this possibility, in the gingiva or granulation tissues. Protein antigens from periodontal bacteria or their products may induce one subset of B cells to switch subclasses that are clustered in the 5' region of the Ig heavy chain gene. Another switching pathway of Ig constant regions may occur in the 3' region (γ2- γ-4- ε -α2-3') which seem to be predominantly involved in LPS-specific responses. It has been reported that the protein and lipid carbohydrate antigens of *P. gingivalis* induce two distinct patterns of IgG and IgA subclass responses in inflamed gingiva (Ogawa et al. 1989a). Therefore it is possible that the change in IgG and IgA subclass obtained is due to the change of microbial antigens or possible other mitogens involved at various stages of periodontitis.

B cell differentiation and the regulation of the switch to different Ig isotypes is cytokine driven (Kishimoto & Hirano 1988; Kitani & Strober 1994). TGF-β1 and/or IL-4 increase the level of a germ-line transcript, and then B cells are induced to switch from membrane-$IgM^+$ to membrane-$IgA^+$ (Wakatsuki & Strober 1993). We do see a fall in the proportion of IgM expressing plasma cells in the gingiva, and an increased proportion of secretory IgA expressing cells. TGF-β1 but not IL-4 mRNA was detected by the reverse transcription-polymerase chain reaction method in inflamed gingival tissues (Lundqvist et al. 1994). Therefore TGF-β1 could influence IgA subclass switching in periodontitis gingiva although we have not been able to detect TGF-β1 mRNA using the *in situ* hybridisation method described in this study (data not shown). However we have occasionally detected some IL-4 expressing T cells in our sections (unpublished observation). Thus a mechanism of this type could be in opperation in diseased periodontal tissue. Recently it has been

reported that germ-line transcripts are relevant to the Ig class and isotype switch recombination in the *in vivo* immune response in humans (Islam et al. 1994). In addition to the influence of cytokines, direct cell to cell interaction may also be involved in isotype switching mechanisms. IgA subclass-specific mRNA bearing plasma cells were seen in sections as discrete cells, i.e. not clustered. We have demonstrated that B cells do not proliferate , but accumulate in the tissues. IgA mRNA bearing plasma cells, however, were often noted in contact with IgG mRNA expressing plasma cells, as detected by double-target *in situ* hybridisation.

The findings of this study support previous work in this area, and also demonstrate that the immune mechanisms involved in periodontal disease are influenced by tissue location, i.e. components of both systemic and mucosal immune responses are involved in periodontal disease. There are definite differences in the components of humoral immunity within diseased periodontal tissues. The gingival margin seems to have retained many features of a mucosal immune response, whereas the deeper granulation seems to be more predominantly systemic. What this means for the disease process is yet to be elucidated. Future studies will no doubt shed more light on these aspects of the immune and inflammatory mechanisms involved in periodontitis.

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| Tissue | IgG | Stdev | Cells/field IgA | Stdev | IgM | Stdev |
|---|---|---|---|---|---|---|
| Granulation | 216 | 32 | 63 | 8 | 9 | 1 |
| Gingival | 225 | 28 | 90 | 9 | 3 | 1 |
| | | | P=0.043 | | P=0.009 | |

| Tissue | IgG | Stdev | Ratios IgA | Stdev | IgM | Stdev |
|---|---|---|---|---|---|---|
| Granulation | 72 | 11 | 21 | 2 | 3 | NA |
| Gingival | 75 | 9 | 30 | 3 | 1 | NA |
| | | | P=0.043 | | P=0.009 | |

| Tissue | IgG1 | | IgG Subclasses IgG2 | | IgG3 | | IgG4 | |
|---|---|---|---|---|---|---|---|---|
| Granulation | 65.3% | 4.7% | 25.1% | 6.8% | 4.0% | 2.5% | 5.6% | 2.3% |
| Gingival | 63.1% | 3.4% | 22.8% | 4.2% | 3.3% | 0.7% | 9.8% | 1.6% |
| Serum conc | 65% | | 23% | | 8% | | 4% | |

| Tissue | IgA1 | Stdev | IgA Subclasses IgA2 | stdev | |
|---|---|---|---|---|---|
| Granulation | 81.4% | 4.3% | 18.6% | 4.3% | P=0.035 |
| Gingival | 68.7% | 1.3% | 31.3% | 1.3% | |
| Serum conc | 90% | | 10% | | |

| Tissue | % of J chain +ve Total IgA | Stdev | IgA2 | | |
|---|---|---|---|---|---|
| Granulation | 1.2% | 1.0% | 6.0% | 5.1% | P=0.019 |
| Gingival | 7.6% | 1.9% | 25.3% | 7.8% | |

| | | | Cells/field | | | |
|---|---|---|---|---|---|---|
| Tissue | IgG | Stdev | IgA | Stdev | IgM | |
| Granulation | 216 | 32 | 63 | 8 | 9 | 1 |
| Gingival | 225 | 28 | 90 | 9 | 3 | 1 |
| | | | P=0.043 | | P=0.009 | |

| | | | Ratios | | | |
|---|---|---|---|---|---|---|
| Tissue | IgG | Stdev | IgA | Stdev | IgM | Stdev |
| Granulation | 72 | 11 | 21 | 2 | 3 | NA |
| Gingival | 75 | 9 | 30 | 3 | 1 | NA |
| | | | P=0.043 | | P=0.009 | |

| | | | IgG Subclasses | | | | | |
|---|---|---|---|---|---|---|---|---|
| Tissue | IgG1 | | IgG2 | | IgG3 | | IgG4 | |
| Granulation | 65.3% | 4.7% | 25.1% | 6.8% | 4.0% | 2.5% | 5.6% | 2.3% |
| Gingival | 63.1% | 3.4% | 22.8% | 4.2% | 3.3% | 0.7% | 9.8% | 1.6% |
| Serum conc | 65% | | 23% | | 8% | | 4% | |

| | | | IgA Subclasses | | |
|---|---|---|---|---|---|
| Tissue | IgA1 | Stdev | IgA2 | stdev | |
| Granulation | 81.4% | 4.3% | 18.6% | 4.3% | P=0.035 |
| Gingival | 68.7% | 1.3% | 31.3% | 1.3% | |
| Serum conc | 90% | | 10% | | |

| | % of J chain +ve | | | | |
|---|---|---|---|---|---|
| Tissue | Total IgA | Stdev | IgA2 | | |
| Granulation | 1.2% | 1.0% | 6.0% | 5.1% | P=0.019 |
| Gingival | 7.6% | 1.9% | 25.3% | 7.8% | |

# Cell division, Synthetic capacity and Apoptosis in Periodontal Lesions Analysed by *In Situ* Hybridisation and Immunohistochemistry

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Running title:

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## Abstract

In this study we investigated the synthetic and proliferative activity of infiltrating mononuclear cells in sections of granulation tissue from periodontitis lesions in both adult periodontitis (AP) and early onset periodontitis (EOP) patients. We also investigated the role of Apoptosis in the remodeling of the inflammed tissue.

We also used a Ki-67 antigen specific antibody and a histone messenger RNA (mRNA) probe to detect cells undergoing cell division in the sections. Furthermore, we used oligonucleotide probes for 28S ribosomal RNA and for the detection of poly A mRNA to detect cells with synthetic capacity. Apoptosis was determined using terminal transfease labelling of fragmented DNA with Biotin labelled dUTP.

Biopsies of granulation tissue were obtained from 9 patients with adult periodontitis (AP), from 10 patients with early onset periodontitis (EOP) and for comparative purposes, biopsies of gingival tissue from 4 patients with AP. There were no differences regarding the relative proportions cells with synthetic capacity or in the numbers of dividing cells in the periodontitis tissue sections. Apototic cells consisted mainly conective tissue cells; fibroblasts and some epithelial cells. few if any leukocytes were Apoptotic. However, we observed an increase in the number of dividing cells in the granulation tissues compared to the gingival sections.

## Introduction

Complex inflammatory and immune responses are involved in the progression of periodontitis. Tissue activity within the diseased periodontium comprises epithelial and connective tissue turnover and the cellular activity associated with the infiltrating inflammatory cells [1]. Previous work has suggested that B cells and T cells accumulate in large numbers in the periodontal tissues although until recently we have had little information on cellular synthetic activity and proliferation of these cells.

Using *in situ* hybridisation methodology we have recently investigated the cellular activity in gingivitis lesions. The messenger RNA (mRNA) content of various cells *in vivo* was assessed with synthetic oligonucleotides [13], we used poly-deoxyribothymidine oligo d(T) to detect the poly-adenosine (poly-A) sequences of messenger RNA (mRNA)[14]. Since, the number of total mRNA molecules and their species distribution is dependant on the level of the cell activity, including the protein production of the cell, and its stage in the cell cycle [15,16]. We were able to show that the plasma cells were among the most active secretory cells in the gingiva. Fibroblasts and endothelial cells were moderately stained, whereas lymphocytes were weakly stained or not stained at all. The detection of ribosomal RNA (rRNA) yielded the same results, because rRNA is present in every cell that synthesises proteins, in quantities related to the synthetic activity of cells [17].

Gemmell and Seymour [18] have shown an increased proportion of γδ T cells with increasing size of infiltrate in diseased gingival tissues. Others [19] have shown an increase in γδ T cell numbers in peripheral blood and it was suggested that homing of these cells to the gingiva may occur during the disease process. However, an alternative possibility exists, that specific T cells proliferate locally within certain tissues (in response to local antigenic stimulation) giving rise to characteristic T cell clones for these regions. Clearly, information on leukocyte proliferation in the diseased gingival tissues may solve the debate as to whether specific leukocytes predominate in the gingival tissue through selective homing mechanisms or by proliferation.

In our recent study on periodontitis gingiva we demonstrated that the infiltrating lymphocytes were more likely to arise through selective homing than by local cell

division [21]. We used an oligonucleotide probe to detect histone mRNA and a specific murine monoclonal to detect Ki-67. Histone is a short-lived cytoplasmic protein which is significantly upregulated during the S-phase of the cell cycle [22,23]. The synthesis of histone protein is coupled with DNA replication and the presence of its mRNA accurately defines proliferating cells within the S-phase of the cell cycle. In contrast, Ki-67 is produced in the nuclei of cells during $G_1$, S, $G_2$ and M phases of the cell cycle, but is absent from resting stage $G_O$ and is a well-recognised nuclear proliferation marker [24,25,26].

The purposeof this study was to extend our investigations on the diseased periodontitis gingiva and examine the cell proliferation activity within the diseased periodontitis granulation tissue in order to determine if lymphocytes proliferate in the inflamed tissue or proliferate at a distant site and then home back to these tissues. By examining cell synthetic activity we aimed to show the activity of plasma cells, B cells and T cells within the periodontal tissue by molecular means. In this study, leukocyte synthetic activity and proliferation were demonstrated using *in situ* localisation of poly (A), 28S rRNA, histone mRNAs and Ki-67 antigens in the periodontitis gingiva. In addition, the localisation patterns of T cells, B cells and monocyte/macrophage were also investigated by immunohistochemistry using specific monoclonal antibodies raised to cluster of differentiation (CD) antigens. Which are known to be specific for these cell types [27,28,29,30]. The AP and EOP sections were also categorised into their diagnostic groups to look for potential differences in their pathogenesis.

## Materials and methods

### Tissue preparation

The tissues used in this study consisted of 10 biopsies of granulation tissue from 9 patients (ages 42-62 yrs) with adult periodontitis (AP) and 10 biopsies of granulation tissue from 10 patients (ages 24-39 yrs) with generalised early onset periodontitis (EOP). diagnostic criteriafor EOP were according to Hart et al [31] and were as follows: Patients under the age of Thirty five years of age, exibiting attachment loss (AL) of at least 5mm on at least eight teeth at least three of which were not first molars or incisors and at least one of which was a first molar. All the patients included in the study had advanced forms of periodontitis, with the exception of one patient with AP who had moderate to severe disease. The pocket depths of the sites biopsied ranged from 6.0-9.0 mm in patients with AP and 5.8-9.6 mm in patients with EOP. We also included for comparative purposes 6 sections of gingiva from 4 patients with AP all taken from periodontitis sites. The tissues were removed under local anesthesia during routine periodontal surgery within the Glasgow Dental Hospital and immediately fixed in 10% neutral buffered formalin at room temperature. They were embedded in paraffin wax and 5 μm serial sections were collected on silane-coated glass slides. Serial sections were used for *in situ* hybridization and immunohistochemistry. Two sections in each sample were used for morphological examination which was based on haematoxylin and eosin (H&E) staining before *in situ* hybridization and immunohistochemistry.

### Oligonucleotide probes

Digoxigenin-labelled oligo d(T) oligonucleotide (30-mer) and fluorescein-labelled histone probe cocktails were purchased from R&D Systems Europe LTD (Abingdon, UK) and Novocastra Laboratory (Newcastle upon Tyne, UK), respectively. To detect rRNA, we used the 28S rRNA oligonucleotide probe (34-mer) as described previously [17] which was synthesized using the cyanoethyl phosphoramidite method on an applied biosystems automated DNA synthesizer. The 28S rRNA probe was labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) according to the manufacturer's guidelines. Briefly, 1-2 μg probe was 3'-end labelled with a mixture containing 1.0 μl digoxigenin-11-dUTP, 4 μl 5 x reaction buffer (potassium cacodylate, 0.5 M pH 7.2; 10 mM $CoCl_2$ mM DTT) and 2.0 μl 30 units terminal deoxynucleotidyl transferase (TdT;

Gibco BRL, UK). The labelling reaction was performed at 37 °C for 3 - 5h. Labelled probes were then purified through a Sephadex column (DNA grade, Pharmacia Biotech, Uppsala, Sweden) and probe labelling was confirmed by dot-blot analysis. The labelled probes were precipitated with ethanol and the pellet dissolved in TE buffer (0.01M Tris/HCl, 0.01M EDTA, pH 7.5) and stored at -20 °C.

***In situ* hybridization**

All solutions were prepared with 0.1% diethyl pyrocarbonate (Sigma) treated double-distilled water (DDW) or phosphate buffered saline (PBS).

Briefly, slides were deparaffinised in xylene, hydrated through descending isopropyl alcohol concentrations, and washed in DDW. The slides were washed in PBS twice for 5 min and then digested with proteinase K (Sigma;15 μg/ml, for the granulation tissue and 60 μg/ml for the pieces of gingiva) in dilution buffer (0.1 M Tris/HCl, 50 mM EDTA, pH 8.0) for 30 min at 37 °C. They were washed twice for 5 min in PBS and, at this stage, pretreatment with RNase A type 1A (Sigma) at 100 g/ml in 2 x standard saline-citrate (SSC)/ 10 mM $MgCl_2$ at 37°C for 1 h was performed as a negative control. Post-fixation was performed by incubation in 4% paraformaldehyde at 4°C for 5 min, followed by washing twice for 5 min in PBS. The slides were immersed in prehybridisation buffer (x2 SSC and 50% formamide) for 2 h at 37°C.

Hybridization mixtures for oligo d(T) and 28S rRNA probes were prepared and hybridization was performed as described in a previous study [21]. Hybridization for fluorescein-labelled histone probes was performed according to manufacturer's guidelines (Novocastra Laboratories LTD). As a negative control, sections were incubated with hybridization buffer only or with sense probes. To determine the specificity of probe binding to tissue RNAs, sections were digested with RNase A prior to hybridization. The slides were rinsed in x4 SSC, sequentially, in x2 SSC at room temperature twice for 20 min, x0.1 SSC at 37 °C twice for 20 min, then washed in x2 SSC for 10 min at room temperature.

**Immunological detection**

The immunological detection of the digoxigenin or fluorescent labelled oligonucleotide-mRNA complex was performed as described in previous reports [13,21]. In summary, the slides were placed for 3h in PBS containing 0.01 % Tween 20 (Sigma, PBST) and 5 % non-fat dry milk (PBSTM) including alkaline phosphatase (AP)-conjugated sheep anti-digoxigenin (1: 100; Boehringer Mannheim) or AP-conjugated anti-FITC antibodies (Novocastra Laboratory LTD) at room temperature. After thorough rinsing in a PBST buffer and 5 min pre-incubation in an alkaline buffer solution, the AP complex was revealed with a freshly prepared solution of nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolylphosphate (Sigma) including levamisole (Sigma) in an alkaline buffer solution for 30-60 min for oligo d(T) and 28S rRNA probes and overnight for histone probe in dark at room temperature, and then washed in DDW 3 times. Finally, the slides were counterstained with 1% neutral red, washed in DDW 3 times and mounted in aqueous mounting medium (Dako).

**Immunohistochemistry**

The sections were deparaffinised as described above and immersed in DDW. To inhibit endogenous peroxidase, they were incubated with 3% $H_2O_2$ in methanol at 4°C for 15 min. Monoclonal Antibody (MoAb) Ki-67 (Ki-67) were purchased from Dako LTD (Glostrup, Denmark). For Ki-67 detection, deparaffinised sections were placed in plastic box filled with a 0.01M tri-sodium citrate solution (pH 6.0), and heated in a conventional microwave oven for 18 min (4 x 4.5 min) at 650W to unmask the antigen [32]. While undergoing microwave processing, slides were always covered with the solution. After heating, slides were permitted to cool down to room temperature over a period of 30-60 min. After washing in PBS (pH 7.4) and blocking, slides were incubated with monoclonal antibodies. The fluorescein conjagated monoclonal Antibody for Ki-67 was applied over night to tissue sections at 4°C. After washing with PBS, slides were sequentially

incubated with biotin-labelled goat anti-florescein antibody (Novocastra) for 30 min. After washing with PBS, they were treated with avidin peroxidase for 30 min, then treated with DAB substrate kit (Vector Lab) for 2-7 min under microscopic observation. After washing with DDW, slides were finally counterstained with hematoxylin and mounted in aqueous mounting medium.

**DNA *in situ* end labelling assay**

The labelling of DNA breaks *in situ* was performed according to the *in situ*-end labelling method (ISEL) described by Gavrieli *et al.* [ ]which utilises the fact that DNA fragmentation occurs as an early event in apoptosis. In brief, after dewaxing and rehydrating, sections were washed in DDW, incubated with 15 μg/ml proteinase K for 30 min at 37 °C, and then washed 4 times in DDW for 2 min. The sections were incubated with TdT buffer (30 mmol/l Tris-HCl buffer, pH 7.2, 140 mmol/l sodium cacodylate, 1 mmol/l cobalt chloride) containing TdT (0.3 U/ml, Gibco BRL) and biotin-labelled 16-dUTP (Boehringer Mannheim) in a humid atmosphere for 1 h at 37 °C. The reaction was terminated by transferring the slides to buffer containing 300 mmol/l sodium chloride and 30 mmol/l sodium citrate for 15 min at room temperature. The sections were rinsed with DDW, and detection performed using the Vecstain ABC elite kit. The dark brown precipitate, an indication of the presence of DNA fragmentation, was examined by bright-field microscopy. In negative controls, the TdT was omitted from TdT buffer containing biotin-labelled-16-dUTP.

**Cell Counting**

Generally, when counting the numbers of cells stained with each of the markers, twelve microscopic fields which showed the strongest cell infiltration, from each serial section of all 26 biopsies, were chosen for counting using a meshwork eyepiece (0.172 mm x 0.172 mm; 0.0296 $mm^2$. The number of positive cells present in a given field were counted in

duplicate, mostly by two different individuals, who then compared the results. Positive cells were also counted using the Kontron 300 Image Analysis System and KS300 software programmed to maximize sensitivity and specificity of positive cell detection (Kontron Elektronik Eching, Germany).

The major criterion was that each area included was present in all of a series of sections. Otherwise the total number of common undamaged fields available were analyzed. The minimum number of fields available in any section included in the study was five. Great care and considerable effort was expended to identify the corresponding areas in series of sections. We tested the efficiency of our method for relocating the same areas in serial sections. This was done by counting the cells in the same area after: removing the slide from the microscope, repositioning the slide holder and relocating the area after reinserting the slide. Capturing an image of the original field using the Kontron System enabled relocation of the same area again with precision. The results of replicate cell counts that differed by less than or equal to 5% were deemed acceptable.

## Results

Cell synthetic activity

Sections were initially examined with the oligo d(T) and 28S rRNA probes for the detection of hybridisable total mRNA and 28S rRNA, which was found in all sections tested. Strong staining was observed in plasma cells and epithelium, slight to moderate staining were seen in fibroblasts, macrophages, giant cells, endothelial cells and lymphocytes. Almost all PMN were negative, a few positive PMN were found in blood vessels (Figure 1a). *In situ* hybridisation with the 28S rRNA probe gave similar results to that of the oligo d(T) probe (Figure 1b). Close examination of adjacent serial sections stained with both markers confirmed that they could be detected in the same cells.

The strongest signals were frequently seen in plasma cells within connective tissue and granulation tissue. With respect to epithelial cells within the epithelium the staining intensity appeared to decrease from the basal towards the superficial epithelial layers. Plasma cells and basal epithelial cells always stained positively, when present, acting as an in-built positive control. Fibroblasts in connective tissue sites were surrounded by bundles of collagen fibrils, and the majority of these fibroblasts in many areas were moderately stained by both probes (Figure 1), whereas in other sites almost all fibroblasts were poorly stained although the cell shapes were similar. Some giant cells were also moderately stained, whereas other cells were negative (data not shown). Endothelial cells were stained slightly to moderately (data not shown). In contrast, lymphocytes close to the epithelium also gave a mixed reaction, ranging from a few strongly stained through weakly positive to negative. All control sections of the periodontal tissues without probes showed no background signal. Messenger RNA was not detected in the RNase-treated control sections (data not shown). There were no significant differences in the numbers of synthetic cells, as assessed by the 28S rRNA probe or with Oligo d(T), between any of the tissue groups (fugure 3a).

Cell Proliferation

A few histone mRNA-expressing cells were detected in basal and suprabasal epithelial cells (data not shown) and/or mononuclear cells in only 4/20 cases (2 AP & 2 EOP) of granulation tissue and in none of the gingival tissue sections tested. and their reactivity was weak (Figure 2a). In contrast, Ki-67 positive cells were also found in epithelium (data not shown), mononuclear cells and fibroblasts in all samples (Figure 2a). We used the microwave method described in our earlier paper (Takahashi *et al* 1996) to unmask the Ki-67 antigen. Fine tuning of the microwave treatment time required to totally unmask the antigen reduced it to 18 minutes, however, there was still some physical disruption of some of the smaller pieces of tissue. In all cases the number of Ki-67 positive cells was consistently and significantly higher (Mann Whitney U-test; $p<0.001$) than the number of histone mRNA-positive cells (data not shown). Significantly, greater numbers of Ki-67 positive cells were observed in the AP granulation tissue than in the AP gingival sections (Figure 3b ;Mann Whitney U-test; $p< 0.05$).

Apoptosis

The characteristic morphological features in apoptosis, such as shrinkage of cells, condensation of chromatin and apoptotic body formation were not clearly observed in H&E sections, whereas nuclear fragmentation of PMN were clearly shown with the ISEL methodology (Figure 2b). With ISEL methodology, apoptotic cells were detected in all tissue sections. Control sections of tonsil tissue were used to confirm the eficacy of the technique, and a positively stained section is shown in Figure 2c. the numbers of apototic cells rarely exceeded 20 cells per field under a x40 objective (x400). There was no difference in the numbers of apoptotic cells Apoptotic cells between the AP and EOP tissue sections and only a slight but non-significant increase in apoptotic cells in AP granulation tissue compared with the AP gingiva (data not Shown).

## Discussion

The results presented in this paper here extend our earlier study on periodontitis gingiva [13]. Recently, Moskow and Polson [33] have expressed doubt that experiments performed on the superficial gingiva were sufficient to give a clear picture of the disease, inflammatory and immune processes in severe periodontitis. In an attempt to address this point we have studied the extent of cell proliferation, their synthetic cell activity and Apoptosis in the deeper periodontitis granulation tissue from two different forms of periodontitis, AP and EOP, and included gingival tissues for direct comparison. In addition to the qualitative approach to the histological data presented in our earlier paper. We have extended the analysis and performed a quantitative analysis of cells with synthetic capacity, the proliferating types and the numbers of apoptotic cells present in a set of serial sections. We have also looked at these cell profiles in different diseased periodontitis tissues and within the different areas of the biopsies.

Cells with synthetic capacity were detected using *in situ* hybridisation. Oligonucleotides, oligo d(T) and 28S ribosomal RNA probes were used to detect cells containing poly adenylated mRNA and 28S ribosomal RNA (28S rRNA). Both these markers provide an insight into the synthetic activity of cells. Apart from the endothelial cell the plasma cell was the most heavily stained cell type in all of the sections. Other cell types stained to a lesser extent confirming the observations of the previous study. Further studies are underway to investigate the role of plasma cells in periodontitis.

We were able to detect Ki-67 positive cells in all of the sections studied. On close examination of adjacent sections we were able to discern that the majority of mononuclear cells that stained for the CD antigens were not stained for Ki-67 or with the histone probe suggesting that the majority of B cells, T cells, and monocytes/macrophages were not proliferating (e.g. were at the Go phase of cell division).

**to be continued .....**

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Legends

Figure 1:

Serial sections of granulation tissue from a patient with Early-onset Periodontitis (EOP) showing: a) The hybridisation signal of the 28S ribosomal RNA oligonucleotide probe. Moderate to strong staining can be observed in plasma cells, fibroblasts and a few endothelial cells (x400). Whereas, only moderate to weak staining is observed in lymphocytes. b) The hybridisation signal for Oligo d(T) used as a probe for polyadenylated mRNA. The strongest signals can be seen in plasma cells, more moderate staining is seen in fibroblasts and other cell types (x400). Similar results were obtained on sections of tissues of all the patients in the study.

Figure 2:

Different fields from the same section as figure 1 are shown here illustrating:
a) The hybridisation signal with the probe for mRNA encoding histone proteins, the synthesis of which occurs during the cell division. Very few cells were stained with this marker of cell division (x400). b) Immunohistochemical staining with the Ki-67 monoclonal antibody as the primary antibody shows that very few mononuclear cells were positive for this proliferation marker. Whereas a number of epithelial cells can be seen to be positive (x400). Similar results were obtained for the tissues of all the patients in the study.

Figure 3:

Serial sections from the same granulation tissue from the patient with EOP ,as for figure 1&4, showing the corresponding fields and demonstrating : a) Immunohistochemical staining with a murine monoclonal antibody to Human CD 20 antigen, demonstrating a large number of B cells (x400). b) Immunohistochemal analysis with a murine monoclonal to the human CD 68 antigen, showing the presence of a few monocytes / macrophages (x400). Similar results were seen in the tissues of all the patients in the study.

Figure 4:

Serial sections from the same granulation tissue from the patient with EOP, as for figures 1&3, showing the corresponding fields and demonstrating : a) Immunohistochemical staining with a murine monoclonal antibody to Human CD 3 antigen, a pan-T cell marker, demonstrating a large number of T lymphocytes (x400)in the section. b) Immunohistochemal analysis with a murine monoclonal to the human CD 45RO antigen,

a showing the presence of many memory T cells in the section. (x400). Similar results were seen in the tissues of all the patients in the study.

Figure 5:

The number of positively staining cells per field in serial sections of granulation tissues from patients with AP(n=10) and EOP(n=10), and sections of gingiva (Ging) from patients with AP (n=6) detected with: a) 28S ribosomal RNA probe (28SrRNA), Oligo d(T) probe, Ki-67. b) Monoclonal antibodies to CD 20 (B cells), CD 3 (pan-T cells), CD 45RO ( memory T cells). The results are represented on the chart as the median value (black bar) surrounded by a box, the, the lower and upper limits of which show the 1$^{st}$ and 3$^{rd}$ quartiles of the data set, respectively. The fine lines projecting from the bottom and the top of the box and terminating with a bar represent the quartile range. Outliers are shown also.

Figure 6:

The ratios of cells stained in sections of granulation tissue from patients with AP (n=10) and EOP (n=10) and in sections of gingival tissue (Ging) from patients with AP (n=6) utilising the following markers: a) CD 20 (B cells), CD 45RO (memory T cells) and CD 68 (monocytes/macrophages) to the population of CD 3 positive cells (pan-T cells). b) CD 20 (B cells), CD 3 (T cells) and CD 68 (monocytes/macrophages) to the sum of the CD 20, CD 3 & CD 68 positive cells. The results are represented on the chart as the median value (black bar) surrounded by a box, the lower and upper limits of which show the 1$^{st}$ and 3$^{rd}$ quartiles of the data set, respectively. The fine lines projecting from the bottom and the top of the box and terminating with a bar represent the quartiles range. Outliers are shown also.